Chromosome Territory Position and Active Relocation in Normal and Hutchinson-Gilford Progeria Fibroblasts

A thesis submitted for the degree of Doctor of Philosophy by

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Abstract:

Radial chromosome positioning in interphase nuclei is non-random and can alter according to developmental, differentiation, proliferation or disease status. The aim of this thesis is to understand how chromosome re-positioning is elicited and to identify the nuclear structures that assist this re-localisation event.

By positioning all human chromosomes in primary fibroblasts that have left the proliferative cell cycle, the study within this thesis has demonstrated that in cells made quiescent by reversible growth arrest, chromosome positioning is altered considerably. Upon removal of serum from the culture medium, chromosome re-positioning took less than 15 minutes, required energy and was inhibited by drugs affecting the polymerization of myosin and actin. The nuclear distribution of nuclear myosin 1β was dramatically different in quiescent cells as compared to proliferating cells. If the expression of nuclear myosin 1β was suppressed using interference RNA procedures the movement of chromosomes after 15 minutes in low serum was inhibited. When high serum was restored to the serum starved cultures chromosome repositioning was only evident after 24-36 hours that coincided with a return to a proliferating distribution of nuclear myosin 1β.

Hutchinson-Gilford Progeria Syndrome (HGPS) is a premature ageing syndrome due to a mutation in *LMNA* gene that codes for lamin A protein – a critical component of the nuclear lamina and the nuclear matrix. The mutation causes this protein to be accumulated in a farnesylated form (also known as progerin) within the nuclei of HGPS patient cells. Interestingly, the positions of chromosome 10, 13 and 18 territories, in proliferating HGPS fibroblasts, are different from proliferating control cells and in fact resemble that of normal quiescent fibroblasts. Unlike control

fibroblasts, the repositioning of chromosome territories in response to serum withdrawal is dysfunctional in HGPS patient cells and interestingly this defect coincides with atypical distribution of nuclear myosin 1β. Moreover, the effect of accumulation of progerin is not only restricted to the nuclear lamina, but has more widespread effects with the presence and distribution of some nucleolar proteins also adversely affected in progeria cells. Treatment of HGPS patient cells with a drug FTI-277 that inhibits farnesylation of the lamin A protein, also restores the chromosome territory dynamics and the nucleolar antigen localisation within the interphase nuclei, thus reaffirming that functional lamin A protein is indeed vital for maintenance of interphase chromosome territory organisation, chromosome behaviour and regulation of nucleolar proteins.

In summary, the studies within this thesis have demonstrated that chromosome repositioning within interphase nuclei is rapid upon withdrawal of serum, but repositioning after readdition of serum requires a longer time and passage of cells through mitosis upon re-stimulation of serum starved cells. Importantly, the nuclear lamina and the nuclear motors comprising nuclear myosin 1β appear to be vital for this global reorganisation of interphase chromosome territories.

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Declaration:

I hereby declare that the work presented in this thesis has been performed by me unless stated.

Ishita S Mehta.

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Abbreviations:

2D – Two dimensional

3D – Three dimensional

2DD – Human dermal fibroblasts

^{o}c – Degree centigrade

A – Adenine

Acto-NMIβ – actin-Nuclear myosin Iβ

AG01972 – Cell line from a patient suffering from HGPS

AG11498 - Cell line from a patient suffering from HGPS

AG11513 - Cell line from a patient suffering from HGPS

APD – Accumulated population doubling

AT – Adenine - thymine

ATP – Adenosine triphosphate

AWS – Atypical Werner's syndrome

B23 – Nucleophosmin

BAF – Barrier to autointegration factor

BDM - 2, 3-Butanedione 2-Monoxime

BLAST – Basic local alignment search tool

BSA – Bovine Serum Albumin

C – Cytosine

CCD - Cooled charged-coupled device

cDNA – Complementary deoxyribonucleic acid

CDK – Cyclin dependent kinase

CHO – Chinese hamster ovary

CLSM – Confocal laser scanning microscope

cm – Centimeter

CMT – Charcot-Marie-Tooth Dystrophy

CO_2 – Carbon dioxide

CT – Chromosome territory

CT-IC – Chromosome territory – interchromatin comapartment

Cy3 – Cyanine 3

Cy5 – Cyanine 5

DAPI - 4, 6-diamidino-2-phenylindole

DCM – Dialated cardiomyopathy

DFC – Dense fibrillar component

DMEM - Dulbeco's modified Eagles Medium

DMSO – Dimethyl sulphoxide

DNA – Deoxyribonucleic acid

DOP-PCR - Degenerate oligonucleotide primed polymerase chain reaction

dUTP – Deoxyuridine trinucleotide phosphate

E358K – Cell line from limb-girdle muscular dystrophy patient

EDMD – Emery-Dreiffus muscular dystrophy

EDTA - Ethylenediaminetetra-acetic acid

FBS – Foetal calf serum

FC – Fibrillar centres

FISH – Fluorescence *in situ* hybridisation

FITC – Fluorescein isothyiocynate

FPLD – Dunnigan familial partial lipidystrophy

FRAP assay - Ferric reducing antioxidant power assay

FTI – Farnesyltransferase inhibitor

g – Grams

G – Guanine

G_0 – Quiescence

G1 – Gap phase 1

G2 – Gap phase 2

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GC – Granular component

GC – Guanine-cytosine

GLD – Generalized lipidystrophy

GGTI – Geranylgeranyltransferase inhibitor

G_S - Senescence

GTP – Guanine triphosphate

HDF – Human dermal fibroblast

HGPS – Hutchinson Gilford Progeria Syndrome.

hnRNP – Hetero nuclear ribonucleoproteins

HRP – Horseradish peroxidase

HSA – *Homo sapien* autosome

ICD – Interchromatin domain

ICN – Interchromatin network

IMP – Integral membrane proteins

INM – Inner nuclear membrane

KCl – Potassium Chloride

kDa - Kilo Dalton

KH_2PO_4 – Potassium dihydrogen orthophosphate

LAP2α – Lamin associated protein 2α

Lat A – Latrunculin A

LDHCP – Lipoatrophy with diabetes, hepatic steatosis, hypertrophic cardiomyopathy and leukomelanodermic papules.

LGMD – Limb girdle muscular dystrophy

LINT25 - LAP2α interactor protein

LMNA – Lamin A

M – Mitosis phase of the cell cycle

M – Molar

MAA - Methanol : acetic acid

MAD – Mandibuloacral dystrophy

MADA – Mandibulacral dysplasia

MAR – Matrix attachment region

MHC – Major histocompatibility complex

mg – Milligrams

ml – Millilitres

mm – Milimeters

mM – Milimolar

mRNA – Messenger ribonucleic acid

MW – Molecular weight

N/A – Not applicable

Na_2HPO_4 – Disodium hydrogen orthophosphate

NaCl – Sodium Chloride

NCS – New born calf serum

nm - Nanometer

NMIβ – Nuclear myosin 1β

No. – Number

NOR – Nucleolar organising region

NPC – Nuclear pore complexes

NPM – Nucleophosmin

ONM – Outer nuclear membrane

PC3 – Prostrate cancer cell line

PCR – Polymerase chain reaction

PD – Population doubling

pKi-67 – Nucleolar protein

PML – Promyelocytic Leukaemia

PBS – Phosphate buffered saline

Rb – Retinoblastoma protein

RD – Restrictive dermopathy

rDNA – Ribosomal DNA

RNA – Ribonucleic acid

RPM –Rotations per minute

RT – Room temperature

S – DNA synthesis phase of the cell cycle

SAR – Scaffold attachment region

SAHF – Senescence associated heterochromatic foci

SD – Standard deviation

SEM – Standard error of mean (Standard deviation/ square root of sample size)

siRNA – Small interfering RNA

snRNP – Small nuclear ribonucleoproteins

SSC - Sodium Saline Citrate

T – Thymine

TRF – Telomere binding protein

TRITC – Rhodamine

Tween 20 – Polyoxyethylene sorbitan monolaurate

µg – Microgram

µl – Microlitre

µM – Micromolar

UV – Ultraviolet

VSMC – Vascular smooth muscle cell

v/v – Volume / volume

w/v –Weight/ volume

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Chapter 1: Introduction

1.1. The eukaryotic cell cycle:

Most eukaryotic cells, for growth and regeneration, will proceed through an ordered series of events in which the cell duplicates its contents and then divides into two cells. The system which takes the cells sequentially through the phases of duplication and division is termed as the cell cycle. A perfect synchronisation of DNA synthesis and chromosomal segregation is vital for every cell undergoing this process of self-reproduction.

1.1.1. Proliferative cell division cycle

The proliferative stage of a cells life-span is a period when the cells duplicate their DNA and then divide into two cells by cycling through a cell division cycle. This cell division cycle, in eukaryotic cells consists of four tightly regulated phases, including G1, S, G2 and M phase (Figure 1.1). Mitosis or M-phase is where chromosomal segregation and nuclear division takes place resulting into division of one cell into two. Replication of DNA occurs during the synthesis or S-phase. DNA synthesis and cell division are separated by two gap phases including the G1 and G2 phases whereby the cell prepares for its entry into the S and M-phases (Figure 1.1). Transition of the cell from one phase of the cell division cycle to another is carefully controlled by cyclin-CDK (cyclin dependent kinase) protein complexes which form various checkpoints present in all of these 4 phases.

The G1, S and the G2 phase of the cell cycle are collectively termed as an interphase stage (Figure 1).

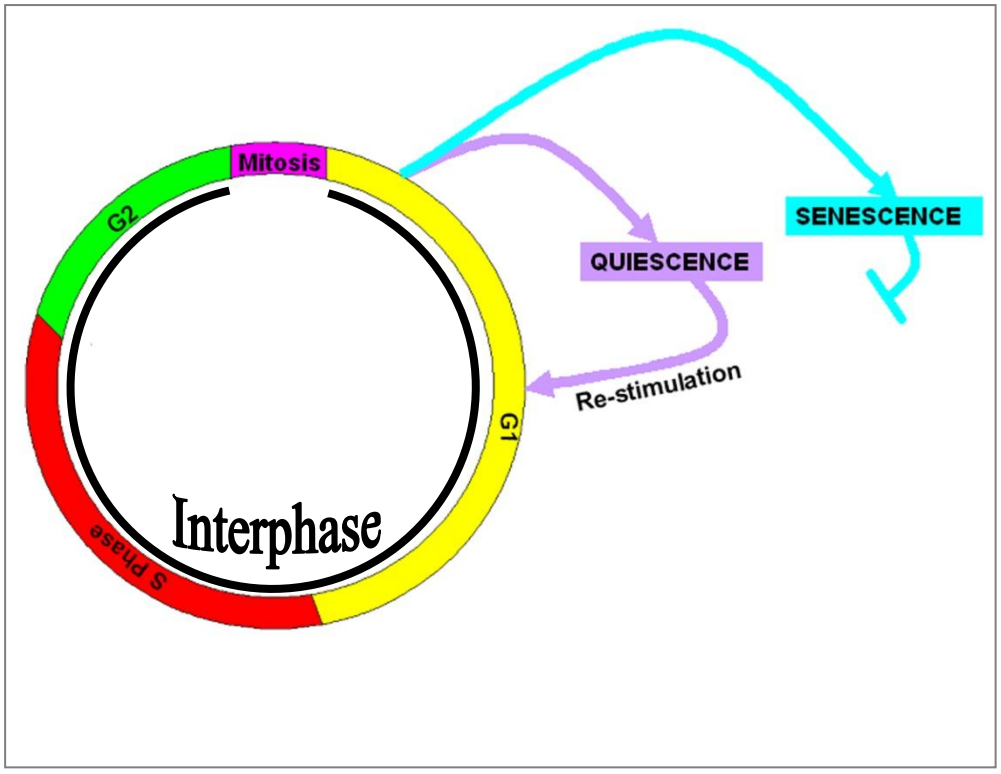


Figure 1.1. Eukaryotic cell cycle: The proliferative cell division cycle is consists of four phases including G1, S, G2 and M phase, where G1, S and G2 phases are collectively termed as interphase. Cells can often exit the proliferative cell division cycle to enter non-proliferative states such as quiescence or senescence.

1.1.2. Non-proliferative stages in eukaryotic cells life-span:

All living cells, *in vivo* and *in vitro* (Pardee, 1974) are capable of exiting the proliferative cell division cycle and are viable in alternate modes of existence, collectively known as the non-proliferative phases. There are two very distinct non-proliferating states known – namely quiescence or G_0 and senescence or G_s.

1.1.2.a. Quiescence or G_0

In 1962, Mortimer Mendelsohn was one of the first to put forward the idea of 'growth fractions' whereby he first suggested that not all cells are cycling at all times and that some cells die and some leave the cell cycle and then return (Mendelsohn, 1962). This idea was further extrapolated to state that when cells leave the cell cycle, they enter into a distinct state outside the normal cell cycle which was initially termed as 'state A' (Smith and Martin, 1973) or 'G_0'(Burns and Tannock, 1970). This distinct state, now widely known as quiescence / G_0 is an active process whereby cells are viable in a reversible non-proliferative state (Coller et al., 2006).It is an inherent property of most eukaryotic cells to remain in a quiescent state throughout most of their lifespan and would only enter the cell cycle in response to extra cellular stimuli or injury (Coller et al., 2006, Liu et al., 2007).

In addition to the cessation of DNA synthesis and generation of mitotic spindles, quiescent cells exhibit numerous discrete properties that differentiate them from proliferating cells. Quiescent cells have reduced cell size and show reduced motility as compared to their proliferating counterparts (Yusuf and Fruman, 2003). G_0 cells also exhibit metabolic variations, as well as differences in their tendency to undergo differentiation, and in the regime of genes expressed as compared to proliferating cells (Venezia et al., 2004, Coller et al., 2006, Liu et al., 2007, Gos et al., 2005, Glynne et al., 2000, Viatour et al., 2008, Chechlinska et al., 2009, Kipling et al., 2009, Mizuno et al., 2009). Interestingly, it has also been demonstrated that quiescent cells lack proteins required for the association of pre-replicative complexes and components with chromatin (Coller et al., 2006, Madine et al., 2000). Further, G0 cellsdisplay differential positioning of chromosomes within their nuclei (Bridger et al., 2000). Quiescence is thus a protective phase when cells are protected 2

protected in ssame sentence from metabolic damage, genetic changes and are resistant to apoptosis (Yusuf and Fruman, 2003).

Entry of cells into quiescence is regulated by growth arrest signals. Some of these signals that are commonly used to induce quiescence include serum withdrawal / mitogen deprivation, contact inhibition (Abercrombie, 1970, Abercrombie and Heaysman, 1954), and loss of cell substratum adhesion (Coller et al., 2006) which may be considered as a type of stress to the cells. Various studies have now revealed that although the cells respond to each of these signals by entering a quiescent state where they do not show many differences in their proliferative status, the mode of entry into quiescence and gene expression profiles differ depending on the type of signal (Coller et al., 2006, Gos et al., 2005, Liu et al., 2007, Yusuf and Fruman, 2003, Coller, 2007, Coppock et al., 1993). Combination of two or more of the above signals is observed to cause the cells to enter the state of quiescence more rapidly (Coller et al., 2006).

Quiescence, which was initially denoted as the dormant state of the cell, is in fact a highly dynamic state (Coller et al., 2006) whereby the cells are undergoing distinct changes so as to maintain themselves in reduced resources (Yusuf and Fruman, 2003) and to transverse efficiently through the proliferating cell cycle when required (Coller, 2007). Although, quiescence is a state in which some somatic cells spend most of their life-span, there is very little known about the regulation, nuclear architecture, changes in the chromatin or the distinct mechanisms by which the cells move between the G_1 to G_0 states.

1.1.2.b. Senescence or G_s

The concept of cellular senescence was first introduced by August Wisemann when he suggested that "death occurs because of a simple reason that a worn-out tissue cannot renew itself forever (Weismann, 1889)." This idea was further supported by experimental data by Leonard Hayflick and Paul Moorhead who observed that normal human fibroblasts enter into an irreversible state of growth arrest (now termed as replicative senescence) following serial cultivation in culture (Hayflick and Moorhead, 1961). Thus cellular senescence can be defined as a state of an irreversible growth arrest that the cells attain after a finite and a predictable number of population doublings (Ben-Porath and Weinberg, 2004, Campisi, 2003b, Mehta et al., 2007). This process of replicative senescence appears to be an evolutionary conserved process, i.e. cells from mammals, birds, reptiles, *D.melanogaster* and yeast all have been shown to undergo growth arrest and exhibit senescent phenotypes after repeated doublings (Stanulis-Praeger, 1987, Lanza et al., 2000, Shiels et al., 1999, Whikehart et al., 2000, Campisi, 2001).

As the process of cellular senescence is described as a gradual accumulation of non-dividing cells throughout the reproductive life span of culture (Kill et al., 1994) it is a major obstacle to continuous propagation of cells, and thus is often regarded as a tumour suppressing mechanism (Campisi, 2003b, Campisi, 2003a). Various studies showing a co-relation between increasing number of senescent cells (Dimri et al., 1995, Li et al., 1997, Pawelec et al., 1999) and decreasing activity of stem cells (Collado et al., 2007) with the age of tissue or organism, are a step towards confirming the speculated link between the cellular senescence and organismal ageing (Campisi, 2003b, Hayflick and Moorhead, 1961, Collado et al., 2007, Smith and Kipling, 2004).

Senescent cells exhibit an altered phenotype as compared to their proliferating counterparts. For instance, senescent fibroblasts display a larger, flatter morphology (Bowman et al., 1975, Sherwood et al., 1988) with a larger nucleus (Mehta et al., 2007, Mitsui and Schneider, 1976), increased adhesion to the extra cellular matrix and fewer cell-cell contacts (Campisi, 2000, Narita et al., 2003, Ben-Porath and Weinberg, 2004) and increased aneuploidy (Sherwood et al., 1988, Benn, 1976, Mukherjee et al., 1995). In addition to this, senescent cells display dramatic changes in their chromatin structure with formation of senescence associated heterochromatin foci (SAHF) (Narita et al., 2003), a differential regime of gene expression profile (Kipling et al., 2009, Shelton et al., 1999, Zhang et al., 2003, Schwarze et al., 2005, Burton et al., 2009) and alteration in the spatial organisation of genome within interphase nuclei compared to their proliferating counterparts (Mehta, 2005, Mehta et al., 2007, Bridger et al., 2000).

These cells to some extent, respond to mitogenic stimuli and by entering the cell cycle to transverse through S-phase and G_2 expressing proliferating markers, but they cannot go through cell division and may subsequently be blocked in G_2 (Kill and Shall, 1990, Kill et al., 1994). Cells in a senescent state can remain viable indefinitely in culture. This is true in most cases *in vivo* (Michaloglou et al., 2005) as well as *in vitro* with a very few exceptions where some senescent tumour cells of liver carcinomas phagocytose rapidly (Xue et al., 2007).

Replicative senescence in mammalian cells is thought to be induced by various intrinsic factors including telomere shortening whereby there is a progressive loss of telomeric repeats with each cell division cycle (Canela et al., 2007, Cawthon et al., 2003, Ogami et al., 2004, Ben-Porath and Weinberg, 2004, Blackburn, 2001, Allsopp et al., 1995, Harley et al., 1990a, Kill, 1998, Masutomi et al., 2003, Davis

and Kipling, 2005) as the DNA replication machinery is unable to ???? RNA primers at the ends of the lagging strand - a process often referred to as the end replication problem (Blackburn, 1991). Thus, with ongoing cell divisions, telomeres shorten to a critical limit beyond which leads to loss of telomeric function (Allsopp and Harley, 1995). This is further thought to induce a DNA double strand break damage response that leads to a p53 dependent growth arrest or replicative senescence (d'Adda di Fagagna et al., 2003). Thus, telomere shortening acts as a counting measure or a "replicotmeter" to determine the number of divisions a cell has gone through (Harley et al., 1990b, Allsopp et al., 1995, Allsopp et al., 1992, Levy et al., 1992, Ben-Porath and Weinberg, 2004, Davis and Kipling, 2005). In addition to telomere shortening, other stimuli such as accumulation of DNA damage (Parrinello et al., 2003, te Poele et al., 2002, Bartkova et al., 2006, d'Adda di Fagagna et al., 2003, Di Micco et al., 2006) and de-repression of the *INK4a/ARF* locus (Krishnamurthy et al., 2004, Zindy et al., 1997, Chkhotua et al., 2003, Ressler et al., 2006). Mode of entry into senescence through each of these pathways is governed by the proteins p53 and retinoblastoma (Rb) (Ben-Porath and Weinberg, 2004, Collado et al., 2007, Davis et al., 2005).

The changes in the nuclear architecture and its effect on genome behaviour are two of the least studied issues with respect to senescent cells. A further understanding of these aspects may help provide stronger evidence to relate the mechanism of senescence to organismal ageing or tumour suppression.

1.2. Nuclear architecture:

The cell nucleus, a defining feature of most eukaryotic cells, is a highly complex organelle that contains and protects the genetic material of the cell. In addition to this, the nucleus co-ordinates a variety of functions and processes, including transcription and processing of deoxyribonucleic acid (DNA), DNA replication and repair, regulation of gene expression and ribosome assembly. Synchronized and efficient working of all these processes requires the nucleus to be a highly organized cellular component. Thus the nucleus is divided into several nuclear compartments (van Driel et al., 1991, Strouboulis and Wolffe, 1996), including the genome, nuclear envelope, nucleoli, nuclear bodies, nuclear matrix and the newly discovered nuclear motors, which form the nuclear architecture and perform overlapping but distinct functions (Figure 1.2.). Nuclear structure maintains nuclear integrity by allowing the different nuclear compartments to interact enabling the nucleus to work efficiently. Any aberrations or disruption of nuclear structure, could lead to severe disease conditions including cancer and nucleopathies (Foster and Bridger, 2005).

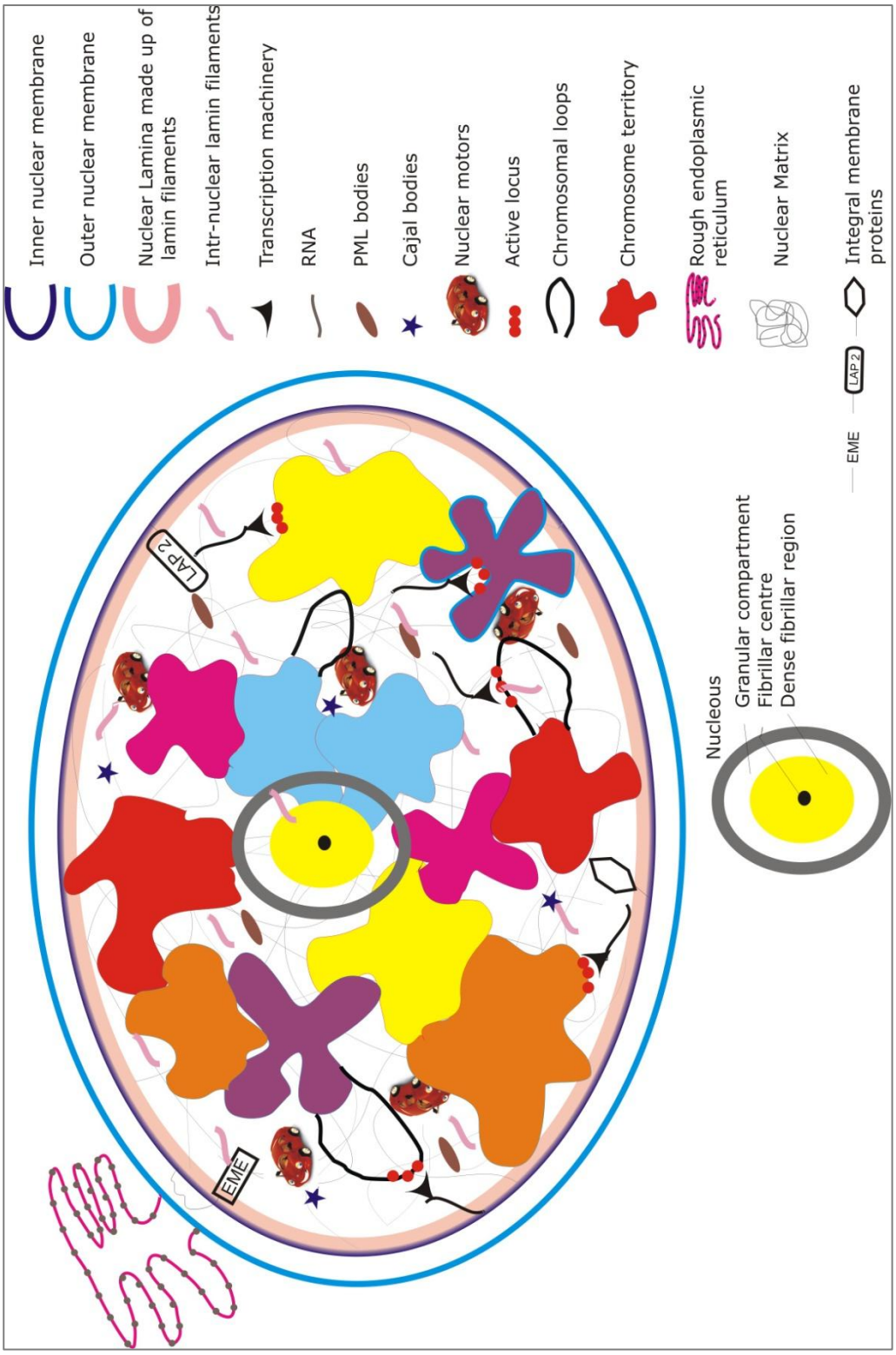


Figure 1.2. Cartoon displaying the components of an interphase nucleus:

Organisation of nuclear sub-structures including the nuclear envelope, nuclear bodies, nuclear lamina, nuclear motors and the genome within a mammalian interphase nucleus is displayed in this figure.

The studies within this thesis concentrate on the interaction between nuclear components such as the nucleolus, the nuclear lamina, nuclear motors and the genome; hence much of this introduction is focussed towards these features of the nuclear architecture.

1.2.1. Nuclear envelope, the lamina and lamin proteins:

The nucleus of an eukaryotic cell is separated from the cytoplasm by a selectively permeable barrier called the nuclear envelope which is made up of the nuclear membranes, the nuclear pore complexes and the nuclear lamina (Gerace and Burke, 1988). The nuclear membrane is double layered, in which the outer nuclear membrane is continuous with the endoplasmic reticulum, thus facing the cytoplasm (Voeltz et al., 2002). The inner nuclear membrane in turn faces the nuclear interior and can be identified by the subset of integral membrane proteins (IMPs) it contains (Schirmer et al., 2003). Sixty seven of these proteins have been now identified by proteomic analysis (Schirmer et al., 2003), which include lamin B receptor, lamin associated polypeptide 1 and 2, emerin, MAN1, nesprins and barrier to autointegration factor (BAF). The inner nuclear membrane is vital for maintaining the nuclear shape and integrity of the nuclear envelope (Bridger et al., 2007, Aebi et al., 1986) and is interspersed by nuclear pore complexes (NPCs) where the inner and outer nuclear membranes join. The NPCs are involved in transportation of molecules between the nucleus and the cytoplasm (Goldberg, 2004) (Figure 1.6.).

The inner nuclear membrane is lined by a meshwork of lamin polymers which form a structure known as the nuclear lamina (Gerace and Burke, 1988, Gruenbaum et al., 2005, Herrmann and Aebi, 2004, Moir et al., 2000, Stuurman et al., 1998, Aebi et al., 1986). The nuclear lamina is made up of type V intermediate

filament proteins called ‘nuclear lamins’ (Fisher et al., 1986, McKeon et al., 1986) that contain a central α-helical rod domain flanked by a globular N-terminal head at one end and a C-terminal tail domain on the other end (Strelkov et al., 2004). The lamin proteins help in attachment of the lamina to the nuclear envelope and interact with the IMPs, chromatin binding proteins and also directly with DNA/chromatin (Gruenbaum et al., 2003, Zastrow et al., 2004). Thus, the nuclear lamina helps to tether regions of genome to the inner nuclear membrane (Guelen et al., 2008, Bridger and Bickmore, 1998, Bridger et al., 2007, Hutchison et al., 1994, Pickersgill et al., 2006, Towbin et al., 2009).

1.2.1.a. Lamin genes and expression

Mammals, including humans have three lamin genes including *LMNA*, *LMNB1* and *LMNB2* which code 7 distinct lamin proteins. *LMNA* codes A-type lamins including lamins A, AΔ10, C and C2 (McKeon et al., 1986, Lin and Worman, 1993, Machiels et al., 1996, Fisher et al., 1986, Furukawa et al., 1994). *LMNA* gene undergoes alternative splicing to form lamins A and C proteins such that lamin A has 98 extra amino acids than lamin C (Berrios et al., 1991). Lamin C on the other hand has 6 unique amino acids at its C-terminal end (Fisher et al., 1986, McKeon et al., 1986). Lamin AΔ10 is identical to lamin A protein but has the whole of exon 10 spliced (Broers et al., 2006, Machiels et al., 1996) while lamin C2 is a form of lamin A that is found only in germ line cells (Furukawa et al., 1994). On the other hand, lamin B1 is derived from *LMNB1* gene and lamins B2 and B3 are splice variants derived from *LMNB2* gene locus (Pollard et al., 1990, Biamonti et al., 1992, Furukawa and Hotta, 1993, Stuurman et al., 1996).

The expression of A-type lamins is developmentally regulated and their appearance in cell types is correlated with differentiation (Lehner et al., 1987, Riemer et al., 1995, Stick and Hausen, 1985, Rober et al., 1989), with lamin AΔ10 present only in very low amounts in all cell types (Machiels et al., 1995, Machiels et al., 1996). The presence of lamins A and C have also been demonstrated in early embryos including in mammalian oocytes (Schatten et al., 1985, Prather et al., 1989, Houliston et al., 1988) and during late stages of embryogenesis (Prather et al., 1989, Stewart and Burke, 1987, Foster et al., 2007, Lee et al., 2007) but not in blastocytes (Schatten et al., 1985, Prather et al., 1989, Stewart and Burke, 1987). On the other hand, lamins B1 and B2 are widely expressed in most somatic cells both in adults and in embryos (Vergnes et al., 2004). Expression of certain lamin proteins including lamin C2 is restricted to the testis and during meiosis (Alsheimer and Benavente, 1996) while lamin B3 is only present in sperm cells and in oocytes (Stick and Hausen, 1985, Benavente et al., 1985, Furukawa and Hotta, 1993).

1.2.1.b. Post-translational modifications and formation of lamin proteins

Precursor mRNA molecules for all lamins except lamin C (which does not undergo post-translational processing) contains a CaaX motif at the C-terminal end that instigates farnesylation of the cysteine residue by the enzyme farnesyltransferase (Beck et al., 1990) (Figure 1.3.).

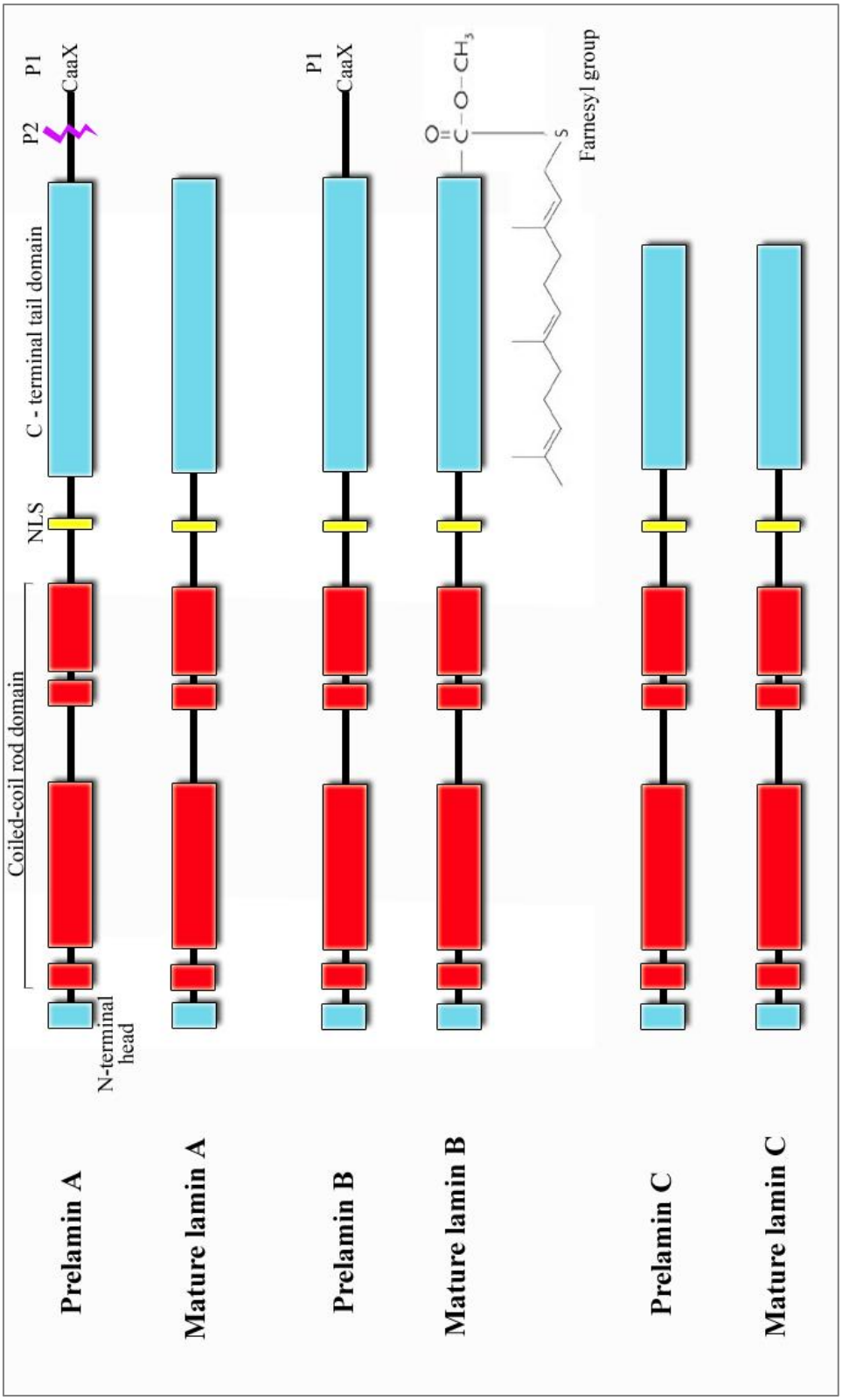


Figure 1.3. Basic structure of mammalian lamins: Lamins A and B have a CaaX motif (P1) at the C-terminal end which signals farnesylation of these proteins. Lamin A, further has a site for endoproteolytic cleavage that is recognised by ZMPSTE24-FACE1 enzyme (P2) which cleaves the protein and removes the farnesylated cysteine. Lamin C, on the other hand does not undergo any post-translational modifications.

The presence of farnesyl group at the C-terminal end, along with the CaaX motif, promotes the association of pre-lamin proteins to the nuclear membrane and hence is vital for correct localisation of the mature protein (Hennekes and Nigg, 1994). After their localisation at the nuclear membrane, lamin B proteins have completed their post-translational modifications and accumulate within the nucleus in an isoprenylated form throughout the cell's life-span (Young et al., 2005, Prufert et al., 2004, Ralle et al., 2004) (Figure 1.3.). In contrast, lamin A proteins have an additional cleavage site for endoprotease cleavage whereby the protein then loses the terminal three aaX amino acids followed by methyl esterification of the C-terminal cysteine residue. Finally, the protein undergoes a second endo-proteolytic cleavage by an enzyme ZMPSTE24-FACE1 metalloproteinase (Pendas et al., 2002), resulting in a loss of 15 amino acids at the C-terminal end including the farnesylated cysteine and thus leading to the formation of a mature lamin A protein (Broers et al., 2006) (Figure 1.4.). The final step of this post-transcriptional modification process, whereby the farnesyl group is lost, is thought to be important for insertion of the mature lamin A into the nuclear lamina (Capell et al., 2005). Cartoon demonstrating the post-translational processing of lamins is displayed in figure 1.4.

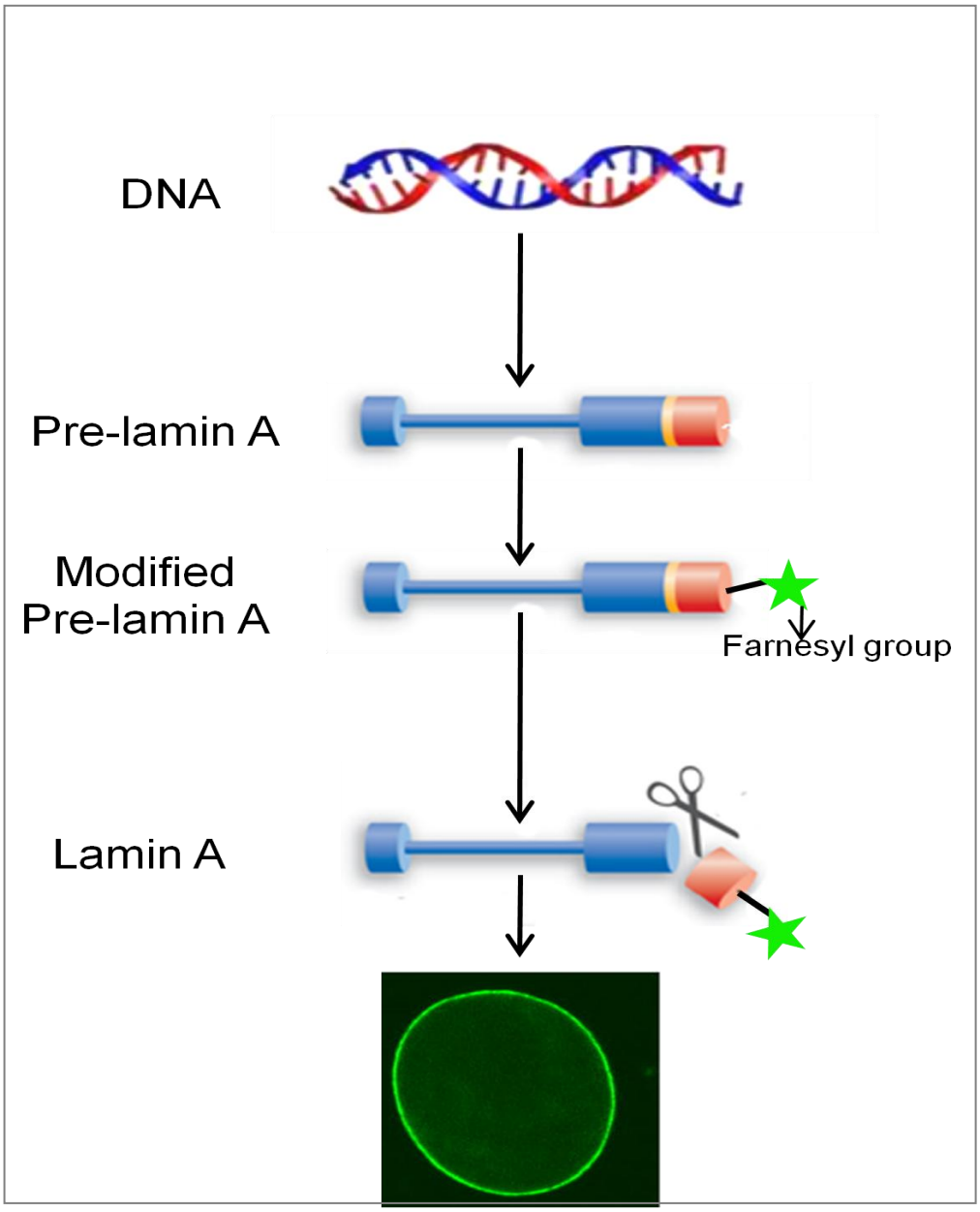


Figure 1.4. A figure demonstrating the post-transaltional modifications that pre-lamin A protein undergoes to form a mature lamin A protein: Processing of pre-lamin A to a mature lamin A protein involves several post-transalational modifications including attachment of a farnesyl group and an endoproteolytic cleavage by ZMPSTE24-FACEI enzyme. B-type lamins are also processed and undergo farnesylation but do not undergo cleavage. Lamin C does not undergo any post-translational processing. After the post-transalational processing is complete, these lamins are incorporated into the nuclear lamina.

1.2.1.c. A-type lamins' binding partners

Apart from their localisation at the nuclear periphery, both A- and B-type lamins have been observed distributed in the nucleoplasm as intranuclear lamin foci and as lamin filament network which are the essential components of the nuclear matrix (Barboro et al., 2002, Goldman et al., 1992, Lutz et al., 1992, Bridger et al., 1993, Broers et al., 1999, Moir et al., 1994, Hutchison et al., 1994, Jackson and Cook, 1988, Jackson, 2004, Hozak et al., 1995, Bridger, 1994). Within the nucleus, A-type lamins are found in association with chromatin, histones (Zastrow et al., 2004), DNA replication sites (Kennedy et al., 2000, Broers et al., 2006) and RNA splicing speckles (Muralikrishna et al., 2001) as well as in nuclear areas of increased transcription (Spann et al., 2002), within intranuclear tubules (Broers et al., 2006, Maske and Vaux, 2004) and in the nucleolus (Hozak et al., 1995, Zimber et al., 2004, Martin et al., 2009). The list of binding partners for A-type lamins is extensive and is growing continuously (Maraldi and Lattanzi, 2005) (Figure 1.5.). At the nuclear periphery, several LEM domain containing proteins including emerin (Sakaki et al., 2001), lamina-associated proteins 2 (LAP2) (Dechat et al., 2000) and MAN1 (Mansharamani and Wilson, 2005) have been identified as lamin binding partners. Other INM proteins such as nesprins not only associate with A-type lamins *in vitro* (Mislow et al., 2002), but also require correct expression of lamin A for their localisation at the nuclear envelope (Libotte et al., 2005). A-type lamins interact with SUN domain proteins, which thus form a structural bridge allowing indirect associations of these lamins with cytoplasmic proteins (Haque et al., 2006). Indeed, indirect associations between lamins and cytoplasmic proteins such as actin (Broers et al., 2006), microtubules (Gruenbaum et al., 2005) as well as other intermediate filaments such as vimentin (Broers et al., 2004, Tolstonog et al., 2002, Bloom et al.,

1996) are mediated via nesprins, SUN proteins and NPC complex proteins respectively. Interactions between A-type lamins and lamin B receptor (LBR) (Worman et al., 1988) as well as LAP1 (Dechat et al., 2000) at the nuclear periphery have also been identified. A-type lamins interact with chromatin *in vivo* and *in vitro*, via their DNA binding domain and indirectly via LEM domain proteins (Rzepecki and Fisher, 2002, Clements et al., 2000, Lee et al., 2001, Martins et al., 2000, Goldberg et al., 1999). Chromatin binding regions are also present on lamin C tail domain which binds histones (Taniura et al., 1995). Various domains of the lamin protein that do not have any DNA sequence specificity have also been demonstrated to interact with chromatin (Stierle et al., 2003). A-type lamins tether telomeres to the nuclear matrix (Shoeman and Traub, 1990, de Lange, 1992, Masny et al., 2004) as well as interact with signal transduction proteins (Rao et al., 1997, Tang et al., 2000, Martelli et al., 2002, Parnaik, 2008) and transcription factors (Ozaki et al., 1994, Dreuillet et al., 2002, Lloyd et al., 2002, Markiewicz et al., 2002, Johnson et al., 2004, Ivorra et al., 2006). It is not yet clear if there are any interactions between A- and B-type lamins, but a recent study has demonstrated that the networks made by both these lamins are distinct but interconnected and that B-type lamins are important for structural organisation and maintenance of A-type lamins within the interphase nucleus (Shimi et al., 2008).

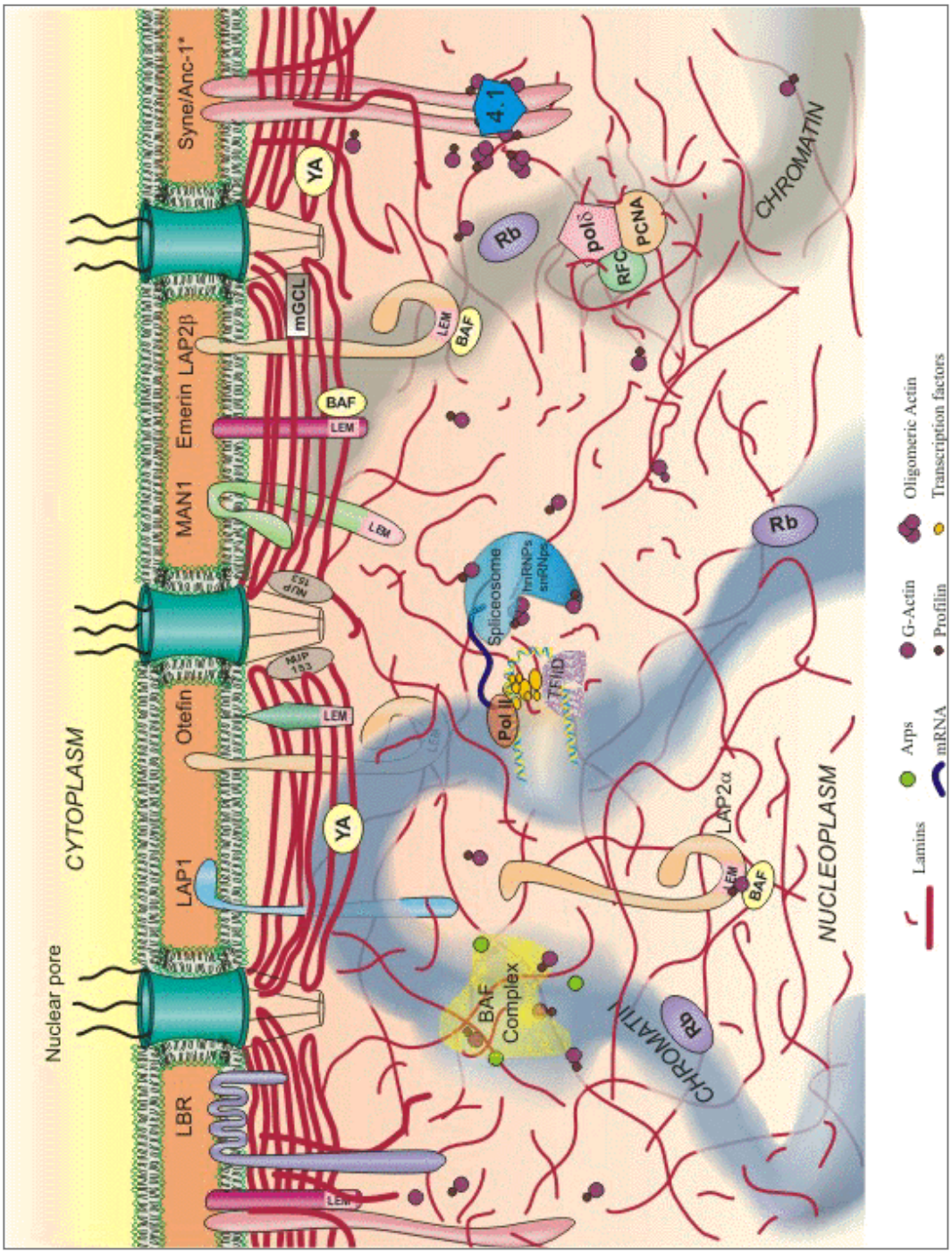


Figure 1.5. Structure of the nuclear lamina and lamin binding proteins: The figure displays INM, ONM and the nuclear lamina underneath the INM made up of nuclear lamins. Various proteins associated with INM are lamin binding partners including nesprins, emerin, BAFs, LAP2, LBR, HP1 and MAN1. Figure adapted from (Goldman et al., 2002).

1.2.1.d. Cell cycle and A-type lamins

The distribution of lamins in the nucleus differs with cell cycle status and thus different patterns of lamin expression are observed in G_1, G_2 and S phase of the cell cycle (Bridger et al., 1993). A-type lamins are present in DNA pre-replication foci along with p150, pRb and PCNA during early G_1 phase and as the cell reaches S-phase these associations with the DNA replication foci, as well as the intranuclear lamin foci are lost (Kennedy et al., 2000, Bridger et al., 1993, Broers et al., 2006). Indeed, the nucleoplasmic pool of lamins along with LAP2α is important for G_1-S phase transition (Naetar and Foisner, 2009).

In addition to undergoing cellular division, all living cells are capable of exiting the proliferative cell cycle and are viable in alternate non-proliferating states (Pardee, 1974), namely quiescence (G_0) or reversible growth arrest (Mendelsohn, 1962) and replicative senescence (G_s) or irreversible growth arrest attained after a predicted number of cellular divisions (Hayflick and Moorhead, 1961). Some very early studies performed in rat liver cells reveal that the lamin proteins are present only at the nuclear periphery and thus there are no visible intranuclear foci of lamins in G_0 state of the cell cycle (Gerace et al., 1978, Gerace et al., 1984) Accumulation of more lamin A/C, prelamin A and some other derivatives of lamin A have been reported in senescent cells (Ukekawa et al., 2007). It is suggested that the increase in lamin A molecules in the nucleus causes a stoichiometric imbalance in the number of molecules interacting with lamin A which further may have a role in causing irreversible growth arrest (Ukekawa et al., 2007). It has also been observed that accumulation of different forms of pre-lamin A causes differential re-localisation of heterochromatin proteins (Lattanzi et al., 2007). It has also been proposed that lamin A along with another lamin binding protein LAP2α control the phosphorylation of

retinoblastoma protein which then interacts with various cyclins to maintain the cell in a proliferating state (Pekovic et al., 2007, Naetar and Foisner, 2009). Cells lacking lamin A or LAP2α are observed to undergo a cell cycle arrest due to lack of phosphorylated forms of Rb present in the nucleus (Pekovic et al., 2007, Naetar and Foisner, 2009). A decrease in expression of LAP2α has been also correlated with the exit of the cell from the cell cycle as well as decrease in nucleoplasmic pool of lamin A during G_1 phase and it has been proposed that the interaction of LAP2α and LAP2 α interactor-25 (LINT-25) regulate the transition of the cells from a proliferating state to a non-proliferating growth arrest state (Naetar et al., 2007, Naetar and Foisner, 2009).

1.2.1.e. Lamins and diseases

Lamins and lamin binding proteins are vital for maintaining nuclear integrity as well as being involved in nuclear functions such as cell cycle regulation, DNA replication and transcription, cell differentiation, signal transduction, apoptosis and ageing. Hence, a mutation in lamin genes or mis-regulation in lamin expression disrupts these normal nuclear activities and can further manifest into a disease condition. Indeed lamins along with their binding partners have been implicated in various diseases including several muscular dystrophies and cancers.

Lamins and cancers

Different forms of pre-lamin A and mature lamin A have been implicated in regulation of cell-cycle control and DNA repair mechanisms whereby they activate a proliferative arrest/apoptotic gene p53 (Varela et al., 2005). Hence alterations in composition of lamins within cell nuclei result in loss of proliferative control and hence may be involved in tumour progression (Burke and Stewart, 2006, Venables et al., 2001). A-type lamins are usually absent in small cell lung cancer cell lines, but

abnormal expression of these proteins (Kaufmann et al., 1991) as well as absence of B-type lamins (Broers et al., 1993) was observed in non-small cell lung cancer cell lines. Differential expression of A-type lamins has also been reported in testicular germ cell tumours (Machiels et al., 1997), Hodgkin's disease (Jansen et al., 1997), cancerous prostrate tissues (Coradeghini et al., 2006), skin cancers (basal and squamous cell carcinomas) (Oguchi et al., 2002, Venables et al., 2001) and in gastrointestinal neoplasms (Moss et al., 1999). Atypical levels of lamins were also detected in some cases of acute myeloid leukaemia, chronic myeloid leukaemia, acute lymphocytic leukaemia (Kaufmann, 1992) and in other haematopoietic malignancies (Prokocimer et al., 2006). In addition to this, various recent studies have demonstrated that reduced levels or atypical expression of A-type lamins are linked to poor disease prognosis for gastric carcinoma (Wu et al., 2009) and colorectal cancer patients (Willis et al., 2008) and hence the levels of these proteins may be important for diagnostic purposes.

Laminopathies

The group of disorders due to mutations in genes encoding nuclear lamins or lamin associated proteins are termed 'laminopathies.' Laminopathies are a diverse group of tissue specific diseases. Table 1.1. summarises the list of most of the laminopathies known to date with the gene that is mutated in the respective diseases.

B-type lamins are expressed in all types of somatic cells and thus are thought to be essential for survival *in vivo*. B-type lamin knock-out mice complete development but die soon after birth (Vergnes et al., 2004). In humans, no diseases have yet been linked to the absence of B-type lamins, but there is some evidence that over-expression of B-type lamins causes autosomal dominant leukodystrophy also

known as Barraquer-Simons syndrome (Padiath et al., 2006). Some of the recent studies performed on fibroblasts from lamin B1 knockout embryos show that the nuclei of these cells rotate 90 degrees, thus stressing the role of lamin B1 in maintaining the position of the nucleus with the cytoplasm (Ji et al., 2007).

Over 300 mutations spanning the entire *LMNA* gene have been identified (Lammerding et al., 2004, Neveling et al., 2007, Dechat et al., 2008) and this gene is regarded as the gene associated with the most growing number of mutations in anay gene to date. Since the mutations are found in different regions of the gene resulting in different laminopathies (Figure 1.6.), the distinct phenotype of each laminopathy highlights the wide variety of processes and functions that A-type lamins are involved in (Hutchison et al., 2001).

Table 1.1. List of laminopathies known to date, with the gene affected and the year when they were identified:

Syndrome	Mutation in	Identified in
Atypical Werner syndrome	Lamin A/C	2003
Barraquer-Simons syndrome	Lamin B	2006
Buschke-Ollendorff syndrome	LEM domain containing protein 3 (lamin-binding protein)	2004
Cardiomyopathy, dilated, with quadriceps myopathy	Lamin A/C	2003
Charcot-Marie-Tooth disease, axonal, type 2B1	Lamin A/C	2002
Emery-Dreifuss muscular dystrophy	Emerin (lamin-binding protein)	1996
X-linked -Emery-Dreifuss muscular dystrophy		2000
Emery-Dreifuss muscular dystrophy, autosomal dominant (EDMD2)	Lamin A/C	1999
Emery-Dreifuss muscular dystrophy, autosomal recessive (EDMD3)	Lamin A/C	2000
Familial partial lipodystrophy of the Dunnigan type (FPLD)	Lamin A/C	2002
Greenberg dysplasia	Lamin B receptor	2003
Hutchinson-Gilford Progeria Syndrome (HGPS)	Lamin A/C	2003
Limb-girdle muscular dystrophy type 1B (LGMD1B)	Lamin A/C	2000
Lipoatrophy with diabetes, hepatic steatosis, hypertrophic cardiomyopathy, and eukomelanodermic papules (LDHCP)	Lamin A/C	2003
Mandibuloacral dysplasia with type A lipodystrophy (MADA)	Lamin A/C	2002
Mandibuloacral dysplasia with type B lipodystrophy (MADB)	Zinc metalloprotease STE24 (prelamin-processing enzyme)	2003
Pelger-Huet anomaly (PHA)	Lamin B receptor	2002
Pelizaeus-Merzbacher disease, adult-onset, autosomal dominant	Lamin B	2006
Tight skin contracture syndrome, lethal	Lamin A/C or Zinc metalloprotease STE24 (prelamin-processing enzyme)	2004

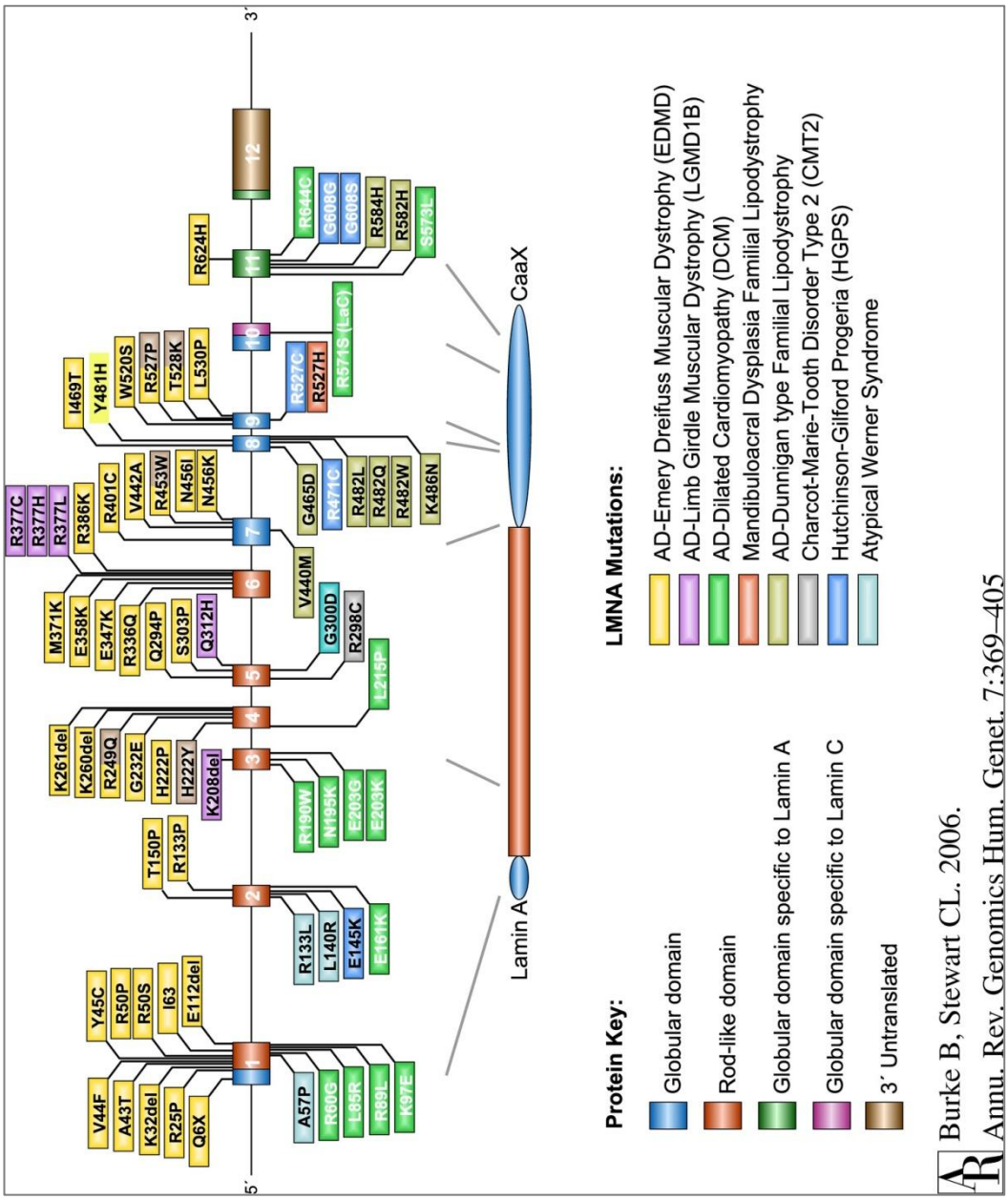


Figure 1.6. A cartoon displaying of *LMNA* gene with 12 exons and some of the associated laminopathies: Some examples of location of mutations in this gene are labeled. AD-EDMD - autosomal dominant Emery–Dreifuss muscular dystrophy; AR-EDMD, autosomal recessive Emery–Dreifuss muscular dystrophy; AWS, atypical Werner syndrome; CMT2B1,Charcot–Marie–Tooth disorder, type 2B1; DCM1A, dilated cardiomyopathy, type 1A; FPLD, Dunnigan familial partial lipodystrophy; GLD, generalized lipodystrophy; HGPS, Hutchinson–Gilford progeria syndrome; LGMD1B, limb girdle muscular dystrophy, type 1B; MAD, mandibuloacral dysplasia; RD, restrictive dermopathy (adapted from (Burke and Stewart, 2006) .

Since some of the studies within this thesis involve Hutchinson-Gilford Progeria Syndrome patient cells, aspects of this disease have been discussed below.

Hutchinson-Gilford progeria syndrome

Hutchinson-Gilford progeria syndrome (HGPS), first identified by Jonathan Hutchinson and Hastings Gilford in 1886 and 1904 respectively (Gilford, 1904, Hutchinson, 1886), is an extremely rare, dominantly inherited, disorder that affects children causing them to age prematurely (De Sandre-Giovannoli et al., 2003, Capell and Collins, 2006, Cao and Hegele, 2003, Eriksson et al., 2003).

Mutation and its effects:

The most common mutation associated with HGPS is a single base-substitution in codon 608 of exon 11 on *LMNA* gene which results in formation of a cryptic splice site resulting in a truncated pre-lamin A protein (progerin), lacking terminal 50 amino acids at the C-terminus end (De Sandre-Giovannoli et al., 2003, Eriksson et al., 2003). This deletion does not affect the CaaX motif and the protein pre-progerin undergoes normal farnesylation, but the protein lacks the ZMPSTE24-FACE1 recognition site necessary for the final cleavage step and hence remains farnesylated (Eriksson et al., 2003, Fong et al., 2004). Retention of the farnesyl group and accumulation of the farnesylated protein at the nuclear envelope compromises the nuclear integrity, disrupts the nuclear lamina including the underlying heterochromatin and leads to the formation of abnormally shaped nuclei, a prominent characteristic seen in HGPS (Goldman et al., 2004, Scaffidi and Misteli, 2005, Bridger and Kill, 2004). Progerin acts in a dominant negative manner on the nuclear functions of cell types that express lamin A, i.e. in majority of differentiated cells (Goldman et al., 2004, Reddel and Weiss, 2004, Scaffidi and Misteli, 2005) and

hence the phenotype of the disease cannot be rescued by transfection of wild-type lamin A (Scaffidi and Misteli, 2005).

Apart from the mutation in codon 608 of exon 11, various other mutations including E145K (De Sandre-Giovannoli et al., 2003, Eriksson et al., 2003), G608S, 2036C>T (Cao and Hegele, 2003), K542N (Plasilova et al., 2004), S143F (Kirschner et al., 2005) on the *LMNA* gene have also been associated with HGPS. In addition to this, a patient with double mutation in *LMNA* gene including R471C and R527C have also been identified (Cao and Hegele, 2003).

Clinical features:

HGPS patients appear normal at birth and the disease symptoms are only observed at 9 - 12 months old (Kieran et al., 2007). Clinical features of this disease include alopecia, growth retardation, extremely aged appearance, loss of subcutaneous fat, progressive artherosclerosis, micrognathia, hypoplasia, alopecia, short stature bone deformations and cardiovascular diseases (Baker et al., 1981, Capell et al., 2005, DeBusk, 1972, Sarkar and Shinton, 2001). Most of these patients have a median age of 13 and die due to myocardial ischemia, infarction or a stroke (Kieran et al., 2007). Mutation in lamin A surprisingly appears to affect only some but not all organs and tissues within these patients, for example presence of the truncated protein has a severe effect on the teeth and heart but has very little effect on the brain, liver, kidney, lungs, gastrointestinal tract and bone marrow (Csoka et al., 2004, McClintock et al., 2006, Lans and Hoeijmakers, 2006, Kieran et al., 2007). Certain phenotypes observed in HGPS patients are similar to that in a normal ageing individual (Gilford, 1904, Kipling et al., 2004, Hutchinson, 1886, Kieran et al., 2007), but there are notable exceptions such as no evidence of neurosensory decline,

dementia and cancer have been detected in these patients (Ackerman and Gilbert-Barness, 2002, Puzianowska-Kuznicka and Kuznicki, 2005) and hence premature ageing observed in HGPS patients is often termed as "segmental" (Pereira et al., 2008, Kieran et al., 2007).

Nuclear architecture in HGPS patient cells

The presence of a mutant lamin A within the cell nucleus not only leads to disrupted nuclear lamina, it might potentially also affect other nuclear functions such as transcription, cell division, chromatin organisation and DNA replication (Foster and Bridger, 2005, Gruenbaum et al., 2005). Indeed, cell cycle deregulation including hyperproliferation (Bridger and Kill, 2004), delayed onset and progression of cytokinesis (Dechat et al., 2007), accumulation of nuclear envelope defects during rebuilding after mitosis (Dechat et al., 2007) and increased apoptosis (Bridger and Kill, 2004) has been documented in cells derived from HGPS patients. Two recent studies also demonstrated abnormal accumulation of progerin at the nuclear membrane during onset of mitosis and the mutated protein further interfered with Rb mediated entry of cells in to S-phase, thus leading to impaired cell cycle progression (Dechat et al., 2007, Dechat et al., 2004). Progerin aggregates have also been detected in the cytoplasm of HGPS patient cells during mitosis and have been thought to cause to chromosomal segregation and binucleation (Cao et al., 2007). Abnormal nuclear shape with herniations, lobulations (Goldman et al., 2004) (Navarro et al., 2005, Navarro et al., 2004), increased micronucleation (Meaburn et al., 2007, Meaburn et al., 2005), thickening of the nuclear lamina (Goldman et al., 2004), defects in heterochromatin organisation (Lattanzi et al., 2007, Columbaro et al., 2005), alteration in epigenetic modifications (Shumaker et al., 2006), clustering of the nuclear pores and uncoupling of nucleo-cytoplasmic protein transport (Busch

et al., 2009) are some of the other effects that are caused due to presence of mutant lamin A protein within the cell nucleus. Defects in DNA repair (Liu et al., 2005, Manju et al., 2006), recruitment of DNA repair factors to damage sites (Liu et al., 2005) and ATR signalling pathways (Manju et al., 2006), accumulations in double strand breaks (DSB), genomic instability (Liu et al., 2005, Manju et al., 2006) and reduction in some DSB repair proteins including Rad51, Rad50, 53BP1 (Liu et al., 2005) and reduced phophorylation of γ-H2AX (Manju et al., 2006) is also commonly found in HGPS patient cells. In addition to this, HGPS patient cells also display alterations in spatial organisation of the genome (Meaburn et al., 2007), accelerated telomere shortening and reduced telomere length (Decker et al., 2009) as well as deregulation of gene expression profiles (Ly et al., 2000, Scaffidi and Misteli, 2005, Dahl et al., 2006, Csoka et al., 2004) including altered expression of mitotic genes (Ly et al., 2000). Moreover, Wnt and Notch signalling pathways are also affected in HGPS patients, which further may implicate in rapid exhaustion of stem cells (Espada et al., 2008, Scaffidi and Misteli, 2008) and thus leads to disease pathology including rapid muscle wasting, tissue degeneration and DNA damage (Hofer et al., 2005, Gotzmann and Foisner, 2006, Halaschek-Wiener and Brooks-Wilson, 2007). This indeed explains the segmental ageing phenotypes observed in patients suffering from this disease whereby only tissues that require constant cell renewal and repair such as skin are affected in HGPS individuals (Halaschek-Wiener and Brooks-Wilson, 2007, Gotzmann and Foisner, 2006).

Studies so far have concentrated on the defects in the nuclear lamina and functions related with the lamina in HGPS cells. Presence of lamins in the intranuclear regions and their possible interactions with other nuclear structures

suggest that components of nuclear architecture other than the lamina may also be affected in cells with mutation in *LMNA* gene.

Treatments for HGPS patients

Research on HGPS over the years has suggested that the accumulation of the farnesylated form of lamin A within cell nuclei is toxic and is the main culprit in disease manifestation. This has been formally proven in a study involving ZMPSTE24 deficient mice, whereby breeding ZMPSTE24 $^{-/-}$ mice with LMNA $^{+/-}$ mice reduced prelamin A by 50% which then decreased the number of misshapen nuclei – a characteristic of HGPS cells, significantly within the litter population (Fong et al., 2004). Hence, several different therapeutic approaches have been tried by different laboratories in order to minimise the amount of progerin accumulation or reduce the toxicity of the mutant protein.

One of the first therapeutic interventions to treat HGPS was targeted at blocking the abnormal splicing of lamin A and thus reduction in the quantities of progerin produced in these patient cells (Scaffidi and Misteli, 2005). Using antisense 25-mer morpholino oligonucleotide (exo11) targeted to the cryptic splice site, these studies indeed showed that splicing correction reduces the cellular defects as well as levels of progerin in HGPS patients (Scaffidi and Misteli, 2005). A similar approach, whereby reduction of mutant protein accumulation as a result of decrease in truncated lamin A mRNA levels using short hairpin RNA (shRNA) also correlates with reversal of cellular defects observed in patient cells (Huang et al., 2005).

Retention of the farnesyl moiety at the end of progerin interferes with the accumulation of this protein in the nuclear lamina and may be implicated in the dominant negative activity of this protein (De Sandre-Giovannoli et al., 2003,

Eriksson et al., 2003). Thus, a concept that blocking farnesylation of pre-progerin protein might help ameliorate disease pathology seen in HGPS cells was put forward in 2003 (Young et al., 2005). To test this hypothesis, a class of drugs called farnesyltrasnferase inhibitors (FTIs), which inhibit attachment of a farnesyl group to the protein by irreversibly binding to the CaaX domain (Adjei, 2005), was employed.

Encouragingly, the first few studies demonstrated that treating HGPS patient cells with FTI prevents the accumulation of progerin at the nuclear envelope and reduces the frequency of abnormally shaped nuclei in culture (Capell et al., 2005, Glynn and Glover, 2005, Mallampalli et al., 2005, Toth et al., 2005, Yang et al., 2005). In their study Glynn and Glover demonstrated an improved morphology and a reduction in percentage of abnormal nuclei in culture by 70% in HGPS cells after treatment with low doses of FTIs (10nM) over a 14-day period (Glynn and Glover, 2005). Significant dose-dependent reduction in nuclear blebbing as well as redistribution of mutant protein from the nuclear envelope into the nucleoplasmic envelope was reported within 48-72 hours of FTI treatment on patient cells (Capell et al., 2005), ZMPSTE24 $^{-/-}$ mouse embryonic fibroblasts (Toth et al., 2005), *Lmna* $^{HG/+}$ and *Lmna* $^{HG/HG}$ mouse fibroblasts (Yang et al., 2005). Patient cells treated with FTIs for 72 hours show improved nuclear stiffness to levels almost comparable to normal cells and significant restoration of directional persistence with regards to cell migration and thus improvement in wound healing ability (Verstraeten et al., 2008).

With promising results from *in vitro* studies with regards to the ability of FTIs in reversing nuclear abnormalities, various laboratories then focussed on animal models of HGPS to test these drugs further. The most common models available for progeria are ZMPSTE24 $^{-/-}$ and *Lmna* $^{HG/+}$ mouse models, both of which show an accumulation of farnesylated prelamin A. (Yang et al., 2005, Yang et al., 2006).

Treatment of ZMPSTE24 $^{-/-}$ mice with FTI, beginning at 5 weeks of age, showed presence of non-farnesylated prelamin A, improved growth curves, bone integrity and body weight, reduced abnormalities in the grip (Fong et al., 2004), and reduction in the frequency of rib fractures, (Fong et al., 2006, Meta et al., 2006, Yang et al., 2005, Yang et al., 2006). In a subsequent study, (Fong et al., 2006) demonstrated that FTI treatment improved body weight, bone structure in *Lmna* $^{HG/+}$ with improvement in bone mineralisation and cortical thickness (Fong et al., 2006). A more recent study that uses a transgenic mouse model carrying the human G608G - *LMNA* mutation and displaying a cardiovascular phenotype, a characteristic reason that causes death in most HGPS patients, demonstrated that FTI treatment reduces vascular smooth muscle cell (VMSC) loss and proteoglycan accumulation and thus prevents the onset, as well as progression, of cardiovascular diseases in these mice (Capell et al., 2008).

Although, FTI treatments have been very successful and are been currently used in the clinical trials to treat HGPS patients (Kieran et al., 2007), some groups have questioned the effect of these drugs on other farnesylated proteins such as lamin B (Rusinol and Sinensky, 2006). In addition, farnesylated proteins such as lamin A and Ras have been shown to undergo alternative prenylation such as geranylgeranylation (See figure 1.7.) when farnesylation is blocked (Kilic et al., 1997, Rusinol and Sinensky, 2006, Basso et al., 2006). FTIs and geranylgeranyltransferase inhibitors (GGTIs) have also been demonstrated to induce cell-cycle arrest in the G_1 phase and hence may cause further damage to patient cells (Efuet and Keyomarsi, 2006). Moreover, FTI treatment do not ameliorate all the abnormalities observed in HGPS patients (Varela et al., 2008), for example no reduction DNA damage accumulation, which is thought to be an important factor

leading to disease phenotype, is observed in patient cells after FTI treatment (Liu et al., 2006). In order to address these issues, various other enzymes involved in prenylation pathway have been targeted (Figure 1.7.). Indeed, using a combination of statins and bisphosphonates that are implicated in the prenylation pathway, not only reduces the amount of progerin, but also decreases nuclear abnormalities observed in HGPS patients (Varela et al., 2008). Another study that uses a combination of mevinolin statin and a chromatin modification inhibitor such as trichostatin A (a histone deacetylase inhibitor), also observe similar results with decreased quantities of progerin, reversal of nuclear abnormalities and restoration of altered distribution of heterochromatin organisation (Columbaro et al., 2005) and thus support the study performed by Varela, et al (Varela et al., 2008).

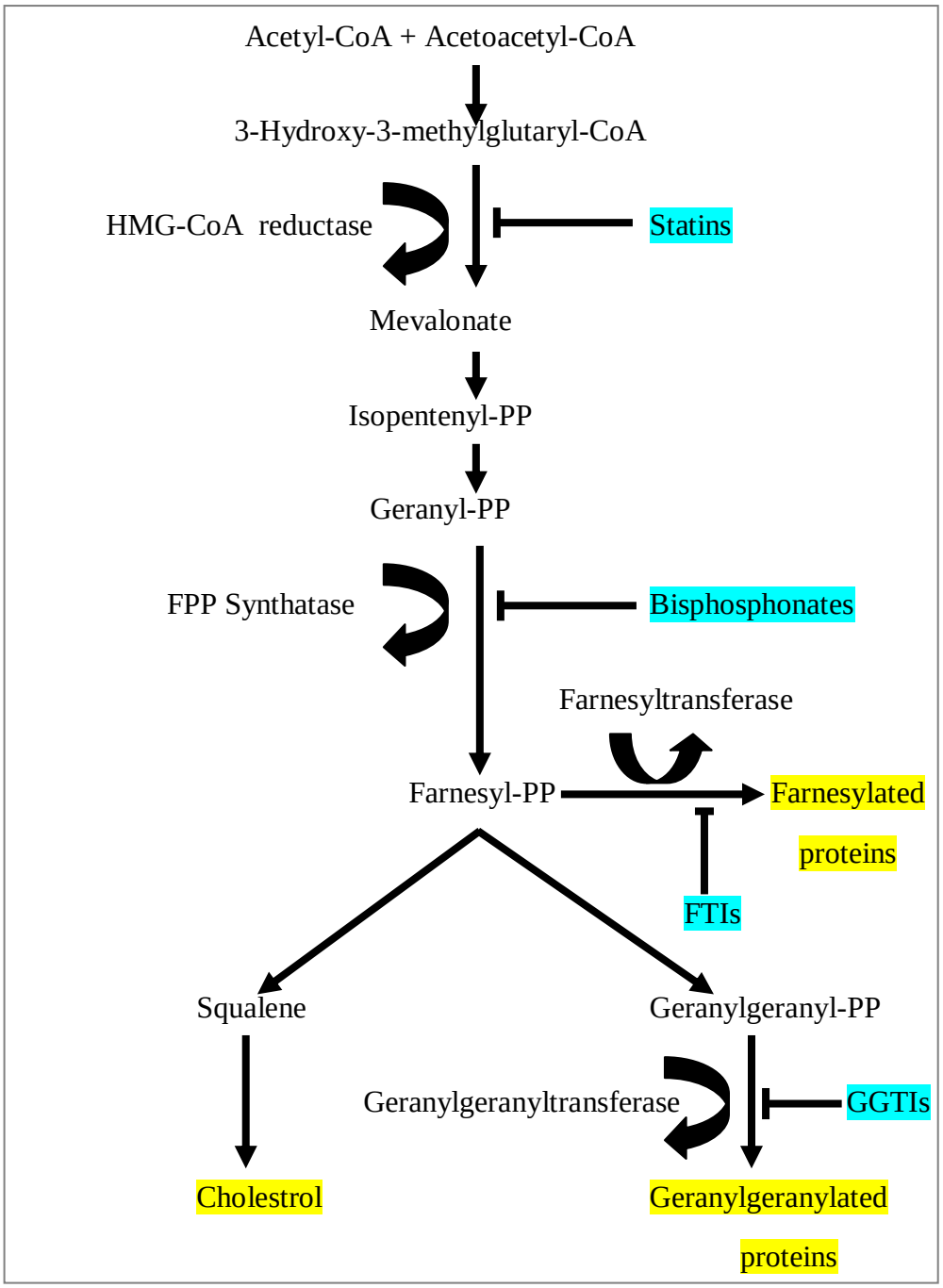


Figure 1.7. The isoprenylation pathway and possible therapeutic interventions for HGPS: For farnesylation of pre-lamin A, the farnesyl moiety, i.e. farnesyl-pyrophosphase (farnesyl-PP) is synthesised via melovante pathway. The end-products of this pathway including geranylgeranyl pyrophosphate that leads to geranylgeranylation of proteins, cholesterol and farnesyl-PP which farnesylates proteins are highlighted in yellow. The agents that can be used as therapeutic agents such as bisphophonates, FTIs , GGTIs and statins have been highlighted in blue. The figure is adapted from (Capell and Collins, 2006).

1.2.2. The Nucleolus:

The nucleolus is a membrane-less and active nuclear compartment involved in RNA biogenesis. The nucleolus, in the past was proposed to be the paradigm of nuclear functional compartmentalization (Strouboulis and Wolffe, 1996). It has always been considered a crucial nuclear structure because of its efficiency in performing nucleolar functions as well as aiding in communication with other parts of the cell (Olson and Dundr, 2005).

1.2.2.a. Structure and functions of the nucleolus

Ultra-structure of a nucleolus consists of three sub-compartments: the fibrillar compartment (FC), dense fibrillar compartment (DFC) and granular compartment (GC) (Jordan, 1984), each of which have a distinct but related functions (Hernandez-Verdun, 1991) (Figure 1.8.). The FCs form distinct structures; present throughout the nucleolus and are connected via a network of DFCs. Both FC and DFC are embedded within the GC which is composed of granules of 15 – 20 nm in diameter (Junera et al., 1995). Ribosome synthesis begins in the DFC with nascent RNA accumulated in this nucleolar domain and in the junctions between DFC and FC (Hozak et al., 1994, Cmarko et al., 2000, Puvion-Dutilleul et al., 1997, Shaw et al., 1995). The process of ribosome synthesis then continues in the intranucleolar regions and then in the GC (Sollner-Webb, 1996, Fatica and Tollervey, 2002, Fromont-Racine et al., 2003, Tollervey, 1996). Depending on what stage of ribosome synthesis they are involved in, the machinery required for the process of ribosome biogenesis including nucleolar proteins, are segregated in these nucleolar compartments. For example fibrillarin (Ochs et al., 1985) and nucleolin (Bugler et al., 1982), that participate in early stages of ribosome biogenesis, are located in DFC,

and DNA topoisomerase I and RNA polymerase I are localised within the FC (Guldner et al., 1986, Reimer et al., 1987). While proteins that are important for late stages of rRNA processing such as nucleophosmin-B23 and Nop52 are localised in GC (Spector et al., 1984, Schmidt-Zachmann et al., 1987, Biggiogera et al., 1989, Gautier et al., 1994).

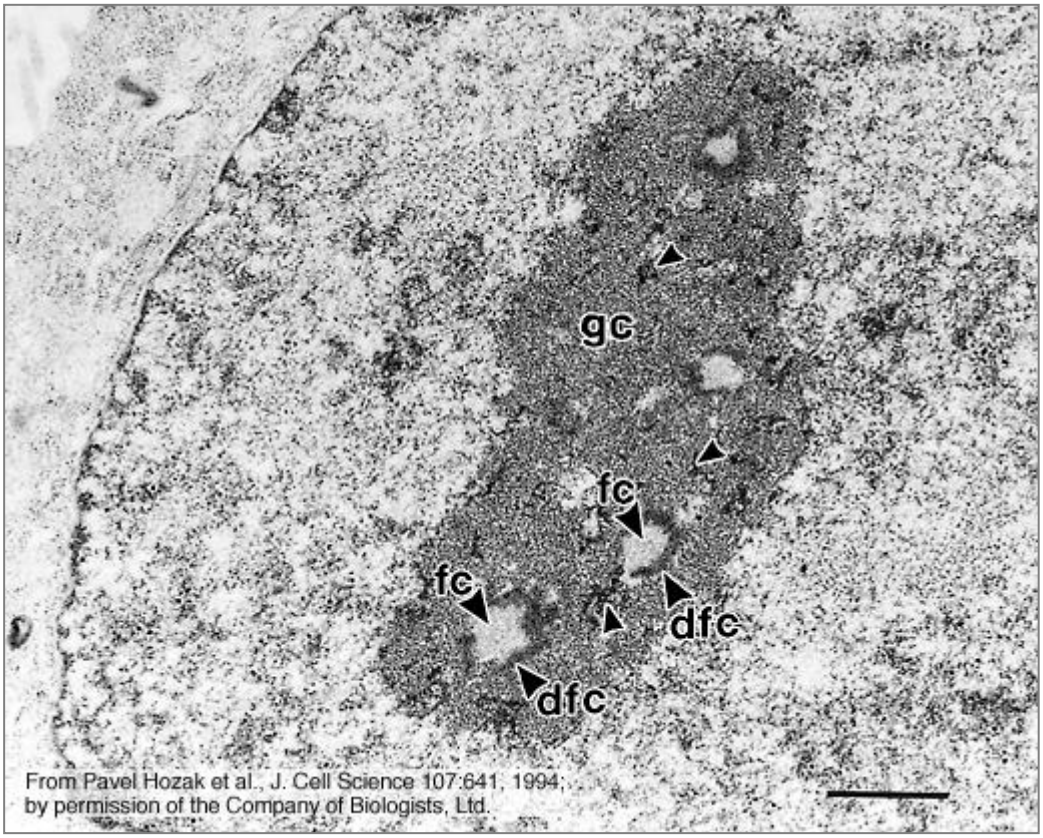


Figure 1.8. Ultrastructure of the nucleolus: Nucleoli within the nuclei of eukaryotic cells are compartmentalised into three different domains, namely the fibrillar compartment (FC), dense fibrillar compartment (DFC) and granular compartment (GC). This image is adapted from Hozak, et al 1994 (Hozak et al., 1994).

In recent studies, it has been found that nucleolar compartments are populated with more than 700 nucleolar proteins which are involved in diverse cellular functions (Boisvert et al., 2007) apart from ribosome biogenesis. Proteins such as Cdc 14 (Shou et al., 1999, D'Amours et al., 2004) and protein phosphatase 1γ (Vagnarelli et al., 2006, Trinkle-Mulcahy and Lamond, 2006) that are important for

exit from mitosis, chromosome segregation and cell division, are sequestered in the nucleoli of cells during interphase in order to limit the cells from undergoing an uncoordinated mitotic exit. Telomere reverse transcriptase, that is vital for addition of telomeric repeats at the ends of newly synthesised chromosomes, is also present in the nucleolus bound to the nucleolar protein nucleolin until the onset of S-phase of the cell cycle. These data suggest that nucleolus and the nucleolar proteins may play a role in cell cycle progression and regulation (Visintin and Amon, 2000), cytokinesis (Trinkle-Mulcahy and Lamond, 2006) and telomerase assembly and activity (Yang et al., 2002, Lukowiak et al., 2001, Heiss et al., 1999, Wong et al., 2002, Maser and DePinho, 2002, Vega et al., 2003). In addition to this, the p14ARF, that is implicated in p53 degradation and thus modulates stress response, is a nucleolar protein and is also thought to sequester HDM2 in the nucleoli in response to cellular stress inhibiting p14ARF-HDM2 mediated p53 degradation complex, implying that the nucleolus may be a part of the stress sensing machinery (Rubbi and Milner, 2003, Mayer and Grummt, 2005). Early studies in yeast nucleoli have demonstrated that Sir complex genes relocate from the telomeres to the nucleoli with age of the organism, suggesting a role for this nuclear organelle in organismal ageing (Guarente, 1997). Chromosomes containing active nucleolar organising regions (NORs), inactive NORs as well as other chromosomes assimilate into the nucleolus and hence this nuclear compartment is thought to be important for spatial organisation of the genome within the nucleus (Manuelidis and Borden, 1988, Bridger et al., 2000, Bridger et al., 1998b, Sullivan et al., 2001, Weipoltshammer et al., 1999). Proteins such as SUMO1, UBF(Casafont et al., 2007), *sentrin*/SUMO specific proteases, SENP3 and SENP5 (Nishida et al., 2000, Gong and Yeh, 2006), vital for sumoylation, are predominantly located in the nucleolus and studies have

demonstrated that knockdown of *SENP5* leads to abnormal nuclear morphology and cell division, which further implies that the nucleolus might be important for regulation of certain post-translational modifications and maintenance of nuclear integrity (Kroetz, 2005, Shou et al., 1999, Gong and Yeh, 2006, Di Bacco et al., 2006).

Thus, the nucleolus is involved in various unconventional cellular functions apart from its primary role in ribosome biogenesis.

1.2.2.b. Nucleolus and the cell cycle

The nucleolus undergoes several cell cycle dependent changes (Visintin and Amon, 2000, Bachant and Elledge, 1999) such as alterations in the structure and number of nucleoli as well as differential expression of nucleolar proteins depending on the cell cycle status of the cell (Boisvert et al., 2007, Hernandez-Verdun, 1991). The rate of ribosome biogenesis is linked to the size of the nucleus and hence with variable rates of ribosome production depending on the metabolic activity of the cell, the size of the nucleolus changes with cells progression through the cell cycle (Derenzini et al., 1998, Peculis, 2002, Hernandez-Verdun, 2006). This is evident from the observation that cycling / proliferating cells need more ribosome production in order to form two daughter cells and so have much larger nucleoli as compared to quiescent or resting cells (Schmidt, 1999). In addition to this, the function of the nucleolus is also influenced by p53 and Rb, both of which are involved in cell cycle progression whereby p53 is required for G_2 arrest and induction of apoptosis while Rb is important for passage through the G_1 phase checkpoint (Derenzini et al., 2009). Interestingly, tumour cells lacking p53 or Rb have significantly larger nucleoli which may be intrinsic to the abnormal cell proliferation associated with cancer cells (Trere

et al., 2004, Derenzini et al., 2004). Regulated sequestration of several cell-cycle check point proteins such as Mdm2, Cdc14 and Pch2 as well as DNA polymerase is another known function of the nucleolus whereby it can influence the cell cycle progression (Pederson, 1998, Carmo-Fonseca et al., 2000, Bardin et al., 2000).

An initial visual comparison between nucleoli of the proliferating and senescent cells was that senescent cells have single but larger nucleolus whereas younger cells show a larger number of small nucleoli (Bemiller and Lee, 1978, Buschmann and LaVelle, 1981). A further study of the nucleoli in senescent cells demonstrated that apart from just the morphological change, the nucleoli also show a drastic difference in their primary function of ribosome biogenesis with senescent cells having a low number of NORs and thus reduced ribosomal gene activity as compared to proliferating cells (Buys et al., 1979, Denton et al., 1981, Lezhava, 2001, Pedrazzini et al., 1998). Also, there are a number of proteins that are involved in cellular senescence that reside in nucleoli. Proteins like p14 ARF and nucleophosmin, which is present in the nucleolus as well as the nucleoplasm, are involved in releasing and facilitating p53 stability and thus might play a role in maintaining the proliferating or the senescent status of the cell (Daniely et al., 2002, Zimber et al., 2004, Colombo et al., 2002). Another important nucleolar protein; nucleolin; has been proven to be a key player in maintaining the proliferating status of the cell. Silencing of the nucleolin gene in cultured fibroblasts make them more prone to apoptosis and are growth arrested in the G2 phase of the cell cycle (Ugrinova et al., 2007). Nucleoli are not only affected by the process of cellular senescence, but they also play a role in promoting the cell towards cellular senescence (Rosete et al., 2007).

Thus, it will be interesting to understand the changes that the nucleolus and the proteins within it go through when the cells exit the cell cycle as well as in cells derived from patients suffering from premature ageing diseases such as HGPS.

1.2.3. Nuclear motors:

Structures within mammalian nuclei undergo repositioning around the nucleoplasm during development, differentiation, in response to external environmental stimuli. Whether it is genes on chromosome loops moving to the transcription factories (Branco and Pombo, 2006, Osborne et al., 2007), nuclear bodies traversing through the nucleoplasm (Boudonck et al., 1999, Platani et al., 2000) or long range movement of chromosomal region (Chuang et al., 2006, Mehta, 2005, Mehta et al., 2008, Dundr et al., 2007, Hu et al., 2008) in response to environmental cues; all of these events require movement. The presence of actin and myosin within cell nuclei has been detected and various studies have proposed that these two proteins act in concert to form what are called as nuclear motors (de Lanerolle et al., 2005, Hofmann et al., 2006a). Like cytoplasmic motors that facilitate muscle contraction, these nuclear motors may be involved in movement of various nuclear structures throughout the nucleoplasm (Hofmann et al., 2006a).

1.2.3.a. Nuclear actin

The presence of actin in the nucleus was first suggested as early as 1969 when Lane first observed fibrillar bundles in oocytes treated with actinomycin (Lane, 1969). This study was followed by many other studies where actin filaments were isolated from nuclei of different cell types from different organisms (Clark and Rosenbaum, 1979, Nakayasu and Ueda, 1983, Jockusch et al., 1974). Studies by Clark, et al then reaffirmed the presence of actin in the nucleus by careful hand

isolation of nuclei from *Xenopus* oocytes so as to avoid any contamination with cytoplasmic proteins (Clark and Merriam, 1977, Clark and Rosenbaum, 1979). Early studies from Rosenbaum and Ueda groups also demonstrated functional relevance of nuclear actin in maintenance of the nuclear envelope via its attachment to the nuclear matrix (Clark and Rosenbaum, 1979, Nakayasu and Ueda, 1983, de Lanerolle et al., 2005). Following this, a study performed on Hela cells suggested that nuclear actin may act as a transcription factor for RNA polymerase B and thus may be involved in the process of transcription (Egly et al., 1984). Direct evidence that proved this hypothesis was provided when inhibition of chromosome condensation (Rungger et al., 1979) and transcription (Scheer et al., 1984) was observed following microinjection of anti-actin antibodies in the nucleus. However, detection of nuclear actin in the nucleus was not well accepted and was thought to be just a contamination of cytoplasmic actin (Ankenbauer et al., 1989). These reasons were enough to disregard all the early work on the presence of actin in the nucleus as just a result of unclean preparations. Yet, all efforts to convince the scientific community of the presence of nuclear actin failed as any attempts to detect actin in the nucleus by fluorescence staining using anti-actin antibodies or actin binding compounds failed. Also, early attempts to detect the most common actin binding partner, myosin in the nucleus were also not fruitful (de Lanerolle et al., 2005).

This point of view regarding the presence of actin has changed radically in last few years with many more studies linking actin to various nuclear functions. The first study that gave convincing evidence of actin's involvement in nuclear processes showed the association of β-actin with chromatin modeling complexes like BAF in activated T-lymphocytes (Zhao et al., 1998). The first evidence of different forms of actin present in the nucleus came from a FRAP assay which showed that actin is

present as a dynamic mixture of monomeric, oligomeric and polymeric fibres in the cell nucleus (McDonald et al., 2006). Moreover, the actin polymers observed in the nucleus are very different to those found in the cytoplasm and are also much more dynamic in nature (McDonald et al., 2006). The dynamic nature of the actin in the nucleus was further supported by fluorescence staining of actin in the nucleus whereby studies in two labs using anti-actin antibodies, i.e. 2G2 and 1C7 displayed different patterns of actin staining in the nucleus and suggesting the presence of both G- and F-actin within nuclei of cells (Gonsior et al., 1999, Schoenenberger et al., 2005).

Following these studies, there was a sudden surge of reports implicating actin with various nuclear functions including chromatin remodeling (Olave et al., 2002, Pederson, 2000), transcription by RNA polymerases (Zhang et al., 2002, Hofmann et al., 2004, Philimonenko et al., 2004, Hu et al., 2004, Kukalev et al., 2005), nuclear transport of RNA (Percipalle et al., 2002, Percipalle et al., 2001, Hofmann et al., 2001, Kimura et al., 2000), controlling the entry of the cells into mitosis (Lee and Song, 2007), partitioning of active nuclear components within the nucleoplasm (Andrin and Hendzel, 2004) and chromosome decondensation (Gieni and Hendzel, 2008). In addition to this, the actin cytoskeleton is also thought to provide a physical bridge between the cytoplasm and the nucleus via its interactions with various INM and ONM proteins such as nuclear lamins and nesprins; and thus might be involved in transmitting mechanical signals from the extracellular matrix to the nucleus (Gieni and Hendzel, 2009). Structural components of the nucleus including Cajal bodies and nuclear speckles have been demonstrated to contain a pool of actin which are important for the maintenance and mobility of these structures (Wang et al., 2006, Gedge et al., 2005).

Thus, with all the emerging views about functions of actin in the nucleus, the dynamic status of actin in the nucleus and the significance of this with respect to nuclear functions need further investigation.

1.2.3.b. Nuclear myosin

Myosins are actin-activated motor molecules that use energy from ATP hydrolysis to generate force and move along actin filaments (Pollard and Korn, 1973, Pollard et al., 1973) to instigate various cytoplasmic processes such as cell crawling, cytokinesis, phagocytosis, growth cone extension, maintenance of cell shape, organelle/particle trafficking (Berg et al., 2001), signal transduction (Bahler, 2000), establishment of polarity (Yin et al., 2000) and actin polymerisation (Evangelista et al., 2000, Lechler et al., 2000, Lee et al., 2000). Although first identified as a cytoplasmic protein, the presence of myosin in nuclei of cells has also been discussed over the last 23 years. In 1986, Hagen et al demonstrated the presence of a nuclear protein that cross-reacted with a monoclonal antibody to *Acanthaamoeba castellanii* myosin 1 (Hagen et al., 1986). Following this study, a myosin II heavy chain like protein was also found near nuclear pore complexes inside the nucleus in *Drosophila melanogaster* (Berrios et al., 1991). Nevertheless, this early work demonstrating the presence of myosin isoforms in the nucleus, like nuclear actin, was met by scepticism and doubt owing to a belief that presence of myosin in the nucleus was also due to contamination by cytoplasmic myosins (Pederson and Aebi, 2002, Nowak et al., 1997). Technical advances leading to the identification of various different isoforms of myosin in the nucleus in recent years has changed this point of view dramatically. Two studies from de Lanerolle's group has revealed the presence of nuclear isoform of a myosin 1 (nuclear myosin 1β; NMIβ) in the nucleus which was very similar to myosin 1C found in the cytoplasm (Pestic-Dragovich et

al., 2000, Nowak et al., 1997) and is conserved in vertebrates (Kahle et al., 2007). Following on from this, a number of myosin isoforms have been located in the nucleoplasm i.e. myosin VI (Vreugde et al., 2006), myosin XVIb (Cameron et al., 2007) and myosin Va (Pranchevicius et al., 2008). These findings have increased interest in understanding nuclear myosin's involvement in processes such as transcription, chromatin remodelling and the movement of chromatin around nuclei (Hofmann et al., 2006a).

Nuclear myosin 1β (NMIβ)

The gene MYO1C present on human chromosome 17p13, encodes three myosin isoforms including myosin 1C isoforms a, b and c. Out of the three isoforms, myosin 1C isoform b, also known as myosin 1β is the shortest isoform and has a unique N-terminal domain as compared to the other isoforms. This isoform of myosin has been demonstrated to be present in the nucleus (Nowak et al., 1997). At least 4 different sub-classes of myosin I have been identified, all of which, including NMIβ consists of a globular head and a single heavy chain (Gillespie et al., 2001) and possesses the signature properties of a myosin 1 molecule including affinity for actin binding and K^+-EDTA ATPase activity (Nowak et al., 1997).

NMI, a ~ 120 kDa protein is evolutionarily conserved in vertebrates (Kahle et al., 2007) and is a member of myosin I subfamily of the myosin superfamily (Gillespie et al., 2001) that are usually found as monomers within the cell nucleus (Roberts et al., 2004). Distinguishing it from the cytoplasmic myosin 1 (CMI) molecule, NMIβ molecule has a unique 16-residue amino acid extension on its N-terminal end. These extra amino acid residues do not have a homology to nuclear localization sequence but cleaving this extension off the nuclear myosin 1 isoform

(NMI) results in retention in the cytoplasm, thus signifying the importance of this extension for nuclear entry and retention of NMI (Pestic-Dragovich et al., 2000).

NMIβ, when first observed by Nowak, et al was found to be evenly distributed in the nuclei throughout the nucleoplasm and was also observed to co-localise with nucleolar structures (Nowak et al., 1997) which was suggestive of possible involvement of this protein in transcribing ribosomal genes. Indeed, in a study performed by de Lanerolle's group, association of NMIβ with RNA polymerase I was identified in both *in vivo* and *in vitro* systems (Philimonenko et al., 2004). Further, this study demonstrated that inhibition of NMIβ using RNA interference technique led to production of pre-rRNA in HeLa cells, while over-expression of this protein increased the pre-rRNA production in a dose-dependent manner (Philimonenko et al., 2004). In addition to this, the *in vitro* studies demonstrated that NMI was essential for the maximum activity of RNA polymerase I (Philimonenko et al., 2004). Interactions between NMI and RNA polymerase II by co-precipitation assays have also been identified (Pestic-Dragovich et al., 2000). *In vivo* and *in vitro* studies demonstrated that blocking NMI inhibits RNA polymerase II transcription (Pestic-Dragovich et al., 2000, Hofmann et al., 2006b). Further, by using an abortive transcription assay and a detailed analysis of different steps in early transcription, NMI was shown to be involved in formation of the first phosphodiester bond in RNA polymerase II mediated transcription (Hofmann et al., 2006b). Thus, the involvement of NMI and another isoform of myosin in the nucleus; myosin Vb and myosin VI, in transcription by RNA polymerases I, II and III has been illustrated (Lindsay and McCaffrey, 2009, Fomproix and Percipalle, 2004, Pestic-Dragovich et al., 2000, Philimonenko et al., 2004, Grummt, 2003, Vreugde et al., 2006, Hu et al., 2008, Cavellan et al., 2006). Mass spectrometry and

immunoprecipitation studies have allowed identification of interactions between NMI and WSTF-SNF2h components of chromatin remodelling complex WICH (Cavellan et al., 2006, Percipalle and Farrants, 2006, Percipalle et al., 2006), suggesting a probable role of NMI in recruiting these chromatin remodelling complexes to rDNA. NMI has also been functionally linked to topoisomerase II (Smukste et al., 2006).

1.2.3.c. Acto-NMI complex and intra-nuclear transport

Force is required to perform movement within the cell nucleus. The movement of myosin filaments over actin filaments generates force within the cytoplasm which facilitates muscle contraction (Sellers, 2000). Thus, with presence of actin and myosin within the cell nucleus, it is highly likely that these two proteins interact in the intra-nuclear environment and thus may be involved in movement of various nuclear entities around the nucleoplasm. Although, there is very little evidence of interaction between these two proteins within the nucleus and this is partly because the nuclear isoform of myosin 1C is an unconventional myosin that does not form filaments (Mermall et al., 1998) as well as the filamentous forms of nuclear actin being different as compared to the cytoplasmic actin filaments (McDonald et al., 2006).

The role of nuclear actin and myosin in nucleo-cytoplasmic transport has been known for several years (Schindler and Jiang, 1986) but recent studies have also identified the importance of acto-NMI complex in intra-nuclear transport of activated chromosome regions (Chuang et al., 2006). In their study, Chuang, et al demonstrated that activation of a transcriptionally inactive region at the nuclear periphery is accompanied by association of nuclear myosin with this gene locus.

Further, using various different techniques, i.e mutant cell lines for both actin and myosin and inhibitors to myosin, they have elegantly shown an active and regulated, actin and myosin dependent translocation of this gene locus to the nuclear interior from its initial location at the nuclear periphery (Chuang et al., 2006).

Actin dependent intranuclear repositioning of gene loci was also observed in case of U2 small nuclear (sn)RNA genes (Dundr et al., 2007). Upon transcriptional activation U2 gene loci undergo a directed long range (2 – 3 μm) linear movement and interact with the nearest Cajal bodies. This movement of the U2 array is not observed in mutant cell lines that cannot polymerise different forms of actin. These data suggest that movement of U2 gene loci to Cajal bodies and then its transcription is an actin dependent mechanism (Dundr et al., 2007). Another study that demonstrated acto-NMI dependent movement of genomic regions within interphase nucleus employed fluorescence *in situ* hybridisation and chromosome painting techniques (Hu et al., 2008). In their study, Hu, et al demonstrated that gene loci present on two separate chromosomes that are distant from each other, interact and move to a common nuclear location upon steroid induction (Hu et al., 2008). This movement of genomic regions is rapid and dependent on acto-NMI complex activity and can be reversibly inhibited by microinjection of NMI antibodies, by incorporation of β-actin or by drugs that interfere with actin and myosin polymerisation such as latrunculin A or jasplakinolide (Hu et al., 2008).

In order to elucidate the mechanism as to how this acto-NMI complex may function within the nuclear environment to facilitate movement, a model was put forward by Hofmann, et al; 2006 (Hofmann et al., 2006a). This model suggests that NMI could bind via its tail to the nuclear entity that needs to be transported around and then actin binds to the globular head of the NMI, and this nuclear motor like

cytoplasmic actin-myosin motor would then translocate the nuclear entity along the tracks of highly dynamic nuclear actin (Hofmann et al., 2006a) (Figure 1.9.). However, whether the association of actin and myosin is required for their role in transcription or if they work independently is still unclear.

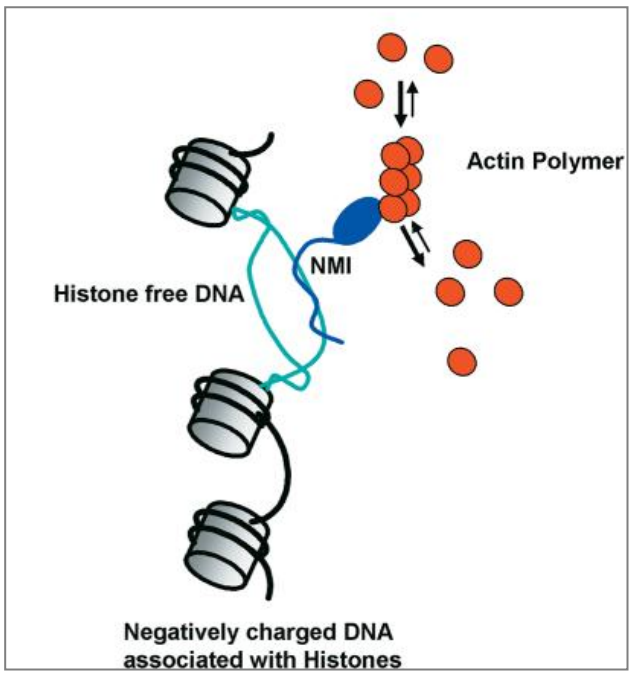


Figure 1.9. Acto-NMI complex in intra-nuclear movement of genomic regions:

This figure is adapted from (Hofmann et al., 2006a). In order to facilitate movement of genomic regions within the cell nucleus, a model proposed by Hofmann, et al suggests that NMI binds to the negatively charged histone free DNA via its tail and to actin via its head. The NMI molecule then moves along the tracks of actin polymer and facilitates the movement of the genomic entity.

1.3. Genome organisation:

The human genome consists of 22 pairs of autosomes and a pair of sex chromosomes. The interaction of the genome with other components of the nuclear structure, its organisation within the chromosomes as well as the spatial organisation of chromosomes within the cell nucleus, together help to influence and control the genome function (Bridger et al., 2007, Foster and Bridger, 2005, Parada and Misteli, 2002).

In late eighties and early nineties of the 19th century, Rabl (1885) and Boveri (1888, 1909) suggested that the chromosomes in an interphase nucleus of a non-dividing cell remained separated as they are in mitosis but with their centromeres on one side and the telomeres on the other (Rabl configuration) (Foster and Bridger, 2005). In addition, territorial organisation of the genome within the nucleus and its putative role in genome function was suggested by the discovery of the Barr body (Barr and Bertram, 1949). It has now been widely accepted that chromosomes in interphase nuclei occupy discrete three-dimensional regions known as chromosome territories. These were first discovered in Chinese hamster ovary cells in 1970s, when Cremer, et al and Zorn, et al demonstrated that irradiation of interphase nuclei with a UV microbeam induced DNA repair in only a few chromosomes, suggesting that chromosomes may not undergo completely decondensation following mitosis (Cremer et al., 1974, Zorn et al., 1979). Indeed the presence of chromosome territories was further confirmed by using fluorescence *in situ* hybridisation techniques using libraries of fluorescently labelled chromosome paints (Cremer et al., 1988, Manuelidis, 1985, Lichter et al., 1988, Pinkel et al., 1988).

1.3.1. Features regulating positioning of genomic regions in interphase nuclei:

Chromosome territories occupy a non-random and a radial distribution within interphase nuclei (Bridger and Bickmore, 1998, Cremer and Cremer, 2001, Parada and Misteli, 2002). This organisation is not only observed for whole chromosome territories but also exists with regard to chromosome arms and bands, (Dietzel et al., 1998a, Dietzel et al., 1998b, Sadoni et al., 1999, Visser and Aten, 1999). This radial organisation of chromosome territories within an interphase nucleus is conserved in organisms throughout evolution from simple organisms such as early embryos of *Drosophila melanogaster* (Marshall et al., 1996), to chicken (Tanabe et al., 2002a, Habermann et al., 2001), to mice (Mayer et al., 2005), to pigs (Foster et al., 2005, Federico et al., 2004) and humans (Bridger and Bickmore, 1998, Cremer and Cremer, 2001, Parada and Misteli, 2002). Such conserved arrangement of chromosome territories has been associated with importance of this spatial organisation to genome function (Parada and Misteli, 2002, Foster and Bridger, 2005, Fraser and Bickmore, 2007, Meaburn and Misteli, 2007, Takizawa et al., 2008b, Cremer et al., 2006).

There have been conflicting views on what determines the position of chromosome territories, sub-chromosomal domain and individual genes in an interphase nucleus. Evidence suggested is that of gene density which suggests that there is a strong correlation between the gene density of the chromosome and its location in the nucleus (Boyle et al., 2001, Bridger and Bickmore, 1998, Gilbert et al., 2005, Kreth et al., 2004) (Figure 1.9.). This theory was first proposed, when chromosome territories of two similar sized chromosomes; chromosome 18 and 19

were localized differently in an interphase nucleus, with the gene rich chromosome 19 in the nuclear interior whilst the gene poor chromosome 18 was located at the nuclear periphery (Croft et al., 1999). Thus, according to this proposal the chromosome territories with higher gene density would be found in the nuclear interior while the chromosome territory with a lower gene density would localize at the nuclear periphery. Studies whereby positioning of all human chromosome territories were performed in interphase nuclei of lymphoblasts (Boyle et al., 2001) and fibroblasts (Boyle et al., 2001, Meaburn et al., 2007) revealed that this was indeed the case. This gene density type of organisation of chromosome territories in interphase nucleus is also observed in primates, Old World monkeys, chickens and mice, thus is thought to be conserved over evolution (Foster and Bridger, 2005, Tanabe et al., 2005, Tanabe et al., 2002b, Mayer et al., 2005). This theory thus suggests presence of gene rich loci within the nuclear interior, thus making this region of the nucleus transcriptionally very active, whereas tethering a gene loci to the nuclear periphery would lead to its silencing and thus this nuclear area was associated with transcriptional inactivation (Brown et al., 1997, Ferreira et al., 1997, Foster and Bridger, 2005, Strouboulis and Wolffe, 1996, Andrulis et al., 1998, Cockell and Gasser, 1999, Lukasova et al., 2002).

Some studies have demonstrated the distribution of the genome in nuclei based on its GC-content, whereby GC-rich open chromatin associated with high gene density is located within the nuclear interior while GC-poor more compact and gene poor chromatin is distributed at the nuclear periphery (Ferreira et al., 1997, Sadoni et al., 1999, Saccone et al., 2002, Tanabe et al., 2002a, Boutanaev et al., 2005, Petrova et al., 2006, Federico et al., 2008, Shopland et al., 2006). Gene rich/GC-rich telomeric regions on chromosome 9 (Saccone et al., 2002) as well as segments on

chromosome 7 (Federico et al., 2008) localise in the nuclear interior as opposed to the gene-poor/GC poor segments of chromosome 7 and the telomere on homologous chromosome 9, which are positioned towards the nuclear periphery in an interphase nucleus (Federico et al., 2008, Saccone et al., 2002).

In addition to this, evidence demonstrating that early replicating regions of the genome preferentially occupy an interior location in nuclei whilst late replicating chromatin regions localise at the nuclear or nucleolar periphery, suggests that replication timing of a particular chromatin domain may regulate its position within an interphase nucleus (Gilbert et al., 2005). This has been indeed demonstrated in a study whereby early replicating regions of chromosomes 7 (Federico et al., 2008), 2, 5 and 17 (Grasser et al., 2008) and 12 (Nogami et al., 2000) have been found within the nuclear interior while late replicating regions are located towards the nuclear periphery.

Chromosome regions or individual gene loci have also been demonstrated to occupy preferential location in the nucleus based on their transcriptional activity. *CFTR* gene (Zink et al., 2004), MHC cluster genes (Volpi et al., 2000), the EDC cluster (Williams et al., 2002), the *Hoxb* complex (Chambeyron and Bickmore, 2004, Chambeyron et al., 2005), β-globin locus (Brown et al., 2006, Ragoczy et al., 2006), *GATA2* locus (Szczerbal et al., 2009) and 11p15.5 region (Gilbert et al., 2004, Mahy et al., 2002) localise in the nuclear interior when transcriptionally active, whereas are found at the nuclear periphery when inactive. The idea of gene activity affecting radial position of chromatin regions was further strengthened by a study that compared the location of active and inactive copies of a monoallelically expressed *GFAP* locus (Takizawa et al., 2008a). Data from their study demonstrated that the active copy of the *GFAP* allele preferentially occupied a more internal location as

compared to its inactive copy within the same interphase nucleus (Takizawa et al., 2008a).

Thus, data from the studies discussed so far, demonstrate that organisation of the genome within an interphase nucleus may be regulated by its local gene density, transcriptional activity, GC-content or replication timing. Although, recent studies have demonstrated conflicting evidence suggesting that replication timing or transcriptional activity do not regulate organisation of chromatin regions within an interphase nucleus, but are instead controlled or regulated by nuclear position of chromosomal DNA (Kupper et al., 2007, Takizawa et al., 2008b).

In contrast to the gene density or GC-content based positioning of chromosomal DNA, there are some studies that suggest that the chromosomes in the interphase nucleus are positioned according to their size with larger chromosomes at the nuclear periphery and the smaller chromosomes in the nuclear interior (Bolzer et al., 2005, Cremer and Cremer, 2001, Sun et al., 2000) (Figure 1.10.). But the cells used in all these studies were either 90-95% confluent in which case most of the cells in culture would become quiescent due to contact inhibition (Abercrombie, 1970, Abercrombie and Heaysman, 1954) or were made quiescent by serum starvation. It has also been observed that chromosomes from chicken, proliferating embryonic fibroblasts and non-proliferating neurons, fit this size theory of chromosome positioning (Habermann et al., 2001). Chromosome organisation from some other animals like murine chromosomes and pig chromosomes fit both the size as well as gene-density theory of chromosome positioning (Mayer et al., 2005) {Foster, et al in prep + Bridger pers. comm.}; Interestingly, Bickmore's group also observed that two chromosomes; chromosome 18 and 19 were positioned according to their size when the cells are made quiescent as opposed to the localization of these chromosomes

based on their gene density in proliferating cells (Bridger et al., 2000). Size-dependent organisation of chromosomes has also been observed in fibroblasts when they are in a state of replicative senescence (Mehta, 2005, Mehta et al., 2007, Bridger et al., 2000, Meaburn et al., 2007).

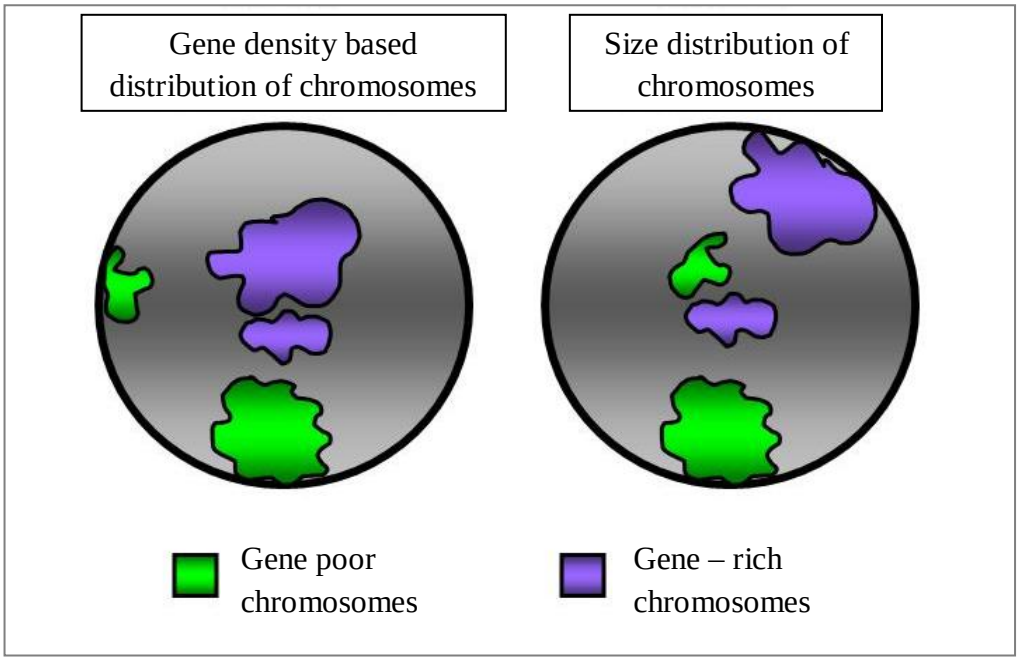


Figure 1.10. Cartoon demonstrating gene density based distribution and a size distribution of chromosome territories in an interphase nucleus.

Although, there is controversy within the field over how the chromosomes are positioned within the interphase nucleus, a possibility that a combination of two or more of the above discussed factors may together influence the nuclear organisation of chromatin regions. Further, specific factors may influence the positioning of some but not all chromosomes and chromosomal regions. Other factors such as the higher order structure of the genome, the temporal transcriptional status of a particular chromosome in the nucleus or pre-requisite attachment of a particular chromosome territory to various nuclear structures at particular time are may also dictate the localization of chromosome territories in the cell nucleus.

1.3.2. Models of chromosome organisation within interphase nuclei:

DNA within each chromosome territory also displays an ordered arrangement within interphase nuclei. Indeed genes as well as sub-chromosomal regions have been demonstrated to occupy non-random positions within an interphase nucleus. Various studies have also demonstrated several inter- and intra-chromosomal associations which might have an impact on genome function (Bacher et al., 2006, Osborne et al., 2004, Tolhuis et al., 2002, Ling et al., 2006, Lomvardas et al., 2006, Spilianakis et al., 2005, Spilianakis and Flavell, 2004, Xu et al., 2006, Wurtele and Chartrand, 2006, Simonis et al., 2006, Zhao et al., 2006). Specific regions of the genome have been demonstrated to loop out of the parent chromosome territory, thus facilitating long range chromatin interactions and leading intermingling between different chromosome territories (Branco and Pombo, 2006, Tolhuis et al., 2002). Three distinct models including the chromosome territory – interchromatin compartment (CT-IC), lattice and interchromosomal network model, have been put forward in order to understand how these interactions between DNA from different chromosomes are established.

CT-IC model, initially known as interchromosome domain (ICD) model was the first to address the significance of chromosome position to genome function within an interphase nucleus (Cremer et al., 1993). In order to assess the role of spatial location of chromosomes in transcription and splicing, a study performed by Cremer group simultaneously labelled chromosome territories and splicing speckles or the transcript of an integrated human papilloma virus genome (Zirbel et al., 1993). The results from this study demonstrated that both splicing speckles and the

transcript localised at the edges of the chromosome territories, accumulating in chromatin free regions of the nucleus (Zirbel et al., 1993), which was initially termed as inter-chromosome domain (Cremer et al., 1993) and then changed to inter-chromatin domain (ICD) (Cremer and Cremer, 2001). Further evidence suggesting the presence of ICD came from studies performed by Lichter and Singer group, whereby they demonstrated that introduction of *Xenopus* vimentin containing a nuclear localisation signal (Bridger et al., 1998a) or polyA RNA (Politz et al., 1999) into nuclei of human cells, led to their localisation in chromatin free area of the nucleus, i.e. in the ICD (Politz et al., 1999, Bridger et al., 1998a). The CT-IC model suggests that active genes localise at the periphery of the chromosome territories so that they are accessible to the transcriptional factories present in the ICD, while inactive genes are present in more interior locations of a chromosome territory (Cremer et al., 1993, Cremer and Cremer, 2001). Indeed, detailed two-coloured 3D-analyses demonstrated localisation of 3 coding regions at the periphery of the chromosome territories while a non-coding region was in the interior of a chromosome territory (Kurz et al., 1996). Similarly, transcriptionally active *ANT2* and *ANT3* gene loci were shown to be positioned at the periphery of the active X chromosome, while *ANT2* was found in a more interior location in the inactive chromosome X territory (Dietzel et al., 1999). These results were further supported by various other studies showing transcriptionally active gene loci at the edge of the chromosome territory while inactive chromatin regions localising more interiorly within the territory (Eils et al., 1995, Scheuermann et al., 2004, Tajbakhsh et al., 2000). Moreover, evidence of chromosome looping away from the parent chromosome territory was also demonstrated, thus suggesting recruitment of the chromatin regions that requires to be transcribed to the ICD (Chambeyron et al.,

2005, Volpi et al., 2000) (Branco and Pombo, 2006). Another report supporting this evidence demonstrates that the activation of a B-type lymphocyte cell is accompanied by translocation of certain genes such as *Myc* and *Igh* to the transcriptional factories (Osborne et al., 2007). In addition to transcription factors, the ICD also contains RNA transcripts (Bridger et al., 2005, Zirbel et al., 1993, Lawrence et al., 1989, Jimenez-Garcia and Spector, 1993), nuclear bodies including Cajal and PML bodies (Zirbel et al., 1993, Matera, 1999, Bridger et al., 1998a, Bridger et al., 2005) and splicing factors (Misteli, 2001).

Although, observations made by some studies contradict or appear not to fit into the CT-IC theory. A study performed by Bickmore group demonstrated that coding regions of some transcriptionally active DNA are located at an interior location within a chromosome territory (Mahy et al., 2002). How these regions come in contact with the transcription machinery which is present in the ICD is not clear. Moreover, CT-IC model suggests that there are little or no intra-chromosomal interactions with each chromosome territory occupying a distinct domain within an interphase nucleus (Munkel et al., 1999, Visser and Aten, 1999, Cremer et al., 2000, Visser et al., 2000) and hence fails to explain the recent evidence demonstrating the extensive intermingling between regions of two or more distinct chromosome territories (Branco and Pombo, 2006).

A second model describing the aspects of interphase chromosome positioning is the lattice model which suggests the presence of an array or lattice organisation of 10 – 30 nm DNA fibres across the nucleoplasm (Dehghani et al., 2005). Further, these chromatin fibres occupy distinct nuclear locations to form a chromosome territory but intermingling between fibres from two or more chromosomes is possible (Dehghani et al., 2005). Studies demonstrating the presence of chromatin

packaged as 10 – 30 nm fibres within the nucleus (Bazett-Jones and Hendzel, 1999) as well as showing evidence of macromolecules and nascent RNA transcripts throughout the nucleus (Verschure et al., 1999, Visser et al., 1998, Verschure et al., 2003) support this lattice model for organisation of interphase chromosomes. In contrast to the CT-IC model, this model suggests that transcription can occur within the chromosome territories as demonstrated by various studies (Mahy et al., 2002, Verschure et al., 1999), and is not restricted to the IC.

None of the above models takes into account the long range interactions observed between chromatin regions from distinct chromosome territories, nor do they address the association of chromosomal DNA with other structures of the nuclear architecture including the nucleolus, Cajal and PML bodies, nuclear envelope, transcription factories and the nuclear matrix (Branco and Pombo, 2007). The ICN model addresses these issues and suggests a presence of a network of intra and inter-chromatin interactions, which further regulate the spatial distribution of chromosomes within the nucleus (Branco and Pombo, 2006). Moreover this model also explains the cell-type (Spilianakis et al., 2005, Harmon and Sedat, 2005, Bantignies et al., 2003) and tissue specific (Osborne et al., 2004, Tolhuis et al., 2002, Spilianakis and Flavell, 2004, Palstra et al., 2003, Lee et al., 2005) expression of genes by postulating that intermingling and thus inter- as well as intra-chromosomal interactions are dependent on the transcriptional status of the gene locus and changes when the chromatin region undergoes transcription (Branco and Pombo, 2006). Thus, this model suggests that a chromosome territory is an individual entity and a very open structure with many folds such that transcription machinery can come in contact with the gene needed to be transcribed even though it is in the inner fold of the chromosome territory. On the other hand, in cases when regulatory regions of

particular genes are located at a distance from the gene, it might be the case that entire gene loci would loop out of the chromosome territory and making the interaction between different parts of the genome easier (Branco and Pombo, 2006, Fraser and Bickmore, 2007). There are clusters of certain genes are found to co-localize with transcription factories consistently (Chakalova et al., 2005, Sproul et al., 2005) while there are other genes that are more dynamic and localize differently within or out with the chromosome territory depending on their transcriptional status (Osborne et al., 2004). In addition to interactions between regions of the genome, the ICN model also takes into considerations the functional associations between genome and other nuclear sub-structures including the lamina, nucleolus, nuclear bodies, transcription factories and the nuclear matrix (Branco and Pombo, 2007).

1.3.3. Cell cycle and interphase chromosome positioning:

The foundation for interphase positioning of chromosome territories is thought to be laid down during anaphase as first discovered by Rabl (Parada and Misteli, 2002). During the first 3 hours of cells entry into the G_1 phase, chromosome positioning within the interphase nucleus is random (Bridger et al., 2000, Belmont and Bruce, 1994). During this period, condensed mitotic chromosomes decondense, undergo diffusional motion and interact/attach to nuclear structures including the nucleolus, nuclear matrix and the nuclear envelope. This was suggested by live cell imaging studies performed using a fluorescently tagged locus which displays motility during early G_1 phase in CHO cells whereby it translocates from the nuclear periphery to the nuclear interior before finally settling at the nuclear periphery (Tumbar and Belmont, 2001). This was further supported by Walter, et al whereby

they demonstrated that in Hela cells, interphase chromosome territory positions were established in early G_1 phase of the cell cycle, when more pronounced movement of chromosomes was observed as compared to late G_1, S and G_2 phases (Walter et al., 2003). Chromosome territories, once positioned during early G_1, are maintained in the same nuclear location and undergo restricted large-scale movements throughout the interphase (late G_1, S and G_2 phases), until completion of G_2 phase (Thomson et al., 2004, Zink et al., 1998, Walter et al., 2003, Abney et al., 1997, Manders et al., 1999, Vazquez et al., 2001, Dimitrova and Gilbert, 1999).

Extensive reorganisation of chromosome territory positions is observed as cells exit the proliferative cell cycle to enter into G_0 or G_s phase. This was first suggested by Bridger, et al, whereby they demonstrate differential localisation of chromosome 18 in non-proliferating (quiescent and senescent) fibroblasts as compared to their proliferative counterparts (Bridger et al., 2000). Further, by determining positions of all human chromosomes, it is clear that chromosome territory organisation in senescent fibroblasts is based on chromosome size (Mehta, 2005) as opposed to gene density based organisation observed in proliferating fibroblasts (Croft et al., 1999, Boyle et al., 2001). These data suggest movement or repositioning of chromosome territories upon cells exit from the proliferative cell cycle to enter a non-proliferative state. Such re-organisation of whole chromosome regions have also been observed during differentiation (Kuroda et al., 2004, Galiova et al., 2004, Kim et al., 2004, Parada et al., 2004, Foster et al., 2005, Panning and Gilbert, 2005, Casolari et al., 2005, Szczerbal et al., 2009).

1.3.4. Interaction of the genome with other components of the nuclear architecture:

Constraints on diffusional movement of chromatin *in vivo* (Marshall et al., 1997b), evidence of chromosomal DNA existing within the nucleus in a non-random, organised manner and not a tangled mess (Marshall et al., 1997a) as well as maintenance of chromosome territory positions throughout interphase (Thomson et al., 2004, Zink et al., 1998, Walter et al., 2003, Abney et al., 1997, Manders et al., 1999, Vazquez et al., 2001, Dimitrova and Gilbert, 1999, Croft et al., 1999) all suggest interaction of genomic regions with nuclear substructures such as nuclear matrix, nucleoli or the nuclear lamina (Parada and Misteli, 2002, Chubb et al., 2002, Heun et al., 2001, Zink et al., 1998).

Indeed various studies have demonstrated association of specific chromosome loci with the nuclear envelope and the nuclear lamina during interphase (Paddy et al., 1990, Marshall et al., 1996, Foster and Bridger, 2005, Guelen et al., 2008, Pickersgill et al., 2006). Most of these interactions occur via lamins (Glass et al., 1993, Luderus et al., 1994, Taniura et al., 1995, Hoger et al., 1991, Goldberg et al., 1999, Stierle et al., 2003) and lamin binding partners (Zhang et al., 2005, Ye and Worman, 1996, Foisner and Gerace, 1993, Zhang et al., 2001) which have been demonstrated to bind mitotic chromosomes, naked DNA (Luderus et al., 1994), histones (Taniura et al., 1995, Goldberg et al., 1999) as well as heterochromatin proteins (Ye and Worman, 1996). Further, since lamins are not only found at the nuclear periphery, but are also distributed in intranuclear regions, a possibility exists that chromosomes interact with and might be tethered by internal lamins (Goldman et al., 2002, Bridger et al., 1993, Pickersgill et al., 2006). In addition to their

interaction with the nuclear lamina, regions of the DNA also interact with nuclear pore complexes at the nuclear envelope (Sukegawa and Blobel, 1993, Galy et al., 2000, Laroche et al., 1998). Interestingly a recent study by Brown, et al demonstrated association between three human chromosomes, including HSA 5, 7 and 16 with nucleoporin 93 (NUP93), a component of the nuclear pore complex (Brown et al., 2008). Further, another study using ChIP-on-chip analysis has demonstrated interactions between nucleoporins and gene loci upon activation of transcription in human cells (Casolari et al., 2005).

Acrocentric chromosomes containing NOR regions have been shown to be embedded in the nucleolus (Weipoltshammer et al., 1999). This is also true for some chromosomes carrying inactive NORs (Sullivan et al., 2001, Bridger et al., 1998b) as well as for chromosomes that do not contain NORs at all (Bridger et al., 1998b, Bridger et al., 2000, Manuelidis and Borden, 1988). A study from Bickmore group demonstrated that chromosomes and genomic regions associated with the nucleolus undergo restrained movement and the restriction on the movement can be overcome upon disruption of the nucleoli using chemicals (Chubb et al., 2002). In addition to this, chromosome 18 which is usually at the nuclear periphery in proliferating cells (Sullivan et al., 2001, Croft et al., 1999) has been demonstrated to reposition to the nuclear interior where it is embedded within the nucleoli in non-proliferating cells (Bridger et al., 2000, Mehta, 2005), thus suggesting that association of this region of chromatin with the nucleolus may alter as the cell exits the proliferative cell cycle. The influence of attachment to the nucleolus on genome function is still elusive.

Another nuclear structure that has been found to closely interact with DNA is the nuclear matrix, which forms the filamentous structural framework of the nucleus (Jackson and Cook, 1988). Various different regions of chromatin including matrix

attachment regions (MARs) (Dijkwel and Hamlin, 1988), scaffold attachment regions (SARs) (Laemmli et al., 1992, Heng et al., 2004), telomeres (de Lange, 1992, de Lara et al., 1993, Luderus et al., 1996, Okabe et al., 2004, Markova et al., 1994) as well as centromeres / kinetochores (Chaly et al., 1985, Markova et al., 1994, Liao et al., 1995) have been demonstrated to interact with the nuclear matrix (Foster and Bridger, 2005, Elcock and Bridger, 2008). Ma et al has demonstrated strong attachments of various human chromosomes, including chromosomes 1, 2, 4, 7, 9, 11, 14 and 22 to the nuclear matrix (Ma et al., 1999). Transcriptionally active and early replicating DNA have been demonstrated to preferentially attach to the nuclear matrix and are found in the residual nucleus in nuclear halo preparations – a biochemical technique used remove all the soluble proteins leaving just the DNA and nuclear matrix behind (Gerdes et al., 1994, Jackson et al., 1998). These associations of chromatin regions with the nuclear matrix alter in different stages of the cell cycle (Gerdes et al., 1994). Further, gene-rich chromosome 19 is tethered to the nuclear matrix while gene-poor chromosome 18 is not retained within the residual nuclei after high salt extraction procedures and is found in the nuclear halo, implicating its weak attachments with the nuclear matrix (Croft et al., 1999). This attachment of the genome to the nuclear matrix has been suggested to be vital for DNA replication, chromosome organisation and transcription (Stein et al., 1994).

Interestingly, nuclear bodies such as PML and Cajal bodies have been also shown to have associations with DNA. PML bodies make extensive contacts with the local chromatin environment that it occupies (Eskiw et al., 2004, Eskiw et al., 2003), particularly interacting with transcriptionally active loci (Wang et al., 2004). Actively transcribing MHC I locus on chromosome 6 have been demonstrated to associate with PML bodies (Shiels et al., 2001). Association of MHC cluster to these

nuclear bodies is essential since chromosome 18, after artificial insertion the MHC cluster also shows association with PML bodies (Shiels et al., 2001). PML bodies associate with MARs and special AT-rich sequence binding protein 1 (SATB1) and organises the MHC I locus in loops within the interphase nucleus, thus implicating a role of these nuclear bodies in organisation of chromatin regions (Kumar et al., 2007). Cajal bodies have been implicated in organisation of specialised genes (Osborne and Eskiw, 2008). Histone gene clusters on human chromosomes 1 and 6 as well as U1 and U2 small nuclear RNA genes interact with Cajal bodies within the nucleus (Frey et al., 1999, Schul et al., 1999a). In addition to this, a recent study demonstrated movement of U2 small nuclear RNA loci in order to form associations with Cajal bodies during S-phase of the cell cycle (Dundr et al., 2007). These studies suggest a possible role of Cajal bodies in genome organisation within an interphase nucleus.

1.3.5. Functional significance of genome organisation in interphase nuclei:

The most convincing evidence of possible influence of interphase chromosome location to transcription status of the genome came from movement of individual gene locus observed during differentiation. Indeed, there have been reports of preferential relocation of a particular gene locus to the nuclear interior when it requries to be transcriptionally active and the localization of the same gene locus at the nuclear periphery when it is transcriptionally silent. The movement of *Igh* (Immunoglobulin heavy chain) gene locus was analysed in mammalian B-lymphocytes. This gene locus is preferentially positioned at the nuclear periphery but during B-cell differentiation when this gene is required to undergo transcription, the

gene locus re-positions to the nuclear interior. Similar examples of repositioning of activated genes at the nuclear interior has been observed in the case of CD4 genes locus during T-lymphocyte differentiation (Kim et al., 2004), *c-maf* gene locus during T-cell differentiation (Hewitt et al., 2004) and in the C*ftr* gene locus in adeno-carcinoma cells (Zink et al., 2004).

On the other hand, links have been demonstrated between preferential positioning of the gene locus in proximity to constitutive heterochromatin region (Brown et al., 1999). In other words, activation of certain genes is accompanied by its movement away from the constitutive heterochromatin regions (Delaire et al., 2004, Hewitt et al., 2004). Apart from gene activation, studies on yeast and *Drosophila* have demonstrated that localizing chromatin to the nuclear periphery leads to silencing of genes encompassed in that chromatin (Csink and Henikoff, 1998, Andrulis et al., 1998, Dernburg et al., 1996, Maillet et al., 1996). This is also true in case of β-globin gene locus as well as other lymphoblast specific genes, whereby their transcriptional activity is silenced concomitant to their localisation near heterochromatin (Brown et al., 1999, Francastel et al., 1999). Presence of activated genes within the nuclear interior and association of transcriptionally inactive regions with the nuclear periphery is also a feature of genes involved in porcine adipogenesis (Szczerbal et al., 2009).

Although, some studies have argued against strong links between location of genomic region and gene activity and have suggested that correlation between localisation in the nuclear interior with gene expression and at the nuclear periphery with gene silencing is a very simplified view of looking at regulation of transcription which may not be true for all genes (Takizawa et al., 2008b, Lanctot et al., 2007). For example, no relocalisation of the CD8 locus was observed in T-cell

differentiation (Kim et al., 2004) and the gene locus of interferon γ always localized at the nuclear periphery irrespective of its transcriptional activity (Hewitt et al., 2004). No correlation between gene expression and radial position was found in a study involving 11 randomly selected genes studied under growth and differentiation conditions (Meaburn and Misteli, 2008). Moreover, artificially tethering a chromatin region to the nuclear periphery does not affect the transcriptional status of all genes present on it (Kumaran and Spector, 2008, Finlan et al., 2008). This, thus suggest that the model co-relating nuclear localization and repositioning of a particular gene locus to the transcriptional status of that gene may apply only on a sub-set of mammalian genes. Presence of heterochromatin at the nuclear periphery and interaction of genes with it leads to its silencing has led to the belief that the nuclear periphery is a region of transcriptional but it is vital to consider that the presence of heterochromatin is not restricted to the nuclear periphery but is found distributed throughout the nucleus. In addition to this, there is evidence suggesting the presence of transcriptionally active regions at the nuclear pore complexes in the nuclear lamina (Casolari et al., 2004) as well as some other studies demonstrating the presence of active transcription sites all throughout the nucleoplasm, and not only in the nuclear interior (Wansink et al., 1993).

In addition to their non-random organisation within an interphase nuclei, chromosomes also have preferential location within an interphase nucleus with respect to each other, where by some heterologous chromosomes are juxtaposed more frequently than others (Parada and Misteli, 2002, Parada et al., 2004). This radial distribution of interphase chromosome territories appears to be cell-type specific (Boyle et al., 2001) and also varies between different tissue types (Parada et al., 2004). One of the reasons for these specific chromatin neighbourhood, might be

that co-regulated regions of the genome aggregate to form what are thought be transcription factories which contains transcription complexes and serve multiple genes at the same time (Fraser and Bickmore, 2007). Chromatin also has preferred neighbourhoods in terms of other nuclear structures. For example, genomic regions containing U2 and histone genes associate with Cajal bodies (Frey et al., 1999, Schul et al., 1999b), chromosomes containing NOR regions are embedded in the nucleolus (Sullivan et al., 2001, Weipoltshammer et al., 1999), while MHC locus on chromosome 6 preferentially localise with PML bodies (Shiels et al., 2001). These nuclear neighbourhoods might be important for genome function and may implicate in regulation of transcription of selected genes (Lemon and Tjian, 2000, Francastel et al., 2000).

Thus, the relationship between interphase positioning of chromosomal DNA and genome function is complex and requires further understanding.

1.3.6. Genome organisation and disease:

The first evidence of perturbed positioning of chromosomes within the nucleus and its implication in causing a diseased condition was demonstrated in epilepsy patients (Borden and Manuelidis, 1988). Positions of chromosomes 1, 9 and Y were altered slightly while position of chromosome X showed a drastic repositioning in neurons derived from these patients (Borden and Manuelidis, 1988). Further, mislocalisation of chromosome 18 territories was found in several tumour cell lines, whereby chromosome 18 occupied a more internal location as compared to normal cells (Cremer et al., 2003). In addition to this, the proximity of chromosomes and interactions between two or more chromosome territories has been suggested to cause specific translocations which might further manifest into tumours (Parada and

Misteli, 2002, Kozubek et al., 1999, Bickmore and Teague, 2002, Roix et al., 2003). Thus is indeed true in case of translocations observed in some thyroid cancers (Nikiforova et al., 2000), chronic myeloid leukaemia (Neves et al., 1999, Kozubek et al., 1999), liposarcomas (Kuroda et al., 2004) and for Robertsonian translocations (Therman et al., 1989). In addition to this, a recent study has demonstrated altered positioning of chromosomes in laminopathy patients that contain a mutation in lamin A or lamin A binding proteins. Chromosome 13 and 18 occupy an internal location within interphase nuclei of fibroblasts derived patients suffering from various laminopathies including Emery Dreifuss muscular dystrophy (EDMD), Hutchinson-Gilford progeria syndrome (HGPS), limb-girdle muscular dystrophy (LGMD) and Charcot-Marie-Tooth disorder type 2B (CMT2B), as opposed to their localisation at the nuclear periphery in control fibroblasts (Meaburn et al., 2007).

1.4.Overview:

Understanding the genome and its function is vital in the field of biomedical research. The human genome sequencing project has laid the foundation towards this goal whereby the sequence of 3 billion bases of human DNA was determined and approximately 30,000-40,000 protein coding genes in the human genome have been identified (Lander et al., 2001, Venter et al., 2001). The enormous amount of information available from this sequencing project provides a platform for understanding of how the human body may function as well as for treatment of diseases. Although, it is important to remember that genomes are not a single dimensional entity and elucidation of DNA sequence was only the starting point of genomics research. In reality, to understand the implications of the genome on life and diseases, it is vital to extrapolate the DNA sequence information from the human genome project onto genome function; which is the major goal of the post-genomic era.

DNA is folded and compacted in order to occupy the limited space within the cell nucleus whereby the genome replicates itself, transcribes and translates to form proteins. Interestingly, parallel to the human genome sequencing project, another important revelation in the field of genomics came from the finding that the genomes are positioned in a non-random manner in the cell nucleus (Croft et al., 1999). The organised genome and the machineries required for its maintenance and function within the nucleus, along with other nuclear components make up the nuclear architecture. The relationship between linear sequence of DNA, chromatin structure and the functional organisation of chromosomes in the cell nucleus, together are vital factors for genome regulation. The organisation of the genome within the nucleus

and its interaction with nuclear components change during development and in disease; and thus this non-random chromosome positioning within the architectural framework of the nucleus is thought to be a critical dimension of genome function (Bridger et al., 2007, Foster and Bridger, 2005, Parada and Misteli, 2002). What factors and mechanisms determine the positions of specific chromosomes in the cell nucleus is an important question in the field, the answer to which would further provide an insight into machineries required for genome organisation as well as elucidate whether the position of the genome influences its regulation and function.

The spatial distribution of chromosomes within interphase nucleus changes as the cells undergo differentiation or as the cells enter the state of replicative senescence. The response time as to when these alterations occur once the cells receive environmental cues is an important question. Elucidation of when this reorganisation of the nuclear architecture actually occurs would be highly relevant not only for understanding the processes of differentiation, cellular senescence and human ageing, but also for biotechnological studies such as somatic cloning and gene therapy. Chromosomes are large nuclear entities and reorganisation of whole chromosome regions within cell nucleus may be a complex event. What factors or nuclear structures drive this process of alteration in chromosome positions in response to various cytoplasmic signals? How vital is it for cells to be able to reorganise their genome and the manifestations on cellular functions in case this process is dysfunctional would also provide important clues towards genome function.

Thus, understanding when the reorganisation of genome occurs, identifying the machinery involved in this repositioning event as well as revealing the manifestations of aberrant chromosome positioning and reorganisation; would

elucidate the ultimate quest of linking chromosome positions within the cell nucleus to genome function. Knowledge of functional interplay between genome organisation and its activity would make the genome a more controllable entity and thus can be of vital importance for therapeutic purposes.

Chapter 2: Chromosome territory organisation in quiescent fibroblasts

- Some of the contents of this chapter are in press for Genome Biology under the title "Rapid chromosome territory relocation by nuclear motor activity in reposnse to serum removal in primary human fibroblasts" – Ishita S Mehta, Manelle Amira, Amanda J Harvey and Joanna M Bridger.

- Some of the contents of this chapter are in preparation for a publication in Ageing Cell – "Chromosome territory positions and nuclear motors in senescent fibroblasts." - Ishita S Mehta, Martin Figgitt, Ian R Kill and Joanna M Bridger.

2.1. Introduction:

Cellular quiescence is a state of reversible growth arrest and is thought to be induced by various anti-mitogenic signals including topoinhibition, serum starvation and due to stress induction (Abercrombie, 1970, Abercrombie and Heaysman, 1954, Coller et al., 2006). Each of these signals induce entry of cells into quiescence but this may be via different pathways (Coller et al., 2006, Gos et al., 2005, Liu et al., 2007, Yusuf and Fruman, 2003, Coller, 2007, Coppock et al., 1993).

Exit from the proliferative cell cycle and entry into quiescence is accompanied by differential regime of gene expression (Coppock et al., 1993, Yusuf and Fruman, 2003, Gos et al., 2005, Coller et al., 2006, Coller, 2007, Liu et al., 2007), lack of nuclear lamin foci (Dyer et al., 1997) with all the lamins sequestered at the nuclear periphery (Gerace et al., 1984, Gerace and Burke, 1988), increased protein levels of lamin A and C (Pugh et al., 1997) and diffuse appearance of PML bodies (Terris et al., 1995). In addition to these, alterations are also observed in epigenetic chromatin markers in facultative chromatin and invariant constitutive heterochromatin surrounding the centromeres (Grigoryev et al., 2004). The numbers of telomere associations between telomeric ends of dicentric chromosomes are increased in quiescent cells as compared to their proliferating counterparts (Nagele et al., 2001). Moreover, increased centromere clustering and kinetochore movements are observed in G_0 cells as compared to proliferating cells; whereby most kinetochores localise at the nuclear periphery or adjacent to the nucleoli (Solovei et al., 2004). Thus large scale changes in nuclear organisation accompany the transition between quiescence and proliferation.

Interestingly, the spatial organisation of whole chromosomes is distinctly different between quiescent and senescent cells as compared to proliferating cells (Bridger et al., 2000, Mehta, 2005). Positioning of chromosome 18 and 19 territories of chromosome in senescent and quiescent cells revealed that territories of chromosome 18 were localised towards the nuclear interior in these non-proliferating states in contrast to their locations at the nuclear periphery in proliferating human dermal fibroblasts (HDFs), while chromosome 19 was present in the nuclear interior in both proliferating and non-proliferating HDFs (Bridger et al., 2000). Further, positioning of all human chromosomes in senescent HDFs suggested that the organisation of chromosome territories in interphase nuclei still remains radial, but the territories of some chromosomes such as 1, 5, 6, 8, 10, 12, 13, 15, 16 and 18 re-localise and alter positions as the cells enter a state of senescence (Mehta et al., 2007, Mehta, 2005). Observing the complete data set (Table 2.1), it is clear that in senescent cells, the larger chromosomes are localized closer to the nuclear periphery while the smaller chromosomes are clustered in the nuclear interior (Mehta et al., 2007, Mehta, 2005). Data from these studies (Bridger et al., 2000, Mehta et al., 2007, Mehta, 2005) suggests that the chromosomal organisation within the interphase nucleus shifts from a gene-density based organisation to a size-dependent organisation as the cell transits from a proliferating to a senescent state.

To assess whether global spatial chromosome reorganisation occurs with cells entry into quiescence, similar to that observed during transition of cell from proliferating to a state of replicative senescence; interphase nuclear positions of all human chromosomes has been determined in quiescent primary HDFs. This study would further highlight the presence of any differences in the spatial organisation of chromosome territories between quiescent and senescent cells. Spatial chromosome

positions for selected chromosomes have been assessed in fibroblasts induced to undergo quiescence by different growth arrest signals, namely serum deprivation, stress and topoinhibition. With suggested links between spatial chromosome positions and gene expression, these data will then allow determination if each growth arrest signals employ similar or distinct signalling mechanisms to induce cellular quiescence. In addition to this, the response of senescent cells to growth arrest signals has been assessed by determining the positions of certain chromosomes following serum starvation of these cells.

Indeed results from this chapter demonstrate that spatial chromosome positions undergo re-organisation from a gene density based distribution in proliferating fibroblasts to a size-dependent distribution in quiescent fibroblasts. Differences in nuclear positions of certain chromosomes between quiescent and senescent fibroblasts have also been highlighted. Further, the positions of chromosome territories remain in place once the fibroblasts enter a state of replicative senescence and that senescent cells are unable to respond to growth-arrest signals in terms of chromosome positions.

2.2. Methods and Materials:

2.2.1. Cell Culture:

Human dermal fibroblasts (HDFs) 2DD (Bridger et al., 1993) were maintained in Dulbeco's modified Eagles Medium (DMEM) {Gibco} supplemented with 10% (v/v) new born calf serum (NCS) {Gibco}, 2% (v/v) streptomycin and penicillin antibiotics {Gibco} and 200mM L-glutamine {Gibco}. The fibroblasts were maintained at 37^{o}C, in a humidified atmosphere containing 5% CO_2.

These cells were passaged twice every week. All reagents used for cell culture were preheated to 37ºC before use. The medium was removed and the cultures washed using versene (NaCl 0.8% (w/v), KCl 0.02% (w/v), Na_2HPO_4 0.0115% (w/v), KH_2PO_4 0.02% (w/v), 0.2% EDTA (w/v){Sigma Aldrich}). The cultures was then treated with a solution of 0.25% trypsin{Gibco}: versene (1:10, v:v) to facilitate the detachment of cells from the tissue culture flasks (approximately 5 minutes). The effect of trypsin was neutralized by addition of an equal volume of DMEM medium. This cell suspension was centrifuged at 300 – 400g for 5 minutes. The supernatant was removed and the cells were resuspended in a known volume of fresh medium. To determine the cell numbers an aliquot of the cell suspension was placed on a haemocytometer (Neubauer, 1/400mm^2) with a chamber depth of 0.1 mm and a count was taken for the number of cells in 5 large squares of the haemocytometer under a 10X lens of a phase contrast light microscope. The following equation was used to estimate the number of cells harvested in the cell suspension.

$$\frac{Number\ of cells}{Number\ of\ squares} \times Volume\ of\ cell\ suspension\ \times 10^4$$

The cells were then seeded in 75cm^2 tissue culture flasks {Nunc} at a density of 5 x 10^5 cells per flask. To maintain a stock of cells, 1 million cells were frozen down in medium that the cells were cultured in plus 10% dimethyl sulphoxide (DMSO) (v/v) {Sigma} solution. These cells were then stored in liquid nitrogen (-170^{o}C) until needed. When required, the cells were defrosted rapidly in a water bath at 37^{o}C and as soon as the cells were thawed they were transferred to a tissue culture flask containing appropriate medium pre-warmed at 37^{o}C.

The cells were passaged twice every week until they became a senescent culture. Proliferating cells within the primary cultures were identified using the proliferative marker, anti-pKi-67, which is distributed in a number of different patterns within proliferating human fibroblasts (Kill, 1996, Bridger et al., 1998b, Verheijen et al., 1989b, Verheijen et al., 1989a). A culture was considered to be senescent when the fraction of Ki-67 positive cells in the culture decreased to 10% or less.

Cells were made quiescent by growing them in 10% NCS – DMEM medium for at least two days, washing with serum free medium and then incubating with medium containing 0.5% NCS for 7 days. Cells were also induced to undergo quiescence by topoinhibition by growing young proliferating cells in 10% NCS medium for four weeks (re-feeding with fresh 10% NCS – DMEM medium twice every week) so as to make them reach confluency.

Cells were also treated with DMSO so as to induce them to undergo quiescence due to stress (Srinivas et al., 1991). Young proliferating fibroblasts were

incubated with medium containing 2.5% DMSO for 48 – 72 hours. It is important to note that both, topo-inhibition or DMSO does not cause any damage to the DNA and hence cells do not undergo DNA induced growth arrest (Srinivas et al., 1991, Abercrombie, 1970, Abercrombie and Heaysman, 1954).

2.2.2. Two-dimensional *in situ* hybridisation (2D FISH):

2.2.2.a. Fixation

Before fixation primary HDFs were grown for at least 1 week after being taken out of liquid nitrogen. DMEM was removed and the cells were washed once with versene. The cells were then harvested by incubation in 0.25% trypsin (See section 2.2.1). The effect of trypsin was then neutralised by the addition of at least an equal volume of DMEM. Following centrifugation at 300-400g for 5 minutes, the supernatant was removed and the cells were re-suspended in a small amount of remaining medium. Harvested cells were treated with a hypotonic solution (0.075M KCl) for 15 minutes at room temperature, so as to swell the cells. The cell suspension was centrifuged at 300g for 5 minutes using a bench top centrifuge (Centaur 2 MSE). To fix the cells, the supernatant was removed and the cell pellet was re-suspended in small amount of buffer left so as to avoid cell clumping. Ice-cold methanol: acetic acid (3:1 v/v) was added drop-wise to the cells with gentle shaking. These cells were incubated at 4^{o}C for time duration ranging from at least 1 hour to 18 hours. The cells were then centrifuged again at 300g at 4^{o}C for 5 minutes and the fixation and centrifugation procedures were repeated 4-5 more times but only leaving the cells in the fix solution on ice for 5-15 minutes. Fixed cell suspensions were then stored at 4ºC or -20ºC until required.

2.2.2.b. Slide preparation and denaturation

The fixed cell suspensions were centrifuged at 300-400g for 5 minutes and the supernatant was removed. Cell pellets were resuspended in a small amount of fresh ice-cold methanol:acetic acid (3:1 v/v). The cells were then dropped onto humid or damp SuperFrost TM slides from a height, the slides were air dried and then aged at 70^{o}C for 1 hour or for 2 days at room temperature. The aged slides were passed through an ethanol row of 70%, 90% and 100% ethanol for 5 minutes in each solution, followed by air drying. The slides were then pre-warmed at 70^{o}C for 5 minutes before incubating in a denaturating solution (70% (v/v) formamide, 2X Sodium Saline Citrate ($Na_3C_6H_5O_7$) (300mM sodium chloride, 30mM hydrous tri-sodium citrate, pH 7.0, SSC) at 70^{o}C for 2 minutes. Following the denaturation, the slides were immediately plunged into ice-cold 70% ethanol for 5 minutes and then again passed through the ethanol row (90% and 100% ethanol). The slides were then air-dried and kept warm until hybridization with the probe.

2.2.2.c. Probe preparation and hybridisation

For positioning of all the chromosomes in quiescent cells, directly labelled total human whole chromosome DNA probes {Appligene Oncor} were denatured by incubating at 70^{o}C for 10 minutes. These probes were incubated at 37^{o}C for 10-120 minutes to allow re-annealing of repetitive sequences. 10µls of probe was applied to the denatured slide, the coverslip was sealed with rubber cement {Halfords} and left to hybridise in a humified chamber at 37ºC for at least 18 hours.

However, for all other studies, PCR biotin-16-dUTP labelled probes were used. The chromosome templates 10, 13, 18 and X {a kind gift from Prof. Michael Bittner} were produced by amplifying flow sorted chromosomes by degenerate

oligonucleotide primed polymerase chain reaction (DOP-PCR) (Telenius et al., 1992). These chromosome templates were labelled with biotin-16-dUTP {Roche} by DOP-PCR (Bridger et al., 1998a). The probe mixture was then prepared by ethanol precipitation of the biotin labelled chromosome paint (200-400µg per slide) with the addition of C_ot-1 DNA (7µg per slide) {Roche}, Herring Sperm (3µg per slide) {BDH}, 1/10th volume of 3M sodium acetate {BDH} pH 5.2. This mixture was incubated at -80^{o}C for at least 30 minutes followed by centrifugation at 300 – 400g for 30 minutes at 4^{o}C. The DNA pellet was then washed with ice cold 70% ethanol and centrifuged again at 300-400g for 15 minutes at 4^{o}C. The supernatant was removed and the pellets were allowed to dry at 40^{o}C on a hot block. The probes were then dissolved in 12µls (per slide) of hybridisation mix (50% formamide, 10% dextran sulphate {Helena Biosciences}, 10% 20X SSC, 1% Polyoxyethylene sorbitan monoolaurate (Tween 20) {Sigma Aldrich}) overnight at room temperature. The probes were denatured at 75^{o}C for 10 minutes and then allowed to re-anneal at 37^{o}C for at least 30 minutes. 12 µls of the probe was then applied to the slide, covered with pre-warmed 22 x 32 mm coverslip {Fisher} and sealed with rubber cement {Halfords}. The slides were allowed to hybridise with the probe in a moist chamber at 37^{o}C for at least 18 hours.

2.2.2.d. Washing 2D-FISH

Post hybridisation, the rubber cement was gently removed and the slides were washed three times for 5 minutes each in buffer A (50% (v/v) formamide, 2X SSC, pH 7.0) preheated to 45^{o}C. Slides were then washed in buffer B (0.1X SSC, pH 7.0) pre-warmed at 60^{o}C but placed in 45^{o}C water bath, three times for 5 minutes each before being transferred to 4X SSC at room temperature. For directly labelled probes from Appligene Oncor, the slides were mounted in counter-stain 4, 6-

diamidino-2-phenylindole (DAPI) in Vectashield mounting medium {Vector Laboratories}.

For the slides hybridised with chromosome painting probes made by PCR, after the post hybridisation washes as above (Section 2.2.2.d.), the slides were incubated with a 100µls blocking solution (4% bovine serum albumin; BSA, w/v) for 10 minutes at room temperature. Further to detect the biotin labelled probe, the slides were then incubated in 100µls of 1:200 diluted streptavidin – Cyanine 3 (Cy3) {Amersham Life Science Ltd} for 1 hour at room temperature. The slides were then washed in a 4X SSC solution containing 0.05% of Tween 20 in the dark at 42^{o}C with three changes of the solution every 5 minutes. The slides were then mounted in counter-stain in Vectashield mounting medium containing DAPI.

2.2.2.e. Microscopy and analysis

All slides were examined using 100X Plan Fluoropar oil immersion lens {Leica} or Olympus UPlan FLN 100X oil immersion lens {Olympus} on a Leica fluorescence microscope or on a Olympus BX41 fluorescence microscope respectively. pKi-67 negative or positive nuclei were selected randomly as per the experiment by following a rectangular scan pattern. Grey-scale images of these nuclei were captured using cooled charged-coupled device (CCD) camera {Sensys, Photometrics} or Model viewpoint GS gray scale digital camera {Digital Scientific}. These images were pseudocoloured and merged using Digital Scientific software, the Quips Pathvysion, Smart Capture VP V1.4 or Smart capture 3.0.0 {Digital Scientific Ltd}.

At least 50 – 60 images per slide were captured and converted into TIFF or PICT format. The images were passed through a simple erosion analysis as

described in Boyle, et al; 2001 and Croft, et al; 2001 using IPLab Spectrum Software. The script {used with a kind permission of Prof. Wendy Bickmore and Dr. Paul Perry} is devised to divide each captured nuclei in 5 concentric shells of equal area, the first shell starting from the periphery of the nucleus going to the interior of the nucleus (5^{th} shell) (Figure 2.1). The script measures the pixel intensity of DAPI and the chromosome probe in these five shells and puts the data obtained in a table. The background was removed from the FISH signal by subtracting the mean pixel intensity within the segmented nucleus. The probe signal was normalized by dividing the percentage of the probe by the percentage of DAPI signal in each shell. Thus, normalized proportion of probe was calculated in all 5 shells for at least 50 nuclei.

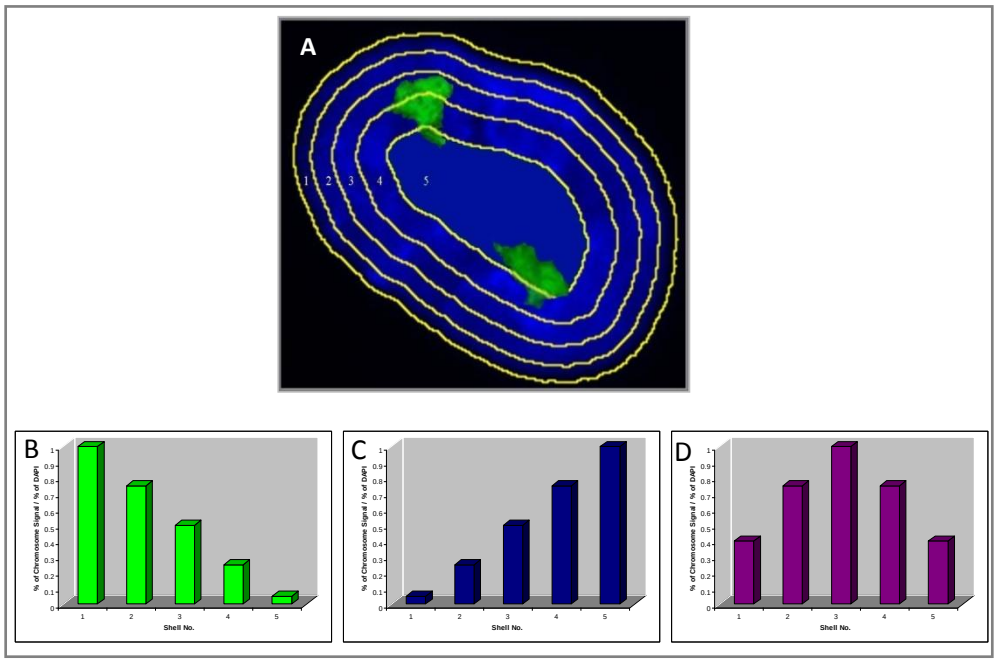


Figure 2.1 Erosion analysis for positioning of chromosome territories: Methanol acetic acid fixed nuclei of 2DD fibroblasts were probed with specific whole chromosome paints. Images were then taken and run through a simple erosion analysis script. The script divides each nucleus into five concentric shells of equal area (A) and then measures the signal intensities of probe and the amount of DNA in each shell. The amount of probe is then normalised with respect to the amount of DNA for each shell and then histograms are plotted which allow us to determine the positions of chromosomes in terms of Peripheral (B), Intermediate (D) and Interior (C).

Histograms were plotted using these data and standard error bars representing the +/- standard error of the mean (SEM: Standard deviation/ square root of n, where n is the sample size) were also plotted using Microsoft Excel software. Simple statistical analyses were performed using the two tailed student *t*-tests.

2.2.3. Three dimensional fluorescence *in situ* hybridization (3D-FISH):

To conserve the 3D structure of fibroblast nuclei, a FISH methodology previously described by (Bridger et al., 1998a) was used.

2.2.3.a. Cell culture and fixation

Cells were prepared for 3D-FISH as previously described in section 2.2.1 but with some exceptions. For 3D-FISH, cells were grown on sterile glass "Superfrost^{TM}" slides at 37ºC, 5% CO_2 at a starting density of 1 x 10^5 cells / slide. The cells were allowed to grow on the slide for at least 2 days, washed in 1X phosphate buffer saline (4.3mM sodium phosphate dibasic, 137mM sodium chloride, 2.7mM potassium chloride, 1.4mM potassium phosphate, pH 7.0, PBS) and then fixed in 4% paraformaldehyde (w/v), 1X PBS for 10 minutes at room temperature. The cells were permeabilised with 0.5% Triton-X100 (v/v) {Sigma} and 0.5% saponin (w/v) {Sigma} in 1X PBS solution for 20 minutes at room temperature and then rinsed with 1X PBS three times for 5 minutes each. Following this, the slides were incubated in a solution of 20% glycerol {Sigma} for at least 30 minutes at room temperature prior to being snap-frozen in liquid nitrogen for 15 - 30 seconds before being stored at -80ºC until required.

2.2.3.b. Probe preparation and denaturation

For 3D FISH, biotin-labelled whole chromosome templates were used. The probes were amplified and labelled with biotin using DOP-PCR and then ethanol precipitated as described in section 2.2.2.c. The probes were denatured at 75ºC for 10 minutes and then allowed to re-anneal at 37ºC for 10 – 120 minutes.

2.2.3.c. Slide preparation and denaturation

The slides were taken out of -80 ºC storage and were allowed to thaw at room temperature. The freeze-thaw process in liquid nitrogen, as described before (in section 2.2.2.a.), was repeated for further 4 – 5 times with soaking the slides in 20% glycerol between each freeze-thaw. Excess glycerol was washed from the slides

using three changes of 1X PBS for 10 minutes each, followed by depurination in 0.1N HCl for 5 minutes at room temperature with shaking. Excess acid was washed away with 2X SSC for 15 minutes with three changes of the buffer and then slides were incubated in 50% formamide, 2X SSC, pH 7.0 solution overnight.

The slides were denatured by incubation in denaturation buffer A (70% formamide, 2X SSC, pH 7.0) pre-warmed at 73ºC for precisely 3 minutes. The slides were then rapidly transferred to denaturation buffer B (50% formamide, 2X SSC, pH 7.0) pre-warmed at 73ºC for 1 minute. During this time, 10µls of previously denatured probes that was allowed to partially re-anneal at 37ºC was added to a pre-warmed 22 x 32mm^2 coverslip. Once the slide was ready after denaturation, excess denaturing solution was removed. The slide was then immediately presented to the probe on the coverslip. The coverslip was sealed on the slide using rubber cement and the probe was allowed to hybridise on the slide in a pre-warmed humified chamber at 37ºC for 2 days.

Post hybridization washes were performed as described in section 2.2.2.d.

2.2.3.d. Imaging and analysis

The images of nuclei prepared by 3-dimensional FISH were captured using a Nikon confocal laser scanning microscope (TE2000-S) equipped with a 60X/1.49 Nikon Apo oil immersion objective. The microscope was controlled by Nikon confocal microscope C1 (EZ – C1) software version 3.00. Stacks of optical sections with an axial distance of 0.2µm were collected from random nuclei. Stacks of 8-bit gray-scale 2D images were obtained with a pixel dwell of 4.56 and 8 averages were taken for each optical image. The positioning of chromosomes in relation to the nuclear periphery was assessed by performing measurements using

Imaris Software (Bitplane scientific solutions) whereby the distance between the geometric centre of each chromosome territory and the nearest nuclear edge was measured. Measurements for at least 20 nuclei were performed for each sample. Frequency distribution curves were plotted with the distance between the centre of chromosome territory and the nearest nuclear periphery on the x-axis and the frequency on the y-axis. Simple statistical analyses were performed using the two tailed student *t*-tests.

2.2.4. Indirect immunofluorescence:

2D and 3D-FISH was combined with indirect immunofluorescence staining for protein Ki-67 to distinguish proliferating cells from non-proliferating cells. pKi-67 is a nucleolar protein that is only present in cells in the proliferative cell cycle (Gerdes et al., 1984, Gerdes et al., 1986, Bridger et al., 1998b).

The coverslips from the slides on which FISH was performed were removed by placing the slides in 1X PBS solution on a shaker for 30 minutes or until the coverslips detach. The slides were incubated with 100µls of rabbit primary anti pKi-67{Novacastra} (1:1500 dilution in 1% NCS/PBS v/v) for 1 hour at room temperature. The excess antibody was then removed using 1X PBS with three changes of buffer every 5 minutes. The slides were incubated for 1 hour at room temperature with 100µls secondary antibody; the Swine anti-rabbit (TRITC) antibody {DAKO} (1:30 dilution in 1% NCS/PBS v/v). The excess antibody was removed with 1X PBS for 15 minutes with three changes of buffer, one every 5 minutes. The slides were then mounted in counter-stain DAPI in Vectashield mounting medium.

2.3. Results:

2.3.1. Positions of chromosomes 10, 18 and X in normal proliferating human dermal fibroblasts:

Positions of all human chromosome territories have been assessed in normal proliferating human dermal fibroblasts (Boyle et al., 2001, Meaburn et al., 2007) and both these studies have demonstrated that in proliferating fibroblasts chromosome territories are organized according to gene density. Before positioning all human chromosomes in normal quiescent fibroblasts, it was vital to recapitulate the positions of at least certain chromosomes in normal proliferating cells.

To determine the nuclear location of specific chromosomes, HDFs were harvested and fixed for standard 2D-fluorescence *in situ* hybridisation (FISH). Proliferating cells within the primary cultures were identified using the proliferative marker, anti-pKi-67, which is distributed mainly in the nucleolus and is shown in red (Bridger et al., 1998b) (Figure 2.2.A-D). Representative images of chromosome territories in proliferating cells are displayed in figure 2.2.A-D.

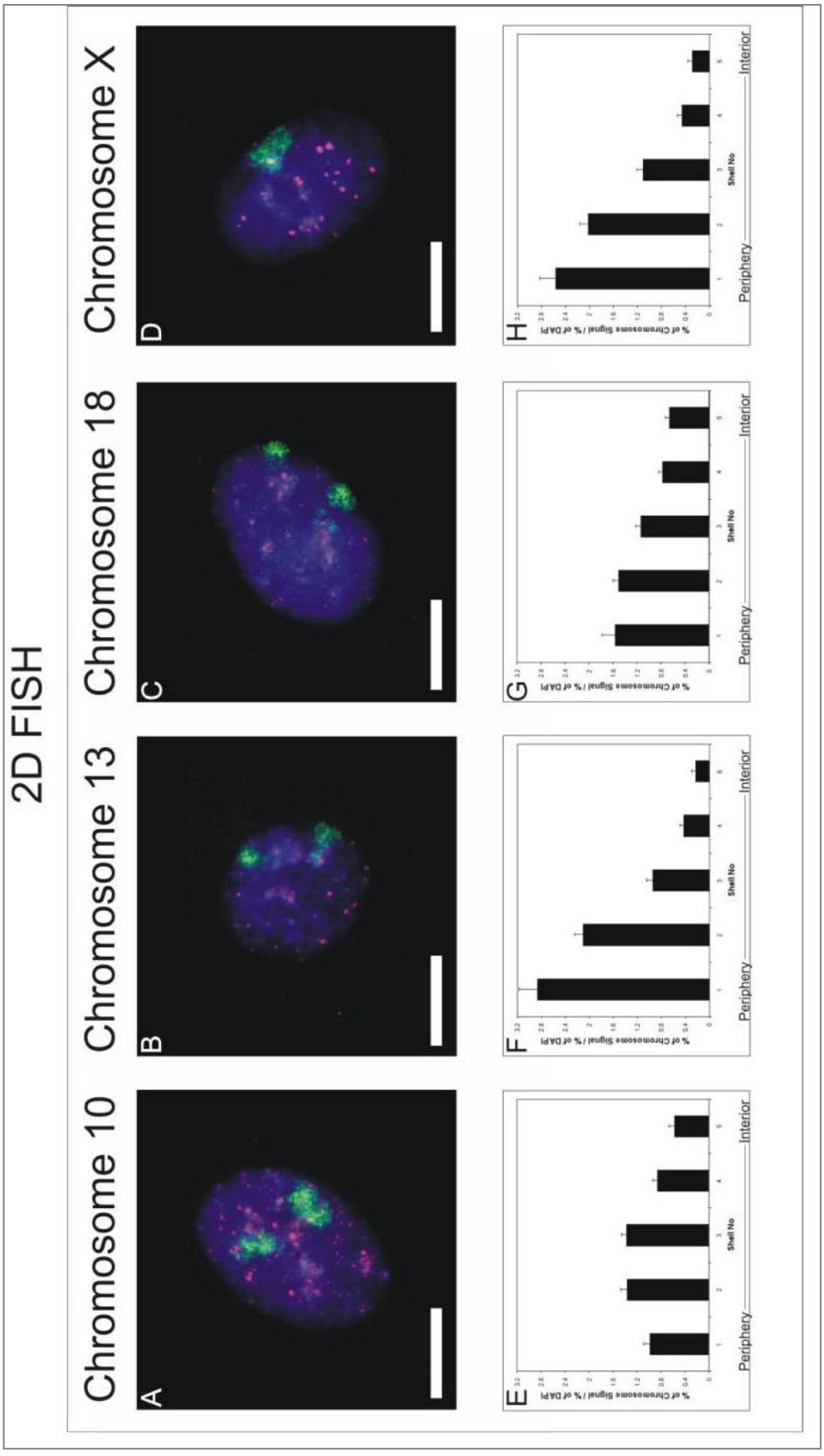


Figure 2.2. Chromosome positioning in interphase nuclei of proliferating HDFs

Figure 2.2. Chromosome positioning in interphase nuclei of proliferating HDFs: Proliferating human dermal fibroblasts (HDFs) cultures were subjected to 2D-fluorescence in situ hybridisation (FISH) in order to delineate and analyse the nuclear location of chromosomes 10, 13, 18 and X. In panels A-D the chromosome territories are revealed in green with a single chromosome territory for chromosome X since the 2DD HDFs are male in origin. The red antibody staining is the nuclear distribution of the proliferative marker anti-pKi-67, whose presence denotes that a cell is in the proliferative cell cycle. DAPI in blue is a DNA intercalator dye and reveals the nuclear DNA. Scale bar = 10μm. The histograms in panels E-H display the distribution of the chromosome signal in 50-70 nuclei for each chromosome for 2D-FISH as analysed by simple erosion analysis. Bars represent the mean normalised proportion (%) of chromosome signal for each human chromosome. Error bars represent standard error of mean (SEM).

The positions of chromosomes 13, 18 and X have been successfully recapitulated as documented by previous studies (Meaburn et al., 2007, Boyle et al., 2001), whereby chromosomes 13, 18 and X are located at the nuclear periphery in young proliferating cells (Figure 2.2. panels B-D, F-H). Moreover, as Figure 2.2. panel A and E demonstrate, chromosome 10 in proliferating fibroblasts unlike chromosomes 13, 18 and X, it is found in an intermediate nuclear position again as demonstrated in data from(Meaburn et al., 2008).

The relative interphase position of chromosomes 10 and X in an intermediate nuclear location and at the nuclear periphery respectively, has also been confirmed in 3D-FISH analyses (Figure 2.3.). Measurements in μm from the geometric centre of the chromosome territories to the nearest nuclear periphery, as determined by the DAPI staining, were taken in at least 20 nuclei. The data have not been normalised for size measurements so that actual measurements in μm can be seen. However, all data have been normalised by a size measurement and this not does alter the relative positioning of the chromosomes in cell nuclei (data not shown).

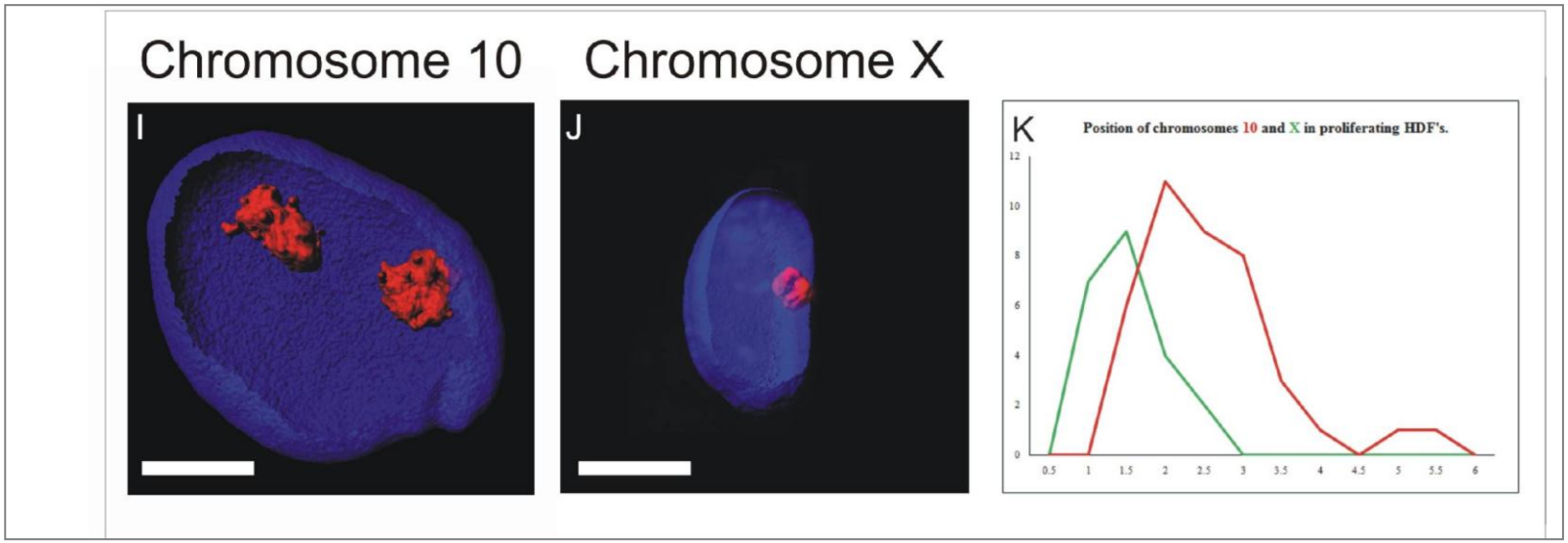


Figure 2.3. Positions of chromosome 10 and X territories in interphase nuclei of proliferating HDFs: Proliferating HDFs were subjected to 3D-FISH analysis in order to delineate the nuclear locations of chromosome 10 and X territories. Panels I and J display 3D projections of 0.2µm optical sections through 3D preserved nuclei subjected to 3D-FISH, imaged by confocal laser scanning microscopy and reconstructed using Imaris 3.0 software. The chromosome territories are displayed in red and proliferating cells were also selected with positive anti-pKi-67 staining (not shown in reconstruction). Scale bar = 10µm. The line graph in panel K displays a frequency distribution of µm from the geometric centre of the chromosome territories to the nearest nuclear periphery, as defined by DAPI staining. Images for 20 nuclei were analysed.

2.3.2. Positions of all human chromosomes in normal quiescent human dermal fibroblasts:

Further, positions of all chromosome territories were determined in quiescent fibroblast nuclei. Representative images of chromosome territories in pKi-67 negative quiescent nuclei are displayed in Figure 2.4.

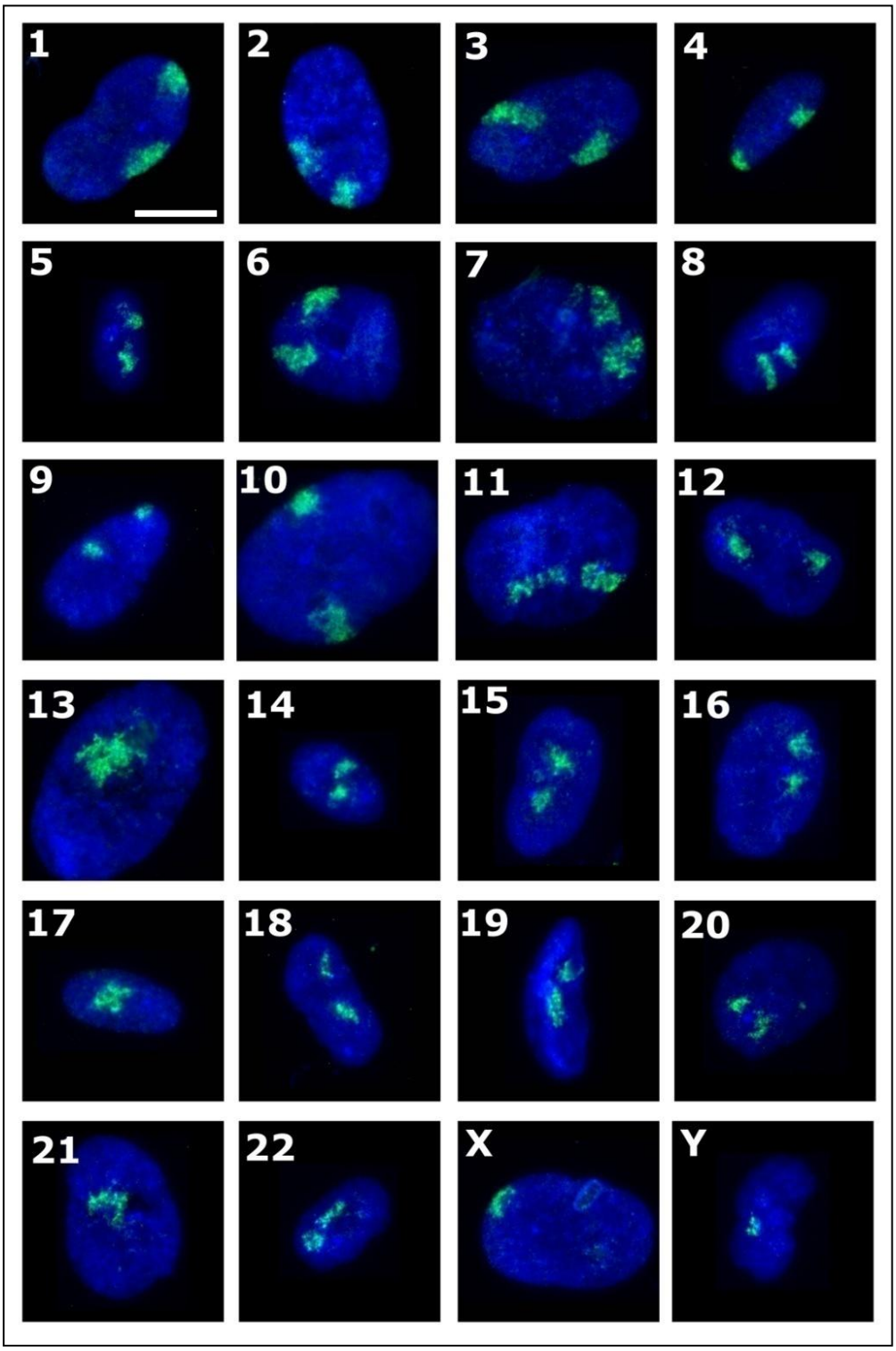


Figure 2.4. Representative images demonstrating the positions of all human chromosomes in normal quiescent fibroblasts: Images displaying the spatial arrangement of each of the human chromosome territories (in green) in normal quiescent (serum-starved) interphase nuclei (stained in blue) of fibroblasts. The numbers by the side of each nucleus indicates the chromosome hybridised to by FISH. Scale bar = 10 μM.

Analysis of the images obtained from 2D- FISH slides was performed as described earlier (Bridger et al., 2000, Croft et al., 1999, Boyle et al., 2001, Meaburn et al., 2007, Meaburn et al., 2005). On performing the positioning analysis on quiescent interphase nuclei (serum-starved) we found that certain chromosomes were in very different positions to where they were in proliferating interphase nuclei i.e. HSA 1, 6, 8, 10, 11, 12, 13, 15, 18 and 20 (Table 2.1., Figure 2.5. and Figure 2.6.).

Figure 2.5. Histograms displaying the positions of all human chromosome territories in normal quiescent fibroblast nuclei

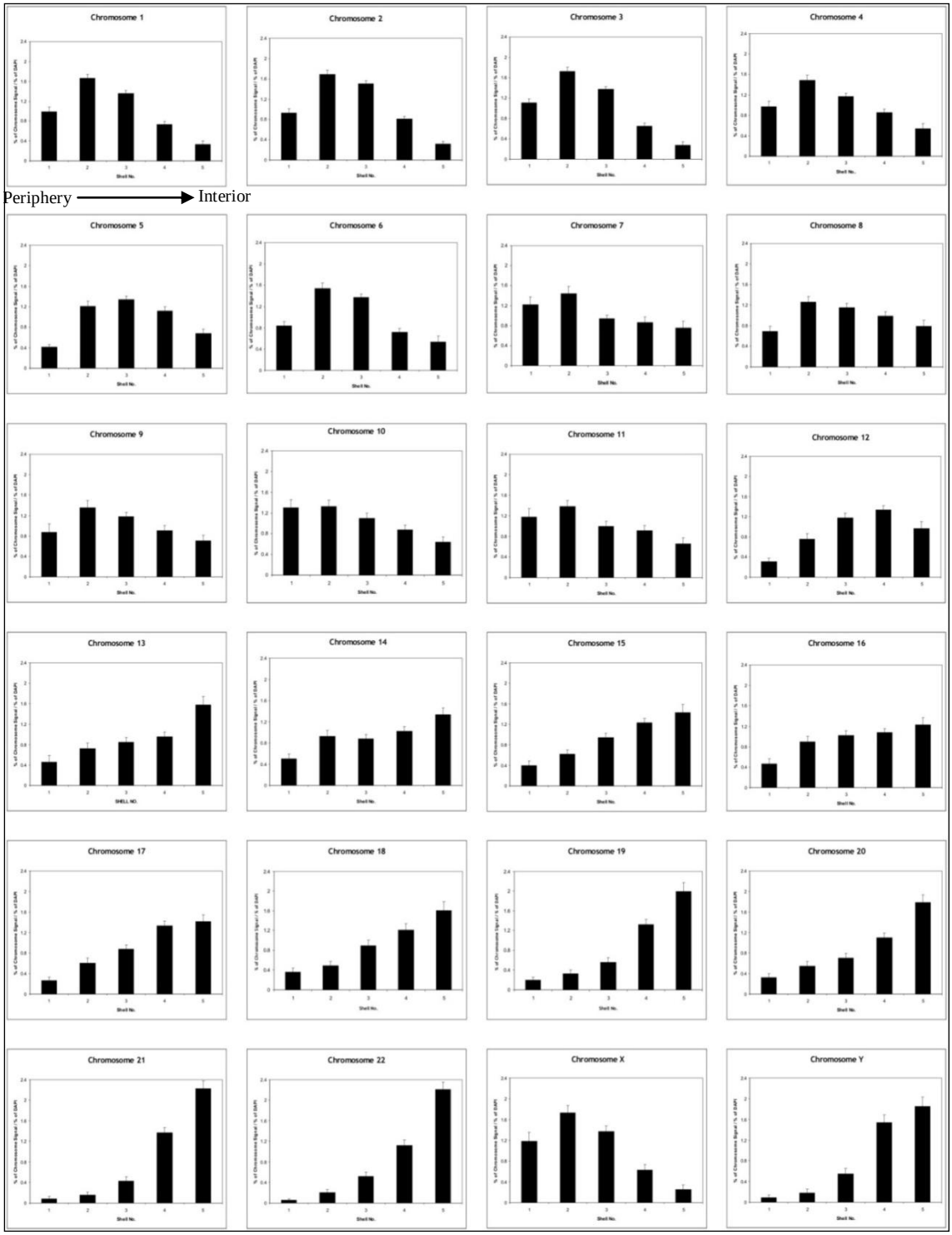


Figure 2.5. Histograms displaying the positions of all human chromosome territories in normal quiescent fibroblast nuclei: Young proliferating HDFs were grown in 10% NCS for 48 hours and then were induced to undergo quiescence by serum starvation (incubation in 0.5% NCS) for 7 days. Standard 2D-FISH assay was performed and at least 50 digital images were analysed per chromosome by simple erosion analysis script. The graphs show the % of chromosome signal of each human chromosome normalized with the % of DAPI in each of the eroded shells (y-axis); and the shell number on the x-axis. The standard error bars representing the standard errors of mean (SEM) were plotted for each shell for each graph.

Table 2.1. Locations of all human chromosomes in interphase nuclei of normal proliferating, quiescent and senescent fibroblasts. The data for proliferating and senescent fibroblasts has been obtained from (Boyle et al., 2001, Meaburn et al., 2007, Mehta, 2005)

Chromosome No. and their Gene Density	Proliferating HDFs	Quiescent HDFs	Senescent HDFs
1 (2776)	Intermediate	Periphery	Periphery
2 (1866)	Periphery	Periphery	Periphery
3 (1473)	Periphery	Periphery	Periphery
4 (1164)	Periphery	Periphery	Periphery
5 (1281)	Intermediate	Intermediate	Periphery
6 (1528)	Intermediate	Periphery	Periphery
7 (1474)	Periphery	Periphery	Periphery
X (1344)	Periphery	Periphery	Periphery
8 (1025)	Intermediate	Periphery	Intermediate
9 (1207)	Periphery	Periphery	Periphery
10 (1094)	Intermediate	Periphery	Interior
11 (1841)	Intermediate	Periphery	Intermediate
12 (1355)	Periphery	Interior	Interior
13 (556)	Periphery	Interior	Interior
14 (1220)	Interior	Interior	Interior
15 (961)	Periphery	Interior	Intermediate
16 (1108)	Interior	Interior	Intermediate
17 (1442)	Interior	Interior	Interior
18 (438)	Periphery	Interior	Interior
19 (1624)	Interior	Interior	Interior
20 (717)	Interior	Intermediate	Interior
21 (367)	Interior	Interior	Interior
22 (756)	Interior	Interior	Interior
Y (322)	Interior	Interior	Interior

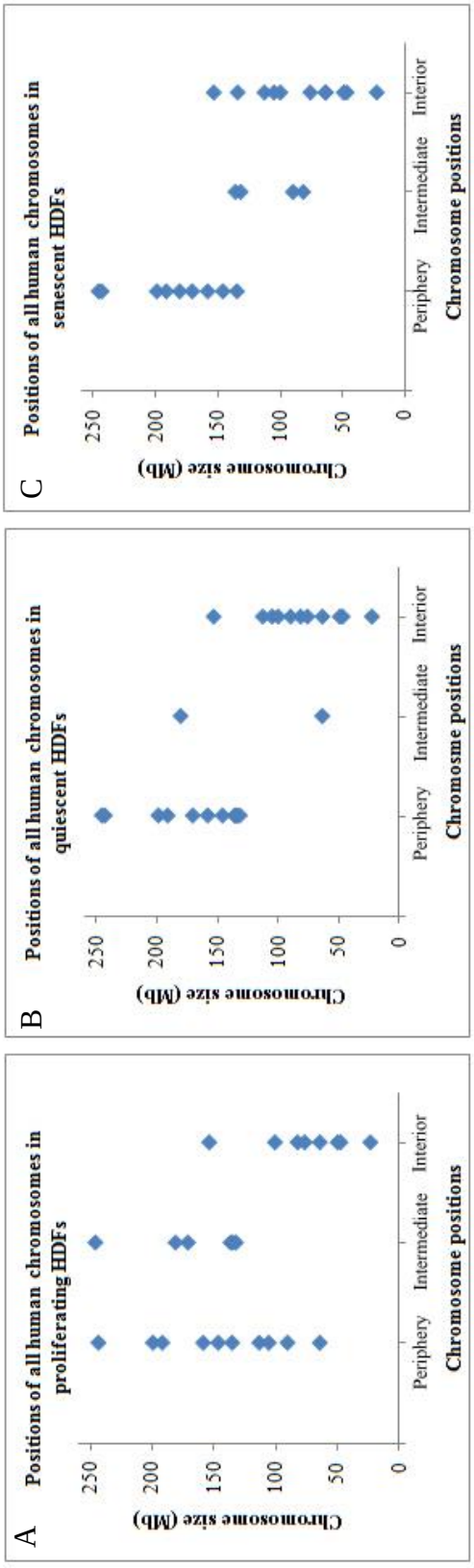
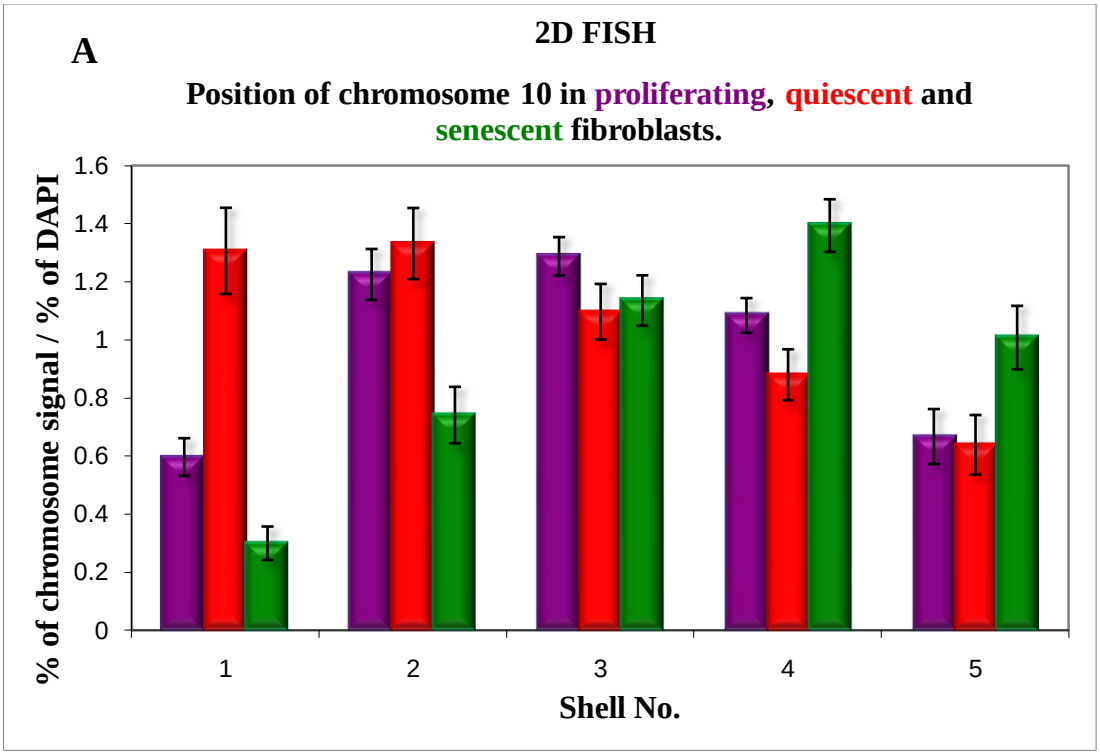


Figure 2.6. Relationship between chromosome size and its position within proliferating, quiescent and senescent HDFs: Graphs display the position of chromosomes with respect to being at the nuclear periphery, at an intermediate location within the nucleus, or in the nuclear interior on the x-axis v/s chromosome size in Mb on the y-axis. Positions of all human chromosomes with respect to size in proliferating, quiescent and senescent cells are displayed in panels A, B and C respectively.

2.3.2. Chromosome 10 occupies differential locations in normal proliferating, quiescent and senescent human dermal fibroblasts:

Analysis of the positions of all the chromosome territories in serum-starved quiescent cells and senescent HDFs showed that some chromosomes such as chromosome 1, 6, 12, 13 and 18 altered their locations as cells go from a proliferating state to either of the two non-proliferating states (Table 2.1.). Interestingly, there were some differences in the position of chromosomes between senescent and quiescent fibroblasts such as between the location of chromosomes 1, 5, 8, 10, 15 and 20 differs between these different non-proliferating states (Table 2.1.). Although, the most notable difference was observed in the location of chromosome 10, which occupies an intermediate position in proliferating cells, a peripheral location in quiescent cells and localises at the nuclear interior in senescent cells (Figure 2.7.).

Figure 2.7. Differential location of chromosome 10 territories in proliferating, quiescent and senescent cell nuclei



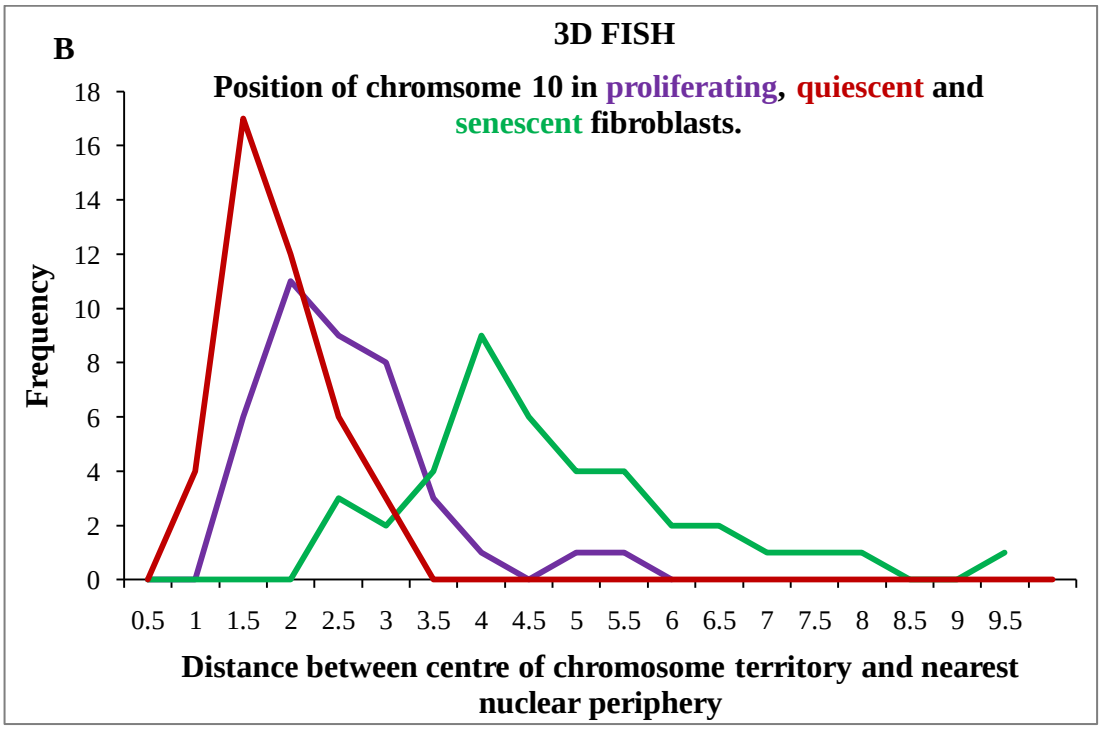


Figure 2.7. Differential location of chromosome 10 territories in proliferating, quiescent and senescent cell nuclei: The mean normalized proportion of chromosome 10 signal derived from proliferating (purple bars), quiescent (red bars) and senescent (green bars) nuclei is displayed as a histogram (A). Error bars represent standard error of the mean (SEM). This data is further confirmed by 3D FISH analysis and is displayed in form of a frequency distribution curve (B). Unpaired, unequal variance, two-tailed student's *t*-test at 95% confidence interval ($p<0.05$) has shown that there is a significant difference between the position of chromosome 10 in proliferating (purple line) and quiescent (red line) fibroblasts, between proliferating (purple line) and senescent (green line) fibroblasts and between quiescent (red line) and senescent (green line) fibroblasts.

Thus, with such alteration in its position, chromosome 10 can be further used as a marker to distinguish between quiescent and senescent cells.

2.3.3. Positions of chromosomes 10 and X in normal human dermal fibroblasts made quiescent by serum starvation, topoinhibition and stress:

To test whether the alteration in location of chromosome 10 in quiescence was a growth factor dependent process or an intrinsic response of all cells made quiescent; some young HDFs were grown until topoinhibition. These cells were allowed to grow to confluency in 10% serum medium for 4 weeks with re-feeding with fresh medium twice every week.

Another mode of entry into quiescence for any living cell is due to stress (Coller et al., 2006). Thus, to determine the location of chromosome 10 in nuclei of cells that have entered quiescence due to stress, young proliferating HDFs were treated with a stress inducing agent DMSO (Srinivas et al., 1991). None of these treatments would have caused DNA damage induced growth arrest.

Thus, the hypothesis was that if the repositioning of chromosome 10 was growth factor dependent, it would occupy an intermediate location in nuclei of cells made quiescence due to topoinhibition or due to stress; similar to its location in nuclei of proliferating cells.

Chromosome 10 demonstrated a shift towards the periphery (Figure 2.8.C) of the nucleus in topo-inhibited cells, but this shift was not as significant as in serum-starved quiescent cells (Figure 2.8.B). Thus, it seems that entry of a cell into quiescence is accompanied by a slight shift in the position of chromosome 10 in the nucleus, but this shift significantly increases in absence of growth factors.

In cells treated with DMSO, i.e. the cells where quiescence response was induced due to stress, chromosome 10 localised in the nuclear periphery (Figure 2.8.D). This location of chromosome 10 resembles that of serum starved quiescent cells (Figure 2.8.B).

Chromosome X localises at the nuclear periphery in cells made quiescence due to serum starvation, topoinhibition and stress (Figure 2.8. D-F).

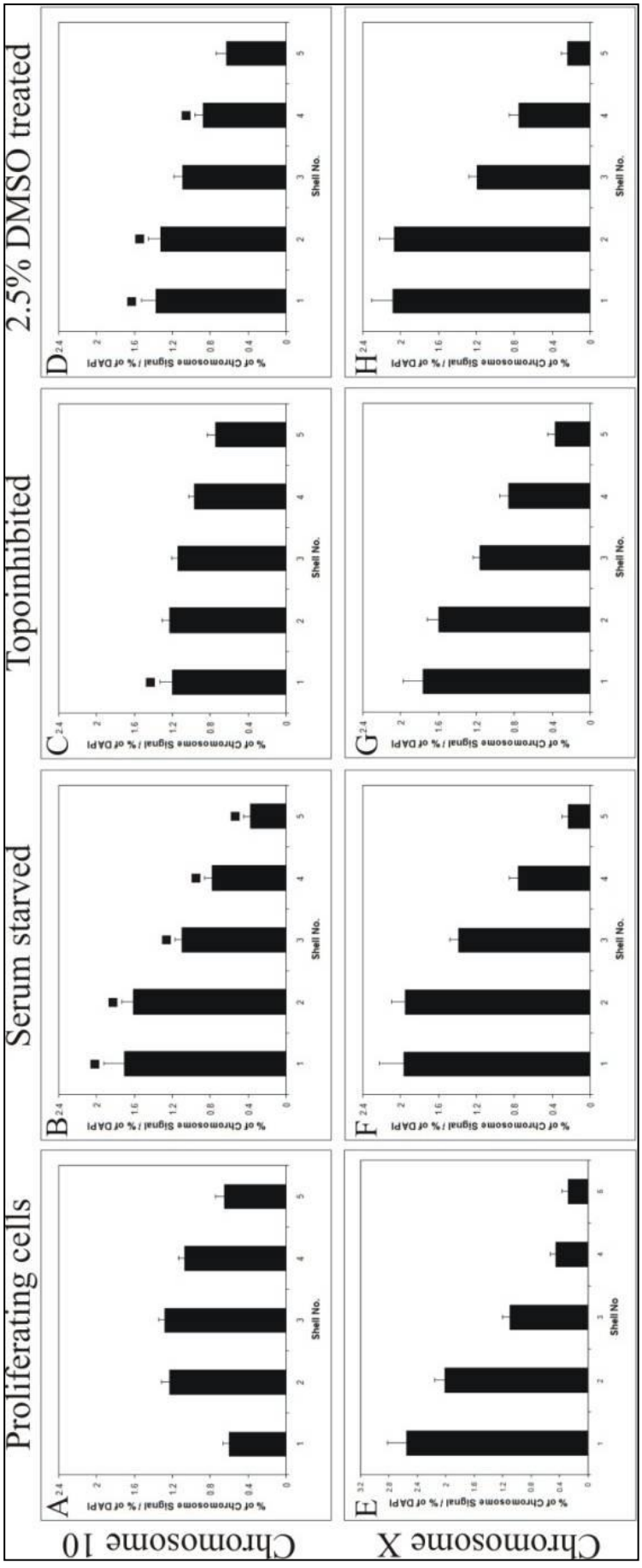


Figure 2.8. Position of chromosomes 10 and X in cells made quiescence due to serum starvation, topoinhibition and stress:

Figure 2.8. Position of chromosomes 10 and X in cells made quiescence due to serum starvation, topoinhibition and stress: Young proliferating HDFs were grown in culture for at least 2 days and then serum starved for 7 days. Young proliferating HDFs were grown in 10% NCS for 4 weeks to grow them to confluency (topoinihibition). To induce quiescence via stress response, young proliferating cells were treated with 2.5% DMSO for 48-72 hours. Positions of chromosomes 10 and X were determined using standard 2D-FISH assay. Chromosome X localises at the nuclear periphery in normal proliferating cells (E) and in cells made quiescent by serum starvation (F), topoinhibition (G) and due to treatment with DMSO (H). Chromosome 10 occupies an intermediate location in normal proliferating cells (A) while a peripheral location in normal cells serum starved for 7 days (B) and in cells treated with DMSO (D). Chromosome 10 shows a slight shift towards the nuclear periphery in cells made quiescent due to topoinhibition (C).

2.3.4. Positions of chromosomes 10 and X in serum-starved senescent cells resembles that of normal senescent cells:

To determine whether senescent cells respond to serum starvation with respect to chromosome positioning, we placed senescent cells in low serum for 168 hours (7 days) and analysed the nuclear positions of chromosomes 10 and X (Figure 2.9.) using a standard 2D-FISH assay. As expected, HSA X does not change its position at the nuclear periphery (Figure 2.9.E-H) However, unexpectedly HSA 10 also did not change its relative nuclear position but displays some movement with removal of serum and remains localised in the nuclear interior (Figure 2.9.D) similar to that in normal senescent fibroblasts (Figure 2.9.C); and does not reposition to the nuclear periphery. This indicates that senescent cells may not reorganise their genome after they have become senescent. Thus, once cells are senescent, they no

longer are able to respond to low serum with respect to chromosome positioning in interphase nuclei.

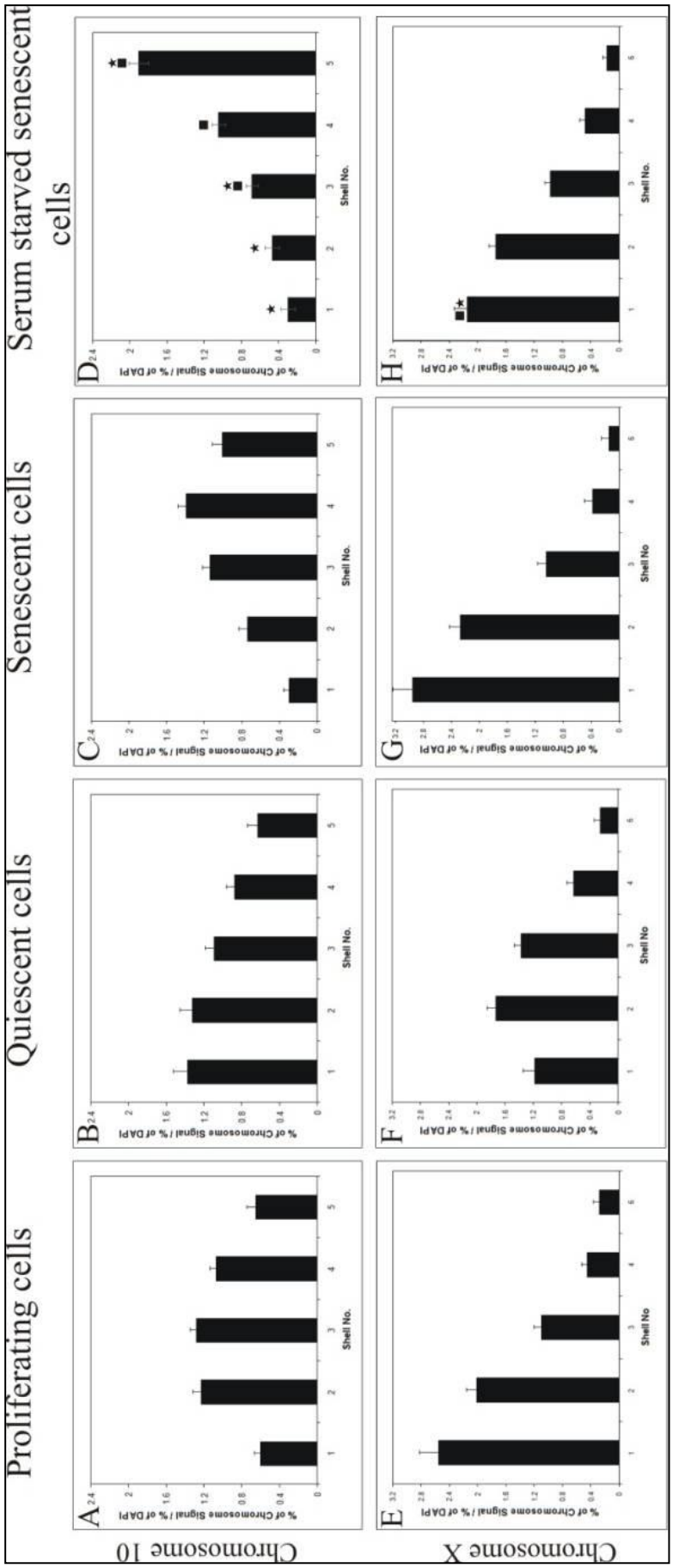


Figure 2.9. Positions of chromosome 10 and X in senescent cells serum starved for 7 days.

Figure 2.9. Positions of chromosome 10 and X in senescent cells serum starved for 7 days: HDFs were grown in 10% NCS until they become senescent (% of Ki-67 positive cells in culture is less than 10%). Senescent cells were serum-starved by incubation with 0.5% NCS for 168 hours (7days). Positions of chromosomes 10 and X were determined using the standard 2D-FISH analysis. Chromosome X territories, in serum-starved senescent cells, localises to the nuclear periphery (H) as they do in normal proliferating (E), quiescent (F) and senescent (G) cells. In serum starved senescent cells, chromosome 10 is positioned in the nuclear interior (D), similar to its position in normal senescent cells (C). The stars indicate statistical difference ($p<0.05$), as assessed by Students *t*-test, to the normal quiescent cells (B and F). The filled-in squares indicate statistical difference ($p<0.05$) to normal proliferating cells (A and E).

2.4. Discussion:

The study in this chapter completes the nuclear positioning of all 24 chromosomes in normal quiescent (serum-starved) primary fibroblasts as assessed by 2D-FISH and erosion analysis. Spatial organisation of chromosome territories in quiescent fibroblasts is dependent on chromosome size or mass, with larger chromosomes located at the nuclear periphery and smaller chromosomes positioned in the nuclear interior. Thus, as the organisation of chromosome territories changes from a gene density based positioning in proliferating cells to a size correlated organisation with induction of quiescence, there are obvious differences between the positions of certain chromosome territories between proliferating and quiescent fibroblasts (Figure 2.6.). These data reconcile with previous data that have observed chromosome territories organised according to a size based distribution in non-proliferating cells (Mehta, 2005, Bridger et al., 2000, Meaburn et al., 2007, Bolzer et al., 2005, Sun et al., 2000). Whether or not this spatial reorganisation of chromosome territories is required for entry into cellular quiescence remains unclear.

Chromosomes that contain NOR regions including chromosomes 13, 14, 15, 21 and 22 (Sullivan et al., 2001, Weipoltshammer et al., 1999) as well as other chromosomes that do not have rDNA including chromosomes 1 (Manuelidis and Borden, 1988, Bridger et al., 1998b), 8 (Bridger et al., 1998b), 9 (Manuelidis and Borden, 1988) and 17 (Bridger et al., 1998b) have been found to associate with the nucleolus in proliferating cells. Four out of these nine chromosomes including chromosomes 1, 8, 13 and 15 are occupy different positions in quiescent fibroblasts as compared to proliferating HDFs. It still remains to be identified if the differential localisations of these chromosomes in quiescent HDFs have an influence on their association with the nucleoli. In addition to this, genomic regions as well as whole

chromosomes are bound to the nuclear matrix (Craig et al., 1997). Thus, repositioning of chromosomes with cells entry into G_0 may also affect the attachment of genomic regions to the nuclear matrix. Alterations in association of chromosomes or genomic regions to these structures of the nuclear architecture may then influence chromosome structure, gene expression, DNA replication, RNA transport and processing, signal transduction and apoptosis (Bickmore and Oghene, 1996, Bode et al., 2000, Djeliova et al., 2001, Wei et al., 1998, Wei et al., 1999).

Reorganisation of chromosome territories occurs in cells induced to undergo quiescence by serum starvation and stress. Although, when cells are induced to undergo quiescence via topoinhibition, the positions of the two chromosomes studied resemble that of proliferating HDFs. It should be noted that even though the cells were cultured until 100% confluency for four weeks, the culture still showed presence of proliferating cells (13% Ki-67 positive cells) and will also have cells that are senescent. Since there are no markers available to distinguish between quiescent and senescent cells, graph displaying the position of chromosomes in Ki-67 negative fraction of cells (representing non-proliferating cells) would be obtained from a mixed population of quiescent and senescent cells.

By assessing the distribution of DAPI across the five shells of the erosion anaylsis script (See figure A1), it is clear that the repositioning of chromosome territories as cells traverses from proliferating to quiescent or proliferating to senescent state, is not just the result of complete reorganisation of all DNA in the cell nucleus.

It is interesting that there are not only differences in chromosomal location between proliferating and non-proliferating cells but there are also some chromosomes displaying differences between quiescent and senescent cells, most

notably chromosome 10. Such a difference in positioning for this chromosome in particular could provide a new bio-marker to differentiate between G_0 and G_S cells. Quiescence and senescence are indeed distinct states; whereby G_0 cells have smaller nuclei (Grigoryev et al., 2004), are capable of undergoing repair and differentiation (Coller et al., 2006, Gos et al., 2005, Liu et al., 2007) as opposed to G_s cells that are usually characterised by larger nuclei (Mehta et al., 2007, Bowman et al., 1975, Sherwood et al., 1988, Mitsui and Schneider, 1976), accumulation of DNA damage (Di Micco et al., 2006, Bartkova et al., 2006, d'Adda di Fagagna et al., 2003, Parrinello et al., 2003, te Poele et al., 2002) and have been implicated in organismal ageing (Campisi, 2000, Campisi, 2001, Campisi, 2003a, Campisi, 2003b, Smith and Kipling, 2004). Thus, although both, G_0 and G_s are non-proliferating states of the cells, functions and processes that occur within the cells when they exist in these states are quite different and thus may require expression of different set of genes. This is further supported by differential gene expression profiles observed in quiescent (Chechlinska et al., 2009, Kipling et al., 2009, Coller et al., 2006, Liu et al., 2007, Glynne et al., 2000, Venezia et al., 2004, Gos et al., 2005, Mizuno et al., 2009, Viatour et al., 2008) and senescent cells (Kipling et al., 2004, Shelton et al., 1999, Zhang et al., 2003, Schwarze et al., 2005). Whether or not differential locations of chromosome territories within interphase nuclei of quiescent and senescent cells contribute to this distinct gene expression is unknown. Experiments involving microarray analysis to identify clusters of genes associated with proliferation, senescence and quiescence as well as interference by blocking the repositioning of particular chromosomes and their effects on entry of cells into the non-proliferative states; may help identification of different pathways that are

activated by the cells when they are either signalled to undergo senescence or quiescence.

Surprisingly, in senescent cells placed in low serum, relative position of chromosome 10 is unaltered and still remains in the nuclear interior. This suggests that the spatial organisation of chromosomes is in place once the cells enter a state of replicative senescence and thus the machinery or factors required for repositioning chromosomes may not be functional in these cells. It might also be possible that senescent cells are unable to respond to serum deprivation. Moreover, the associations of chromosomes and genomic regions to structures within the nuclei, including the nuclear matrix, nuclear lamina and nucleoli may be less flexible in senescent cells; thus restricting the repositioning of chromosome territories.

Thus, spatial organisation of chromosomes within interphase nuclei not only differs between various cell types, but is also distinct as cells traverse from a proliferating to a non-proliferating state in their life-span; thus stressing the role of this differential organisation in genome function. Chapter 3 provides a further insight into factors involved in this reorganisation of the genome within an interphase nucleus.

Chapter 3: Repositioning of chromosome territories - a nuclear motor dependent process

- Some of the contents of this chapter are published in Biochemical Society Transactions (2009) under the title "Nuclear motors and nuclear structures containing A-type lamins and emerin: is there a functional link? – Ishita S Mehta, Lauren S Elcock, Manelle Amira, Ian R Kill and Joanna M Bridger.

- Some of the contents of this chapter are in press for Genome Biology under the title "Rapid chromosome territory relocation by nuclear motor activity in reposnse to serum removal in primary human fibroblasts" – Ishita S Mehta, Manelle Amira, Amanda J Harvey and Joanna M Bridger.

3.1. Introduction:

Interphase chromosome organisation is established within the cell nuclei in early G_1 phase of the cell cycle, whereby chromatin movement is evident to allow spatial positioning of individual chromosome territories (Gilbert et al., 2005, Dimitrova and Gilbert, 1999). Following this, the spatial positions of the chromosomes are maintained through rest of G_1, S and G_2 phases of the cell cycle (Bridger and Bickmore, 1998, Bridger et al., 2000).

Results from chapter 2 and some previous studies in Bridger and Bickmore labs (Bridger et al., 2000, Mehta, 2005) have demonstrated that spatial organisation of chromosome territories is altered when fibroblasts exit the proliferative cell cycle to enter a non-proliferative state. Similarly, chromosome repositioning is also evident and occurs within 30 hours after quiescent fibroblasts are re-stimulated to enter the proliferative cell-cycle (Bridger et al., 2000). This process thus presumably requires the cells to rebuild their nuclear architecture following a mitotic division (Bridger et al., 2000). This repositioning of chromosome territories as cells traverse in and out of the proliferative cell cycle; implies movement of whole chromosomes through the nucleoplasm.

Chromosome movement during mitosis, meiosis and in simple organisms is controlled from the cytoplasm where energy is generated by dynein molecules along the tracks of microtubules (Haque et al., 2006, Chikashige et al., 2007b, Chikashige et al., 2007a, Starr, 2009). This energy is then transferred to KASH domain proteins such as nesprins via bridges created by proteins such as Kms1 and Sad1 in the cytoplasm (Chikashige et al., 2007b, Miki et al., 2004). KASH domain proteins

further interact with SUN proteins and create links to the nuclear lamina at the inner nuclear envelope, thus allowing signals and forces to be translated to the nucleoplasm (Haque et al., 2006, Starr, 2009, Chikashige et al., 2007a).

The presence of actin (Nakayasu and Ueda, 1983, Bettinger et al., 2004, Pederson and Aebi, 2002, Nakayasu and Ueda, 1985, Andersen et al., 2002, Miller et al., 1991, Gonsior et al., 1999, Schoenenberger et al., 2005) and myosin I (NMI) (Hofmann et al., 2006a, Pestic-Dragovich et al., 2000, Grummt, 2003, Fomproix and Percipalle, 2004, Hofmann et al., 2004)], myosin VI (Vreugde et al., 2006), myosin16b (Osborne et al., 2007) and myosin Va (Pranchevicius et al., 2008) have been reported in the cell nuclei. There is also an increasing body of evidence that they co-operate to form a nuclear myosin-actin motor (Hofmann et al., 2006a). Further, nuclear actin and myosin have been implicated in nucleocytoplasmic transport (Schindler et al., 1989) and importantly in movement of chromatin around the nucleus (Chuang et al., 2006, Dundr et al., 2007, Hu et al., 2008) as well as whole chromosomes [(Hu et al., 2008); Mehta et al in submission].

One of the most studied nuclear isoforms of nuclear myosins; nuclear myosin Iβ (NMIβ) is encoded by the gene *MYO1C*. In addition to NMIβ, *MYO1C* gene encodes two other cytoplasmic myosins lacking the 16 amino acid sequence that is unique to NMIβ and is required for its nuclear localisation (Pestic-Dragovich et al., 2000). NMIβ associates with chromatin remodelling complexes along with nuclear actin. Actin binds to SWI/SWF-like BAF remodelling complexes while NMIβ associates with components of the WICH chromatin remodelling complex (Hofmann et al., 2006a). In a model put forward by (Hofmann et al., 2006a) NMIβ is implicated in movement of chromatin within cell nuclei. The model predicts that NMIβ could bind via its tail to the nuclear entity that requires movement, while actin

binds to the globular head of the NMI molecule. This nuclear motor would then translocate the nuclear entity along highly dynamic tracks of nuclear actin (Hofmann et al., 2006a).

The study within this chapter aims to determine the response time within which the repositioning of chromosomes in fibroblast interphase nuclei occur as cells enter a non-proliferative state as well as what instigates the shift and thus causes the re-positioning of the chromosome territories when the cells exit the cell cycle either through quiescence or senescence.

It is very difficult to determine as to when cells exactly undergo senescence, but entry of cells into quiescence via serum starvation can be controlled temporally. Thus, this allows the determination of a response time for chromosome repositioning to removal of growth factors. Chromosome 10 occupies different locations within interphase nuclei of proliferating, quiescent and senescent fibroblasts and the territories of chromosome 10 reposition from an intermediate nuclear position in proliferating HDFs to a peripheral nuclear location in quiescent fibroblasts (Chapter 2). Conversely, chromosome 13 and 18 territories reposition from the nuclear periphery to the nuclear interior upon serum starvation (Chapter 2) (Bridger et al., 2000), while chromosome X territories are located at the nuclear periphery in both proliferating and quiescent HDFs. Hence, in this study, locations of chromosomes 10, 13, 18 and X territories in interphase cell nuclei have been determined at various time points following serum withdrawal. Positions of chromosomes 10, 18 and X have been determined in quiescent fibroblasts following re-stimulation with serum at various time-points, in order to recapitulate the earlier study for chromosome 18 as well as to determine if the same is true for chromosomes 10 and X territories.

To further assess if repositioning of chromosome territories following serum starvation is an active and an energy requiring process, positions of chromosome 10, 18 and X territories were determined in HDFs were treated with inhibitors of ATPase and GTPase followed by serum withdrawal. In order to determine the mechanism of chromosome repositioning following serum removal, HDFs were treated with inhibitors of microtubules, actin polymerisation and myosin polymerisation. Further, RNA interference technique was used to interfere with the expression of *MYOIC* gene that encodes NMIβ.

In summary, the results from this chapter demonstrate that chromosome repositioning following serum withdrawal is a rapid and an active process requiring the activity of nuclear motor proteins including actin and NMIβ. Chromosome repositioning in quiescent cells following serum re-stimulation requires longer time duration than cells traversing through mitosis.

3.2. Methods and Materials:

3.2.1. Cell culture:

Young HDFs were maintained and cultured in 10% NCS-DMEM using the protocol in section 2.2.1.

For "chromosome movement" experiments after induction of quiescence, cells were incubated in 0.5% NCS for 0, 5, 10, 15 and 30 minutes.

To analyse chromosome positions following serum restoration, young HDFs were cultured in 10% NCS-DMEM for at least 2 days, serum starved (0.5% NCS-DMEM) for 7 days and re-stimulated with 10% NCS-DMEM for 0, 8, 24, 32 and 36 hours.

3.2.2. Treatment with inhibitors:

Drugs were used to inhibit the action of microtubules, ATPase, GTPase and to inhibit polymerisation of actin and myosin.

3.2.2.a. Inhibitors

Inhibitors used in this study: Karyomax colcemid solution – 10 µg/ml {Invitrogen}, 2,3-Butanedione 2-Monoxime (BDM) – 500 mgs {Calbiochem}, Latrunculin A, in solution – 1 mM {Calbiochem}, Ouabain octahydrate – 1 g {Calbiochem}, AG10 – 5 mgs {Calbiochem}, Jasplakinolide in solution – 1 mM {Calbiochem}.

3.2.2.b. Treatment with inhibitors

Young proliferating HDFs, strain 2DD, were seeded in tissue culture flasks at a density of 5 x 10^5 per flask and then allowed to grow for 2 days in 10% NCS-DMEM medium. Cells were then incubated with 10% NCS – DMEM medium containing the inhibitor at 37^{o}C.

To inhibit the activity of ATPase or GTPase, cells were treated with 100 μM of ouabain (Calbiochem) for 30 minutes (Senol et al., 2007) or with 100 μM of AG10 (Calbiochem) for 20 minutes prior to serum withdrawal (Soltoff, 2004, Moriya and Linder, 2006), respectively. To inhibit the polymerisation of actin, cells were treated with 1 μM of either Latrunculin A (Calbiochem) (Schoenenberger et al., 2005, Munter et al., 2006) or Phalloidin oleate (Calbiochem) (Laudadio et al., 2005) for 30 minutes. Myosin polymerisation and activity were inhibited by treating cells either with 10mM of 2,3-Butanedione 2-Monoxime (Calbiochem) for 15 minutes (Cramer and Mitchison, 1995, Soeno et al., 1999, Fabian et al., 2007) or 1 μM of Jasplakinolide (Calbiochem) for 60 minutes respectively (McDonald et al., 2006). See table 3.1. For all inhibitors samples for 2D-FISH analyses were harvested before and after incubation in low serum (0.5% NCS DMEM) for 15 minutes.

Table 3.1. List of inhibitors: This table includes inhibitors used, their respective substrates, concentrations at which they are used, the time duration they are used for and the reference from where this information is derived.

Inhibitor	Substrate	Final Concentration	Duration	Reference
Colcemid	Microtubules	1 μg/ml	1 hour	
BDM	Myosin	10 mM	15 minutes	(Cramer and Mitchison, 1995, Soeno et al., 1999, Fabian et al., 2007)
Jasplakinolide	Myosin activity Actin	1 μM	1 hour	(McDonald et al., 2006)
Latrunculin A	Actin	1 μM	30 minutes	(Schoenenberger et al., 2005) (Munter et al., 2006)
Phalloidin Oleate	Actin	1 μM	30 minutes	(Laudadio et al., 2005)
Ouabain	ATPase	100 μM	30 minutes	(Senol et al., 2007)
AG10	GTPase	100 μM	20 minutes	(Soltoff, 2004) (Moriya and Linder, 2006)
Ouabain + AG10	ATPase + GTPase	100 μM of each	30 minutes	(Soltoff, 2004, Moriya and Linder, 2006, Senol et al., 2007)
DMSO (control)	Control	0.1 %	1 hour	Maximum amount added with the inhibitors above

3.2.3. siRNA transfection for MYO1C:

3.2.3.a. Cell culture

Young proliferating HDFs were grown as in section 2.2.1. For optimisation of transfection efficiency, the cells were seeded into 24 well plates at a density ranging from 4 x 10^3 and 9 x 10^3 per well in antibiotic free 10% NCS-DMEM.

For transfections with MYO1C and negative control siRNA sequences, young proliferating HDFs were seeded at 1 x 10^4 per well in antibiotic free 10% NCS-DMEM in a 12 well plate. The cells were allowed to adhere to the substratum for at least 24 hours before performing any transfections.

In order to perform indirect immunofluroscence staining, a 13 mm diameter coverslip {Fisher} was placed in selected wells before seeding of the cells.

3.2.3.b. Optimising the transfection efficiency with siGLO

Transfections were performed using siRNA sequences from ThermoScientific and DharmaFECT 1 transfection reagent kit. Transfection efficiency was tested using siGLO transfections indicators {Thermo Scientific Dharmacon}. 24 hours after seeding cells, 1µl of DharmaFECT 1 transfection reagent was mixed with 49µls serum-free DMEM and incubated for 5 minutes at room temperature. Meanwhile 25µls of 2µM siGLO transfection indicator was added to 25µls serum free medium. The transfection reagent/serum free medium was added to the siRNA/serum free medium and incubated at room temperature for 20 minutes to allow transfection complexes to form. 400µls of complete antibiotic free 10% NCS -DMEM was then added to the mixture to give a total volume of 500µls. The transfections were performed by replacing medium in each culture well with 500µls

of the transfection complexes in complete medium. The cells were then incubated at 37 ºC with 5% CO_2. 6 hours after transfection, the medium in each well was replaced with normal antibiotic-free growth medium containing serum. Transfection efficiency was assessed by fluorescence microscopy 24 hours after transfection

3.2.3.c. MYO1C and negative control siRNA transfection

Cells were grown in 10% NCS – DMEM for 24 hours in 12 well plates. The transfection reagents were prepared as described in 3.2.3.c. 2µls of DharmaFECT 1 transfection reagent was added to 98µls of serum free medium and incubated for 5 minutes at room temperature. 50µls of MYO1C (ON-TARGETplus SMART pool, human MYO1C) {Thermo Scientific} or negative control (ON-TARGETplus Non-targeting Pool) {Thermo Scientific} siRNA (2µM) was added to 50µls of serum free medium. The transfection reagent/serum free medium was added to the siRNA/serum free medium and incubated at room temperature for 20 minutes to allow transfection or RNA-induced silencing complexes (RISC) to form. 800µls of antibiotic free complete medium was added to the mixture. For transfection to take place, medium in each well of the 12-well plate was replaced with 1000µls of transfection complexes in antibiotic-free complete medium. The cells were then incubated at 37 ºC with 5% CO_2. 6 hours after transfection, the medium in each well was replaced with normal antibiotic-free growth medium containing serum to reduce the toxicity caused by the transfection reagents. The negative control siRNA is a non-targeting sequence for the human genome but is incorporated in the RISC that are required for silencing.

24 hours after the first transfection, a second identical transfection was performed to increase the amount of suppression. Myosin suppression was assessed

by indirect immunofluorescence at 24, 48 and 72 hours following the second transfection. Samples for 2D FISH and indirect immunofluorescence staining were fixed after 48 hours as in 2.2.2.a following the final transfection.

3.2.4. 2D-FISH, 3D-FISH and indirect immunofluorescence:

2D-FISH and 3D-FISH were performed using whole chromosome paints and analysed as in section 2.2.2. Indirect immunofluorescence was performed for pKi-67 as in section 2.2.2.e.

3.3. Results:

3.3.1. Alteration in chromosome territory position – a rapid response to serum withdrawal:

In order to determine when the genome is reorganised upon exit from the cell cycle and the rate of the response to removal of growth factors, actively proliferating young cultures of primary HDFs were cultured in 10% NCS-DMEM for at least 2 days following withdrawal of serum by incubation in 0.5% NCS-DMEM. Samples were taken at 0, 5, 10, 15 and 30 minutes post-serum withdrawal for fixation and positions of chromosome 10, 13, 18 and X territories in at least 50 interphase nuclei were determined by 2D-FISH and erosion analysis (Figure 2.1.), for each time point. Graphs in figure 3.1.A-T display the locations of each of these chromosomes at each time point.

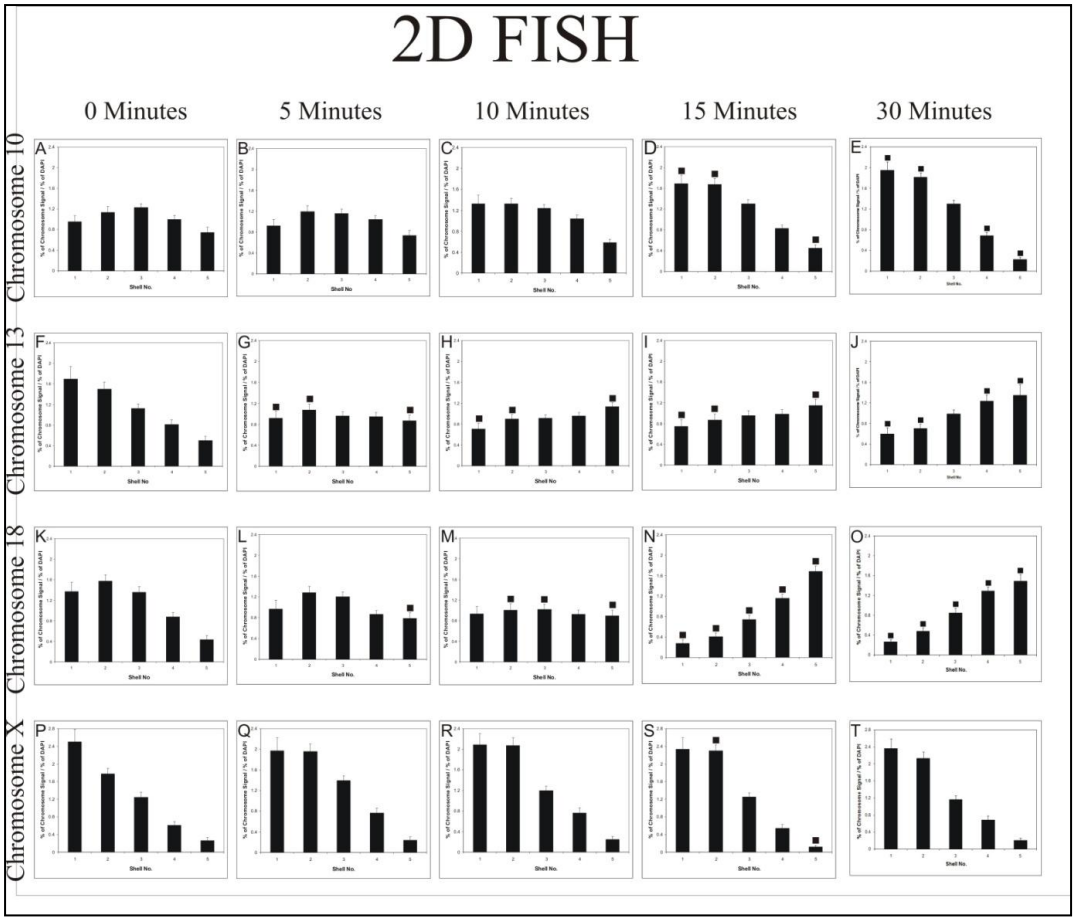


Figure 3.1. Rapid repositioning of chromosomes following removal of serum:

The nuclear locations of human chromosomes 10 (panels A - E), 13 (panels F – J), 18 (panels K – O) and X (panels P – T) were analysed in normal fibroblast cell nuclei fixed for 2D-FISH after incubation in medium containing low serum (0.5%) for 0, 5, 10, 15 and 30 minutes. The filled-in squares indicate significance difference (P<0.05) when compared to control proliferating fibroblast cell nuclei (0 minute time point) (A, F, K and P) as assessed by unpaired, unequal variances two – tailed students *t*-test.

The histograms in figure 3.1. reveal that chromosomes 13 (Figure 3.1. F, I and J) and 18 (Figure 3.1. K, N and O) have moved from the nuclear periphery to the nuclear interior within 15 minutes after incubation in medium containing low serum. Histograms at each time point reveal that both these chromosomes show an intermediate-type nuclear positioning at the earlier time points (5 and 10 minutes) (Figure 3.1.G, H, L and M), indicating the repositioning of chromosome territories

with time. On the other hand, chromosome 10 has repositioned from an intermediate nuclear location (similar to that in proliferating cells) to a peripheral nuclear location (Figure 3.1. A – E), within 15 minutes of incubation in low serum. Chromosome X has not become repositioned in cells incubated with low serum at any of the time-points studied, as expected from previous studies (Boyle et al., 2001, Bridger et al., 2000).

To assess whether chromosome movement that is observed by 2D-FISH assay following serum withdrawal can be replicated by a 3D-FISH assay, samples were collected after 0 and 15 minutes post serum withdrawal. Cells were then fixed in order to maintain the 3-dimensional structure and subjected to a 3D-FISH assay. The distance between the centre of chromosome territories and the nearest nuclear edge were then measured using Imaris software, for at least 20 images captured by confocal laser scanning microscopy (CLSM).

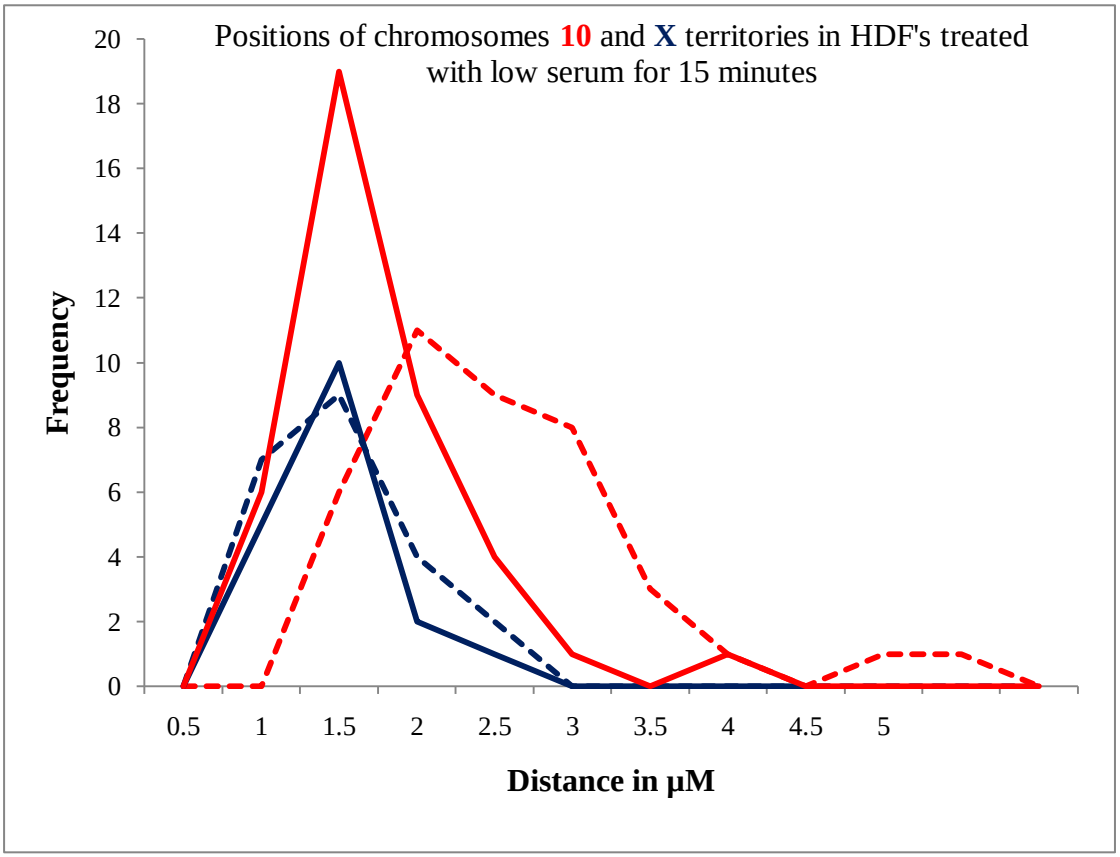


Figure 3.2. 3D-FISH analyses demonstrating rapid chromosome repositioning following serum withdrawal: Position of chromosomes 10 (red line) and X (blue line) territories was assessed using 3D-FISH analyses after incubation in medium containing low serum for 0 (dashed lines) and 15 minutes (solid lines) (panel U).

Similarly, in 3D-FISH analyses by CLSM, hybridisation signals for chromosome 10 were observed in an intermediate location within proliferating cells (0 minutes) (Figure 3.2.), whereas after 15 minutes post serum removal, these signals were found to be localised at the nuclear periphery in most of the nuclei (Figure 3.2.). Hybridisation signals for chromosome X were found located at the nuclear periphery before and after serum removal (Figure 3.2).

In order to address this re-positioning of chromosome territories following serum removal is not due to change in shape of the nuclei, comparisons were made between the shapes of nuclei before and after removal of serum. At least 50 nuclei

per sample were opened in Scion image software and the area, perimeter, major axis and the minor axis was measured for each nucleus. Different attributes of shape such as form factor, roundness, compactness and the aspect ratio were calculated using the formulas below (J.C.Russ).

$$Form\ Factor = (4\pi \times Area) \div (Perimeter)^2$$

$$Roundness = (4 \times Area) \div (\pi \times Maximum\ diameter^2)$$

$$Aspect\ Ratio = Major\ axis \div Minor\ axis$$

$$Compactness = \sqrt{\{(4 \div \pi) \times Area\}} \div Major\ axis$$

Average form factor, roundness, aspect ratio and compactness for HDFs before and after serum removal were then plotted as a graph (Figure 3.3.)

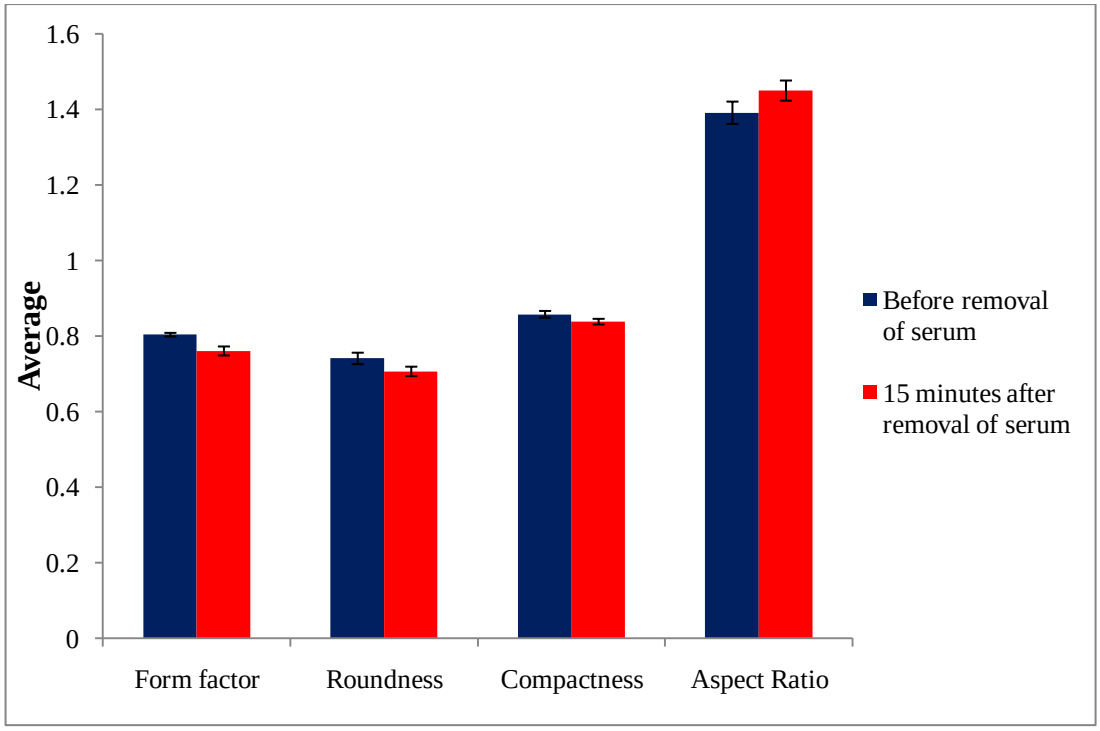


Figure 3.3. Shape analysis for HDFs before and after serum withdrawal: HDFs were cultured in 10% NCS-DMEM medium and then subjected to serum withdrawal by incubation in medium containing low serum for 15 minutes. Samples were collected before and after serum withdrawal, fixed with methanol:acetic acid (3:1) and mounted in DAPI. Various factors that measure the shape of the nucleus including form factor, compactness, roundness and the aspect ratio was measured for at least 50 nuclei per sample. The average for each of these quantities is plotted on the graph. Error bars represent standard error of mean. Unpaired, unequal variances two-tailed students *t*-test was used to determine statistical differences.

There was no statistical differences in form factor, roundness, compactness and aspect ratios between nuclei of HDFs before and after removal of serum (Figure 3.3.), thus suggesting no change in shape of the nuclei within 15 minutes upon withdrawal of serum. In addition to this the volume of DNA in each of the five shells of the eroision analysis script shows negligible difference in HDF nuclei before and after removal of serum (Figure A1).

These data thus demonstrate that whole chromosomes 10 and 18 territories undergo repositioning within 15 minutes upon removal of serum.

3.3.2. Restoration of proliferative chromosome position following re-stimulation of quiescent cells:

Re-positioning of chromosomes 10, 13 and 18 following serum removal is a rapid response. A previous study has demonstrated that relocation of chromosome 18 from the nuclear interior in G_0 cells to the nuclear periphery in serum re-stimulated cells took 30+ hours and appeared to require cells to rebuild their nuclear architecture following a mitotic division (Bridger et al., 2000). In order to recapitulate these data for chromosome 18 and to detect if the same was true for chromosome 10, young proliferating HDFs were grown in 10% NCS for at least two days, incubated in low serum (0.5% NCS) for seven days and then re-stimulated with fresh medium containing 10% DMEM-NCS. Samples were collected at 0, 8, 24, 32 and 36 hours after re-stimulation with 10% DMEM-NCS. Nuclear positions of chromosomes 10, 18 and X were determined by standard 2D-FISH assay and erosion analysis script. Graphs in figure 3.2. displays the positions of chromosome 10, 18 and X territories at these time points and also in young proliferating cells (controls).

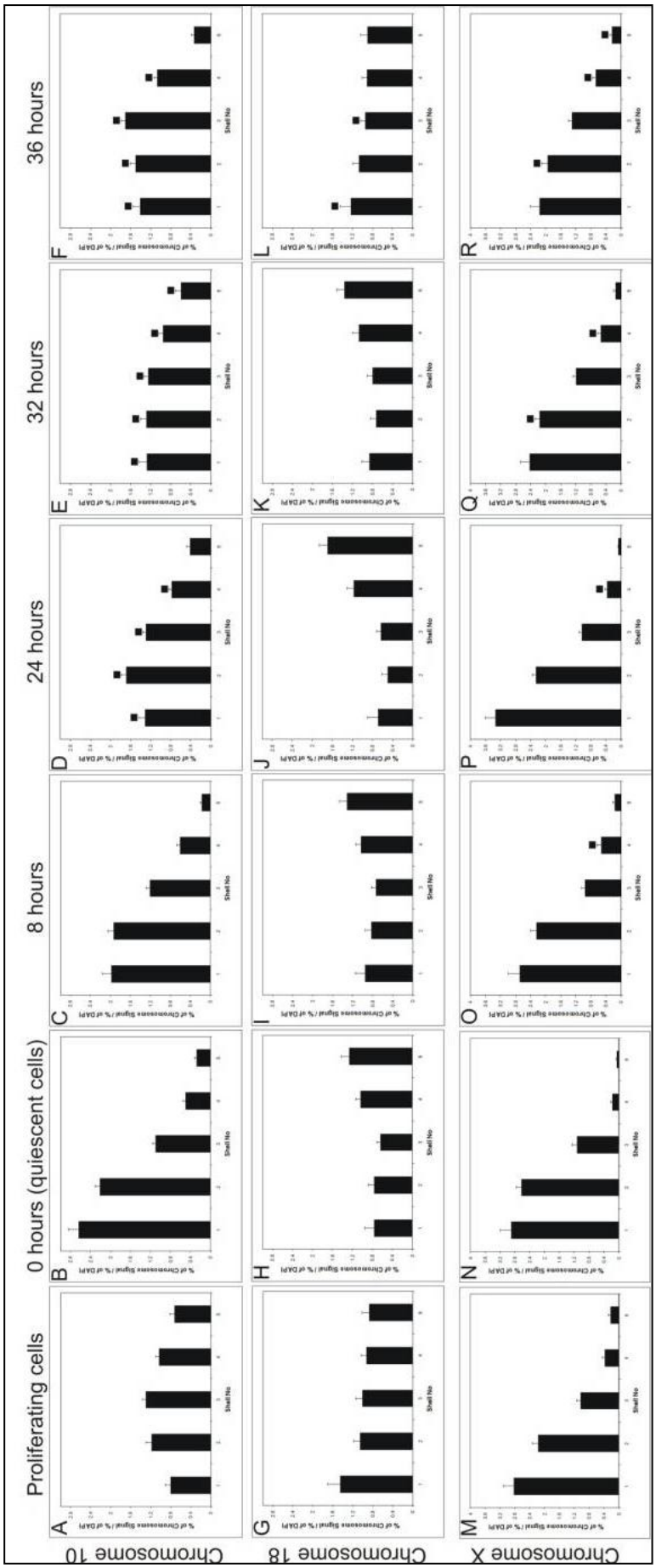


Figure 3.4. Restoration of proliferative chromosome position following re-stimulation of quiescent cells.

Figure 3.4. Restoration of proliferative chromosome position following re-stimulation of quiescent cells: The relocation of chromosomes to their proliferative nuclear location takes more than 24 hours for chromosome 10 and 36 hours for chromosome 18. Proliferating cells (panels A, G, M) were placed in low serum (0.5%) for 7 days (panels B, H, N) and then re-stimulated to enter the proliferative cell cycle with the addition of high serum. Samples were taken at 8 hours (panels C, I, O), 24 hours (panels D, J, P), 32 hours (panels E, K, Q) and 36 hours (panels F, L, R) post-re-stimulation. The graphs display the normalised distribution of chromosome signal in each of the five shells, shell 1 being the nuclear periphery and shell 5 being the inner most region of the nucleus. Statistical differences were measured using unpaired, unequal variances two-tailed students *t*-test. The filled-in squares represent a significant difference ($p<0.05$) for that shell when compared to the equivalent shell for the time 0 data (G_0 data) (B, H and N) for the erosion analysis.

Histograms in figure 3.4. reveal that the re-positioning of chromosome 10 from the nuclear periphery in serum starved cells (Figure 3.4. panel B) to an intermediate nuclear location takes place only at 24-36 hours after re-stimulation of G0 cells with 10% NCS (Figure 3.4. panels D-F). The study was also able to reiterate that chromosome 18 requires almost 36 hours (Figure 3.4. panel L) after re-stimulation, to return to the nuclear periphery. Although chromosome X remains at the nuclear periphery there is a slight change in the distribution of chromosome X at 32-36 hours (Figure 3.4.Q-R).

In order to determine whether chromosome repositioning following serum re-stimulation requires cells to undergo mitosis (Bridger et al., 2000), mitotic index for all time points, i.e. 0, 8, 24, 32 and 36 hours post re-stimulation with serum was determined. At least 1000 nuclei in total were counted at each time point and the fraction of cells undergoing mitosis was plotted against time (Figure 3.5.)

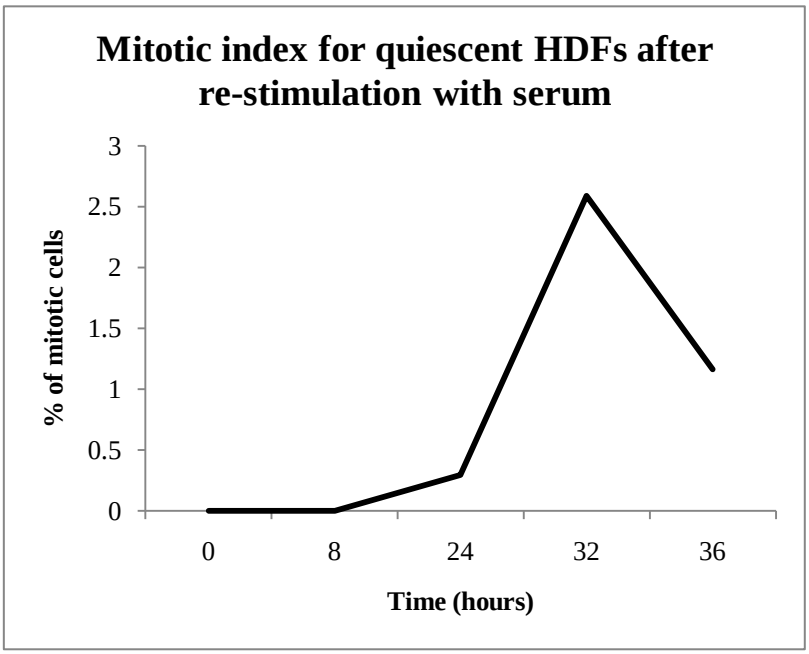


Figure 3.5. Mitotic index for quiescent HDFs after re-stimulation with serum: HDFs were induced to undergo quiescence by incubation in low serum for 7 days, following which the cells were re-stimulated with fresh serum. Samples collected after 0, 8, 24, 32 and 36 hours after addition of serum, fixed with methanol:acetic acid (3:1) and mounted in DAPI. At least 1000 cells were counted per sample and the fraction of cells undergoing mitosis was determined. The graph displayed above displays the percentage of mitotic cells (y-axis) plotted against time after serum re-stimulation (y-axis).

No mitotic cells were detected during the first 8 hours after re-stimulation of quiescent HDFs with serum (Figure 3.5.). A small fraction of mitotic cells (0.29%) were observed after 24 hours post addition of fresh serum, while nearly 2.6% cells underwent mitoses thus showing a peak for mitotic index at 32 hours post serum re-stimulation (Figure 3.5.) . After 48 hours of re-stimulation, the fraction of mitotic cells detected in culture decreased to 1.16% (Figure 3.4.).

From the data presented in section 3.3.1 and this section, it appears that there is a rapid response to the removal of growth factors that reorganises the whole genome within interphase nucleus and this reorganisation is only corrected in

proliferating cells after 24+ hours in high serum, after the cells have passed through mitosis.

3.3.3. Rapid chromosome movement following serum starvation is an energy dependent process:

Chromosome positioning data in cells incubated in serum deprived conditions reveal that chromosomes change their location within 15 minutes of incubation in low serum (See section 3.3.1). Such rapid repositioning of whole regions of the genome implies that this movement may be an active and an energy dependent process.

To test this hypothesis, cells were treated with inhibitors of ATPase (oubain octahydrate), GTPase (AG10) and a combination of both and then incubated them in serum deprived medium for 15 minutes. To ensure that the position of the chromosome territories was not a result of the treatment with the inhibitor, chromosome positioning was performed in a set of internal controls. The cells that served as internal controls were only treated with the inhibitors with no incubation in low serum (Figure 3.6D).

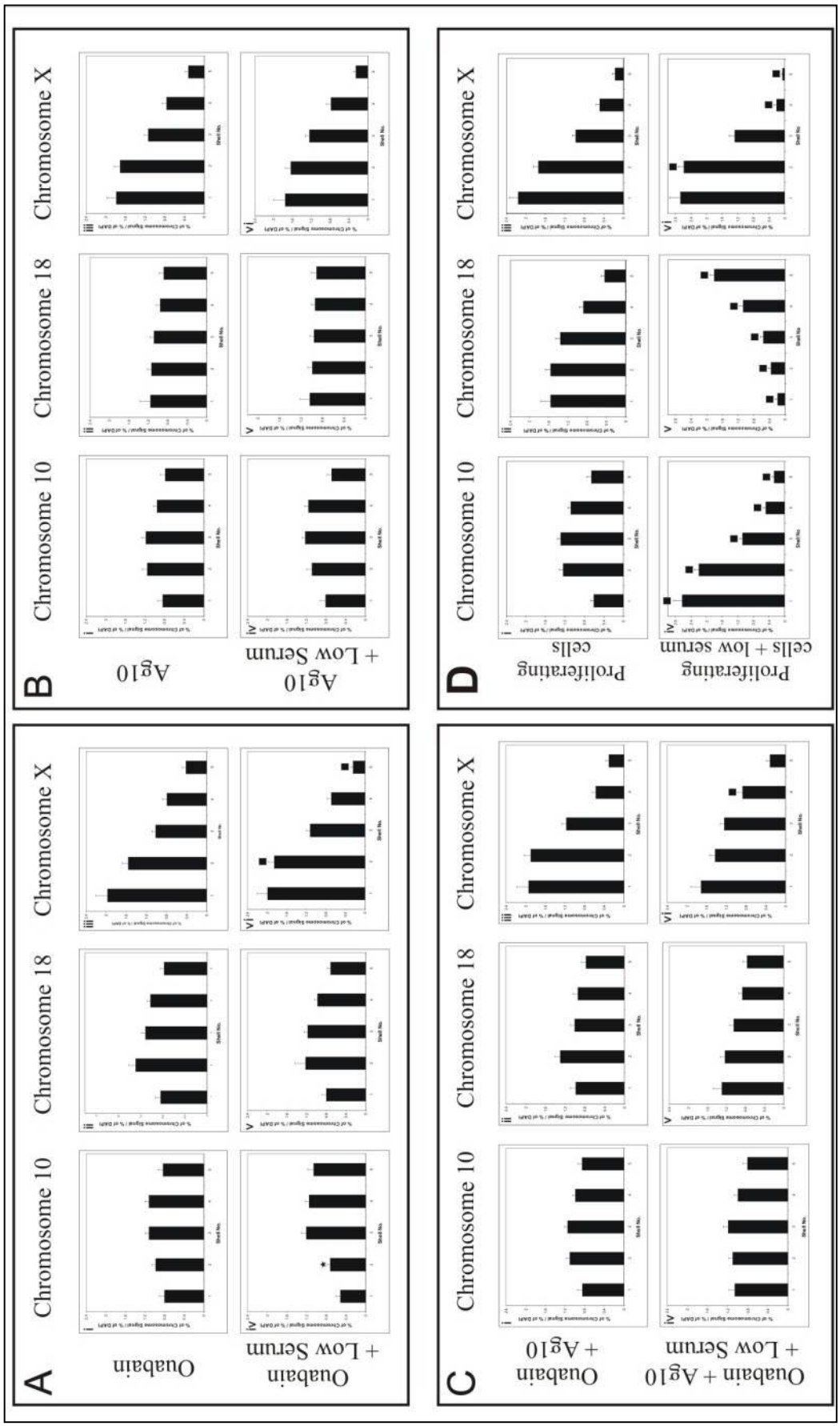


Figure 3.6. Chromosome repositioning requires energy.

Figure 3.6. Chromosome repositioning requires energy: The relocation of human chromosomes 10 and 18 after incubation in low serum is energy dependent. The nuclear location of human chromosomes 10, 18 and X were determined in normal human proliferating cell nuclei without any treatment (D), treated with ouabain (ATPase inhibitor) (A), AG10 (GTPase inhibitor) (B) or a combination of both (C) prior to and after incubation in low serum for 15 minutes. The error bars show standard error of mean. Unpaired, unequal variances two-tailed students *t*-test was used to determine statistical differences in locations of chromosomes 10 and X territories before and after removal of serum. The filled in squares indicate a significant difference ($p<0.05$) to cells treated only with the inhibitor.

Chromosome 10 occupies an intermediate position (similar to that in normal proliferating cells (Figure 3.6. panel Di) in cells treated with either, ouabain (Figure 3.6. panel Ai), oubain and low serum (Figure 3.6. panel Aiv), AG10 (Figure 3.6. panel Bi), AG10 and low serum (Figure 3.6. panel Biv), or a combination of both ouabain and AG10 (Figure 3.6. panel Ci) or ouabain, AG10 and low serum (Figure 3.6. panel Civ). Similarly, in all the cells treated with the above inhibitors with or without low serum, chromosome 18 occupies a peripheral location (Figure 3.6. panels A –C ii and v). Chromosome X occupies a peripheral location at all times, as anticipated (Figure 3.6. panels A-C iii and vi). Positions of chromosomes 10, 18 and X in control proliferating fibroblasts at 0 and 15 minutes after serum removal are shown in figure 3.6. panel D.

HDFs cultured on sterile glass slides, treated with GTPase inhibition AG10 and fixed in order to preserve the 3-dimensional structure, were subjected to a 3D-FISH assay to determine the positions of chromosome 10 and X territories before and after incubation in low serum for 15 minutes. Images of at least 20 nuclei per sample were captured using CLSM and the distance between the centre of each chromosome territory to the nearest nuclear edge was measured using Imaris

software. Graph displaying the distance of chromosome 10 and X territories before and after serum removal for 15 minutes in HDFs treated with GTPase inhibitors, is depicted in figure 3.7.

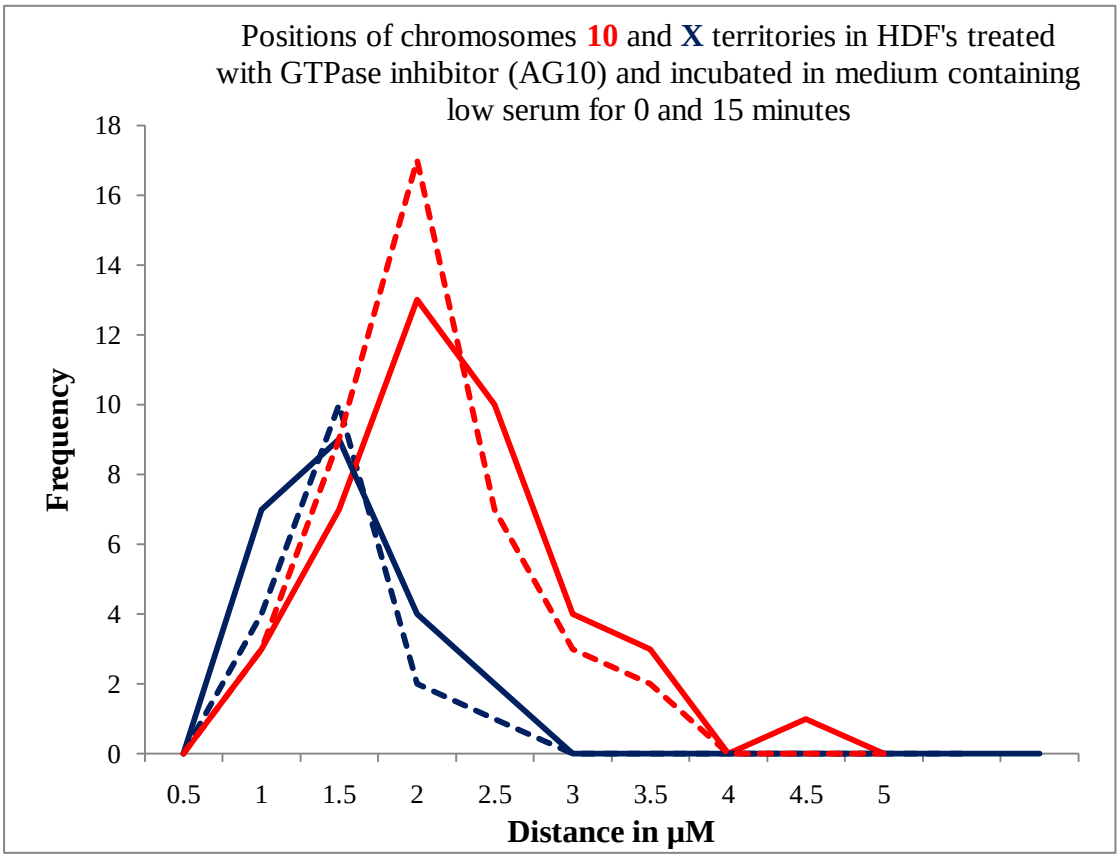


Figure 3.7. 3D-FISH analyses demonstrating that repositioning of chromosomes following serum removal requires energy: Position of chromosomes 10 (red lines) and X (blue lines) territories was determined using 3D-FISH assay in cells treated with AG10 and incubated in low serum for 0 (dotted lines) and 15 minutes (solid lines). Distance between the centre of the chromosome territory and the nearest nuclear edge (x-axis) was measured.

3D-FISH analyses revealed that chromosome 10 territories are localised in an intermediate location within interphase nuclei of HDFs treated with GTPase inhibitor before and after incubation in low serum for 15 minutes (Figure 3.7, red lines). Hybridisation signals for chromosome X territories localised at the nuclear periphery

in cells treated with AG10 and incubated in low serum for 0 and 15 minutes (Figure 3.7, blue lines).

Therefore, the chromosomes (chromosome 10 and 18) that showed movement in response to serum deprivation do not do so when either the enzyme ATPase, GTPase or both are non-functional in the nucleus. Since these enzymes are involved in the break down of ATP and GTP molecules which then releases energy, it can be extrapolated that the rapid response of chromosomes territories to serum deprivation is an energy driven or an energy dependent process.

3.3.4. Effect of microtubule polymerisation inhibition on movement of whole chromosome territories:

Since chromatin is known to be closely associated with microtubules and these are the filaments that are involved in segregation of chromatin during mitosis, it was postulated that these filaments might be involved in the movement of whole chromosome regions even in the interphase nucleus. Thus, to test this further, young fibroblasts were treated with 1µg/ml of microtubule polymerisation inhibitor, colcemid, for 1 hour. One set of these cells was then incubated with medium containing low serum for 15 minutes and the other set was used as a control to ensure that colcemid itself did not have an effect on the chromatin repositioning in the nucleus. 2D-FISH was then performed on these cells treated with colcemid before and after incubation with low serum using whole chromosome paints for chromosomes 10, 18 and X. But when imaging was performed on these cells, it was observed that the chromatin in cells treated with colcemid and low serum was highly disrupted and these nuclei appeared damaged (Figure 3.8. E - H). Moreover, the chromatin in these nuclei seems to be different than in normal fibroblasts, the edges

of the nuclei were not smooth and some nuclei appeared to have undergone tearing (Figure 3.8. F and G). The treatment was repeated two more times and the same results were seen. Also, to ensure that the concentration of colcemid was not too high, images were taken for cells treated with just colcemid (Figure 3.8. A – D) and these nuclei did not appear damaged, were oval in shape and the chromatin distribution in these nuclei was very similar to normal proliferating cells. Also, the number of cells in culture after treatment with colcemid and low serum decreased as compared to the number of cells in culture after treatment with only colcemid.

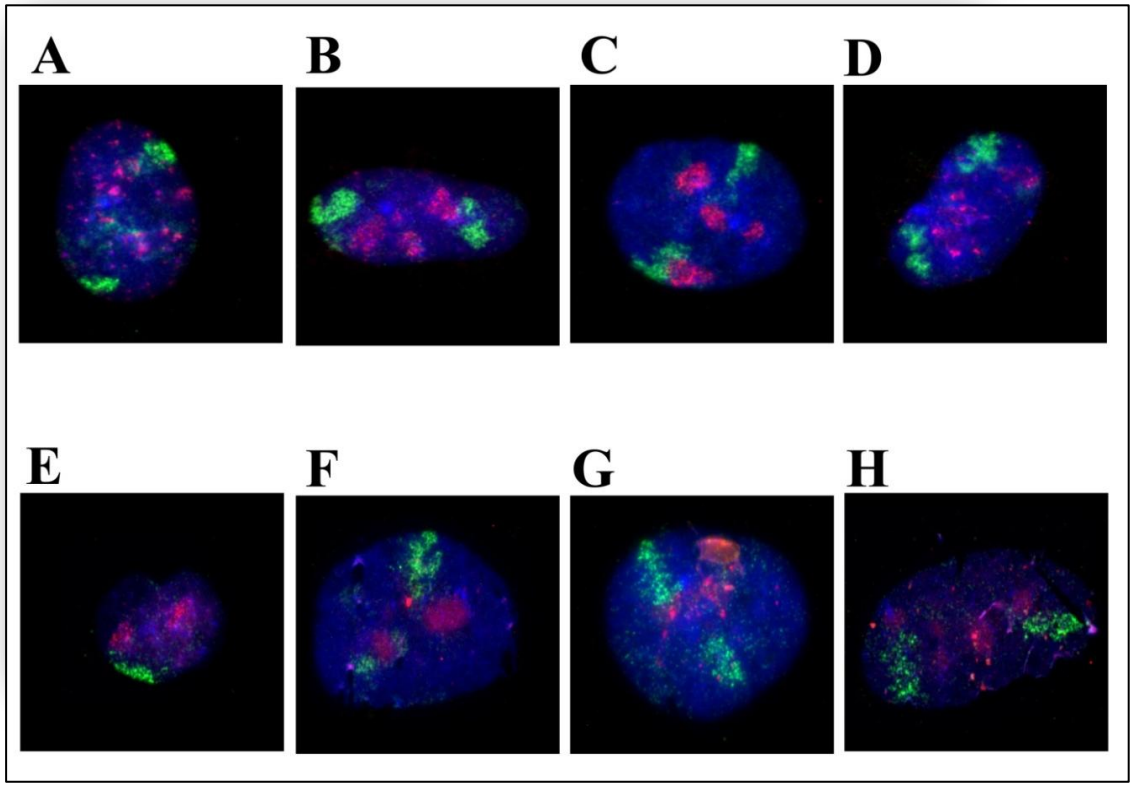


Figure 3.8. Representative images of nuclei of cells treated with colcemid: HDFs cultured in 10% NCS-DMEM were treated with 1µg/ml of colcemid for an hour and then incubated in medium containing low serum. Samples were collected for cells treated with colcemid before and after incubation in low serum, fixed and subjected to 2D-FISH analyses using chromosome 10, 18 and X painting probes. Panel A-D shows representative images of interphase nuclei of cells treated with colcemid before incubation in low serum, while panel E-F displays representative images of interphase nuclei of cells treated with colcemid and then incubated in low serum for 15 minutes.

These data, thus suggest that normal HDFs were intolerant to a combination of colcemid and low serum in culture and may lead to disorganisation of chromatin.

3.3.5. Rapid chromosome movement following serum starvation requires nuclear actin and myosin:

To test if nuclear motors were involved in movement of chromosomes 10 and 18 upon serum removal; normal 2DD fibroblasts were treated with a range of actin and myosin polymerization inhibitors and incubated in low serum for 0 and 15 minutes before performing a standard 2D-FISH analysis (Fig 3.9. A-E).

Figure 3.9. Chromosome repositioning requires nuclear myosin and actin:

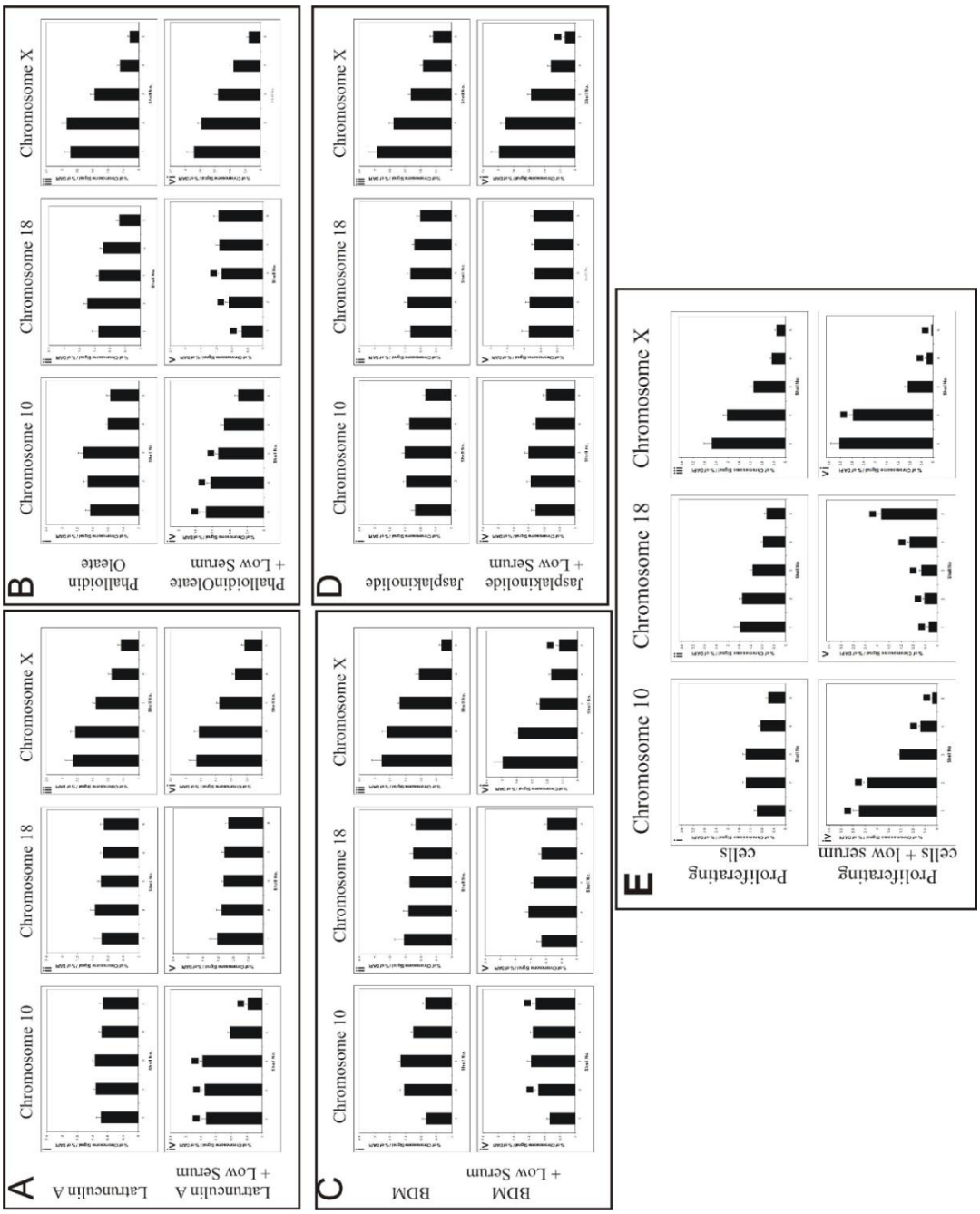


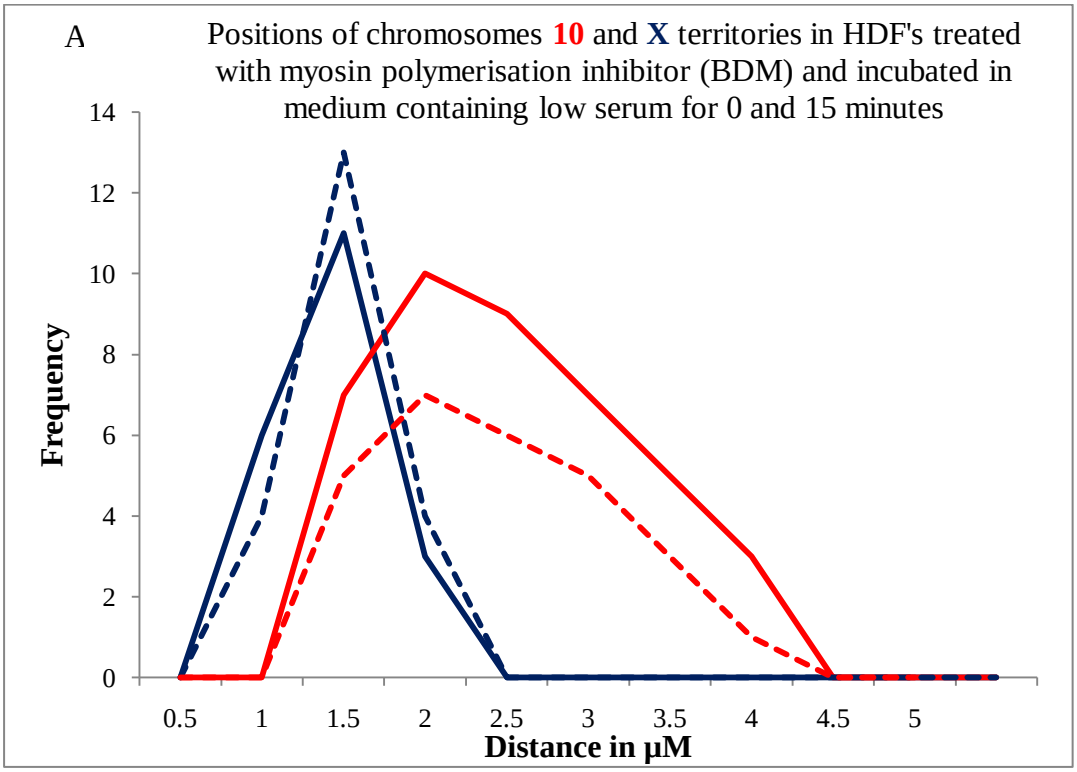
Figure 3.9. Chromosome repositioning requires nuclear myosin and actin: The relocation of human chromosomes 10 and 18 after incubation in low serum is myosin and actin dependent. The nuclear location of chromosome 10, 18 and X were determined in normal human prolifrating cell nuclei treated with latrunculin A and phalloidin oleate (inhibitors of actin polymerisation) (panels A and B) and BDM (inhibitor of myosin polymerisation) (panel C), jasplakinolide (affects both actin and myosin) (panel D) and without any inhibitor treatment (E) before and during incubation in low serum for 15 minutes. Panel E displays the positions of chromosomes 10 and X in HDFs without treatment with any inhibitor before and after 15 minutes incubation in low serum. The error bars show standard error of mean. The stars indicate a significant difference ($p<0.05$) to cells treated only with the inhibitor.

In cells treated with latrunculin A and low serum, chromosome 10 occupies an intermediate nuclear location with a slight repositioning within the same nuclear compartment (Figure 3.9.Aiv) and chromosome 18 occupies a peripheral nuclear location (Figure 3.9.Av) which for both chromosomes is similar to their locations in normal proliferating cells (Figure 3.9.Eiv and v). The same is true of cells treated with BDM and jasplakinolide (Figure 3.9.C - E). Chromosome X, as expected, remains at the nuclear periphery at all times post treatment (Figure 3.9.A-E iii and vi).

In the cells treated with phalloidin oleate (Figure 3.9.B), chromosome territories do respond to low serum and thus exhibit movement when incubated in low serum for 15 minutes. This is not surprising as phalloidin does not penetrate the nucleus unless cells are treated with DMSO; and hence it would not inhibit polymerisation of nuclear actin resulting in no repositioning of chromosome territories.

Positions of HSA 10 and X were further determined using 3D-FISH analyses in HDFs treated with BDM and Phalloidin oleate before and after withdrawal of serum for 15 minutes.

Figure 3.10. 3D-FISH analyses demonstrating positions of chromosome 10 and X territories in HDFs treated with actin and myosin polymerisation inhibitors before and after serum removal.



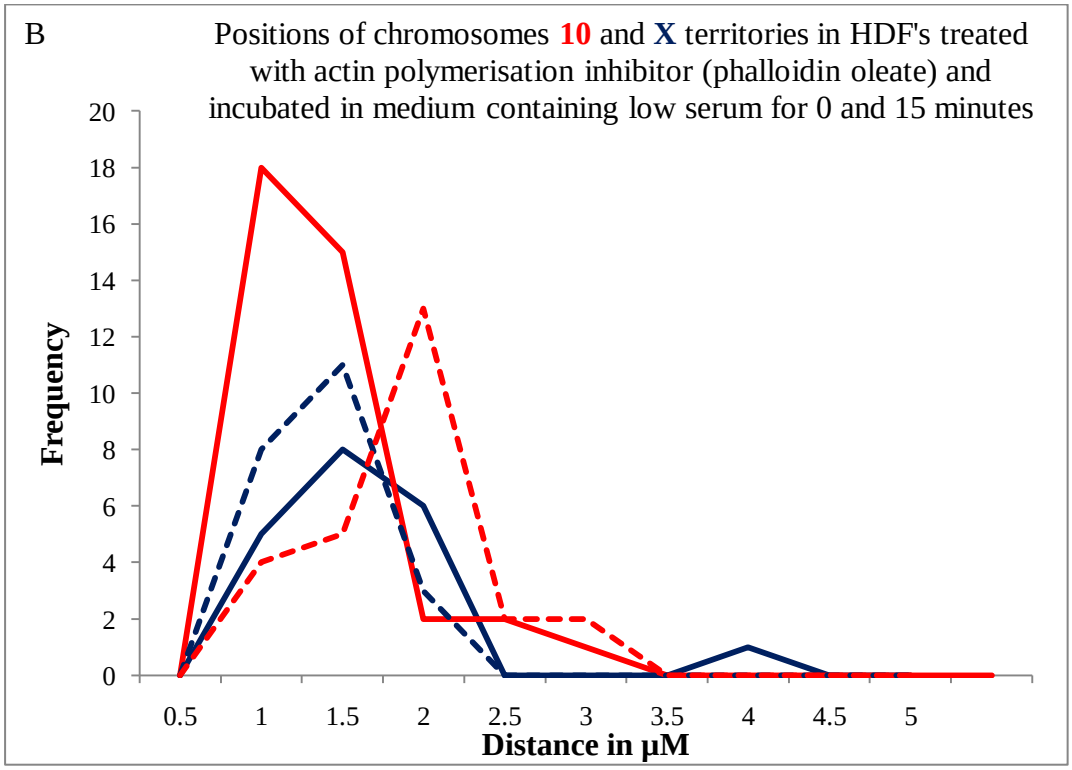


Figure 3.10. 3D-FISH analyses demonstrating positions of chromosome 10 and X territories in HDFs treated with actin and myosin polymerisation inhibitors before and after serum removal: Position of chromosomes 10 (red lines) and X (blue lines) territories was determined using 3D-FISH assay in cells treated with phalloidin oleate (actin polymerisation inhibitor) (A) and BDM (myosin polymerisation inhibitor) (B) following incubated in low serum for 0 (dotted lines) and 15 minutes (solid lines). Distance between the centre of the chromosome territory and the nearest nuclear edge (x-axis) was measured.

Thus 3D analyses reveal that the hybridisation signals for chromosome 10 territories coincide with the nuclear periphery following serum removal in cells treated with phalloidin oleate (Figure 3.10. panel B - red solid line) unlike its intermediate location in these HDFs before serum removal (Figure 3.10. panel B - red dotted line). Chromosome 10 territories localise in an intermediate nuclear location in HDFs treated with BDM before (Figure 3.10. panel A - red dotted line) and after (Figure 3.10. panel B - red solid line) removal of serum. Territories of chromosome X remains at the nuclear periphery in cells treated with BDM (Figure 3.10. panel A blue lines) and phalloidin oleate (Figure 3.10. panel B blue lines) before (dotted lines) and after (solid lines) removal of serum.

These results suggest that the movement of whole chromosomes in response to serum deprivation requires actin polymerization and myosin activity. In other words, the nuclear motors, consisting of nuclear actin and myosin are some how involved in the repositioning of whole chromosome territories in the nucleus.

3.3.6. Movement of chromosome territories following serum removal depends on nuclear myosin 1β:

Rapid, energy dependent chromosome repositioning appears to be dependent on nuclear motors comprising of nuclear actin and nuclear myosin. Various studies have shown presence of several different myosin iso-forms in mammalian cell nuclei, including myosin I, myosin IV, myosin Va, myosin VI, myosin 16b, each isoform being associated with a specific role within the cell nucleus. A recent study (Chaung et al., 2006) and a model proposed by Hoffmann et al., 2006 suggest involvement of nuclear myosin Iβ isoform in movement of gene loci and chromatin regions around the nucleus. This led us to hypothesis that this myosin isoform – nuclear myosin Iβ is involved in the repositioning of chromosome territories following serum removal.

To test this hypothesis, we suppressed the message of MYO1C gene that encodes NMIβ by siRNA and assessed the positions of chromosomes 10, 18 and X using standard 2D-FISH analysis.

3.3.6.a. Optimisation of transfection efficiency for MYO1C siRNA

siGLO transfection indicators are fluorescent oligonucleotides that localise in the nucleus thus permitting visual assessment of uptake into mammalian cells. To optimise the seeding density for achieving maximum transfection efficiency normal HDFs were transfected with siGLO. Samples were collected 24 hours after transfection and the percentage of siGLO positive cells were determined.

Table 3.2. Optimisation of seeding density for high transfection efficiency: The table below summarises the transfection efficiency of siGLO in HDFs seeded at different cell densities.

Cell Density	Number of siGLO positive cells	Number of siGLO negative cells	Total number of cells counted	% transfection efficiency
4×10^3	67	31	98	68.36
5×10^3	74	28	102	72.25
6×10^3	148	62	210	70.47
7×10^3	148	58	206	71.47
9×10^3	155	49	204	75.9

Thus, the transfection efficiency for siGLO was satisfactory when normal HDFs were seeded at a density between 5×10^3 and 9×10^3.

To further optimise the transfection for MYO1C siRNA and to determine the time point when at which maximum suppression was measured, double transfections were performed with MYO1C targeting and negative control siRNA on normal HDFs. Hence HDFs were transfected with each of the sequences and 24 hours after another aliquot of transfection complexes containing MYO1C targeting or negative control siRNA constructs were added to the cells in culture. Samples were fixed for indirect immunofluorescence at 24, 48 and 72 hours post the final transfection and stained for anti-NMIβ (one of the products of MYO1C gene).

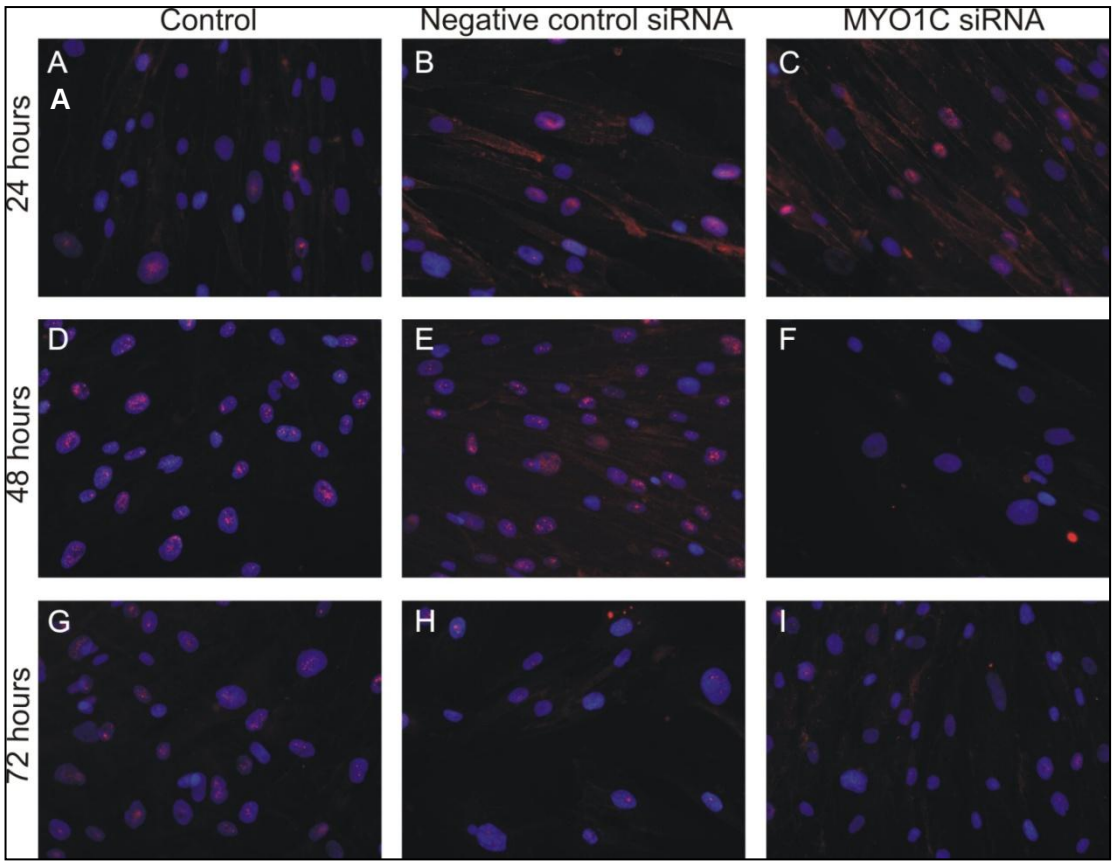


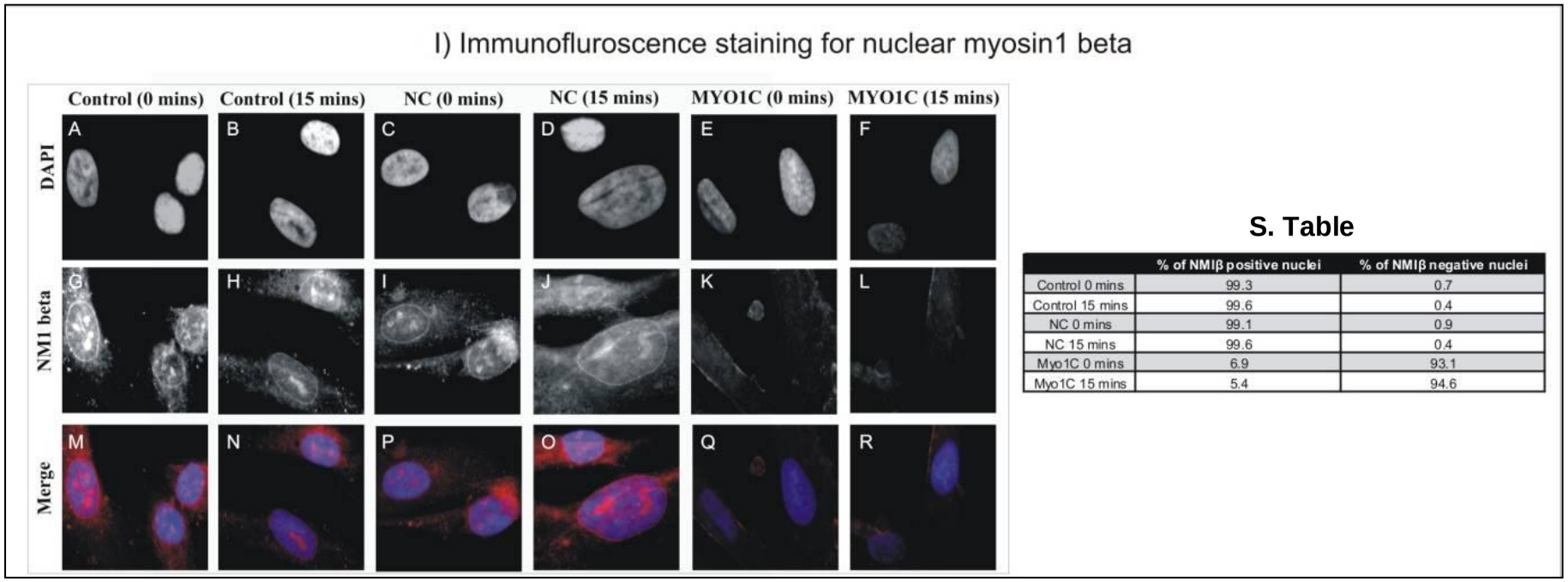
Figure 3.11. Optimisation of transfection frequency: Normal HDFs were transfected with negative control and MYO1C siRNA twice (double transfection). Samples were collected after 24 (A-C), 48 (D-F) and 72 (G-I) hours post final transfection. Indirect immunofluorescence staining for nuclear myosin 1β was performed on these samples.

Several panels of images (one example of which is shown in figure 3.11) were analysed and it was concluded that maximum protein knockdown for MYO1C was seen at 48 hours post final transfection.

3.3.6.b. Repositioning of chromosome territories following serum removal is inhibited by MYO1C siRNA transfection

To determine whether products of MYO1C gene were required for repositioning of chromosome territories following serum removal, young proliferating HDFs grown at a cell density of 1×10^4 in 12 well plates, were

transfected twice with negative control and MYO1C targeting siRNA. Samples for indirect immunofluorescence staining and 2D-FISH were fixed at 48 hours post final transfection, before and after incubation in low serum for 15 minutes. Young proliferating cells grown simultaneously, where no transfection reagents were added, were considered as controls. Indirect immunofluorescence staining for NMIβ (a product of MYO1C gene) (Figure 3.11.) was then employed on all samples and the transfection efficiency was assessed (See table in figure 3.11.).



S. Table

	% of NMIβ positive nuclei	% of NMIβ negative nuclei
Control 0 mins	99.3	0.7
Control 15 mins	99.6	0.4
NC 0 mins	99.1	0.9
NC 15 mins	99.6	0.4
Myo1C 0 mins	6.9	93.1
Myo1C 15 mins	5.4	94.6

Figure 3.12. Nuclear positions of chromosome 10 and X territories in normal HDFs transfected with siRNA constructs: Normal HDFs were transfected with siRNA for negative control and MYO1C (double transfection) and samples for immunofluorescence staining and 2D-FISH were fixed after 48 hours of the final transfection. Representative images of nuclei stained for NMIβ (red) in control (G, H, M and N), cells transfected with negative control siRNA (I, J, O and P) and in cells transfected with MYO1C targeting siRNA (K, L, Q and R) after 0 and 15 minutes of serum starvation are displayed in the figure above. % of nuclei that are positive of NM1β in controls, cells transfected with negative control siRNA and in cells transacted with MYO1C targeting siRNA are displayed in the table S.

To assess the transfection efficiency, at least 1000 nuclei per sample were counted for the presence or absence of staining for NMIβ. Indeed, the protein NMIβ was knocked down in HDFs that were transfected with MYO1C targeting siRNA sequences as more than 90% of the fibroblasts showed no staining for NMIβ in the nucleus, but there was weak staining for NMIβ in the cytoplasm of these cells (Figure 3.12.). The fraction of cells negative for NMIβ in control HDFs and in HDFs transfected with negative control construct was less than 1% (Figure 3.12.).

Further, in order to determine if interphase chromosome repositioning following serum removal required NMIβ, positions of chromosome 10, 18 and X territories were determined using a 2D-FISH analysis. Graphs displaying the locations of chromosome 10, 18 and X in HDFs transfected with no siRNA, negative control siRNA and MYO1C targeting siRNA are shown in Figure 3.13.

Figure 3.13. Nuclear positions of chromosome 10 and X territories in normal HDFs transfected with MYO1C siRNA constructs:

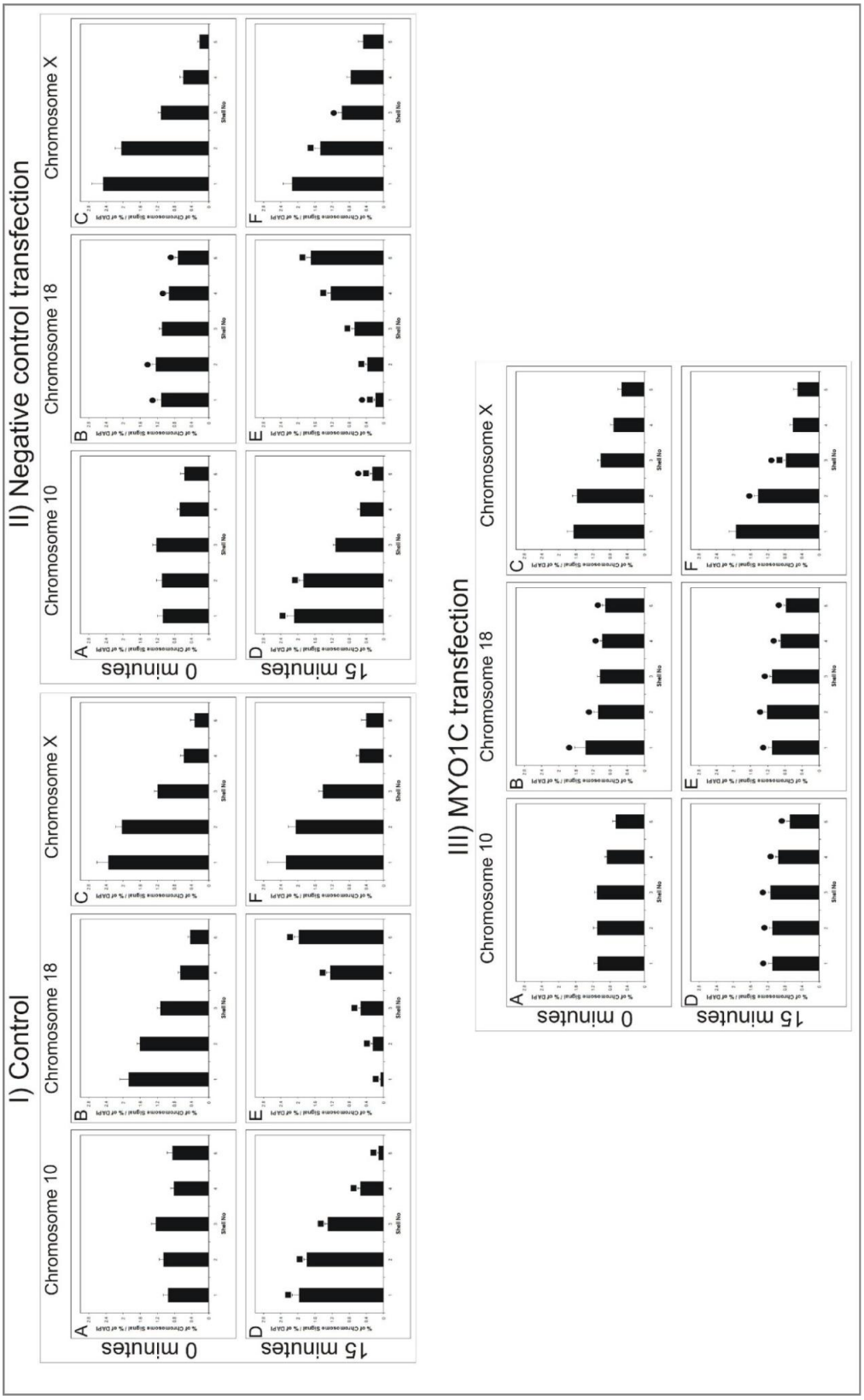


Figure 3.13. Nuclear positions of chromosome 10 and X territories in normal HDFs transfected with MYO1C siRNA constructs: In control HDFs and in HDFs transfected with negative control siRNA chromosome 10 is re-positioned from an intermediate nuclear location (II A and III A) to the nuclear periphery (II D and III D) after 15 minutes of incubation in low serum. Chromosome 18 territories, on the other hand is re-positioned from the nuclear periphery (IIB and III B) to the nuclear interior (II E and III E) after 15 minutes of incubation in low serum in control HDFs and HDFs transfected with negative control siRNA. In HDFs transfected with MYO1C targeting siRNA, chromosomes 10 and 18 (IV) do not show re-positioning following 15 minutes incubation in low serum. Chromosome X remains at the nuclear periphery at all times (IC, F, II C, F, III C, F, IV C and F). Unpaired, unequal variances two-tailed students *t*-tests were performed to assess statistical differences. The filled in squares indicate a significant difference ($p<0.05$) to cells incubated in low serum for 0 minutes and the filled in circles indicate a significant difference ($p<0.05$) to control HDFs.

The hybridisation signals for chromosomes 10 and territories were located in an intermediate nuclear location and at the nuclear periphery respectively, in control HDFs (no siRNA) (Figure 3.13.IA and B), HDFs transfected with both; negative control (Figure 3.13.IIA and B), and MYO1C siRNA (Figure 3.13.IIIA and B), before serum removal. In control HDFs (Figure 3.13.ID and E) and HDFs transfected with negative control siRNA (Figure 3.13.IID and E), chromosome 10 and 18 territories are relocated to the nuclear periphery and within the nuclear interior respectively, upon serum withdrawal. Although, in HDFs transfected with MYO1C targeting siRNA, no re-positioning of chromosome 10 or 18 was observed (Figure 3.13.IIID and E), thus strongly suggesting that NMIβ is required for repositioning of chromosome territories following withdrawal of serum. Chromosome X territories localised at the nuclear periphery at all times (Figure 3.13.I C and F, II C and F and III C and F).

3.4. Discussion:

The results from chapter 2 and some other studies (Meaburn et al., 2007, Bridger et al., 2000, Mehta et al., 2007) indicate that there is a definite alteration in the spatial positions of chromosome within an interphase nucleus as the cell exits the cell cycle to enter a non-proliferating state. How and when the alterations to chromosome positioning occur are two fundamental questions in understanding the role of genome organisation in cell cycle related events. The genome is probably anchored and influenced through a number of interactions with nuclear architecture (Bridger et al., 2007, Mehta et al., 2007, Foster and Bridger, 2005) and so any signalled alterations/modifications to these structures could enable a reorganisation of the position of chromosome territories.

The study in this chapter aims to answer two of these questions. Firstly, to determine when the repositioning of chromosome territories occurs in quiescent cells, serum removal was used as a method to induce quiescence. This method was chosen because it is an easily controllable process where by it is possible to measure time point zero. Thus, analyses from this assay revealed that re-positioning of chromosomes upon serum withdrawal was rapid and complete within 15 minutes of serum removal. After 15 minutes this organisation is in place until the cells are re-stimulated to enter the cell cycle. When serum starved quiescent HDFs (for 7 days) are re-stimulated to enter the cell cycle by the addition of serum, chromosome 10 and 18 (Bridger et al., 2000) are not relocated back to the nuclear periphery until the cells have been through mitosis. The types of repositioning i.e. 1) the rapid response without a nuclear envelope breakdown, and 2) requiring a rebuilding of the nucleus post-mitosis imply that these processes follow different pathways and mechanisms.

To determine how this rapid shift in chromosome position with serum removal is elicited, it was postulated that the repositioning may be either due to detachment of a chromosome from the nuclear periphery due to serum deprivation followed by its transient 'wandering' movement around the nucleus or involvement of an active process which physically causes this movement. The first scenario seemed unlikely with the organisation changing in the time duration of just 15 minutes and also because positions of chromosomes after fifteen minutes are consistent and do not show any intercellular variation. The latter hypothesis and the repositioning of chromosome territories following serum removal being an energy active process was supported by experiments using inhibitors of ATPase and GTPase, two enzymes involved in the process of break down of ATP and GTP to release energy (Figure 3.6. and 3.7).

A recent study demonstrated that forces required to reposition chromosomes within a nucleus are transferred from the cytoplasm via nuclear-envelope bridges made up of SUN proteins and KASH domain proteins (Haque et al., 2006, Chikashige et al., 2007b, Starr, 2009). These forces are generated by dynein along microtubules in the cytoplasm and are transferred to the KASH domain proteins, which are then translated to the nucleoplasm (Chikashige et al., 2007b). Since, microtubules also attach to the chromosomes and are involved in chromosome segregation during mitosis; involvement of microtubules in re-positioning of the chromosomes following serum deprivation was examined. But the results obtained from treating cells with a microtubule inhibitor, colcemid suggested that the combination of colcemid and low serum is probably detrimental to cells, or is somehow disrupting the chromatin organisation, thus implying that microtubules may be required for entry of cells into G_0 state. It might also be the case that the cells

treated with colcemid and low serum cannot attach to the substratum as the microtubule formation is inhibited and there is no serum in the flask. Thus, what exactly happens to the cells when treated with colcemid and low serum needs to be determined and thus the set of experiments on whether microtubules play a role in chromosome territory movement remains inconclusive.

This rapid movement of chromosomes in response to growth factor removal may be elicited through a nuclear motor such as the acto-NMI motor-complex, containing nuclear actin and nuclear myosin I, that has been shown previously to be involved in intra-nuclear movements of chromatin (Chuang et al., 2006, Hofmann et al., 2006a, Dundr et al., 2007). Using inhibitor to polymerization of actin (latrunculin A) and inhibitors to myosin (BDM) and chemicals that stabilise F-actin and disrupt myosin distribution (Jasplakiniloide), the re-localisation of chromosome territories was inhibited in nuclei. Since the drugs used in this study inhibit polymerisation of actin and myosin, it can be indicative that actin and myosin required for repositioning of chromosomes within a cell nucleus has to be in polymeric form. Whether these polymeric actin and myosin molecules in the nucleus resemble the cytoplasmic molecules in its functions still remains unknown. Presence of monomeric forms of actin within the cell nucleus and their involvement of chromosome repositioning cannot be ruled out. This is because in cells treated with jasplakiniloide whereby polymeric forms of actin are stabilised limiting the amount of monomeric form of actin (Holst et al., 2002), repositioning of chromosomes is also inhibited. Whether this is due to limited amounts of monomeric actin in the nucleus or due to disrupted myosin distribution is not clear.

It should be noted that another actin polymerization inhibitor used (Phalloidin oleate) did not inhibit the repositioning in chromosome territories

following serum deprivation. This is important because phalloidin, which is not membrane permeant, has been shown not to bind to nuclear actin unless the cells are treated with DMSO (Sanger et al., 1980), which we had not done. Thus, when cells were treated with phalloidin oleate, this drug would not penetrate the nuclear membrane or would not have been able to inhibit the polymerization of nuclear actin. This would then explain movement of chromosome territories of chromosome 10 and 18 in response to low serum in cells treated with this drug. This is an important finding suggesting that it is indeed actin within the nucleus and not cytoplasmic actin, is required for repositioning of chromosomes.

Very little is known about different isoforms of actin in the nucleus but recent studies have revealed various isoforms of myosin that are present in the nucleus (Hofmann et al., 2006a). Previous studies (Chuang et al., 2006, Hu et al., 2008) and models put forward (Hofmann et al., 2006a) have suggested involvement of a nuclear acto-NMIβ motor, containing nuclear actin and nuclear myosin Iβ in intra-nuclear movement of chromatin. Using an RNA interference technique for MYO1C gene on chromosome 17 that encodes for three isoforms of myosin including NMI, the data in this chapter demonstrates that products of this gene are indeed involved in repositioning of chromosomes following serum removal. One of the products of the gene, myosin 1 beta can be co-transcribed to form either a short cytoplasmic myosin 1 beta or a longer nuclear myosin 1 beta with an additional 16 amino acid at the N-terminal end (Pestic-Dragovich et al., 2000). The additional 16 – amino acid are important for directing this protein into the nucleus (Pestic-Dragovich et al., 2000). Since this is the only gene product of gene *MYO1C* that is nuclear, it can be concluded that at least one of the components of the nuclear motor is nuclear

myosin 1 beta. There is not enough evidence yet to prove that this isoform of nuclear myosin directly binds to nuclear actin, but this is highly likely.

It would be of interest to understand what signalling mechanism is involved in the active nuclear motor dependent movement of chromosomes around the interphase nucleus. Whether or not these signals translated from the cytoplasm involving SUN proteins and KASH domain proteins (Haque et al., 2006, Starr, 2009, Chikashige et al., 2007b) involve nuclear actin or nuclear myosin still remains unclear. The possibility of an intrinsic nuclear signalling mechanism also cannot be ruled out. It would be vital to determine the interactions between nuclear actin and nuclear myosin and other nuclear structures. These interactions might change in a cell-cycle dependent manner and may consequently provide a signal for movement of chromosomes. Whether or not energy and nuclear actoNMIβ are involved in the repositioning of chromosomes following serum re-stimulation of quiescent cells also remains to be unknown.

In summary, rapid energy and nuclear motor dependent chromosome repositioning occurs when fibroblasts undergo quiescence. The chromosome movement assay could be a useful tool to investigate the mechanics of such a nuclear motor.

Chapter 4: Chromosome behaviour in HGPS fibroblasts

- Some of the contents of this chapter are in press for Biochemical Society Transactions under the title "Progeria, the nucleolus and farnesyltransferase inhibitos" – Ishita S Mehta, Joanna M Bridger and Ian R Kill.

- Some of the contents of this chapter are in preparation for Ageing Cell: "Chromosome territory positions and nuclear motors in Hutchison Gilford progeria syndrome patient cells before and after FTI treatment" – Ishita S Mehta, Ian R Kill and Joanna M Bridger.

4.1.Introduction:

Hutchinson-Gilford progeria syndrome (HGPS) is caused due to a mutation in a gene coding for an intermediate filament protein - lamin A. The mutation involves deletion at the C-terminus end of the *LMNA* gene, leading to formation of a truncated protein known as "Progerin." Progerin, unlike normal lamin A is unable to perform the final cleavage step of the post translational modification and hence accumulates within the cell nuclei as a farnesylated protein. Retention of the farnesyl moeity at the C-terminus end of the progerin protein is thought to be the cause leading to various abnormal pathologies observed in the diseased patients, including nuclear abnormalities (McClintock et al., 2006, Bridger and Kill, 2004, Goldman et al., 2004), genomic instability (Liu et al., 2005), disorganised heterochromatin (Scaffidi and Misteli, 2005, Goldman et al., 2004, Columbaro et al., 2005) and alterations to histone methylation regulating heterochromatin (Scaffidi and Misteli, 2005, Shumaker et al., 2006).

Attachment of the farnesyl group at the C-terminus end of lamin A protein requires farnesyltransferase enzyme. In order to reverse the phenotype observed in HGPS patients that is mainly caused due to retention of the farnesyl group, various studies have used chemical inhibitors termed as farnesyltransferase inhibitors (FTIs) which interferes with the function of the enzyme farnesyltransferase and thus inhibits farnesylation of the protein. FTIs, first discovered as potential anti-cancer drugs targeting the *ras* oncogene, are small molecular moieties that inhibit farnesylation of proteins by reversible attachment to the enzyme farnesyltransferase (Meta et al., 2006, Adjei, 2005, Basso et al., 2006). Treatment of HGPS patient cells with FTIs indeed reduces the nuclear deformities (Toth et al., 2005, Capell et al., 2005,

Mallampalli et al., 2005, Glynn and Glover, 2005) and improves wound healing as well as cell migration capacities (Verstraeten et al., 2008) of these diseased cells. In concurrence with results of the treatment observed on patient cells, progeria mice too responded positively to this treatment and displayed improved growth, grip, bone integrity and body weight (Fong et al., 2004) as well as reduction in number of rib fractures (Meta et al., 2006, Yang et al., 2006, Fong et al., 2004, Yang et al., 2005).

One of the shortcomings that FTI treatment has been confronted with, is presence of these drugs within the body may cause alternative post-translational modification of lamin A or progerin. Lamin A and progerin are both alternatively geranylgeranylated by the enzyme geranylgeranyltransferase, when farnesylation is blocked in presence of FTI (Varela et al., 2008). Inhibition of both enzymes, i.e. farnesyltransferase and geranylgeranyltransferase using FTI and geranylgeranyltransferase inhibitor (GGTI) simultaneously, results in accumulation of substantially higher levels of pre-lamin A and reduction in progerin levels (Varela et al., 2008).

The abnormalities observed in cells derived from HGPS patients concur with hypotheses suggesting a putative function of lamin A, along with its binding partners, in interacting with the genome, controlling genome function and organisation (Bridger et al., 2007, Maraldi et al., 2006). Previous studies have indeed demonstrated alterations in chromosome positioning in a number of primary fibroblast lines derived from laminopathy patients (Meaburn et al., 2007), with the nuclear positioning of the chromosomes 13 and 18 within the nuclear interior, alternatively located as compared to control cells. The positioning of these chromosomes in proliferating laminopathy cells is similar to a non-proliferating control cells.

Since HGPS is a premature ageing disorder, displaying phenotypic similarities to normal ageing (Kipling et al., 2004), there is a possibility that these cells, in their proliferative state may have their genome organised like control cells that have entered the state of replicative senescence. Further, evidence suggesting spatial organisation of chromosome regions may be linked to gene expression (Misteli, 2004) makes it of vital importance to be able to distinguish between the two non-proliferating states and to identify whether the chromosome territory organisation in HGPS fibroblasts resemble that of quiescent or senescent control HDFs.

Thus the aim of the study within this chapter is to compare the positions of chromosome territories in HGPS HDFs to that in control HDFs. In addition to this, since lamin A has been suggested to interact with nuclear motor proteins (Mehta et al., 2008), whether or not mutation in *LMNA* gene affects the chromosome repositioning function of nuclear motor (Chapter 3) within HGPS fibroblasts, was assessed. Further, FTI intervention has been used as a tool to try and ameliorate the abnormal chromosome territory organisation and the dysfunctional chromosome territory repositioning response observed in fibroblasts derived from HGPS patients.

4.2. Methods and Materials:

4.2.1. Cell culture:

Control HDFs and HDFs derived from three HGPS patients (cell lines AG11513, AG01972C and AG11498) and limb-girdle muscular dystrophy patient (cell line E358K) (Table 4.1) were cultured in 15% FBS-DMEM with passaging twice every week as in 2.2.1. until they were deemed senescent.

Table 4.1. Characterisation of HGPS cell lines used in the study.

Cell line	Cell type	Mutation	Race	Age	Sex
2DD	Control	N/A	N/A	N/A	N/A
AG11513	Fibroblasts	G608G in *LMNA* gene	Caucasian	8 years	Female
AG01972	Fibroblasts	G608G in *LMNA* gene	Caucasian	14 years	Female
AG11498	Fibroblasts	G608G in *LMNA* gene	Black	14 years	Male
E358K	Fibroblasts	E358K in *LMNA* gene	A kind gift from G. Bonne (Institut de Myologie, France)		

The above cell lines, except 2DD, were obtained from Coriell Repositories.

For chromosome movement experiments after induction of quiescence, cells were incubated in 0.5% FBS for 0, 5, 10, 15 and 30 minutes as in 3.2.1.

Chromosome positions following serum restoration were analysed in HGPS fibroblasts by culturing them in 15% FBS-DMEM for at least 2 days, serum starvation (0.5% FBS-DMEM) for 7 days and restoring serum with incubation in 15% FBS-DMEM. Samples were collected at 0, 8, 24, 32 and 36 hours post serum restoration (See section 3.2.1.).

4.2.2. Treatment with farnesyltransferase I and geranylgeranyltransferase inhibitors:

Farnesylation and prenylation of pre-progerin was inhibited simultaneously or separately by using farnesyltransferase I inhibitor (FTI) and geranylgeranyltransferase inhibitor (GGTI) respectively.

4.2.2.a. FTI and GGTI

Inhibitors for farnesylation and prenylation used in this study: Methyl {N-[2-phenyl-4-N [2(R)-amino-3-mecaptopropylamino] benzoyl]}-methionate, TFA or FTI-277 {Calbiochem}, and 4-[[N-(Imidazol-4-yl)methyleneamino]-2-(1-naphthyl)benzoyl]leucine methyl ester or GGTI-2147 {Calbiochem} were used to inhibit farnesyltrasnferase or geranylgeranyltrasnferase enzymes respectively. The chemical structures of the compounds used are displayed in figure 4.1.

FTI-277 was dissolved in DMSO and stored as 558.5µM stock solution at -20ºC. 531µM stock solution of GGTI-2147 was stored at -20ºC after dissolution in DMSO. Both the inhibitors were protected from light at all times.

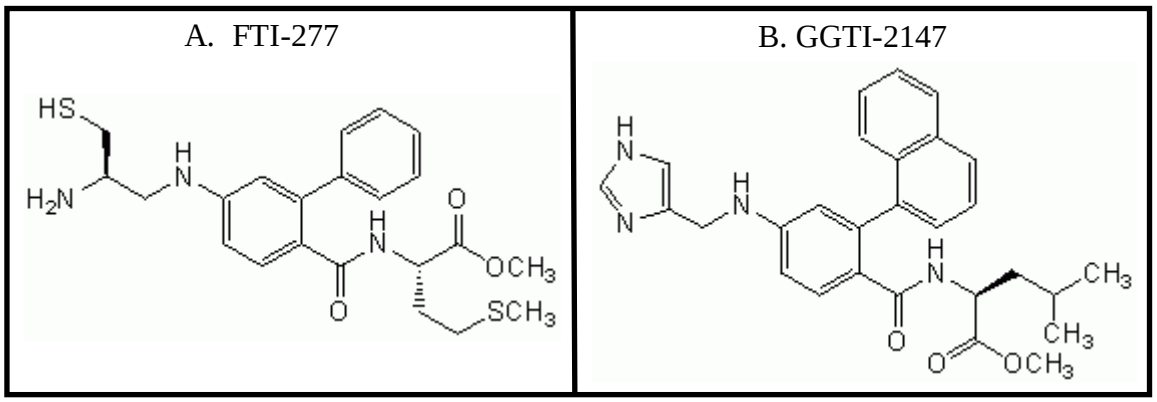


Figure 4.1. Figure displaying the chemical structures of FTI-277 and GGTI-2147: FTI and GGTI are used to inhibit the functions of enzymes farnesyltransferase and geranylgeranyltransferase and hence restrict progerin from undergoing prenylation modification.

4.2.2.b. FTI and GGTI treatment

HDFs derived from HGPS patients were seeded at 2 x 10^5 cells per 10 cm^2 tissue culture dishes and then allowed to grow for at least 2 days in 15% FBS-DMEM. To inhibit the activity of enzymes farnesyltransferase and geranylgeranyltransferase, cells were incubated with 2.5µM final concentration of FTI-277 and 2.5µM of GGTI-2147 containing 15% FBS-DMEM for 48 hours.

4.2.4. 2D-FISH, 3D-FISH and indirect immunofluorescence:

Chromosomes 10, 18 and X territory positions were determined by performing 2D-FISH and 3D-FISH assays using whole chromosome paints as in section 2.2.2 and 2.2.3. respectively. Proliferating cells were distinguished by performing an indirect immunofluorescence assay for pKi-67 as in section 2.2.2.e.

4.3. Results:

4.3.1. Population doubling curve:

Cell growth was measured at each passage by calculation of population doublings (PD) using the following formula

$$\mathrm{PD} = (\log H - \log S)/\log\ 2.0$$

where log H = logarithm of the number of cells harvested

log S = logarithm of the number of cells seeded on first day of passage.

Accumulated population doubling (APD) for control and HGPS HDFs was then plotted (y-axis) against days in culture (x-axis) (Figure 4.2.)

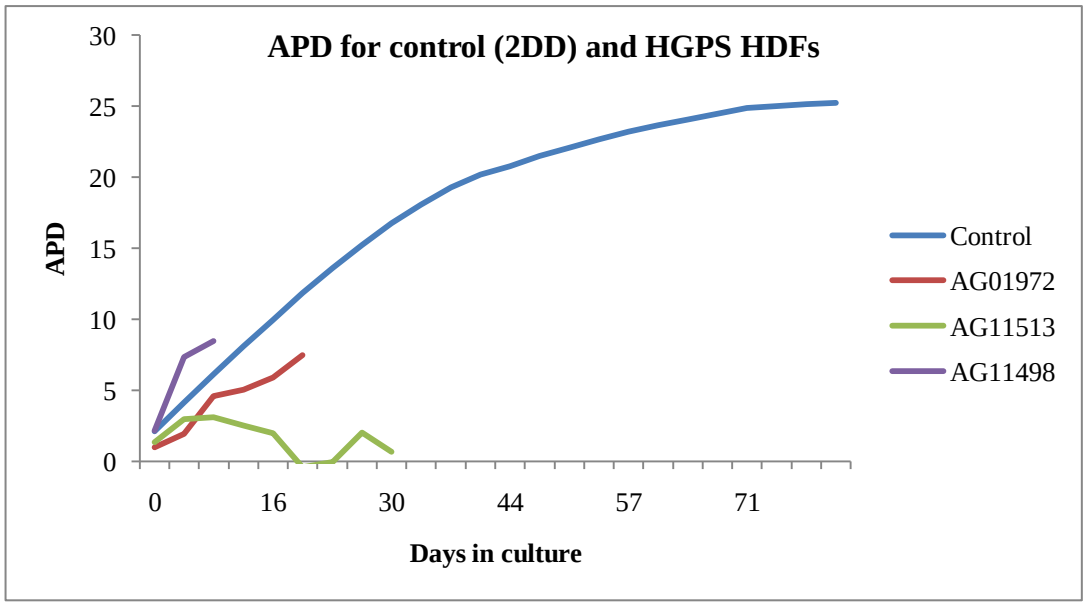


Figure 4.2. Growth rate of control and HGPS fibroblasts in culture: HGPS HDFs and control fibroblasts were cultured in 15% FBS and passaged twice weekly. The number of cells harvested and number of cells seeded were recored at each passage. Cell growth was measured by calculation of accumulated population doublings (APD). In the above graph APD (y-axis) is plotted against days in culture (x-axis).

At early, mid and late passages, HGPS fibroblasts were stained for pKi-67. Thus, fraction of proliferating cells within the culture was determined by the presence of pKi-67 staining (Figure 4.3.).

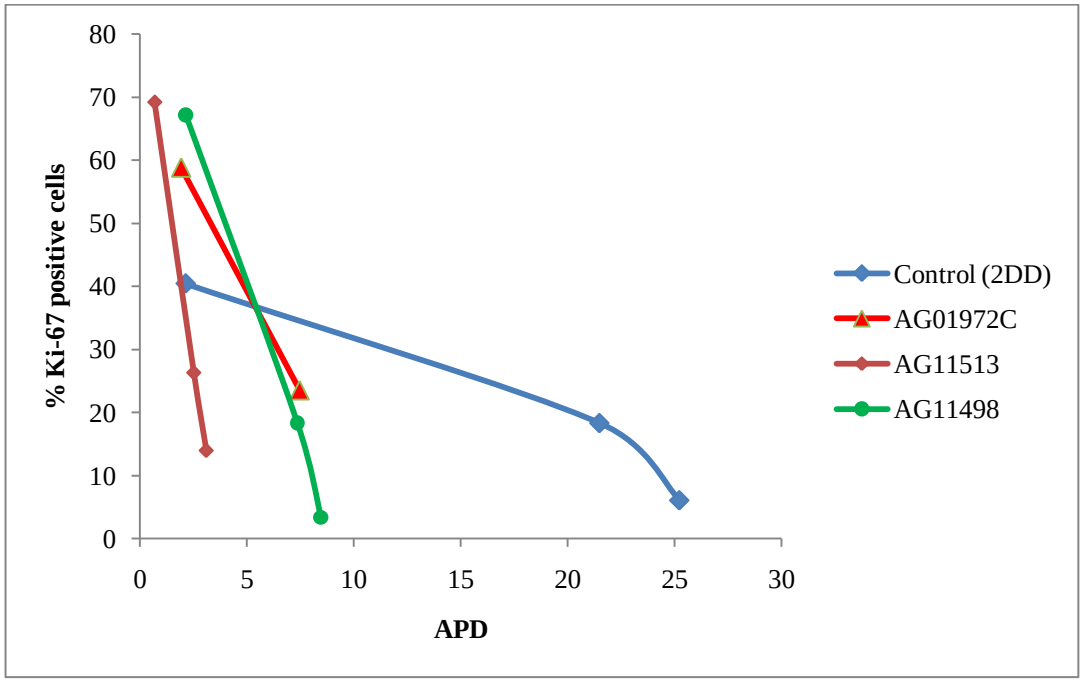


Figure 4.3. Graph displaying the fraction of proliferating cells in early, mid and late HGPS and control HDF cultures: Three *LMNA* mutant HGPS HDF cell lines and a control (2DD) cell line were fixed in methanol:acetone and stained for the presence of pKi-67 at various time points in the culture. At least 200 pKi-67 positive cells were scored. The data were derived from 3 independently treated coverslips for a given fixation. The fraction of pKi-67 positive cells (y-axis) has been plotted against APD (x-axis) to reveal the change in fraction of cells displaying pKi-67 expression over time in culture.

These data, although crude and not performed in age matched culture, demonstrates that HGPS HDFs (AG11513, AG11498 and AG01972C) have a significantly higher fraction of proliferating cells in young cell cultures as compared to control fibroblasts (Figure 4.2. and 4.3.). These data thus is a confirmation of hyperproliferation observed in young HGPS fibroblasts (Bridger and Kill, 2004).

4.3.2. Position of chromosomes 10, 18 and X in proliferating and senescent HGPS fibroblasts:

2D-FISH was performed on cells derived from three HGPS patients – AG01972C, AG11498 and AG11513 and one limb-girdle muscular dystrophy cell line –E358K, using whole chromosome paints for chromosome 10, 18 and X. Positional analysis revealed that chromosome 10 territories, in all four cell lines, were located at the nuclear periphery (Figure 4.4.M, S, Y, AE, P, V, AB and AH) as they would be in the nuclei of normal serum starved quiescent cells. Thus, chromosome territory organisation in proliferating laminopathy cell lines resembles that in normal quiescent cells arrested by removal of growth factors and not normal senescent cells. Chromosome 18 territories were located in an interior location in interphase nuclei for all the four cell lines (Figure 4.4.N, T, Z, AF, Q, W, AC and AI). Chromosome X, on the other hand occupied a peripheral location (Figure 4.4. O, U, AA, AG, R, X, AD and AJ).

Figure 4.4. Representative images and graphs displaying the position of chromosome 10, 18 and X territories in proliferating laminopathy fibroblasts.

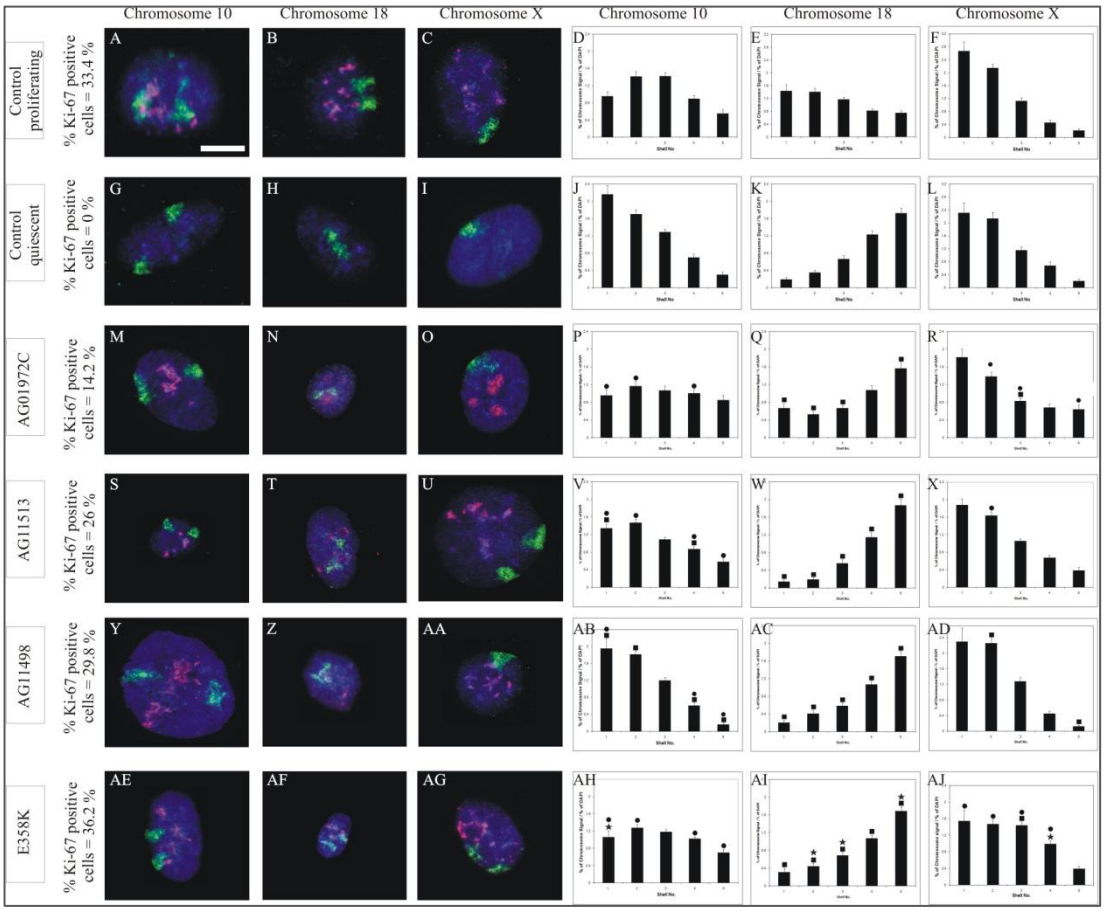
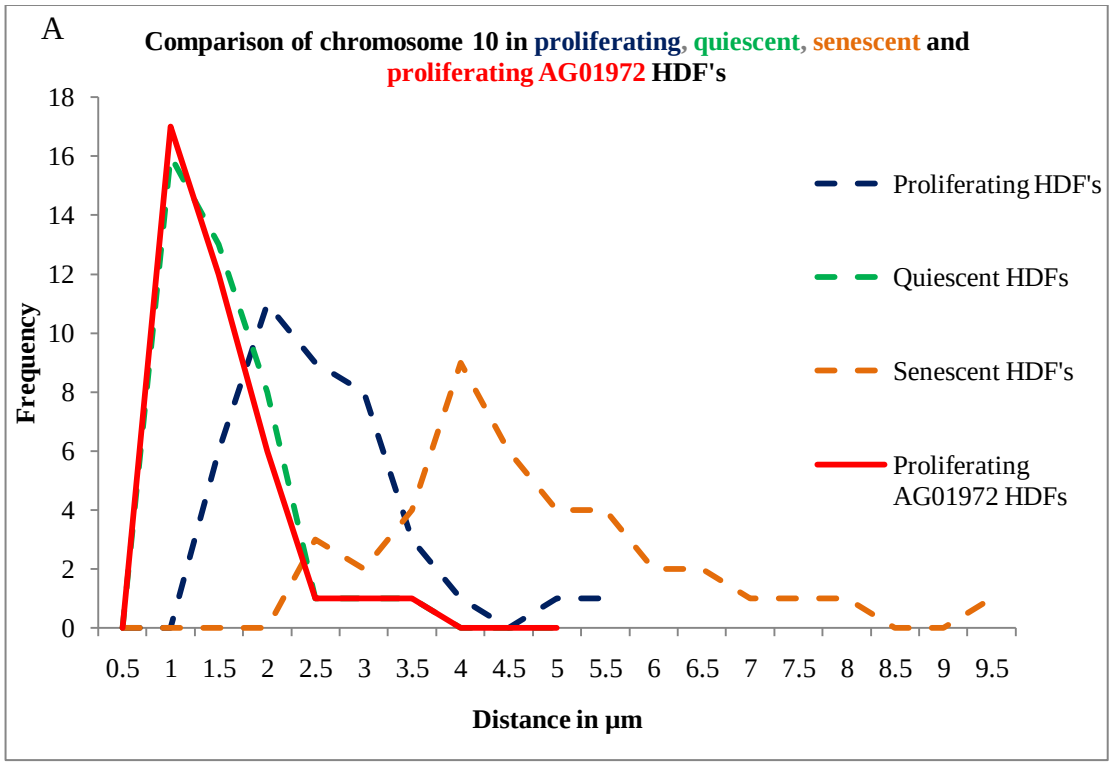


Figure 4.4. Representative images and graphs displaying the position of chromosome 10, 18 and X territories in proliferating laminopathy fibroblasts: HDFs derived from laminopathy patients were subjected to 2D-FISH in order to delineate and analyse the nuclear positions of chromosome 10, 18 and X territories. In panels A-C, G-I, M-O, S-U, Y-A' and AE-AG chromosome territories are revealed in green, nuclear distribution of pKi-67, a proliferation marker is shown in red and blue delineates the nuclear DNA. Scale bar = 10μm. The histograms in panels D-F, J-L, P-Q, V-X, AB-AD and AH-AJ display the distribution of chromosome signal for each chromosome analysed using erosion analysis. Bars represent % mean normalised proportion of chromosome signal for each chromosome. Error bars represent standard error of mean (SEM). Chromosome 10 (M, P, S, V, Y, AB, AE and AH) and chromosome X (O, R, U, X, AA, AD, AG and AJ) occupy a peripheral location while chromosome 18 (N, Q, T, W, Z, AC, AF and AI) is positioned in the nuclear interior, in interphase nuclei of cells derived from all of the above laminopathy cell lines. Unpaired, unequal variances two-tailed students *t*-tests was performed to assess statistical differences between position of chromosomes territories in proliferating HGPS fibroblasts to control proliferating (A-F), quiescent (G-L) and senescent HDFs. Filled in squares indicate a difference when compared to control proliferating HDFs, filled in circles indicate a difference when compared to control quiescent HDFs and filled in stars indicate a difference as compared to control senescent HDFs ($p<0.05$).

In order to ensure that the repositioning in chromosome locations observed is not due to global reorganisation of all the DNA in the nucleus, the distribution of DAPI across the five shells of the eroision analysis script was plotted for control and HGPS HDFs (Figure A1).

Interphase position of chromosome 10 and X territories in proliferating fibroblasts (derived from HGPS patient) was confirmed using 3D-FISH analysis. Distance between the geometric centre of the chromosome territory to the nearest nuclear periphery was then measured in at least 20 nuclei.

Figure 4.5. Relative nuclear positions of chromosome 10 and X territories in proliferating HGPS fibroblasts determined using 3D-FISH:



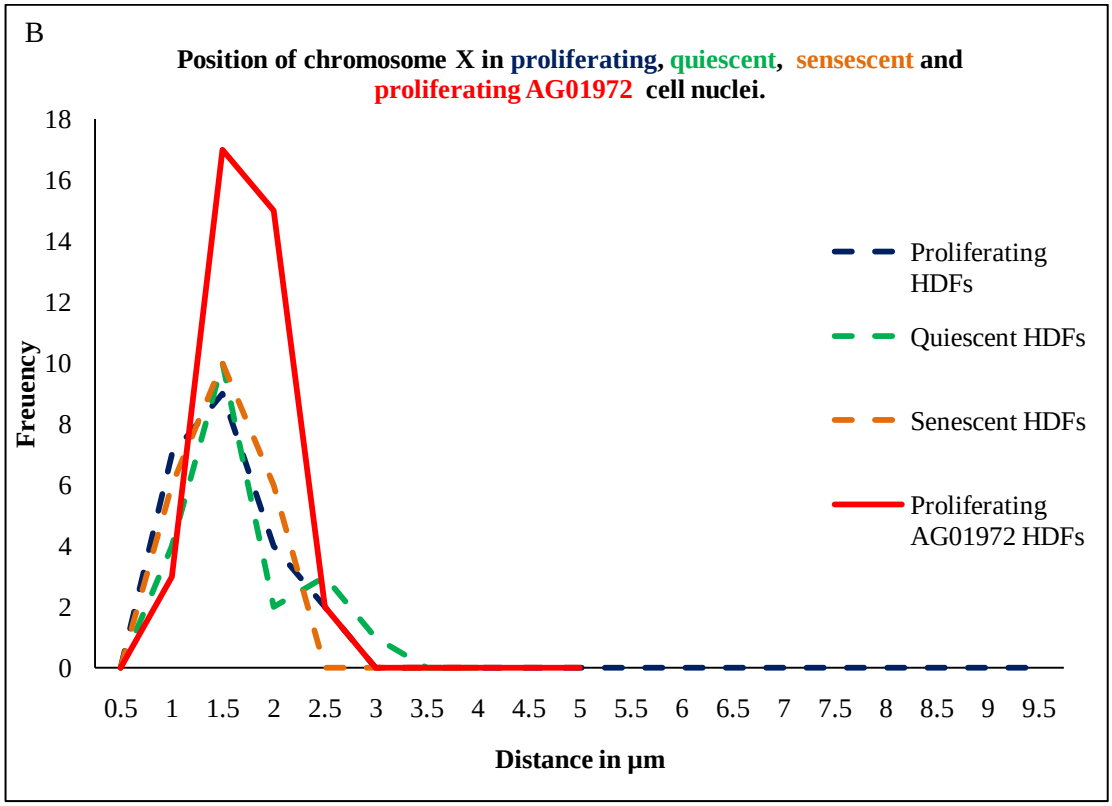


Figure 4.5. Relative nuclear positions of chromosome 10 and X territories in proliferating HGPS fibroblasts determined using 3D-FISH: Position of chromosome 10 and X territories in proliferating AG01972 HDFs – cell line derived from HGPS patient, were analysed using 3D-FISH assay. Cells were fixed using 4% paraformaldehyde to maintain the three dimensional structure and then subjected to a 3D-FISH assay to delineate the area occupied by a particular chromosome territory. Stacks of optical sections with an axial distance of 0.2µm were captured from at least 20 random nuclei. The distance between the geometric centre of the chromosome territory and the nearest nuclear periphery was then measured. Relative distance of chromosome 10 territories (A) and chromosome X territories (B) from the nearest nuclear periphery in proliferating HGPS fibroblasts (red line) have been displayed, which are then compared to relative distance of chromosome 10 (A) and X (B) territories in control proliferating (blue dashed line), quiescent (green dashed line) and senescent (orange dashed line) HDFs.

Thus, data from the 3D-FISH assay are in agreement with the 2D-FISH assay with respect to the positions of chromosome 10 and X territories in proliferating HGPS HDFs. The frequency distribution curves (Figure 4.5.A) display that chromosome 10 territories are not only located towards the nuclear periphery but are also in a very similar location to position of chromosome 10 territories in normal quiescent fibroblasts. Chromosome X localises at the nuclear periphery in control and AG01972 HDFs (Figure 4.5.B).

Further, the position of chromosome 10 territories in senescent HGPS cells was assessed in order to test whether these cells enter senescence in a similar manner to normal proliferating HDFs and thus change their chromosomal organisation. AG11513 cells were maintained in 15% FBS – DMEM medium by passaging them twice every week until the percentage of pKi-67 cells in this culture was below 5% signifying that the culture was truly senescent. The senescent AG11513 cells were

then harvested, fixed and a 2D-FISH was performed using whole chromosome paints for chromosome 10 and X (Figure 4.6.).

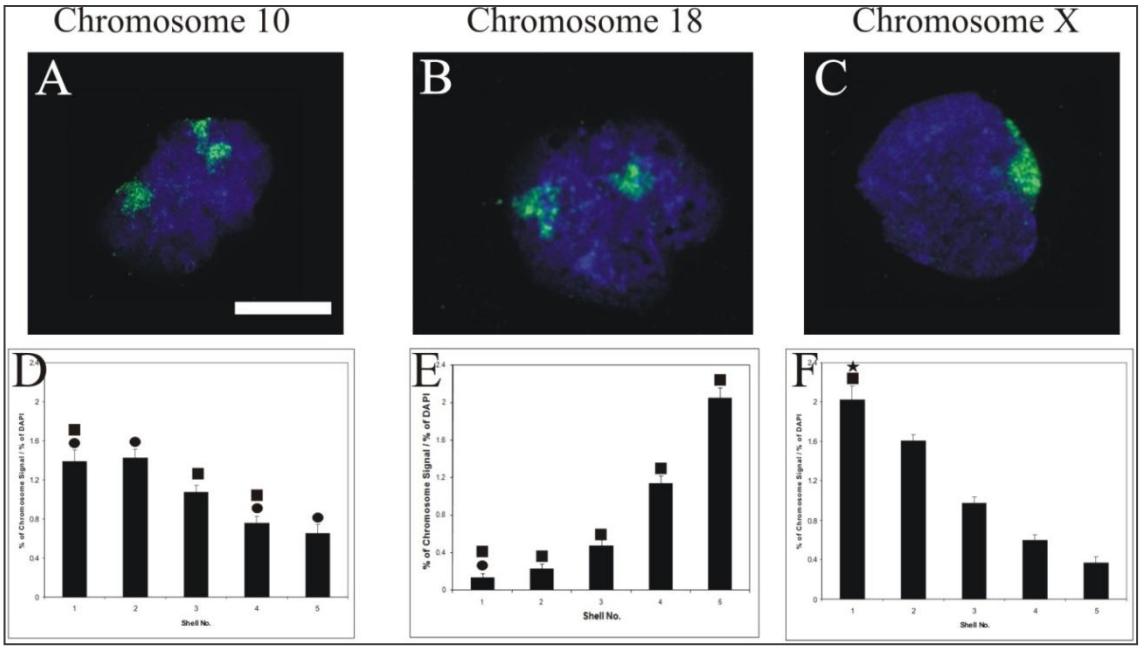


Figure 4.6. Representative images and histograms displaying the position of chromosome 10, 18 and X territories in senescent HDFs derived from a HGPS patient: HDFs derived from a HGPS patient, cell line AG11513 were grown in culture until senescence, i.e. until the level of pKi-67 positive cells in culture were below 5%. Cells were then harvested; fixed and 2D-FISH assay was then employed to determine the positions of chromosomes 10, 18 and X territories. Panel A-C shows representative images displaying locations of chromosomes 10, 18 and X in senescent AG11513 HDFs. Scale bar = 10µm. Histograms in panel D-F display the % mean chromosome signal for each chromosome in each shell as analysed by erosion analysis. Error bars represent standard error of mean. Statistical difference, as assessed by unpaired, unequal variances, two-tailed students *t*-tests, to control proliferating cells is denoted by filled in squares, to control quiescent cells is denoted by filled in circles and to control senescent cells is denoted by filled in stars ($p<0.005$).

Imaging and analysis revealed that the chromosome 10 territories occupy a peripheral location (Figure 4.6.A and D) in the nucleus of senescent AG11513 cells.

Chromosome 18 is located in an interior position in interphase nuclei of senescent AG11513 HDFs (Figure 4.6.B and E) while chromosome X localises to the nuclear periphery in these cells (Figure 4.6.C and F). This thus suggests that although the cells have entered senescence, the chromosomal organisation within their nuclei is still "quiescent-like".

4.3.3. Position of chromosome 10, 18 and X in HGPS fibroblasts following serum withdrawal:

Results from chapter 3 demonstrate that in control HDFs, chromosomes 10 and 18 territories undergo movement in response to serum withdrawal within 15 minutes (Section 3.3.5). To determine whether HGPS patient cells display a similar response to serum withdrawal, AG11498 cells were grown in 15% FBS-DMEM for at least 2 days and then incubated in medium containing low serum (0.5% FBS). Samples were collected 0, 5, 10, 15, 30 minutes and 7 days post serum-withdrawal. 2D-FISH was then employed to determine the interphase position of chromosomes 10, 18 and X at each of these time points. Histograms in figure 4.7. display the positions of chromosome 10, 18 and X in AG11498 HDFs post serum withdrawal.

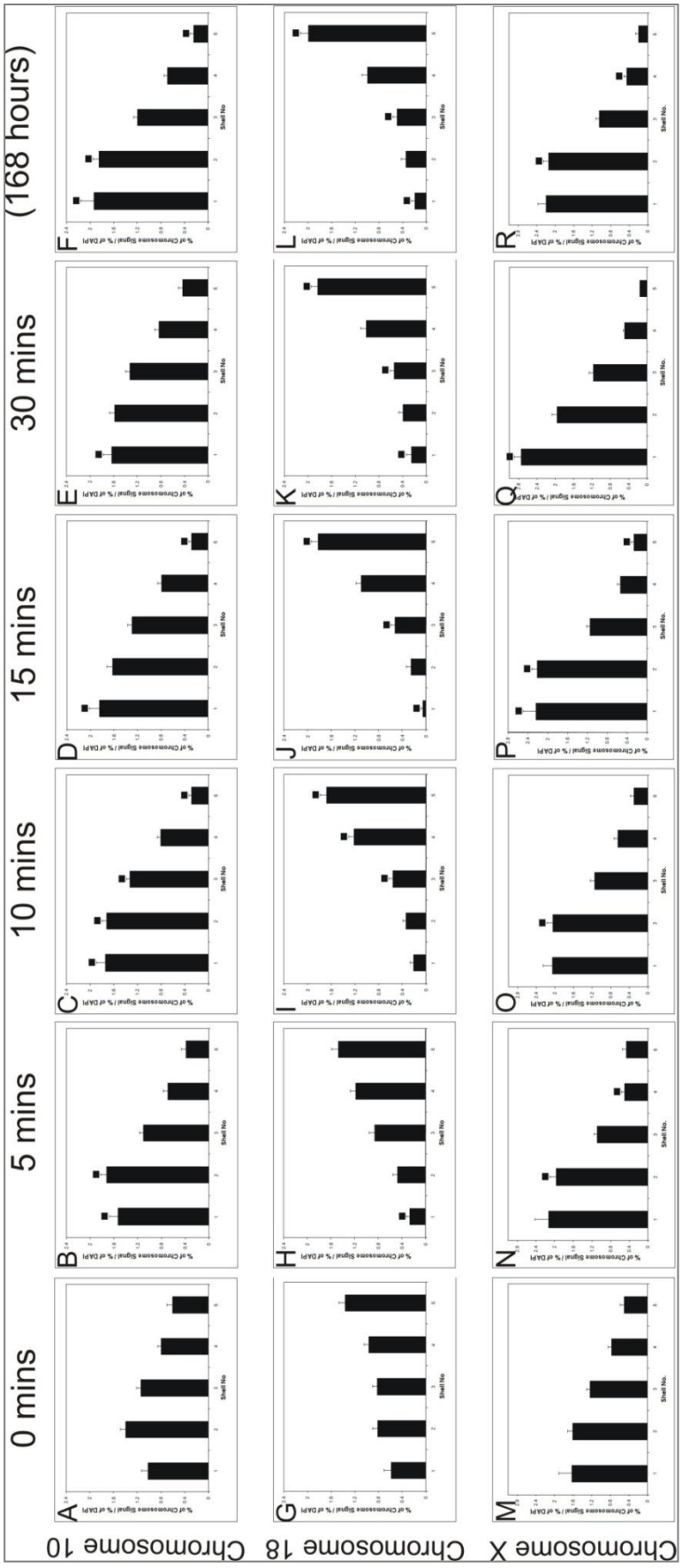


Figure 4.7. Chromosome positioning in HGPS cells following serum removal: Proliferating HGPS cells (AG11498) were placed in low serum (0.5%) for 0 minutes (A, G, M), 5 minutes (B, H, N), 10 minutes (C, I, O), 15 minutes (D, J, P), 30 minutes (E, K, Q) and 7 days (F, L, R). The cells were subjected to a 2D-FISH assay and the nuclear location of chromosome 10 (panels A-F), chromosome 18 (panels G-L) and the X chromosome (panels M-R) assessed by erosion analysis. The filled-in squares represent a significant difference ($p<0.05$) between the cells with the serum removed and the proliferating cells in A, G and M.

Chromosome territories in the laminopathy cells are already positioned in a quiescent-like nuclear location (Figure 4.7. A, G, M). In response to serum withdrawal, chromosomes 10, 18 and X showed no movement from the nuclear compartment they had been assigned to i.e. nuclear periphery, intermediate, nuclear interior respectively (Figure 4.7.). Interestingly, a slight movement in the same nuclear compartment was observed for all three chromosome, i.e. chromosomes 10 (Figure 4.7.B-F) and X (Figure 4.7.N-R) became significantly more peripheral and chromosome 18 (Figure 4.7.H-L) become significantly more interior in response to serum withdrawal.

4.3.4. FTI treatment reduces the nuclear abnormalities in HGPS patient fibroblasts:

Retention of a farnesyl group inhibits the successful localisation of progerin at the nuclear lamina resulting in structural nuclear abnormalities, a characteristic often observed in HGPS fibroblasts (Goldman et al., 2004). About 57% of HGPS fibroblasts (AG11498) used in this study showed abnormally shaped nuclei (Figure 4.8.– untreated). Nuclei displaying blebbing, herniations, micronucleation, abnormal lamina folding and nuclear fragmentation were classified as abnormally shaped nuclei. In agreement with previous studies (Toth et al., 2005, Yang et al., 2005, Glynn and Glover, 2005, Capell et al., 2008, Mallampalli et al., 2005), the fraction of cells with mis-shapen nuclei is significantly reduced to less than 8% in HGPS fibroblasts (AG11498) after 48 hours treatment with 2.5 μM FTI (Varela et al., 2008) (Figure 4.8. – FTI (48 hours)).

Interestingly, in cells that have been kept in culture for 2 passages after a 48-hour FTI treatment, the percentage of abnormally shaped nuclei was significantly

higher as compared to AG11498 cells treated for 48 hours but was significantly lower as compared to untreated AG11498 HDFs (Figure 4.8. – FTI (2 passages)).

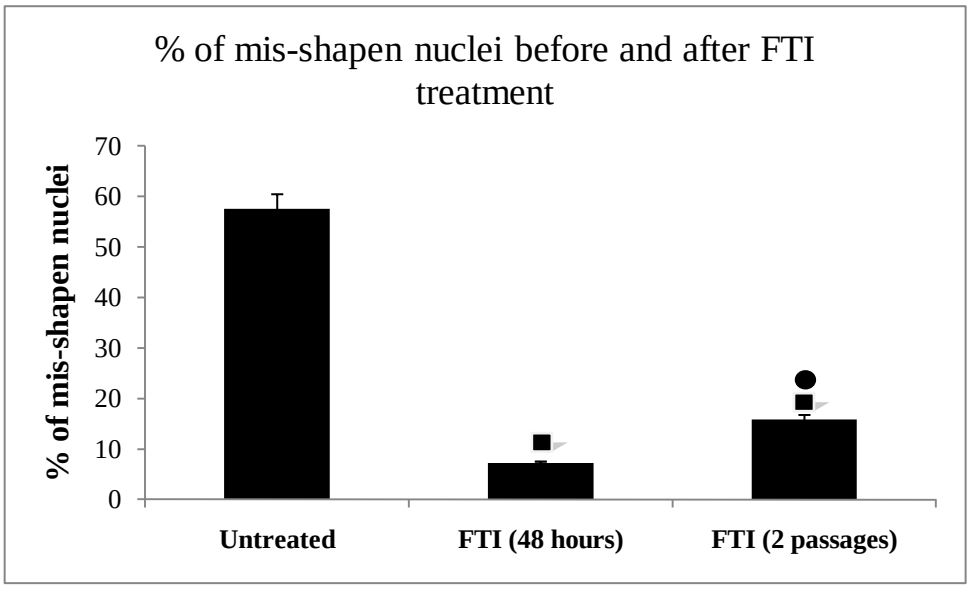


Figure 4.8. Percentage of mis-shapen nuclei in HGPS culture before and after FTI treatment: HDFs derived from HGPS patients (AG11498) were cultured in 15% FBS-DMEM, treated with 2.5 μM FTI-277 for 48 hours, fixed and mounted in Vectashield mounting medium containing DAPI. A sample of HGPS fibroblasts treated with FTI was cultured for 2 more passages without any FTI prior to fixation. Three counts (at least 1000 cells per count) from independently treated coverslips were performed to record the number of mis-shapen nuclei in each sample. Average % of mis-shapen nuclei were plotted for each sample. Error bars represent standard error of mean. Filled-in squares indicate a statistical difference as compared to untreated sample while filled-in circles indicates a statistically significant difference as compared to sample treated with FTI for 48 hours, as assessed by unpaired, unequal variances, two tailed students *t*-tests ($p<0.05$).

4.3.5. FTI:GGTI treatment restores the locations of chromosomes 10, 18 and X to control cell locations in HGPS fibroblasts:

Chromosomes 10, 18 and X territories occupy altered positions in interphase nuclei of proliferating HDFs derived from HGPS patients (Figure 4.4. and 4.5.) as compared to control proliferating HDFs. In order to determine if FTI treatment can restore the locations of these chromosome territories to their normal nuclear locations in proliferating cell, HDFs derived from HGPS patients were treated with 2.5µM FTI-277 for 48 hours. Pre-lamin A and progerin, in presence of FTIs is known to undergo an alternative prenylation modification by the enzyme geranylgeranyltransferase, whereby a geranylgeranyl moiety is attached to the end of the protein instead of farnesyl moiety (Varela et al., 2008). To overcome the limitation of pre-lamin A and pre-progerin becoming prenylated in the absence of farnesyltransferase enzyme, AG11498 fibroblasts were treated with a combination of 2.5µM geranylgeranyltransferase inhibitor (GGTI) and 2.5µM FTI-277 for 48 hours. The effect of GGTI alone and DMSO (amounts equivalent to that used for FTI and GGTI dissolution) on positions of chromosomes in AG11498 HDFs was also determined.

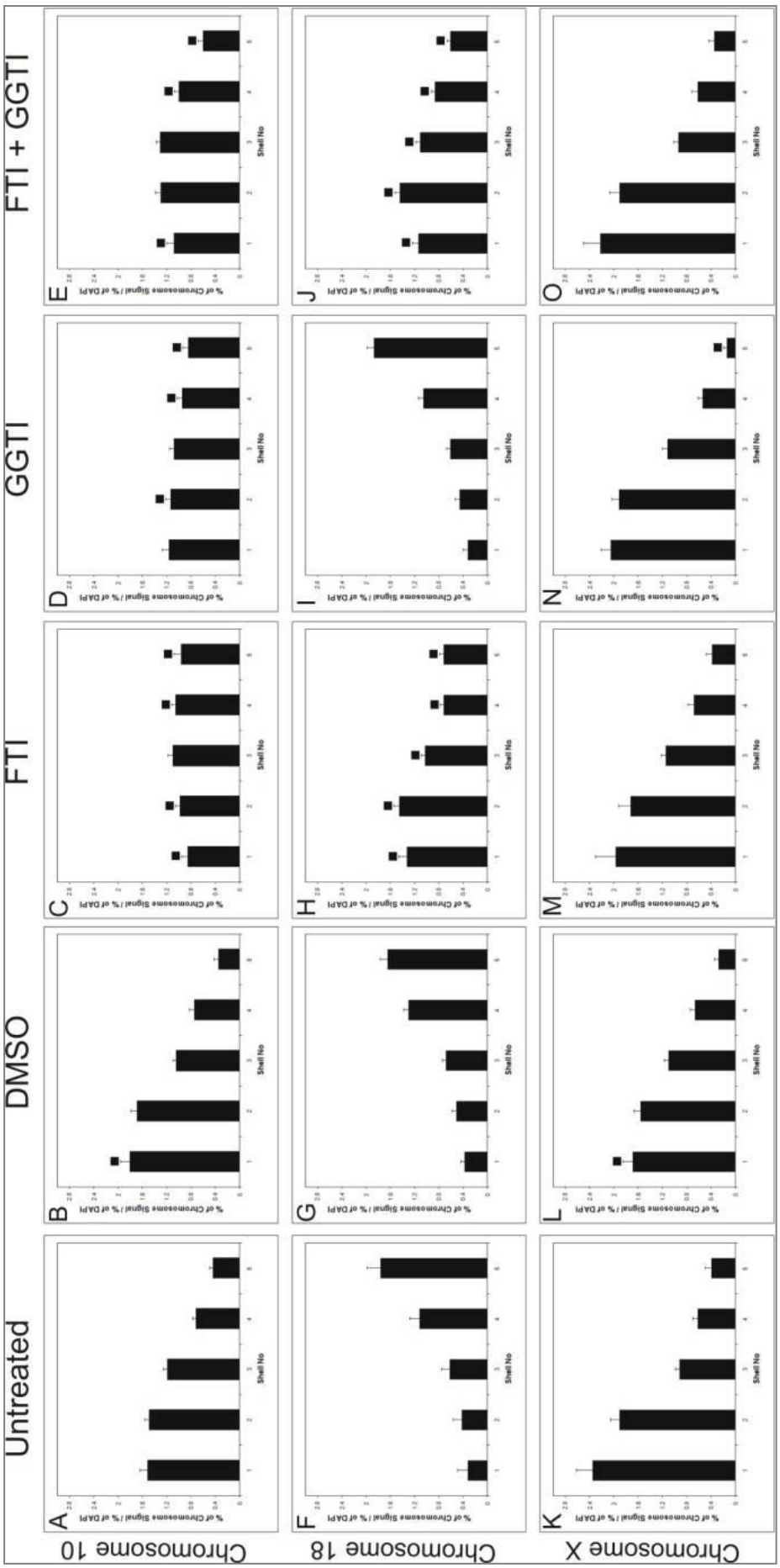


Figure 4.9. Position of chromosome 10, 18 and X territories in AG11498 HDFs treated with a combination of FTI and GGTI for 48 hours.

Figure 4.9. Position of chromosome 10, 18 and X territories in AG11498 HDFs treated with a combination of FTI and GGTI for 48 hours: Histograms display positions of chromosome 10, 18 and X territories in interphase nuclei of untreated AG11498 HDFs (A, F and K), AG11498 HDFs treated with equivalent amounts of DMSO used for dissolution of inhibitors (B, G and L), AG11498 HDFs treated with 2.5μM FTI-277 (C, H and M), AG11498 HDFs treated with 2.5μM GGTI-2147 (D, I and N) and AG11498 HDFs treated with a combination of FTI and GGTI – 2.5μM each (E, J and O). Error bars indicate standard error of mean. Filled in squares indicate statistical difference ($p<0.05$) for that shell when compared to the equivalent shell for untreated sample.

Treatment with FTI and a combination of FTI:GGTI restores the position of chromosome 10 from the nuclear periphery (Figure 4.9.A) to an intermediate nuclear location (Figure 4.9.C and E) and also restores the position for chromosome 18 from the nuclear interior (Figure 4.9.F) to the nuclear periphery (Figure 4.9.H and J). Importantly DMSO (0.01% - equivalent to that added with the inhibitors) has no effect on the positions of chromosomes (Figure 4.9.B, G and L) and hence the restoration of chromosome territories is an effect due to the drug. Treatment with GGTI only does result in the repositioning of chromosome 10 slightly (Figure 4.9.D), but has no significant effect on the position of chromosome 18 (Figure 4.9.I).

To determine if the effect of FTI and GGTI treatments were persistent and that the chromosome positions, once restored, were maintained in these positions, AG11498 HDFs following a 48 hour treatment with FTI, GGTI and a combination of FTI:GGTI, were washed with medium containing no inhibitors and then cultured for next two passages over a period of 9 days. Cells treated with FTI:GGTI went through 1.73 population doublings. After 2 passages, cells were fixed and 2D-FISH was performed to determine the locations of chromosomes 10, 18 and X.

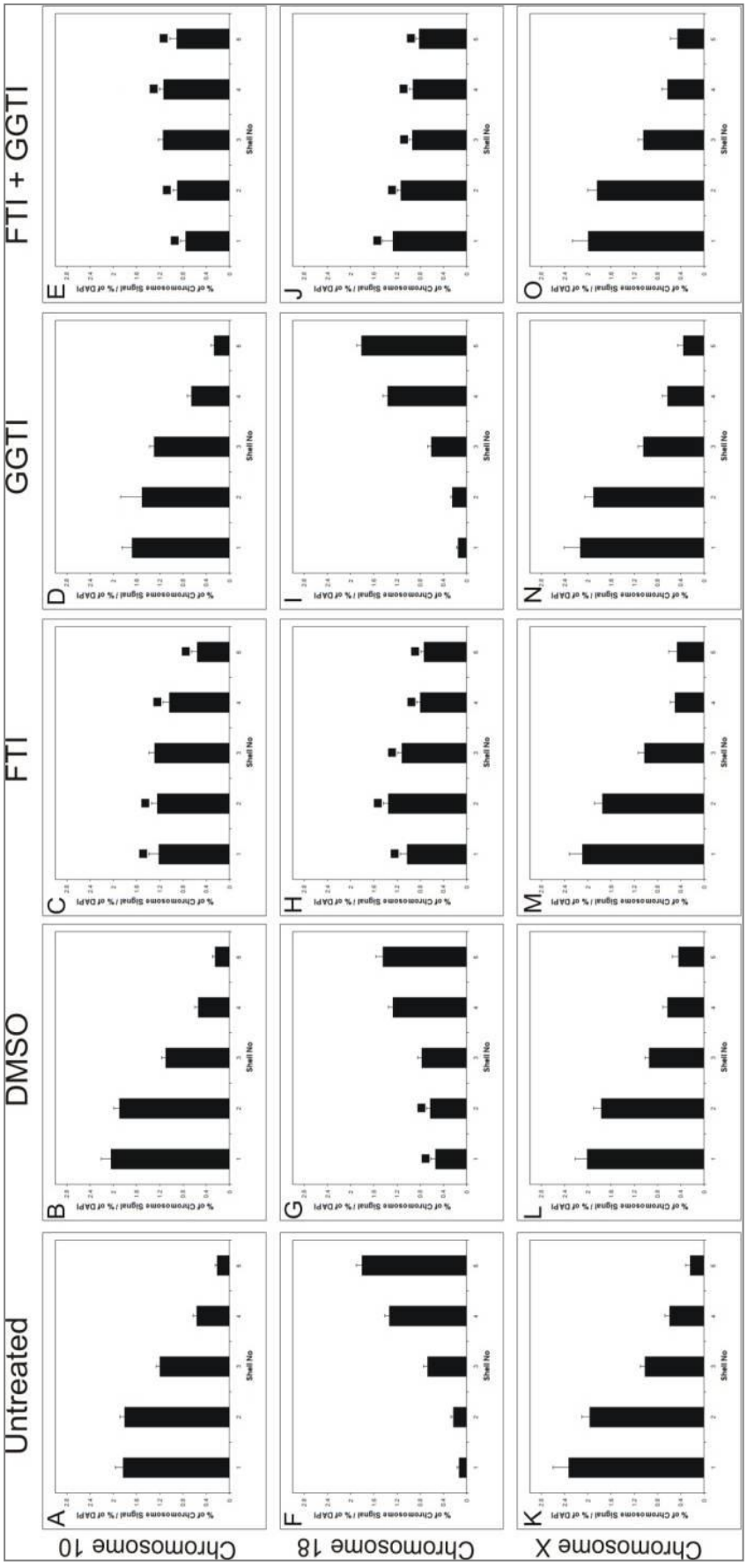


Figure 4.10. Position of chromosome 10, 18 and X territories in AG11498 HDFs after 2 passages post 48 hour treatment with a combination of FTI and GGTI.

Figure 4.10. Position of chromosome 10, 18 and X territories in AG11498 HDFs after 2 passages post 48 hour treatment with a combination of FTI and GGTI:

Positions of chromosome 10, 18 and X territories in interphase nuclei of untreated AG11498 HDFs (A, F and K) as well as in AG11498 HDFs treated with equivalent amounts of DMSO used to dissolve the inhibitors (B, G and L), AG11498 HDFs treated with DMSO (B, G and L), 2.5µM FTI-277 (C, H and M), 2.5µM GGTI-2147 (D, I and N) and a combination of FTI and GGTI – 2.5µM each (E, J and O) for 48 hours and then allowed to grow in culture for 2 passages without the inhibitor are displayed in the histograms. Error bars indicate standard error of mean. Filled in squares indicate statistical difference ($p<0.05$) for that shell when compared to the equivalent shell for untreated sample.

Histograms in figure 4.10. indicates that after two passages; in cells treated with FTI (48 hours treatment) and a combination of FTI:GGTI (48 hours treatment), chromosome 10 and 18 territories are maintained in an intermediate location (Figure 4.10. C and E) and an interior location (Figure 4.10.H and J) respectively, in interphase nuclei of HGPS cells. In HGPS HDFs treated with GGTI (Figure 4.10.D, I and N) and DMSO (Figure 4.10. B, G and L) alone, the positions of chromosomes 10 and 18 are unaltered. Chromosome X remains at the nuclear periphery at all times (Figure 4.10.K-O).

Thus, data from last two sections (4.3.4 and 4.3.5.) demonstrate that FTI treatment reduces nuclear abnormalities and restores chromosomes 10, 18 and X positions to normal nuclear locations. These positions of chromosomes are maintained for at least until 2 passages (9 days) following the removal of the drug from the culture medium.

4.3.6. Chromosome repositioning response to serum withdrawal is restored in FTI:GGTI treated HGPS HDFs:

To determine if FTI treatment restores the chromosome movement ability of HGPS fibroblasts in response to serum withdrawal, AG11498 fibroblasts were treated with a combination of FTI:GGTI (2.5µM each) for 48 hours and then incubated with medium containing low serum (0.5% FBS). Samples were fixed at 0, 15, 30 minutes and 7 days post serum removal and 2D-FISH analysis, to determine positions of chromosomes 10, 18 and X, was employed.

Figure 4.11. Repositioning of chromosomes 10, 18 and X in response to serum withdrawal in interphase nuclei of AG11513 HDFs treated with a combination of FTI:GGTI

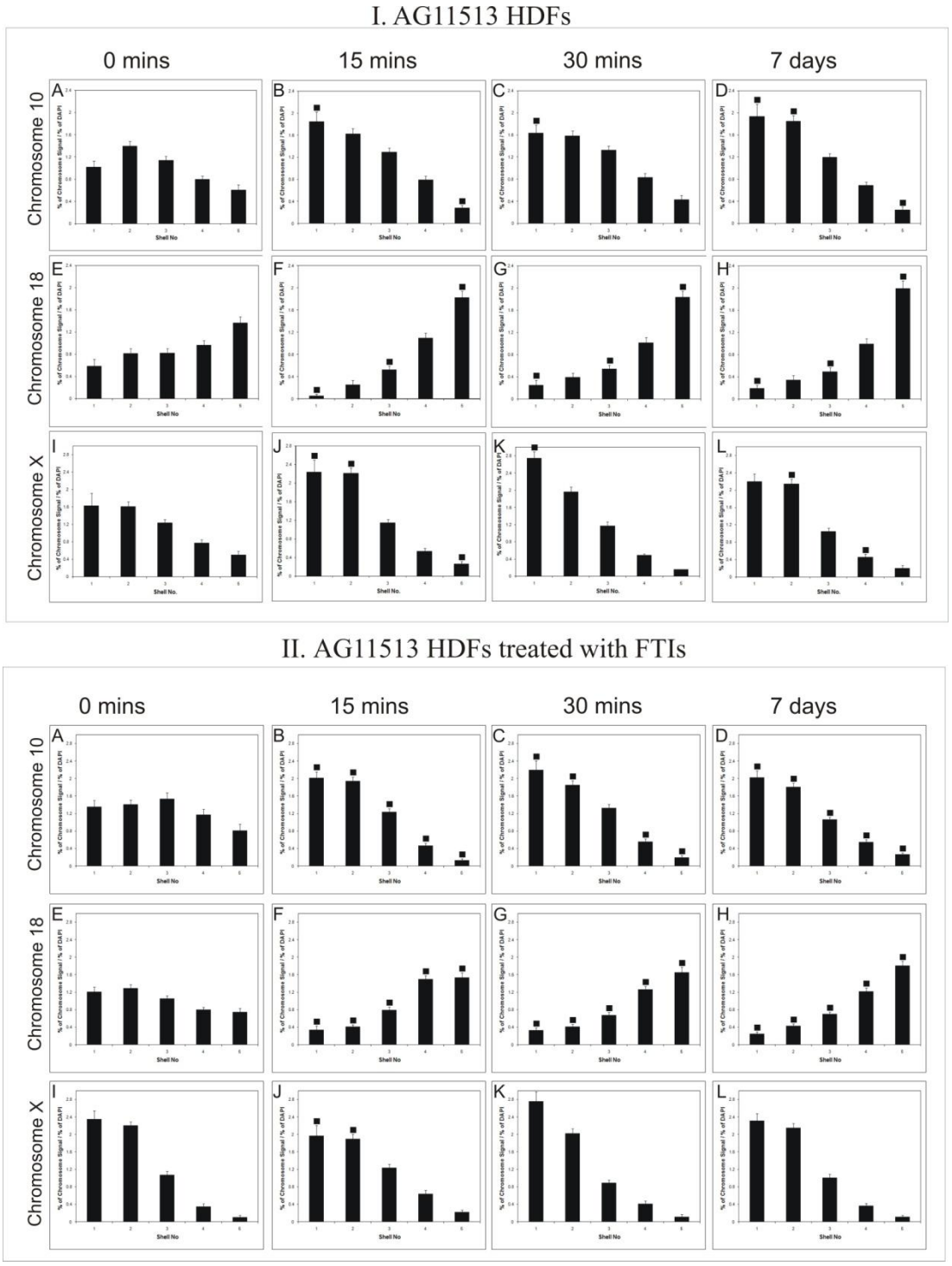


Figure 4.11. Repositioning of chromosomes 10, 18 and X in response to serum withdrawal in interphase nuclei of AG11498 HDFs treated with a combination of FTI:GGTI: Positions of chromosome 10, 18 and X territories in interphase nuclei of proliferating AG11513 HDFs before (I) and after FTI:GGTI treatment (2.5μM each for 48 hours) (II) after 0 (A, E and I), 15 (B, F and J), 30 (C, G and K) minutes and 7 days (D, H and L) following serum withdrawal have been displayed in histograms. Error bars indicate standard error of mean. Filled in squares indicate statistical difference ($p<0.05$) for that shell when compared to the equivalent shell for 0 minute sample (I and II A, E and T).

In HGPS fibroblasts treated with FTI, chromosome 10 territories reposition from an intermediate nuclear location (Figure 4.11. IIA) to a peripheral location (Figure 4.11. IIB) within 15 minutes of serum withdrawal; similar to that in control HDFs (Figure 3.2.). Chromosome 18 on the other hand repositions from the nuclear periphery (Figure 4.11. IIE) to the nuclear interior (Figure 4.11. II.F) again within 15 minutes of serum removal in HGPS after FTI:GGTI treatment but not in HGPS HDFs without treatment (Figure 4.11 I E - H). These positions of chromosomes 10 and 18 at the nuclear periphery and in the nuclear interior respectively are maintained after 30 minutes (Figure 4. 11. IIC and G) and 7 days (Figure 4. 11. II D and H) following serum withdrawal. This repositioning of chromosomes following serum withdrawal is similar to that observed in control HDFs (Figure 3.2.) Chromosome X remains at the nuclear periphery before and after serum withdrawal (Figure 4.11.I and II.I-L).

These data suggest that a combination of FTI:GGTI treatment restore the ability of HGPS fibroblasts to reposition chromosome in response to serum withdrawal.

4.4. Discussion:

A range of degenerative diseases termed laminopathies (Capell and Collins, 2006, Ellis, 2006, Parnaik and Manju, 2006, Rankin and Ellard, 2006) have been found to display many mutations in the lamin A gene, *LMNA*. Altered chromosome positioning has been observed in a number of primary fibroblast lines derived from laminopathy patients (Meaburn et al., 2007), with the nuclear positioning of the chromosomes 13 and 18 within the nuclear interior different to control cells. These data are confirmed in this chapter with altered positions of chromosomes 13 and 18 observed in HGPS fibroblasts as compared to control fibroblasts. In addition to this, the position of chromosomes 10 is also altered in HGPS fibroblasts while position of chromosome X remains unchanged. The nuclear position of chromosome 10 in the four proliferating laminopathy cell lines assessed displayed a "quiescent-like" distribution and thus chromosome organisation in HGPS fibroblasts may resemble that of normal quiescent and not senescent fibroblasts. The hypothesis that lamin A might be involved in cell proliferation (Dorner et al., 2006, Johnson et al., 2004, Pekovic et al., 2007, Van Berlo et al., 2005) and cell cycle regulation (Bakay et al., 2006, Ozaki et al., 1994, Markiewicz et al., 2002, Johnson et al., 2004, Meaburn et al., 2007) and that a mutation in *LMNA* gene may cause uncoupling of these pathways not only explains the hyperproliferation observed in young laminopathy HDFs (Bridger and Kill, 2004), but may also elucidate the reason for the quiescent-like chromosomal organisation observed in these HDFs. The question whether alteration in chromosomal organisation in HGPS patients as demonstrated in this chapter directly influences this change in gene expression profiles remains unanswered. Whether or not there are any similarities between the gene expression profiles of quiescent cells and HGPS cells is addressed in chapter 7. Conversely, it

may also be true that alteration in gene expression profile and the need for certain genes to be expressed may be driving the change in organisation of chromosome territory locations. The result demonstrating that location of chromosomes remains unaltered in senescent laminopathy HDFs reiterates (Chapter 2) that chromosomal organisation is set and thus cannot be reorganised, once the cells enter a state of replicative senescence.

Laminopathy cells do respond to the removal of serum with respect to repositioning of chromosome territories. The population of cells shows more chromosomes in the "quiescent-like". This leads to a presumption that there are a small fraction of cells within the diseased cultures that are behaving more normally than others, perhaps with less progerin, that can still respond to low serum and so there is chromosome movement in these cells. Indeed, there will still be some normal functioning lamin A within these cells and thus some cells could be more affected than others, especially if they have been through more cellular divisions. In addition to nuclear motor dependent chromosome repositioning, re-organisation of chromosome territories is also thought to occur as a result of cytoplasmic signals via SUN proteins and KASH domain proteins. Mutation in *LMNA* gene may result in perturbed interaction of the lamin A protein with nuclear motor or SUN proteins, hence leading to dysfunctional chromosome repositioning response in HGPS patient HDFs.

Thus, in proliferating laminopathy HDFs it appears that quiescent-like genome organisation is a default state and there is no functioning mechanism to place chromosomes in a proliferating location or in a senescence–like location. This could have major implications for cellular differentiation and tissue regeneration in laminopathy patients if genome organization is involved in gene regulation.

With most studies focussing on the effect of FTIs on the structural role of lamins in maintainence of nuclear shape and integrity, effects of these drugs on the functional aspects of lamin A involving chromatin organisation, transcription, DNA replication, DNA damage response and genome stability (Gruenbaum et al., 2005, Lees-Miller, 2006, Worman and Courvalin, 2005, Vlcek and Foisner, 2007) remains to be identified. The study in this chapter demonstrates the effect of FTI treatment on chromosome positions in fibroblasts derived from HGPS patients.

Significant reduction in fraction of abnormally shaped nuclei following FTI treatment in HGPS patient cells was confirmed in this study but an increase in fraction of mis-shapen nuclei over time was observed in cells after the drug was removed suggesting that effect of FTI is reversible. FTI:GGTI treatment restores the positions of chromosome 10 and 18 territories to their normal nuclear locations in HGPS patient cells, thus suggesting that this treatment may also correct aberrant gene expression profiles observed in HGPS patient cells, i.e. if gene expression is linked to chromosome organisation. With chromosome organisation similar to that in control proliferating cells after FTI:GGTI treatment of HGPS fibroblasts, cell cycle regulation and cell proliferation may also be reverted to normal, which may then lower the fraction of cells undergoing apoptosis. Also, after treatment with the inhibitor, the positions of chromosomes are maintained for at least 9 days without the presence of the drug. This reflects on the long-lasting effects of the drug and may also suggest that the drug would work for a longer time duration, thus reducing the number of doses required.

FTI:GGTI treatment also restores the rapid chromosome repositioning response to serum withdrawal in HGPS HDFs. Whether or not nuclear motor proteins involved in this repositioning event are affected due to *LMNA* mutation and

thus corrected after FTI:GGTI treatment is further investigated in chapter 6. With repositioning of chromosomes restored after FTI treatment, HGPS cells may now be able to transverse between proliferating and non-proliferating states more efficiently.

Although the idea of using FTI to ameliorate the disease phenotype in cell culture and animal models has been highly successful till date including in this study, limitations of this treatment cannot be ignored. Treatment with FTIs may interfere with post-translational modifications of other proteins that are required to undergo farnesylation and thus may result in various side effects. FTI treatment may also not be able to revive tissues that have already lost cells or are irreversibly damaged. The argument that FTI treatment does not reduce the amount of progerin and that nonfarnesylated progerin may also be toxic to the cells, cannot be overlooked. Whether or not the improvement seen in HGPS cultures after FTI treatment translates general rescue and reversal of tissue-specific phenotype in HGPS patients requires further long term studies. Various other techniques involving other drugs like statins and bisphosphonates (Varela et al., 2008), recruiting oligonucleotides to specifically target the pathogenic splicing event (Scaffidi and Misteli, 2005) have also successfully reverted some of the phenotypes observed in HGPS cells. Regardless of the criticisms the FTI strategy of treating HGPS appears to be well studied one amongst all the presently available strategies. Moreover, clinical trials have demonstrated that FTIs show little toxicity (Taveras et al., 2003).

In summary, chromosomal organisation in HDFs derived from HGPS patients is similar to that of normal quiescent HDFs and the ability of HGPS HDFs to reposition chromosomes in response to serum withdrawal is perturbed, thus suggesting a role of A-type lamins in chromosomal organisation as well as whole chromosome movement. Interestingly, treatment with FTIs, drugs that are presently

used in clinical trial for treating HGPS patients, restores the chromosomal organisation as well as the chromosome repositioning response of HGPS cells. This would then presumably restore the gene expression and thus reduce the pathology in HGPS patients.

Chapter 5: Nuclear myosin 1β in normal and HGPS patient fibroblasts

- Some of the contents of this chapter are in press for Genome Biology under the title "Rapid chromosome territory relocation by nuclear motor activity in reposnse to serum removal in primary human fibroblasts" – Ishita S Mehta, Manelle Amira, Amanda J Harvey and Joanna M Bridger.

- Some of the contents of this chapter are in preparation for a publication in Ageing Cell – "Chromosome territory positions and nuclear motors in senescent fibroblasts." - Ishita S Mehta, Martin Figgitt, Ian R Kill and Joanna M Bridger.

- Some of the contents of this chapter are in preparation for Ageing Cell: "Chromosome territory positions and nuclear motors in Hutchison Gilford progeria syndrome patient cells before and after FTI treatment" – Ishita S Mehta, Ian R Kill and Joanna M Bridger.

5.1. Introduction:

The coordination of nuclear processes such as DNA replication, regulation of gene expression, processing of RNA and DNA repair is regulated partly through the organisation of the nucleus into functional compartments. Interphase chromosomes occupy specific areas within nuclei called chromosome territories (Cremer and Cremer, 2001) and these territories are non-randomly positioned within the nuclear space by gene-density in proliferating fibroblasts (Croft et al., 1999, Boyle et al., 2001, Meaburn and Misteli, 2008) and by size in non-proliferating cells (Bridger et al., 2000) (Mehta et al., in submission). Until recently whole chromosome regions were thought to be largely immobile but studies performed over last few years have revealed that chromatin regions (Chuang, Carpenter et al. 2006; Dundr, Ospina et al. 2007) as well as whole chromosomes (Hu, Kwon et al. 2008; Mehta, Elcock et al. 2008) (Chapter 3) undergo active movement coordinated by nuclear motors comprising of nuclear actin and NMIβ (Mehta et al., 2008) (Chapter 3).

Identification of nuclear isoforms of myosin and actin and with their involvement in various processes including transcription (Percipalle et al., 2006, Hu et al., 2008, Vreugde et al., 2006, Philimonenko et al., 2004, Hofmann et al., 2004), chromatin remodelling (Percipalle and Farrants, 2006) and movement of chromatin as well whole chromosome territories (Chuang et al., 2006, Mehta et al., 2008, Hu et al., 2008) (Chapter 3), suggests that these proteins may be an integral part of the nuclear architecture.

Nuclear myosin Iβ (NMIβ) isoform of myosin that is found within the nucleus is required for rapid chromosome repositioning, elicited by removal of serum from a proliferating cell culture (Mehta et al., 2008) (Mehta et al., in

submission, Chapter 3). Interestingly, this movement of whole chromosomes in response to serum withdrawal is not observed in HGPS patient cells that have a mutation in *LMNA* gene (Chapter 4). Further, treatment with FTI restore the whole chromosome repositioning response to serum withdrawal in HGPS HDF, thus implying that accumulation of farnesylated form of truncated lamin A (progerin) interferes with re-organisation of chromosome territories. Movement of chromosomes is non-existent in cells after they enter a state of replicative senescence (Chapter 3 and 4). This phenomenon is explained by a model describing formation of a complex between nuclear motor proteins and INM proteins such as lamin A and emerin (Mehta et al., 2008), whereby emerin binds lamin A (Holaska and Wilson, 2006) that can bind to actin (Zastrow et al., 2004), which can bind to both emerin (Holaska and Wilson, 2006) and NMI which can bind emerin (Holaska and Wilson, 2006). With presence of mutant form of lamin A in HGPS patient cells as well as in senescent cells (McClintock et al., 2006), the functionality of this complex including nuclear actin, NMI, lamin A and emerin is compromised and hence chromosome repositioning does not occur in these cells (Mehta et al., 2008).

These results suggest that NMI may have more diverse function in the nucleus apart from transcription and chromatin remodelling and that they may be involved in transport of various nuclear structures through the nucleoplasm or the nucleus. In order to test if this proposition as well as the hypothesis that nuclear motors used for rapid relocation of chromosomes are non-functional in senescent and HGPS patient cells, further detailed characterisation of NMI within the nucleus is essential. The study here is the first step towards this objective whereby the distribution and relative content of NMIβ in control proliferating, quiescent and senescent HDFs is studied. Interestingly, in the vast majority of control non-

proliferating fibroblasts, nuclear myosin 1β is found in large nuclear aggregates, rarely observed in control proliferating cells. In addition to its function in repositioning of chromosomes following serum withdrawal (Chapter 3), the study in this chapter demonstrates evidence that NMIβ may be implicated in establishment of chromosome territory organisation following re-stimulation of quiescent cells.

To test for the presence of the suggested hypothetical complex comprising of lamin A, emerin, NMIβ and actin (Mehta et al., 2008), the distribution and relative content of NMIβ was determined in cells containing a mutant lamin A protein (cell lines derived from HGPS patients). Intriguingly, NMIβ distribution in proliferating HGPS HDFs is similar to its distribution in non-proliferating control fibroblasts. No change in NMIβ distribution is observed when quiescent HGPS fibroblasts are re-stimulated with serum. FTI treatment successfully restores both, the distribution and the relative content on NMIβ in HGPS fibroblasts. Moreover, in HGPS fibroblasts treated with FTI, the change in distribution of NMIβ is observed with entry of cells from G_0 into proliferative cell cycle by re-stimulation of quiescent cells. These data, thus imply that lamin A influences the activity of NMIβ which might be perturbed in HGPS HDFs due to accumulation of farnesylated form of lamin A.

5.2. Methods and Materials:

5.2.1. Cell culture:

Control HDFs, strain 2DD were cultured in 10% NCS-DMEM as described in 2.2.1. until deemed senescent. HDFs derived from HGPS patient – strain AG11498 (section 4.2.1) were maintained in 15% FBS DMEM, passaging twice weekly until they underwent replicative senescence (Section 4.2.1.).

Cells were induced to undergo quiescence by serum starvation, whereby control and HGPS HDFs were incubated in medium containing 0.5% serum for 7 days (Section 2.2.1.).

5.2.2. FTI:GGTI treatment:

AG11498 HDFs, cells derived from HGPS patients, were treated with a combination of FTI and GGTI (2.5μM final concentration each) for 48 hours as in 4.2.2.

5.2.3. Indirect immunofluorescence :

5.2.3.a. Fixation

For indirect immunofluorescence, cells were grown on glass coverslips for at least 48 hours and then washed with 1X PBS with three changes of the buffer. To assess the distribution of NMIβ, cells on coverslips were fixed with methanol:acetone (1:1, v/v) for 10 minutes on ice, followed by three washes in 1X PBS.

5.2.3.b. Antibody staining

Primary antibodies: Mouse (Novacastra) anti-pKi67; rabbit anti-nuclear myosin 1β (Sigma), 1:1000 and 1:50 in PBS/1%NCS (v/v), respectively.

Secondary antibodies: Swine anti-rabbit conjugated with TRITC (Dako) and donkey anti-mouse (Jackson's laboratories) were diluted 1:30 and 1:70 in PBS/1%NCS (v/v) respectively.

Dual staining experiments were performed. Primary antibodies were applied to fixed cells and incubated for 1 hour at room temperature, before extensive washing in 1X PBS. Secondary antibodies were then applied to cells for 1 hour at room temperature in the dark. Following extensive washing of coverslips in PBS, cells were rinsed in distilled water and mounted in Vectashield +/- DAPI (Vector laboratories).

5.2.3.c. Bioimaging and analysis

Digital images of nuclei were captured using a Model viewpoint GS grey scale digital camera, using Smart Capture 3.0.0 (Digital Scientific) via an Olympus BX41 fluorescence microscope using an Olympus UPlan FLN 100X oil immersion lens. Distribution of NMIβ was assessed by counting at least 200 nuclei positive for NMIβ per sample. At least three counts from independently treated coverslips were performed for each sample.

5.2.4. Electrophoresis and Western blotting:

Control (2DD) and HGPS (AG11498) fibroblasts were cultured for 48 hours at least following which cell suspensions, with a known cell number, were centrifuged at 300 – 400g and the supernatents were removed (section 2.2.1.). The

cell pellets were then washed by resuspension in versene and centrifuged again at 300 – 400g. The cells were then lysed in 2X SDS sample buffer (100 mM Tris-HCL pH6.8 (w/v), 4% SDS (v/v), 0.2% Bromophenol blue (v/v), 20% glycerol, 0.2% β-mercaptoethanol (v/v)) boiling at 100 ºC. The samples were stored at -20 ºC until further needed.

Before use, cells in 2X sample buffer were defrosted on ice and then boiled for 10 minutes (at 100ºC) and placed on ice. Cells were loaded at a concentration of either 1 x 10^5 or 5 x 10^4 per well. Whole cell lysates were then resolved on 8% SDS-PAGE gels (30% acrylamide mix (v/v) {Sigma}, 1.5M Tris pH 8.8 {Sigma}, 10% SDS (v/v) {Sigma}, 10% ammonium persulfate (w/v) {Sigma} and TEMED {Sigma}) (5% stacking gel – 30% acrylamide mix (v/v), 1.0M Tris (pH 6.8), 10% SDS (v/v) and 10% ammonium persulfate (v/v), TEMED) in 1X SDS-PAGE running buffer (25mM Tris, 192mM glycine, 0.1% SDS (w/v)) at 90V until the dye front reached the bottom of the gel. Rainbow coloured molecular weight markers {wide range, Sigma-Aldrich} were used to detect protein size.

Proteins were then electrophoretically transferred onto nitrocellulose membrane {Amersham Hybond™-C Extra, Amersham Biosciences} in 1X Towbin transfer buffer (50mM Tris, 380mM glycine, 20% methanol (v/v)) for 1 hour at 300mA. Membranes were incubated in blocking solution (4% (w/v) dried milk powder {Marvel} in 1X transfer buffer) overnight at 4ºC followed by incubation in primary antibody for NMIβ (rabbit) {Sigma} and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (mouse) {Abcam} diluted 1:1000 and 1:5000 respectively in 1% NCS/PBS overnight at 4 ºC. Following three washes in 1X TBS-Tween 20 (50mM Tris pH7.6, 150mM NaCl, 0.1% Tween 20 (v/v)), the membranes were incubated in horseradish peroxidise (HRP) conjugated secondary antibody (Swine

anti Rabbit and Rabbit anti mouse) {DAKO} both diluted 1:1000 for 1 hour at RT. 1X TBS-Tween 20 washes were then repeated and membranes were then subjected to antibody detection. Supersignal West Pico chemiluminiscent substrate kit {Thermo Scientific} was used for antibody detection whereby membranes were incubated in SuperSignal West Substrate Working Solution for 5 minutes in dark. Excess reagent was drained, the blot covered in clear plastic wrap and exposed to X-ray film (Biomax light film {Kodak}). The X-ray film was then developed using standard developer (GBX developer and replinisher {Kodak}) and fixer (GBX fixer and replinisher {Kodak}.

To measure the intensity of the bands, digital images of the Western blots were opened in ImageJ. Each band was outlined individually and the signal intensities in pixels in the area occupied by the band were quantified using the gel analysis tool in ImageJ menu. As a control, this was also repeated for an equivalent area of background for each Western blot. The mean pixel intensity of the background was subtracted from the mean intensity of each band. The band intensity of NMIβ after background subtraction was then normalised with the band intensity of GAPDH (after background subtraction), so as to minimise errors caused during loading samples.

5.3. Results:

5.3.1. NMIβ distribution in normal proliferating, quiescent and senescent HDFs:

In proliferating HDFs, NMIβ was predominantly found distributed along the nuclear periphery near the NE, in the nucleolus and punctuate foci of NMIβ were also observed to be distributed homogenously throughout the nucleoplasm (Figure 5.1.A-C). This distribution has been termed as "proliferating distribution of NMIβ."

In non-proliferating HDFs various nuclear distributions were observed including aggregates of NMIβ in the nucleoplasm, homogenous distribution of NMIβ foci throughout the nucleoplasm, nucleolar only staining (Figure 5.1.D-I), which have all been collectively termed as "non-proliferating distributions of NMIβ." Unlike proliferating HDFs, NMIβ did not localise at the nuclear rim in non-proliferating HDFs (Figure 5.1.D-I).

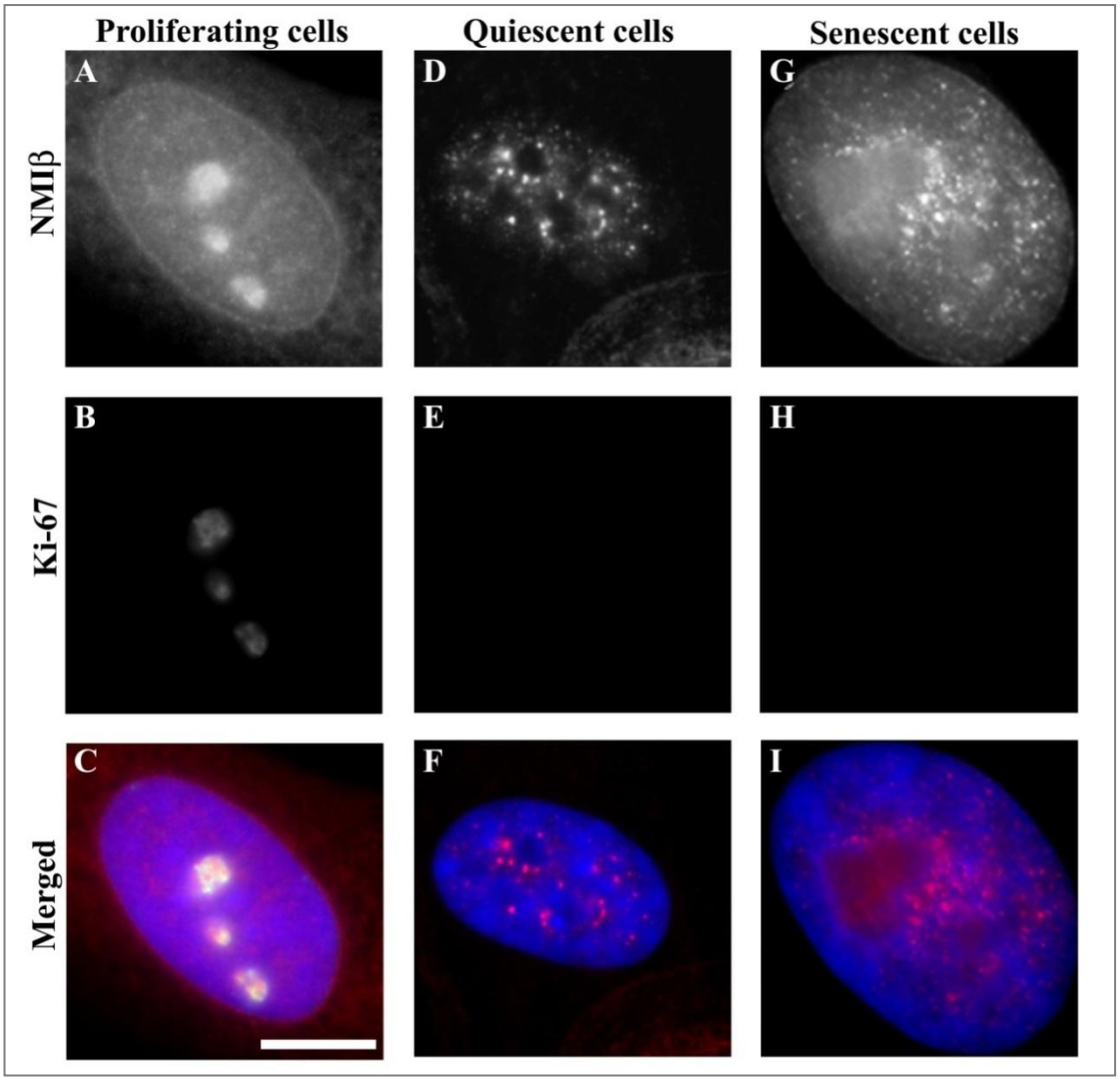


Figure 5.1.Representative images displaying the distribution of NMIβ in proliferating, quiescent and senescent HDFs: Samples were collected from young, old and serum starved HDF cultures, fixed with methanol:acetone and co-stained for anti-myosin 1β (first column) and pKi-67 (second column). DNA is delineated by DAPI (third column). Merged images are displayed in the fourth column. Scale bar =5μm

At least 200 NMIβ positive cells per sample were counted and the distribution of NMIβ was assessed in terms of different distribution patterns i.e. nucleolar + nuclear rim + homogenous, nucleolar only, aggregates, rim + homogenous, homogenous and dull (low intensity staining). The fraction of cells in each category was scored and correlated with the presence of pKi-67 in passage 11

cells (proliferating cells) and the absence of pKi-67 in passage 43 cells (senescent/non-proliferating cells) and in serum starved passage 11 cells (quiescent/non-proliferating). Three counts from independently treated coverslips were performed for each sample.

NMIβ display a range of nuclear distributions and locations in cells fixed with methanol:acetone (Figure 5.1.). 87.2% of proliferating cells displayed normal proliferating distribution of NMIβ in their nuclei (Table 5.1.). The most prevalent staining pattern in senescent cells revealed aggregates of NMIβ (81.4%) (Table 5.1). Even though pKi-67 negative cells were analysed from a passage 43 culture, aggregates were also seen in pKi-67 negative cells in the passage 11 culture, albeit at a much reduced frequency. In serum starved quiescent fibroblasts NMIβ was most predominantly distributed in large aggregates distributed in the nucleoplasm (72.1%) like in senescent HDFs. (Table 5.1). These data indicate that there is a significant difference in the distribution and organisation of NMIβ in non-proliferating fibroblasts as compared to proliferating fibroblasts (Figure 5.1. and Table 5.1.).

Table 5.1. NMIβ distribution in control proliferating, quiescent and senescent HDFs: The table displays % of cells showing a particular pattern of NMIβ staining. Error is denoted by standard deviation.

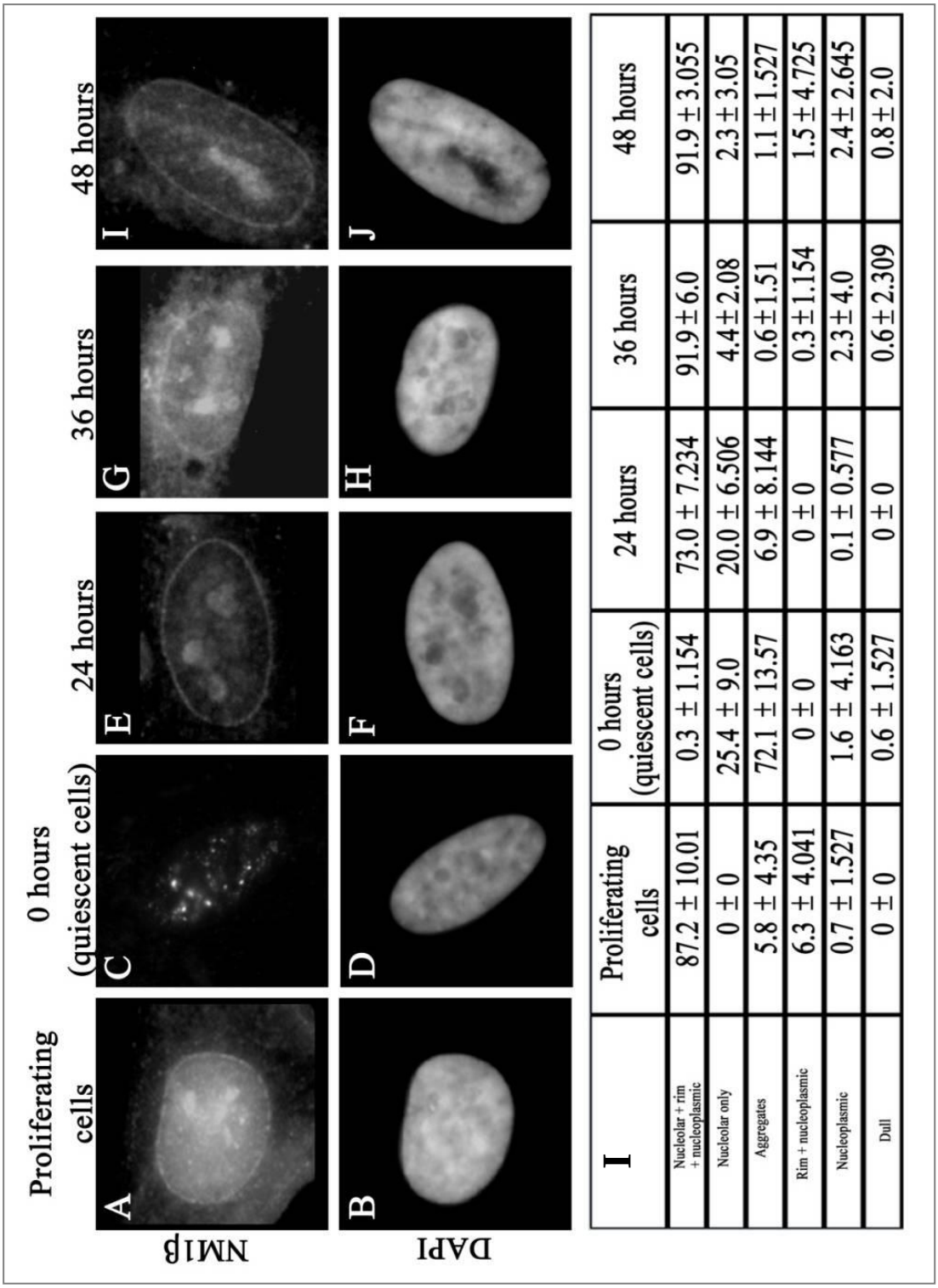
	Proliferating cells	Quiescent cells	Senescent cells
Nucleolar + rim + nucleoplasmic	87.2 ± 10.01	0.3 ± 1.15	2.5 ± 4.16
Nucleolar only	0 ± 0	25.4 ± 9.0	0 ± 0
Aggregates	5.8 ± 4.35	72.1 ± 13.57	81.4 ± 7.21
Rim + nucleoplasmic	6.3 ± 4.04	0 ± 0	10.1 ± 4.58
Nucleoplasmic	0.7 ± 1.53	1.6 ± 4.16	2.3 ± 2.64
Dull	0 ± 0	0.6 ± 1.53	3.6 ± 1.53

5.3.2. Distribution of NMIβ upon re-stimulation of quiescent HDFs:

Distribution of NMIβ in serum starved quiescent HDFs differs from its distribution in proliferating HDFs (Table 5.1 and Figure 5.1). Thus it would be pertinent to determine the time duration in which NMIβ localises at the nuclear envelope, nucleoli and throughout the nucleoplasm as in proliferating cells from the nucleoplasmic aggregates in quiescent cells (Figure 5.1 and Table 5.1) following re-stimulation of quiescent cells.

Control 2DD HDFs were cultured in 10% NCS-DMEM for 48 hours, followed by incubation in low serum (0.5% NCS-DMEM) for 7 days to induce quiescence. Cells were then re-stimulated with medium containing 10% NCS-DMEM and samples were collected 0, 24, 36 and 48 hours post serum re-stimulation. Samples of proliferating cells before serum starvation were also collected. All samples were then fixed with methanol:acetone (1:1) and a standard immunofluorescence assay was performed using anti-NMIβ antibody. At least 200 NMIβ positive cells per sample were counted and the fraction of cells displaying different NMIβ distribution patterns was recorded. The experiment was performed in triplicate for each sample whereby counts were done from 3 independently treated coverslips.

Figure 5.2. Distribution of NMIβ after restimulation of quiescent HDFs.



I	Proliferating cells	0 hours (quiescent cells)	24 hours	36 hours	48 hours
Nucleolar + rim + nucleoplasmic	87.2 ± 10.01	0.3 ± 1.154	73.0 ± 7.234	91.9 ± 6.0	91.9 ± 3.055
Nucleolar only	0 ± 0	25.4 ± 9.0	20.0 ± 6.506	4.4 ± 2.08	2.3 ± 3.05
Aggregates	5.8 ± 4.35	72.1 ± 13.57	6.9 ± 8.144	0.6 ± 1.51	1.1 ± 1.527
Rim + nucleoplasmic	6.3 ± 4.041	0 ± 0	0 ± 0	0.3 ± 1.154	1.5 ± 4.725
Nucleoplasmic	0.7 ± 1.527	1.6 ± 4.163	0.1 ± 0.577	2.3 ± 4.0	2.4 ± 2.645
Dull	0 ± 0	0.6 ± 1.527	0 ± 0	0.6 ± 2.309	0.8 ± 2.0

Figure 5.2. Distribution of NMIβ after restimulation of quiescent HDFs: Normal 2DD HDFs were serum starved for 7 days to induce quiescence. The cells were then restimulated with fresh serum and samples were collected at 0, 24, 36 and 48 hours post serum restoration. Samples were also collected before serum withdrawal (proliferating cells). The samples were then fixed with methanol:acetone (1:1) and distribution of NMIβ was assessed by performing an indirect immunofluorescence assay for NMIβ. Images in panels A, C, E, G and I display the distribution of NMIβ in cells before and after restimulation of quiescent fibroblasts. The table displays the % of cells displaying various patterns of NMIβ staining post re-stimulation. Error is indicated by standard deviation. Scale bar = 10 μm.

Images and table I in Figure 5.2. demonstrates that change of distribution from nucleoplasmic aggregates in quiescent cells (Figure 5.2. C and D) to a proliferating distribution of NMIβ occurs within the first 24 hours post serum re-stimulation (Figure 5.2. E and F and table within), when 73% of cells display proliferating cell-like distribution for NMIβ. The fraction of cells showing localisation of proliferating distribution of NMIβ increases after 36 (91.9%) (Figure 5.2. G and H and table within) and 48 hours (91.9%) (Figure 5.2. I and J) post serum re-stimulation.

With chromosome repositioning occurring between 24 – 36 hours (Chapter 3) post serum re-stimulation and distribution of NMIβ to a proliferating distribution within first 24 hours of serum re-stimulation; it is tempting to hypothesize that chromosome repositioning following serum re-stimulation (Chapter 3) may also be dependent on NMIβ.

5.3.3. Distribution of NMIβ in cells derived from HGPS patients before and after treatment with FTIs:

NMIβ distribution in proliferating HGPS HDFs (figure 5.3.) was different from that in control proliferating HDFs (figure 5.1. and table 5.1.). Unlike control proliferating HDFs, NMIβ does not localise at the NE or in the nucleolus in proliferating HGPS HDFs, instead it is found to be distributed around the nucleoplasm as large aggregates (Figure 5.3.A) as in control non-proliferating fibroblasts (Figure 5.1. and table 5.1.).

Proliferating HGPS HDFs treated with FTIs display a proliferating distribution of NMIβ (Figure 5.3.B). Although like non-proliferating controls and HGPS HDFs without FTI treatment; nucleoplasmic aggregates of NMIβ are also observed in proliferating HGPS HDFs treated with FTI (figure 5.3.B).

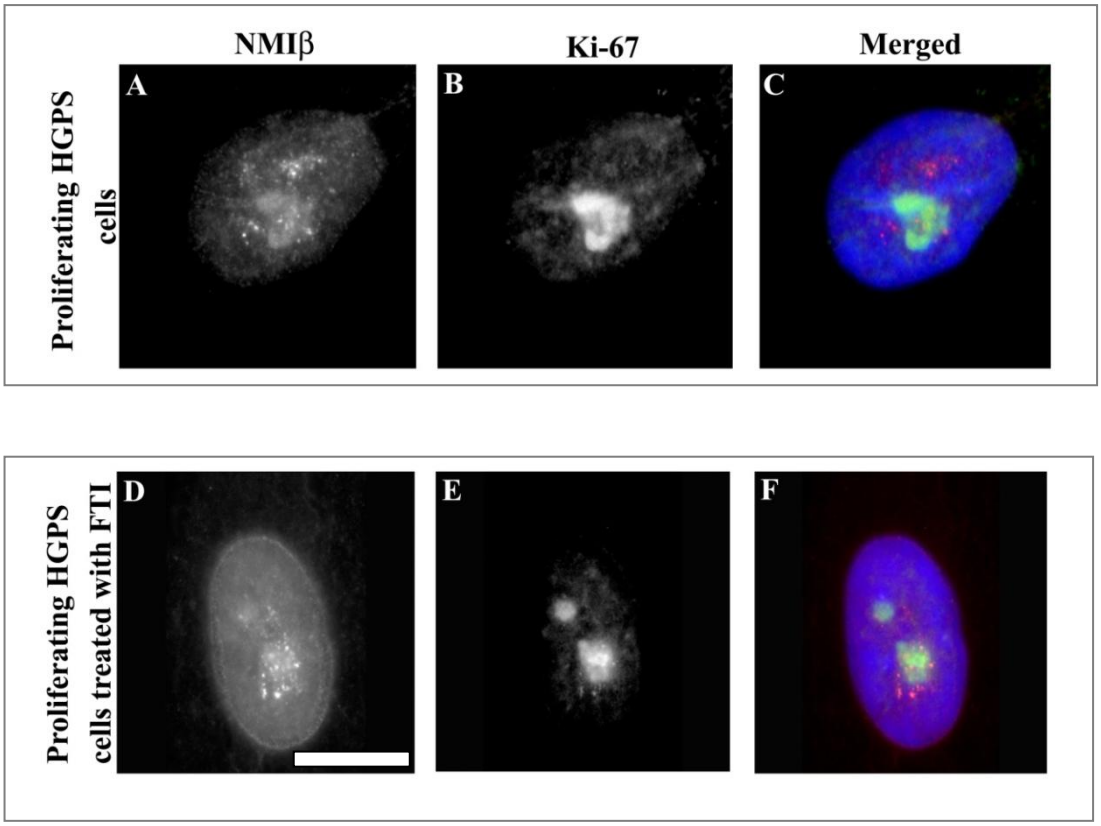


Figure 5.3. Representative images displaying the distribution of NMIβ in HGPS HDFs before and after treatment with FTI-277: Dual coloured immunofluorescence assay for NMIβ (panel A) and pKi-67 (panel B) was performed on HGPS HDFs before (A-C) and after (D-F) treatment with FTI-277. DAPI was used to delineate the DNA in the nucleus. Merged image is displayed in panel C. Scale bar = 10 μm.

To assess the different distribution patterns of NMIβ in HGPS HDFs, independent triplicate counts (at least 200 NMIβ positive cells) per sample were performed. Distribution of NMIβ was also assessed in non-proliferating cells, i.e. in quiescent and senescent HGPS HDFs before and after treatment with FTI-277. Percentage of cells displaying each of the distributions derived from three independent counts are in proliferating, quiescent and senescent HGPS HDFs before and after FTI treatment are shown in Table 5.2.

Most of the proliferating (85.2%), quiescent (77.3%) and senescent (80.1%) HGPS HDFs have NMIβ distributed as large aggregates throughout the nucleoplasm, thus displaying a non-proliferating distribution of NMIβ (Table 5.2.A. and figure 5.1.).

Table 5.2. NMIβ distribution in proliferating, quiescent and senescent HGPS HDFs before and after treatment with FTI-277: The table displays % of cells showing a particular pattern of NMIβ staining. Error is denoted by standard deviation.

A	Proliferating HGPS cells	Quiescent HGPS cells	Senescent HGPS cells
Nucleolar + rim + nucleoplasmic	5.9 ± 3.0	0.8 ± 2.0	3.6 ± 3.21
Nucleolar only	0 ± 0	19.8 ± 6.66	0 ± 0
Aggregates	85.2± 6.11	77.3 ± 6.66	80.1 ± 8.50
Rim + nucleoplasmic	5.2 ± 2.52	0 ± 0	9.6 ± 4.0
Nucleoplasmic	2.9 ± 2.65	1.4 ± 3.51	2.3 ± 2.64
Dull	0.8 ± 1.53	0.6 ± 0.58	4.5 ± 2.51

B	Proliferating HGPS cells treated with FTI	Quiescent HGPS cells treated with FTI	Senescent HGPS cells treated with FTI
Nucleolar + rim + nucleoplasmic	73.8 ± 7.09	2.0 ± 1.53	4.2 ± 1.53
Nucleolar only	0 ± 0	19.3 ± 11.36	0 ± 0
Aggregates	18.6± 6.56	74.2 ± 5.29	81.5 ± 6.24
Rim + nucleoplasmic	4.8 ± 2.08	0 ± 0	8.8 ± 4.51
Nucleoplasmic	1.83± 2.08	2.9 ± 2.08	1.9 ± 3.0
Dull	0.9± 1.53	1.5 ± 1.53	3.5 ± 2.0

Proliferating HGPS HDFs treated with FTI predominantly display nucleolar, nuclear rim and punctuate foci throughout the nucleoplasm distribution of NMIβ (73.8%) (Table 5.2.B). This is similar to the distribution of NMIβ observed in control proliferating HDFs. FTI treatment does not have any effect on the distribution of NMIβ in non-proliferating HGPS HDFs, i.e. in both quiescent and senescent HDFs, where NMIβ is still predominantly observed to be distributed in aggregates throughout the nucleoplasm (Table 5.2.B).

These data fit the hypothesis that presence of farnesylated lamin A interferes with the complex comprising NMIβ, nuclear actin, emerin and lamin A. Due to a mutation in lamin A, its interactions with emerin might be affected which might then affect the interaction between emerin and myosin leading to altered distribution of NMIβ in HGPS HDFs.

5.3.4. Distribution of NMIβ upon re-stimulation of quiescent HGPS HDFs before and after FTI treatment:

In AG11498, a cell line derived from HGPS patient, fibroblasts undergo quiescence like control cells upon serum withdrawal for 7 days, but are unable to enter the proliferative cell cycle upon re-stimulation with serum. The percentage of Ki-67 positive cells in quiescent HGPS cultures is nil and there is a very slight change in this percentage following serum re-stimulation, whereby the percentage of Ki-67 positive fibroblasts increases slightly peaking at 1.22% at 36 hours (Figure 5.5.yellow line). Thus, the fraction of cells displaying presence of pKi-67 following serum re-stimulation in HGPS HDFs (1.22%) is significantly lower as compared to control fibroblasts (38%). This implies that the mutation in lamin A may be uncoupling the pathway that leads the cells in and out of quiescence.

The response to serum re-stimulation and entry into the proliferative cell cycle in quiescent HGPS HDFs is restored by FTI treatment. The percentage of Ki-67 positive fibroblasts in quiescent AG11498 HDFs treated with FTI-277 for 48 hours post serum re-stimulation increases steadily peaking at 22% at 36 hours post serum re-stimulation (Figure 5.5.red line). The response to re-stimulation (peaking at 36 hours) is slower in FTI treated HGPS HDFs than that observed in control fibroblasts (peak at 32 hours). Although, this is not as high as observed in controls

(38%), it is significantly higher than that in HGPS fibroblasts without FTI treatment (1.22%) (Figure 5.5.). The discrepancy observed in percentage of Ki-67 positive cells between control and HGPS fibroblasts after FTI treatment may be because these cultures were not age matched and hence HGPS fibroblasts may have already undergone more PD as compared to younger control HDFs.

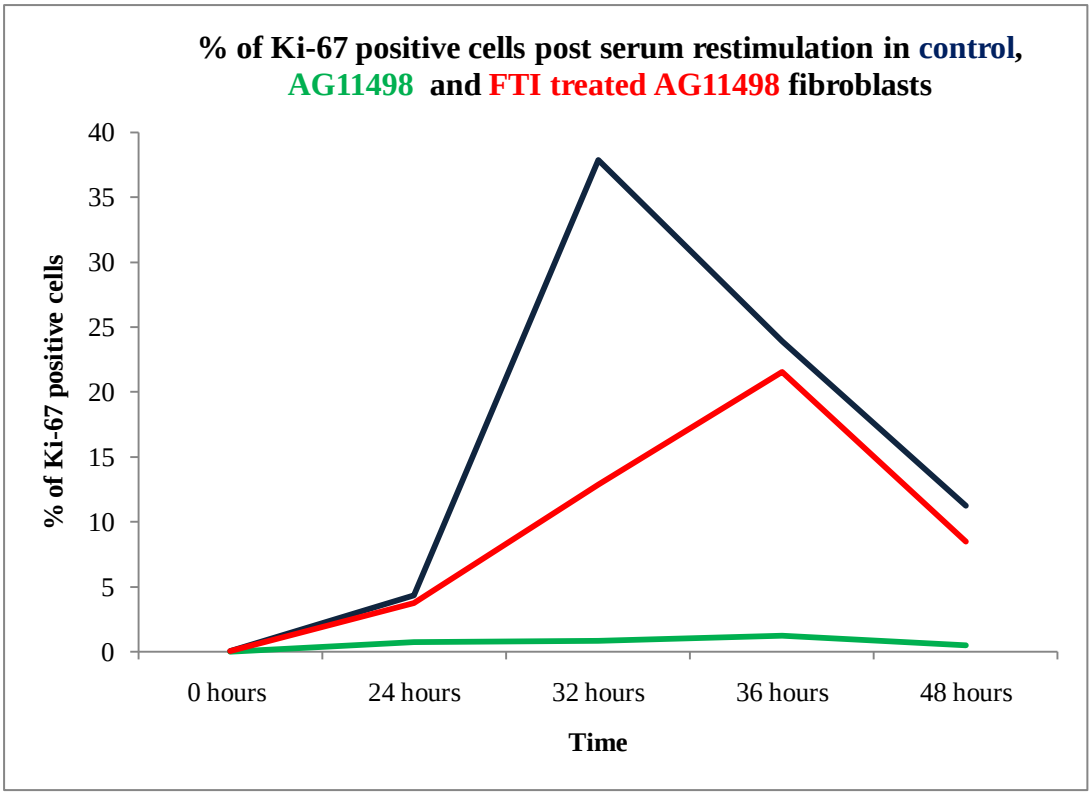
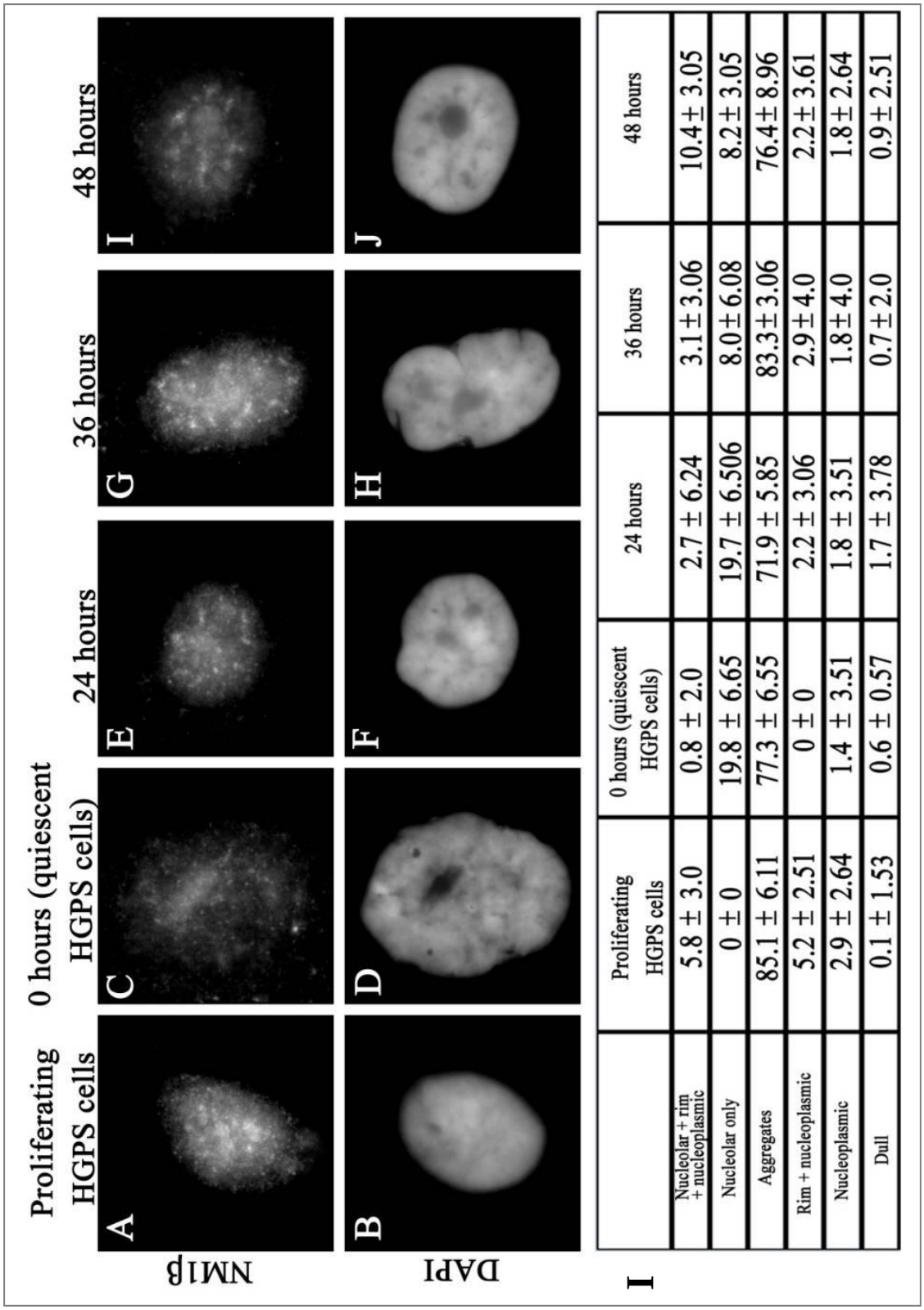


Figure 5.4. Graph displaying the fraction of Ki-67 positive cells in control (2DD), AG11498 and FTI treated AG11498 following serum re-stimulation of quiescent cells: Control (2DD), HGPS (AG11498) and FTI treated HGPS (AG11498) HDFs were serum starved for 7 days to induce quiescence and then were re-stimulated with fresh serum. Samples were collected at 0, 24, 36 and 48 hours post serum re-stimulation, fixed with methanol:acetone (1:1) and a standard indirect immunofluorescence assay was performed for pKi-67. The % of Ki-67 positive cells (representing proliferating cells in culture) (y-axis) in control (blue line), AG11498 (yellow line) and FTI treated AG11498 (red line) HDFs are plotted against time after re-stimulation (x-axis).

Thus, with evidence suggesting that the pathway allowing the re-entry of quiescent HGPS HDFs following serum re-stimulation may be compromised, it would be interesting to identify if there is a change in the distribution of NMIβ following re-stimulation of quiescent cells.

In order to assess the distribution of NMIβ in quiescent HGPS HDFs following serum re-stimulation, serum starved quiescent AG11498 HDFs were re-stimulated with fresh serum and samples were collected at 0, 24, 36 and 48 hours post re-stimulation. The cells were then fixed with methanol:acetone (1:1) and a standard immunofluorescence assay was employed for NMIβ. At least 200 NMIβ positive cells were counted per sample and three counts were performed from independently treated coverslips for each sample. Percentage of NMIβ displaying various distributions of NMIβ was recorded.

Figure 5.5. Distribution of NMIβ after restimulation of quiescent HGPS HDFs.



I

	Proliferating HGPS cells	0 hours (quiescent HGPS cells)	24 hours	36 hours	48 hours
Nucleolar + rim + nucleoplasmic	5.8 ± 3.0	0.8 ± 2.0	2.7 ± 6.24	3.1 ± 3.06	10.4 ± 3.05
Nucleolar only	0 ± 0	19.8 ± 6.65	19.7 ± 6.506	8.0 ± 6.08	8.2 ± 3.05
Aggregates	85.1 ± 6.11	77.3 ± 6.55	71.9 ± 5.85	83.3 ± 3.06	76.4 ± 8.96
Rim + nucleoplasmic	5.2 ± 2.51	0 ± 0	2.2 ± 3.06	2.9 ± 4.0	2.2 ± 3.61
Nucleoplasmic	2.9 ± 2.64	1.4 ± 3.51	1.8 ± 3.51	1.8 ± 4.0	1.8 ± 2.64
Dull	0.1 ± 1.53	0.6 ± 0.57	1.7 ± 3.78	0.7 ± 2.0	0.9 ± 2.51

Figure 5.6. Distribution of NMIβ after restimulation of quiescent HGPS HDFs: AG11498 (HGPS patient) HDFs were serum starved for 7 days to induce quiescence. The cells were then restimulated with fresh serum and samples were collected at 0, 24, 36 and 48 hours post serum restoration. Samples were also collected before serum withdrawal (proliferating cells). The samples were then fixed with methanol:acetone (1:1) and distribution of NMIβ was assessed by performing an indirect immunofluorescence assay for NMIβ. Images in panels A, C, E, G and I display the distribution of NMIβ in cells before and after restimulation of quiescent fibroblasts. The table I displays the % of cells displaying various patterns of NMIβ staining post re-stimulation. Error is indicated by standard deviation.

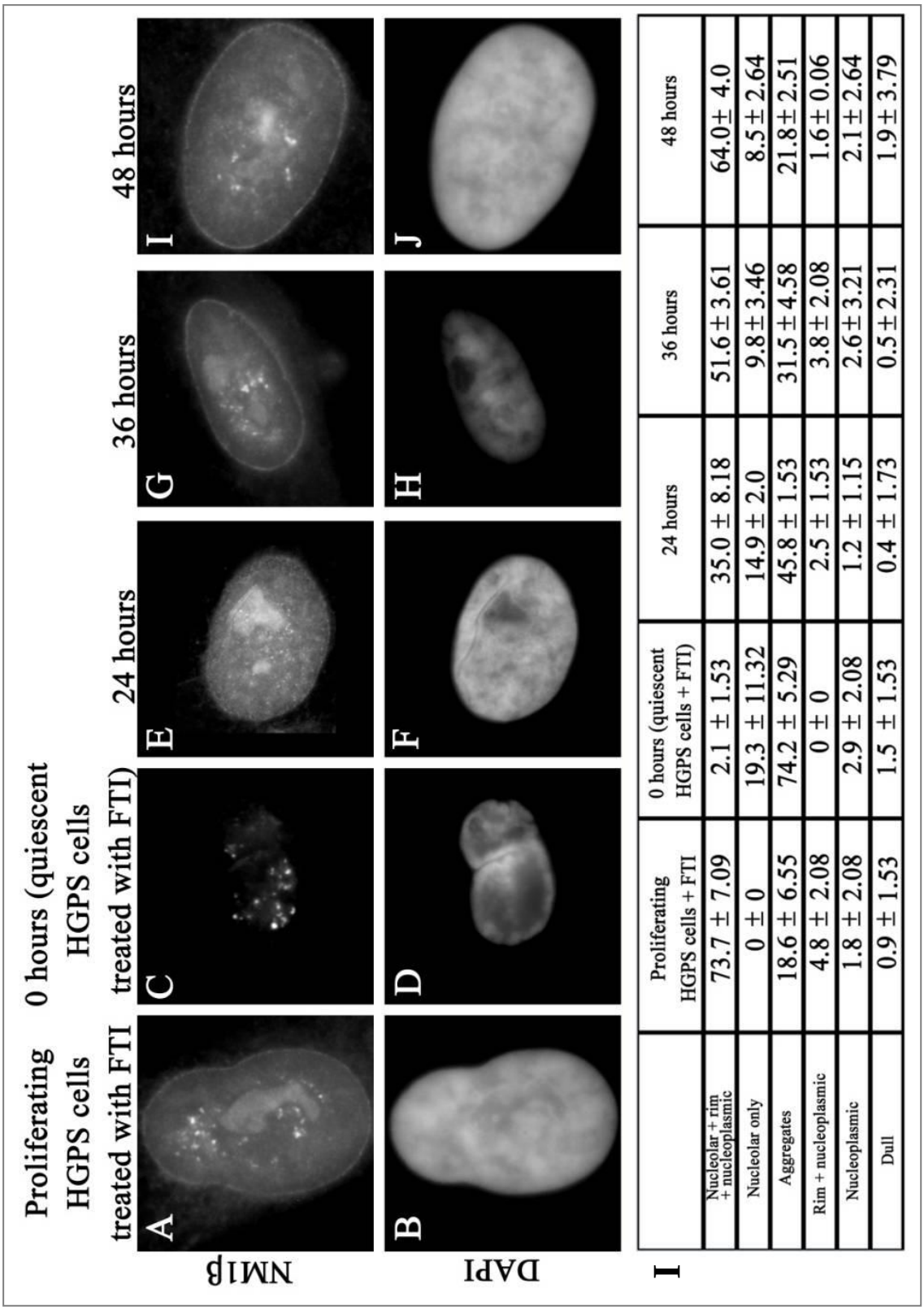
Figure 5.6. displays representative images showing the distribution of NMIβ in quiescent HGPS fibroblasts at 0, 24, 36 and 48 hours following re-stimulation with serum. For all samples, i.e. proliferating HGPS HDFs (85.1%), quiescent HGPS HDFs (77.3%), quiescent HGPS HDFs at 24 (71.9%), 36 (83.3%) and 48 (76.4%) post serum re-stimulation, NMIβ is predominantly distributed in large nucleoplasmic aggregates (Figure 5.6. and table I within) similar to that in control non-proliferating HDFs (Figure 5.6. and table 5.6.).

Results displayed in figure 5.5. demonstrate that FTI treatment re-establishes the ability of quiescent HGPS HDFs to respond to serum re-stimulation and facilitates it re-entry into the proliferative cell-cycle. Whether or not FTI treatment affects the NMIβ distribution in quiescent HGPS HDFs post serum re-stimulation still remains uncertain.

To assess the distribution of NMIβ in quiescent HGPS HDFs treated with FTI, HGPS HDFs were first treated with FTI-277 for 48 hours and then incubated in low serum for 7 days to induce quiescence following which they were re-stimulated with medium containing fresh serum. Samples were collected 24, 36 and 48 hours post serum re-stimulation, fixed with methanol:acetone (1:1) and then stained for

NMIβ using standard indirect immunofluorescence assay. 200 NMIβ positive cells were counted per sample and three counts from independently treated coverslips were performed for each sample. The percentage of cells displaying different NMIβ distributions was recorded.

Figure 5.6. Distribution of NMIβ after restimulation of quiescent HDFs.



I

	Proliferating HGPS cells + FTI	0 hours (quiescent HGPS cells + FTI)	24 hours	36 hours	48 hours
Nucleolar + rim + nucleoplasmic	73.7 ± 7.09	2.1 ± 1.53	35.0 ± 8.18	51.6 ± 3.61	64.0± 4.0
Nucleolar only	0 ± 0	19.3 ± 11.32	14.9 ± 2.0	9.8 ± 3.46	8.5 ± 2.64
Aggregates	18.6 ± 6.55	74.2 ± 5.29	45.8 ± 1.53	31.5 ± 4.58	21.8 ± 2.51
Rim + nucleoplasmic	4.8 ± 2.08	0 ± 0	2.5 ± 1.53	3.8 ± 2.08	1.6 ± 0.06
Nucleoplasmic	1.8 ± 2.08	2.9 ± 2.08	1.2 ± 1.15	2.6 ± 3.21	2.1 ± 2.64
Dull	0.9 ± 1.53	1.5 ± 1.53	0.4 ± 1.73	0.5 ± 2.31	1.9 ± 3.79

Figure 5.7. Distribution of NMIβ after restimulation of quiescent HDFs:

AG11498 HDFs treated with FTI-277 were serum starved for 7 days to induce quiescence. The cells were then restimulated with fresh serum and samples were collected at 0, 24, 36 and 48 hours post serum restoration. Samples were also collected before serum withdrawal (proliferating cells). The samples were then fixed with methanol:acetone (1:1) and distribution of NMIβ was assessed by performing an indirect immunofluorescence assay for NMIβ. Images in panels A, C, E, G and I display the distribution of NMIβ in cells before and after restimulation of quiescent fibroblasts. The table displays the % of cells displaying various patterns of NMIβ staining post re-stimulation. Error is indicated by standard deviation.

FTI treatment restores the NMIβ distribution at the NE in proliferating HGPS HDFs (Section 5.3.3.). In quiescent HGPS HDFs NMIβ is (73.7%) distributed predominantly as nucleoplasmic aggregates similar to that in control quiescent HDFs (Section 5.3.1). After serum re-stimulation, NMIβ is redistributed to the nuclear rim from the nucleoplasmic aggregates in serum starved quiescent HGPS fibroblasts (Figure 5.7.). 24 hours post serum re-stimulation, 35% of cells displayed HGPS distributed at the NE (Figure 5.7.), while the fraction of cells displaying this distribution of NMIβ increased to 51.6% and 64% at 36 and 48 hours respectively after serum re-stimulation (table within figure 5.7.).

Thus, farnesylated form of lamin A present in HGPS patient cells appear to compromise some fundamental cellular processes such as re-entry of the cells in the proliferative cell cycle from quiescence and also affects components of the nuclear architecture other than just the nuclear lamina. Encouragingly, the results here demonstrate that FTI treatment restores the ability of the cells to re-enter the cell cycle from a quiescent state and it also re-establishes the distribution of NMIβ as observed in control fibroblasts.

5.3.5. Relative content of NMIβ in control and HGPS fibroblasts:

Differences observed in the distribution patterns of NMIβ in control proliferating and non-proliferating fibroblasts and between HGPS and control fibroblasts, suggest a possibility that the relative content of the protein in these cells may vary. To test this hypothesis, whole cell lysates were prepared from control proliferating, quiescent and senescent HDFs and also from AG11498 (HGPS) HDFs before and after FTI treatment. The proteins from these lysates were separated on 8% SDS-PAGE gels and electrophoretically transferred onto a nitrocellulose membrane. Amount of NMIβ and GAPDH (positive control) was then assessed by staining the blots with respective antibodies. Figure 5.8. displays images of Western blots displaying the amount of NMIβ and GAPDH in control proliferating, quiescent and senescent fibroblasts and in HGPS fibroblasts before and after FTI treatment.

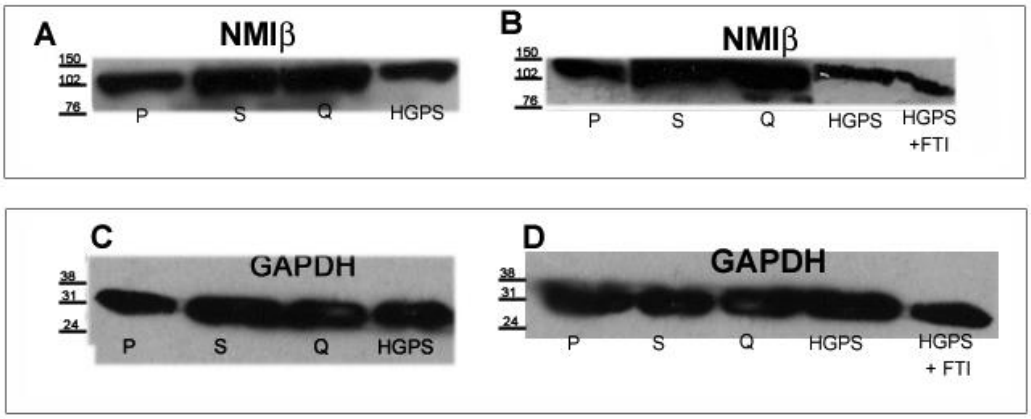


Figure 5.7. Western blot analysis for determining the relative content of NMIβ in control proliferating, quiescent, senescent, HGPS and HGPS + FTI treated HDFs. Whole cell lysates from control (proliferating, quiescent and senescent) HDFs, AG11498 HDFs and AG11498 HDFs treated with FTI were prepared and proteins were separated on 8% SDS-PAGE gels. For blots A and C, the samples were loaded equally at 5 x 10^4 cells per lane. For blot B and C samples were loaded equally at 2 x 10^4 cells per lane. Images for blots of NMIβ (A and B) and GAPDH (Cand D) are shown. {P = control proliferating, Q = control quiescent, S = Control senescent, HGPS = AG11498 p16, HGPS + FTI = AG11498 p16 treated with FTI}.

All samples (Figure 5.8. A and B) have approximately 120kDa band for NMIβ and around 37kDa band for GAPDH. There is a very slight difference in the amount of NMIβ between control proliferating, quiescent and senescent HDFs. Although, it is clear from the Western blots displayed above that the amount of NMIβ in HGPS HDFs is lower as compared to control fibroblasts (Figure 5.8. A and B). Figure 5.8.B also demonstrates presence of NMIβ specific band in AG11498 cells treated with FTI, thus suggesting that FTI treatment restores NMIβ levels closer to that observed in HGPS fibroblasts before treatment.

In order to quantify the differences in the relative content of NMIβ as described above, the area of the band was measured using ImageJ for each sample,

background subtracted and then normalised with the area of GAPDH band for that particular sample. Thus normalised area occupied by NMIβ in control proliferating, quiescent and senescent cells and in HGPS cells (all loaded at 1 x 10^5 cells per well) are displayed in table 5.4.

Table 5.3. Relative content of NMIβ in control (proliferating, quiescent and senescent) and AG11498 HDFs:

Cells	Normalised area occupied by NMIβ band
Control proliferating	1.45
Control quiescent	1.30
Control senescent	1.41
AG11498	0.31

Normalised area occupied by NMIβ in control proliferating, quiescent and senescent HDFs, AG11498 HDFs before and after FTI treatment (all samples loaded at 5 x 10^4 cells per well) are displayed in table 5.5.

Table 5.4. Relative content of NMIβ in control (proliferating, quiescent and senescent), AG11498 HDFs and FTI treated AG11498 HDFs:

Cells	Normalised area occupied by NMIβ band
Control proliferating	0.73
Control quiescent	0.61
Control senescent	0.85
AG11498	0.31
AG11498 + FTI	0.52

Thus, indeed the amount of NMIβ in AG11498 (HGPS) HDFs is significantly lower as compared to that in control fibroblasts and FTI treatment of HGPS HDFs restores the relative content of NMIβ much closer to that in control fibroblasts. There are minute differences in the relative content of NMIβ between control proliferating, quiescent and senescent fibroblasts; with quiescent cells have a slightly lower amount of NMIβ and senescent and proliferating fibroblasts having very similar amounts of NMIβ.

5.4. Discussion:

Molecular motors of the cell cytoplasm have been studied for many years. Over the years there have been on/off discussions as to whether there were molecular motors within the nuclei of cells to perform active movement and to be involved in nuclear processes. The discussions started in the 1970s (Lestourgeon et al., 1975, Clark and Rosenbaum, 1979), with a role for nuclear actin and myosin being suggested in heterochromatin formation (Comings and Okada, 1976). In the 1980s evidence was provided that nuclear actin and myosin were involved in nucleocytoplasmic transport (Schindler et al., 1989) and in the 1990s more ultrastructural studies were performed, localising actin and myosin in the nucleus (Milankov and De Boni, 1993, Amankwah and De Boni, 1994). Over the last few years various isoforms of myosin have been localised in the nucleus and studies are more focussed on the functional relevance of presence of these molecular motors within the cell nuclei (Mehta et al., 2008, de Lanerolle et al., 2005, Hofmann et al., 2006a). In order to understand all nuclear processes that might involve these molecular motors within the nucleus, it is important to systematically characterise the components of these motors in terms of their distribution, relative content, binding partners, regions of localisation and attachments as well as their interactions with other components of the nuclear architecture within the cell nuclei.

The study in this chapter aims to initiate the characterisation of one of the isoforms of myosin found in the nucleus, NMIβ. In particular, the distribution and relative content of NMIβ in proliferating and non-proliferating HDFs has been assessed. In addition to this, to identify any interaction/cross-talk between lamin A, a component of the nuclear lamina, and NMIβ (Mehta et al., 2008), an indirect

approach has been employed, whereby the distribution and relative content of NMIβ in a lamin A mutant cell line has been compared to that in control cells. Previous studies employing immunoelectron microscopy (Nowak et al., 1997) and indirect immunofluroescence (Fomproix and Percipalle, 2004) have demonstrated the presence of NMI within dense fibrillar compartment and granular compartment of the nucleolus, where it has been shown to interact with RNA polymerase I (Fomproix and Percipalle, 2004, Percipalle et al., 2006, Philimonenko et al., 2004, Kysela et al., 2005) and thus influences transcription (Philimonenko et al., 2004, Percipalle and Visa, 2006). In addition to this, NMIβ has also been documented to be located within regions condensed and decondensed chromatin and it also colocalises with transcription sites (Kysela et al., 2005). In control proliferating HDFs, NMIβ is indeed localised in the nucleolus. By performing dual staining experiments with pKi-67 which is known to localise in the DFC domain of the nucleolus (Kill, 1996), the presence of NMIβ within this nucelolar domain has also been confirmed. Whether or not NMIβ colocalise or interact with nucleolar proteins such as nucleolin or Ki-67 present in the same nucleolar domain still remains to be explored. In addition to the nucleolar distribution, NMIβ is also distributed at the nuclear rim by the nuclear envelope in proliferating HDFs as well as foci throughout the nucleoplasm. The functional relevance of presence of NMIβ at these nuclear sites still remains unclear, but hypotheses suggesting interaction between nuclear envelope protein, namely emerin and lamin A, and NMIβ has been put forward (Mehta et al., 2008). NMIβ has been implicated in nucleocytoplasmic transport, hence it is a possibility that NMIβ may interact with components of nuclear pore complexes or with other INM and ONM proteins such as SUN domain proteins, nesprins, some of which have an affinity towards cytoplasmic myosins (Zhang et al.,

2005). NMIβ, being a part of WICH (WSTF-SNF2h complex), and being involved in movement of chromatin regions (Dundr et al., 2007, Chuang et al., 2006) and whole chromosomes (Chapter 3) (Hu et al., 2008) across the nucleus, the nucleoplasmic pool of NMIβ may be facilitating chromatin remodelling and intranuclear chromosomal repositioning. NMIβ colocalises with nuclear actin in plant cell nuclei and is present in the nucleolus, with putative transcription foci and in an intranuclear network (Cruz and Moreno Diaz de la Espina, 2009).

Interestingly, in control non-proliferating (both quiescent and senescent) HDFs, no nuclear rim or nucleolar localisation of NMIβ is observed, instead large nucleoplasmic aggregates of this protein are present. Although, the nature of these aggregates is not clear, they may be composed of protein that is to be removed from the nucleus, since it may no longer be required by irreversibly arrested cells. NM1β has been implicated in transcription and chromatin remodelling; both quiescent and senescent cells proceed to slow down / execute both these cellular processes respectively and so it is not clear whether we are seeing the reorganisation or disorganisation of a nuclear motor component with the aggregates. With aggregates of NMIβ observed in quiescent cells when major DNA repair occurs, are these aggregates a part of DNA repair factories or are marking the site of DNA damage? The functional relevance whereby there would be no interaction between NMIβ, INM and nucleolar proteins remains unclear. Chromosome repositioning is not apparent once cells enter replicative senescence (Chapter 2), thus suggesting that redistribution of NMIβ in non-proliferating HDFs may interfere with their ability to transport nuclear components within the nucleus. NMIβ distribution changes from a non-proliferating cell like distribution to that observed in proliferating cells within first 24 hours post re-stimulation of quiescent cells. With chromosome repositioning

occurring between 24-36 hours post re-stimulation of quiescent cells (Chapter 3), this is indeed an interesting result suggesting that NMIβ is not only required for chromosome repositioning upon entry into quiescence (Chapter 3) but may also be important for the same upon exit from quiescence and entry into proliferative cell cycle. This also leads to the hypothesis that NMIβ may play a vital role in organisation of chromosomes and nuclear architecture in general following mitosis.

In lamin A mutant cell line derived from HGPS patients, NMIβ is predominantly distributed in nucleoplasmic aggregates as observed in non-proliferating control cells and treatment of these cells with FTI (Chapter 4) restores NMIβ to the nuclear rim and nucleolus in proliferating subset of cells. HDFs derived from HGPS patients also have reduced amount of NMIβ as compared to control fibroblasts. If a lower amount of the protein NMIβ has a direct effect on the distribution of the protein is unclear. Thus lamin A is vital for organisation and maintenance of NMIβ within proliferating cell nucleus and presence of farnesylated forms / mis-localisation of lamin A interferes with relative content and distribution of NMIβ. No direct interactions between lamin A and NMIβ have been revealed to date but with both these proteins present at the nuclear rim and as nucleoplasmic foci, this is a very likely possibility. Whether or not transcription is directly affected in HGPS cells due to disorganised NMIβ is unknown. Chromosome positioning is altered in HGPS HDFs (Chapter 4); which may be a result of dysfunctional complex comprising lamin A and NMIβ (Mehta et al., 2008) both of which are organised differently in HGPS HDFs.

Cells with mutant lamin A protein (HGPS patient cells) once induced to undergo quiescence, are unable to re-enter the cell cycle following re-stimulation. This is indeed a surprising result suggesting that signal transduction, in particular

response to growth arrest signals may be perturbed in these cells. Restriction to re-entering the cell cycle may also lead these cells to undergo apoptosis. Cells derived from HGPS patients display quiescent-cell like chromosome organisation (Chapter 4), does this imply that these cells permanently remain quiescent once they enter this state of growth arrest? This would indeed explain the growth curves of the cell lines with mutant lamin A protein where by they are hyperproliferative in culture when young and with age in culture and accumulated DNA damage, more and more cells are forced to enter quiescence, when they cannot re-enter the cell cycle after the DNA damage is repaired, these cells undergo apoptosis. Although, there is a possibility that HGPS HDFs may take much longer time (more than 48 hours) to enter the cell cycle following re-stimulation. Inhibition of farnesylation restores HGPS HDFs from growth arrest state and these cells are able to re-enter the cell cycle,. Thus, confirming the role of lamin A in cell cycle regulation (Johnson et al., 2004, Van Berlo et al., 2005, Dorner et al., 2006, Pekovic et al., 2007) and signal transduction (Rao et al., 1997, Tang et al., 2000, Martelli et al., 2002, Parnaik, 2008).

Treatment with FTI and inhibition of farnesylation restores not only the distribution of NMIβ in the nuclei of cells derived from HGPS patients but also corrects the re-distribution of NMIβ from a non-proliferating cell like distribution (nucleoplasmic aggregates) to a proliferating cell-like distribution (nuclear rim, nucleolar and nucleoplasmic foci) in cells with mutant lamin A. This re-distribution does not occur within first 24 hours post re-stimulation of quiescent cells like in control HDFs but takes much longer (36 hours) after re-stimulation of quiescent cells. This, together with fraction of Ki-67 positive cells post re-stimulation suggests that HGPS fibroblasts, are able to re-enter the proliferative cell-cycle following

inhibition of lamin A farnesylation, but this response is slower as compared to that in control fibroblasts.

Thus, the study within this chapter successfully associates NMIβ with specific nuclear compartments and highlights differences in organisation of NMIβ between proliferating and non-proliferating cells. Using lamin A mutant cell lines, indirect role of lamin A in controlling NMIβ organisation within the nucleus is elicited. Although, this research is suggesting an involvement of NMIβ in various important nuclear functions, many of the potentials of this protein in the nucleus still remains to determined.

Chapter 6: Nucleolar proteins in HGPS fibroblasts

- Some of the contents of this chapter are in press for Biochemical Society Transactions under the title “Progeria, the nucleolus and farnesyltransferase inhibitos” – Ishita S Mehta, Joanna M Bridger and Ian R Kill.

6.1. Introduction:

HGPS is a premature ageing syndrome due to a mutation in *LMNA* gene. The protein products of this gene are important components of the nuclear lamina, hence studies on HGPS cells and models to date have been concentrated on the defects in nuclear lamina and functions related with the lamina. Since lamin A is not only present at the nuclear lamina but is also attached to the nuclear matrix (Goldman et al., 1992, Barboro et al., 2002, Hozak et al., 1995) as well as found within the nuclear interior (Bridger et al., 1993) with sites of replication (Kennedy et al., 2000), transcription factories (Moir et al., 1994) and splicing speckles (Jagatheesan et al., 1999), mutations in *LMNA* gene might affect other nuclear structures and nuclear bodies.

The nucleolus, a prominent sub-cellular structure was first described more than a century and a half ago (Olson and Dundr, 2005). Apart from its primary function of ribosome biogenesis, importantly for this study, the nucleolus has been implicated to play a role in some of the processes that are affected in HGPS patients including cell cycle regulation (Pederson, 1998), genome organisation (Weipoltshammer et al., 1999, Bridger and Bickmore, 1998, Sullivan et al., 2001, Bridger et al., 2000, Chubb et al., 2002, Borden and Manuelidis, 1988) (Chapter 4) and maintenance of proliferative or senescent status of the cell (Ugrinova et al., 2007, Daniely et al., 2002, Zimber et al., 2004, Colombo et al., 2002, Rosete et al., 2007, Mehta et al., 2007). Impaired or irregular functioning of this nuclear domain and its components results in cancer predisposition, genomic instability and may cause diseases such as Diamond-Blackfan anaemia, dyskeratosis congenital and myelodysplastic syndromes (Boisvert et al., 2007). Abnormal nucleolar morphology

and functions have been documented as a result of viral infections as well as in patients suffering from neurodegenerative diseases like Alzheimer's, Huntington's and spinocerebellar ataxias (Boisvert et al., 2007). Interestingly, Werner syndrome protein (WRN) - a RecQ-type DNA helicase, that is implicated in another premature ageing syndrome (Werner's syndrome) has been shown to have strong interactions with nucleoli (von Kobbe and Bohr, 2002, Boisvert et al., 2007, Marciniak et al., 1998). Mutations in genes coding for nucleolar proteins induce premature senescence (Rosete et al., 2007) and hence it would be vital to investigate if nucleolus or the nucleolar proteins contribute to the HGPS pathology.

Nucleolin and NPM are multifunctional nucleolar proteins. Nucleolin occupies the DFC domain of the nucleolus and is involved in controlling cell proliferating and growth (Ginisty et al., 1999, Shimono et al., 2005, Ma et al., 2007), chromatin decondensation (Ghisolfi et al., 1990), chromatin congression at the metaphase plate (Ma et al., 2007) and activation/repression of gene transcription (Borer et al., 1989). Nucleophosmin, on the other hand is present in the GC of the nucleolus and has been shown to associate with DNA and RNA (Dumbar et al., 1989, Wang et al., 1994), acts as a histone (Okuwaki et al., 2001) and a molecular chaperone (Szebeni and Olson, 1999), it interacts with nucleolin (Li et al., 1996) and is implicated in development (Grisendi et al., 2005), cell proliferation (Lim and Wang, 2006) as well as maintenance of genomic stability (Lim and Wang, 2006) and nuclear shape (Amin et al., 2008). Over expression of NPM is associated with several different types of cancers and thus NPM has been thought to play a role in uncontrolled growth of cancer cells (Lim and Wang, 2006). Both nucleolin and NPM are involved in ribosome biogenesis and transportation (Herrera et al., 1995, Savkur and Olson, 1998), regulation of apoptosis (Hirano et al., 2005) and nucleolar

formation (Ma et al., 2007). Fibrillarin, a small ribonucleoprotein present in the DFC domain of the nucleolus, is also involved in ribosome biogenesis - particularly in early processing and modification of pre-RNA (Wu et al., 1998, Lafontaine and Tollervey, 2000, Watkins et al., 2000, Schimmang et al., 1989), ribosome assembly (Tollervey et al., 1993), early embryonic development (Newton et al., 2003), nucleolar assembly (Fomproix et al., 1998), cell viability (Schimmang et al., 1989), cell growth (Newton et al., 2003, Amin et al., 2007) and maintenance of nuclear shape (Amin et al., 2007). Ki-67, although used mainly as a proliferation marker, is a nucleolar protein in addition (Kill, 1996). Ki-67 has been implicated in the regulation of cell cycle (Scholzen and Gerdes, 2000, Starborg et al., 1996), cell proliferation (Scholzen and Gerdes, 2000, Starborg et al., 1996), early stages of rRNA synthesis (Bullwinkel et al., 2006) and protection of chromosome surfaces during mitosis (Kill, 1996, Gautier et al., 1994, Yasuda and Maul, 1990). In addition to these many other putative functions such as maintenance of higher order chromatin structure (Sawhney and Hall, 1992), modulation of nucleolar structure for high rates of ribosomal synthesis (Chatterjee et al., 1987) and maintenance of nucleolar structure (Kill, 1996) have also been linked to this structural protein. Thus, atypical amounts and distributions of nucleolin, NPM, fibrillarin or Ki-67 within the nucleoli of HGPS fibroblasts may result in compromised ability of these cells to perform some or all of the above functions.

With evidence of the presence of lamins in nucleolus suggested (Zimber et al., 2004, Martin et al., 2009, Hozak et al., 1995), it is likely that in HGPS patients the interaction between lamins and some nucleolar proteins may be impaired thereby resulting in some of the phenotypes of the disease including abnormal nuclear shape, hyperproliferation and accelerated apoptosis (Bridger and Kill, 2004). Hence, the

aim of this study is to assess the distribution of major nucleolar proteins, including nucleolin, NPM, fibrillarin and Ki-67, in HGPS fibroblasts and compare their localisations to those in control fibroblasts. Comparisons with respect to relative amounts of nucleolin and fibrillarin were also made between control and these disease fibroblasts by Western blotting. The data reveals atypical distribution and extremely low levels of nucleolin in HGPS fibroblasts and an atypical distribution of NPM in non-proliferating HGPS fibroblasts. Although fibrillarin is distributed in DFC as it would normally be in control cells, there appears to be difference in structural organisation of this protein in the nucleolar domain in HGPS patient HDFs. On treatment with FTIs (See chapter 4), nucleolin distribution and relative content has indeed been restored in HGPS patient fibroblasts. FTI treatment also restores the signal intensity of fibrillarin and structured organisation of fibrillarin in HGPS fibroblasts (Mehta IS, Bridger JM and Kill IR; in press for Biochemical Society Transactions).

6.2. Methods and Materials:

6.2.1. Cell culture:

6.2.1.a. Cell lines

The cell lines used in this study include three human dermal fibroblasts (HDFs) cell lines derived from patients suffering from HGPS (AG11498, AG01972C and AG11513). All of these three cell lines have a typical G608G mutation in *LMNA* gene. All of these cell lines were obtained from Coriell Cell Repositories. For controls, normal 2DD fibroblasts cell line was used (Bridger et al., 1993) (See table 4.1.).

6.2.1.b. Cell culture and FTI treatment

The HDF cell lines (see table 4.1) were maintained as in 4.2.1. For performing indirect immunofluorescence, cells were seeded onto sterile coverslips in 10 cm^2 tissue culture dishes at a density of 2 x 10^5 with 15% FBS-DMEM. Cells were allowed to grow at 37ºC with 5% CO_2 for at least 48 hours before fixing. Cells were made quiescent by incubation in 0.5% serum for 7 days (See 2.2.1).

For FTI treatment, AG11498 fibroblasts were incubated with 2.5 µM FTI-277 for 48 hours as in 4.2.2.a.

6.2.2. Indirect immunofluorescence:

6.2.2.a. Fixation

Cells were grown on sterile 13 mm coverslips for at least 48 hours and then washed with 1X PBS with three changes of the buffer. Cells were fixed using 4% paraformaldehyde (PFA) for 10 minutes at RT, followed by three washes in the 1X

PBS. After this the cells were permeabilised using methanol:acetone (1:1) for 10 minutes on ice, followed by three washes in 1X PBS.

6.2.2.b. Antibody staining

Primary antibodies used in this study: anti-nucleolin (mouse) {Abcam}, anti-nucleophosmin (mouse) – {a kind gift from Pui Chan, Baylor College of Medicine, TX, USA}, anti- fibrillarin (mouse) {a kind gift from Dr. John Aris, University of Florida, USA}, anti-Ki67 (rabbit) {Novacastra Laboratories}.

Secondary antibodies: Donkey anti-mouse (FITC) {Jackson's ImmunoResearch Laboratories}, Swine anti rabbit (TRITC) {DAKO}.

Antibodies were diluted in PBS containing 1% v/v NCS. Cells were co-stained with primary antibodies as mentioned in table 6.2 and incubated at RT for 1 hour. The coverslips were then washed three times with 1X PBS and incubated with respective secondary antibodies (see Table 6.2) at RT for 1 hour. The cells were washed in 1X PBS and rinsed in distilled water before mounting in counterstain DAPI in Vectashield mounting medium.

Table 6.1. Summary of antibodies used for this study:

Primary Antibodies			Secondary Antibodies	
Antigen	Species	Dilution	Antigen + Species	Dilution and fluorescent tag
Nucleolin (Abcam) + Ki-67 (Novacastra)	Mouse Rabbit	1:200 1:1000	Donkey anti-mouse Swine anti-rabbit	1:25 (FITC) 1:30 (TRITC)
Nucleophosmin (Dr. Pui Chan) + Ki-67 (Novacastra)	Mouse Rabbit	1:30 1:1000	Donkey anti-mouse Swine anti-rabbit	1:25 (FITC) 1:30 (TRITC)
Fibrillarin (Dr. John Aris) + Ki-67 (Novacastra)	Mouse Rabbit	1:1000 1:1000	Donkey anti-mouse Swine anti-rabbit	1:25 (FITC) 1:30 (TRITC)
Ki-67 (Novacastra)	Rabbit	1: 1000	Swine anti-rabbit	1:30 (TRITC)

6.2.2.e. Microscopy and analysis

Slides were viewed and imaged on Olympus BX41 fluorescence microscope as in protocol 2.2.2.e. Data were derived from two independently treated coverslips

for each sample. Images were captured at identical exposure settings. Distribution of nucleolar proteins was assessed in terms of presence in nucleoplasm, nucleolus, nucleolar periphery and cytoplasm. Number of nucleoli per cell was also documented.

6.2.3. Electrophoresis and Western blotting:

Whole cell lysates for control and HGPS fibroblasts were prepared as in 5.2.3. Cells were loaded at a concentration of 5 x 10^4 cells per lane and proteins were resolved on a 8% SDS-PAGE gel and then transferred onto a nitrocellulose membrane (See 5.2.3.). Membranes were then blocked in blocking buffer (4% (w/v) dried milk powder {Marvel}, in PBS) overnight at 4ºC. Membranes were then incubated in primary antibodies diluted in 1% NCS-PBS (anti-nucleolin - diluted 1:1000, anti-fibrillarin – diluted 1:1000 and mouse monoclonal anti-GAPDH {Abcam} – diluted 1:5000) (Table 6.2.) at 4ºC overnight. Blots were then washed in 1X TBS-Tween 20 (50mM Tris pH 7.6, 150mM NaCl, 0.1% (v/v) Tween 20) and incubated with secondary antibodies (HRP conjugated rabbit anti mouse or swine anti rabbit {DAKO}, both diluted 1:1000) for 1 hour at RT. The washes with 1X TBS-Tween20 were then repeated and antibody detection was performed as in 5.2.3. The band intensity was quantified as in 5.2.3.

6.3. Results:

6.3.1. Distribution of protein Ki-67 in HGPS fibroblasts:

To assess the distribution of pKi-67, control and HGPS patient cells were fixed with methanol:acetone followed by indirect immunofluorescence staining using anti-Ki-67 antibody. Distribution of pKi-67 was then assessed by counting at least 200 nuclei in total per sample and categorising the pKi-67 nuclei into type 1a, type 1b and type II patterns as done previously (Bridger et al., 1998b). Counts were performed in duplicate from two independently treated coverslips for each sample.

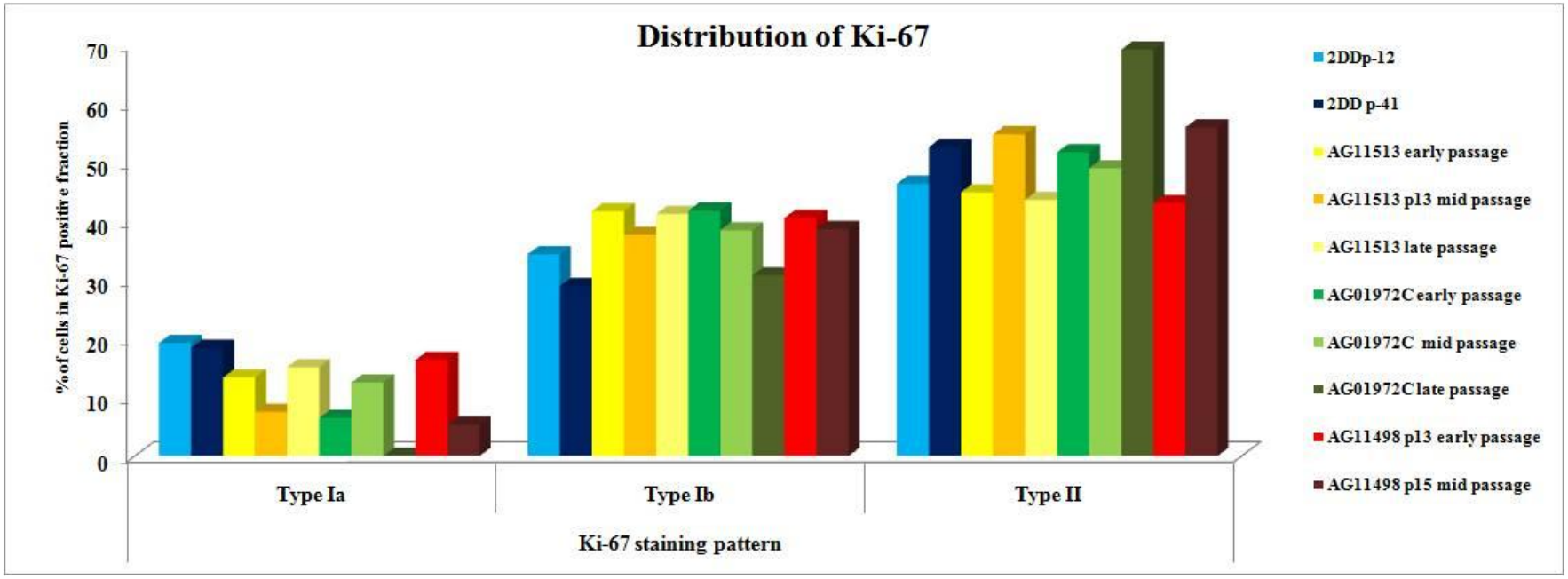


Figure 6.1. Distribution of pKi-67 in HGPS and normal fibroblasts: The % of cells displaying type Ia, type Ib and type II staining for pKi-67 are displayed in the above chart.

Indeed, three distinct populations of cells showing type Ia, type Ib and type II pattern of pKi-67 distributions were observed in both control and HGPS HDFs (Figure 6.1.). The fraction of cells showing type Ia distribution in HGPS fibroblasts was lower than in control fibroblasts and on the other hand the fraction of cells displaying type Ib and type II distribution were similar in both control and HGPS fibroblasts (Figure 6.1.). No atypical pKi-67 distribution was observed in any HGPS fibroblasts.

6.3.2. Distribution and level of fibrillarin in HGPS patient fibroblasts:

To assess the distribution of fibrillarin in HGPS fibroblasts, patient cells were grown on sterile coverslips, fixed and an indirect immunofluorescence assay performed. Early and late passage samples, representing proliferating and senescent cultures, were collected from each of the HGPS cell lines as well as controls. Fibrillarin distribution was assessed by counting at least 200 fibrillarin positive cells and categorising them in typical (nucleolar staining with punctate foci in DFC) and atypical (lack of any punctuate foci of fibrillarin in the nucleolus) patterns. Two independent counts were taken from two separate coverslips for each sample.

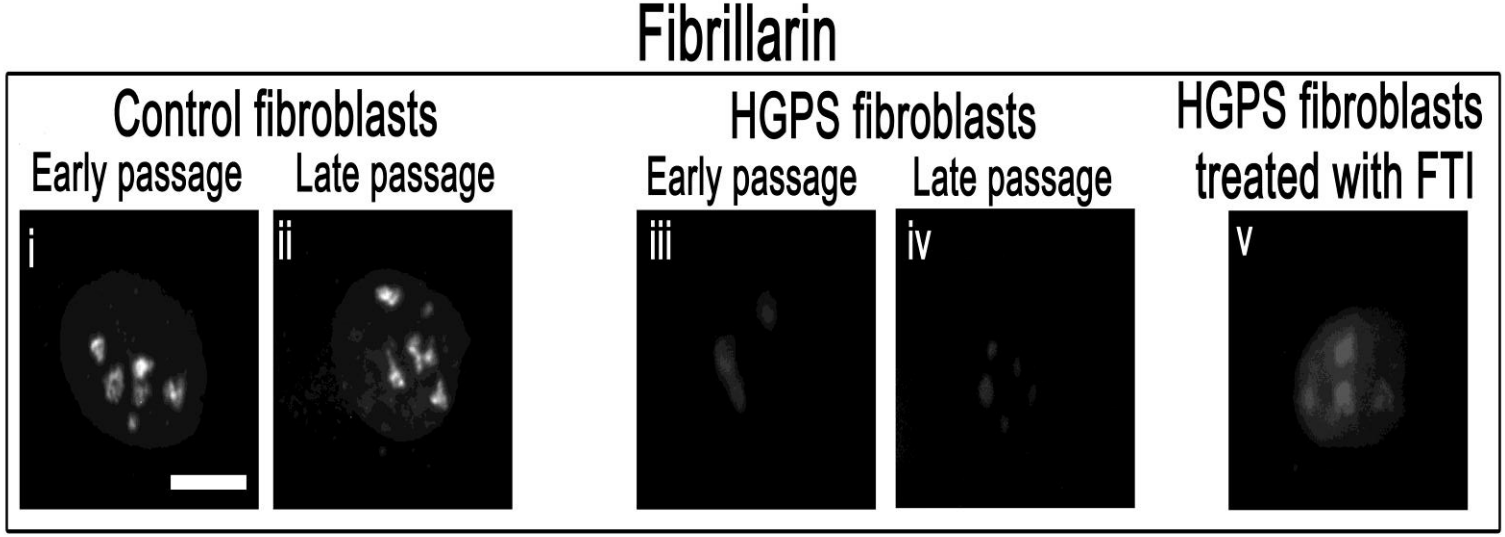


Figure 6.2. Representative images displaying the distribution of fibrillarin: i and ii show representative images of early and late passage control interphase nuclei displaying typical distribution of fibrillarin in DFC. Distribution of fibrillarin in early and late HGPS fibroblasts is representated in iii and iv whilst v shows distribution of fibrillarin in HGPS fibroblast following a 48 hour FTI treatment. Scale bar = 10µm.

Figure 1 displays representative images of fibrillarin distribution in control and HGPS fibroblasts. It is evident from images (Figure 6.2.) as well as analysis of the counts (Table 6.3.) that there was no significant difference in fibrillarin localisation in HGPS fibroblasts as compared to control fibroblasts. The protein was distributed in the nucleolar DFC domain at all times and no cytoplasmic staining was observed (Figure 6.2.). Intriguingly, in control cells fibrillarin is distributed as punctate foci within the DFC domain (typical nucleolar staining) (Figure 6.2. i and ii and Table 6.3.). This structural organisation of fibrillarin in DFC domain of the nucleolus is not observed in HGPS fibroblasts, instead there is a lack of any punctate structure (atypical nucleolar staining) for the protein in the DFC domain (Figure 6.2. iii and iv and table 6.4.). The fraction of cells displaying atypical nucleolar distribution of fibrillarin appears to increase with their age in culture (Table 6.3.). Initial qualitative observations appear to suggest a difference in the signal intensity of the protein in HGPS fibroblasts (Figure 6.2.iii and iv) as compared to control fibroblasts (Figure 6.2.i and ii). This is a preliminary observation visualised when images taken at identical exposure settings were observed. Treatment of AG11498 cells using 2.5µM FTI-277 over a 48-hour period improves the intensity of fibrillarin staining (Figure 6.2.v) as well as the punctate foci distribution of fibrillarin distribution in the DFC domain of the nucleolus, but is still not similar to that of control cells (Table 6.3.).

Table 6.2. Distribution of fibrillarin in control and HGPS fibroblasts:

Staining / Cells	Ki67 positive			Ki67 negative			Total % typical nucleolar staining
	Typical Nucleolar (%)	Atypical Nucleolar (%)	Negative (%)	Typical Nucleolar (%)	Atypical Nucleolar (%)	Negative (%)	
2DD Early passage	42.9	0	0	57.0	0	0	99.9
2DD Late passage	7.2	0	0	92.8	0	0	100
AG11513 Early passage	59.1	0	0	0	40.9	0	59.1
AG11513 Late passage	10.5	0	0	0	89.5	0	10.5
AG01972C Early passage	65.4	0	0	0	33.1	0	65.4
AG01972C Late passage	4.7	0	0	0	95.1	0.2	4.7
AG1148 Early passage	65.9	0	0	0	34.1	0	65.9
AG1148 Late passage	4.6	0	0	0	95.4	0	4.6
AG11498 + FTI	29.3	0	0	23.0	47.7	0	52.3

The differences in intensity of fibrillarin staining suggest that HGPS fibroblasts may have lower amount of this protein. Figure 6.3. displays images of Western blots displaying the amount of fibrillarin and GAPDH (positive control) proteins in HGPS fibroblasts before and after FTI treatment and in control proliferating, quiescent and senescent fibroblasts.

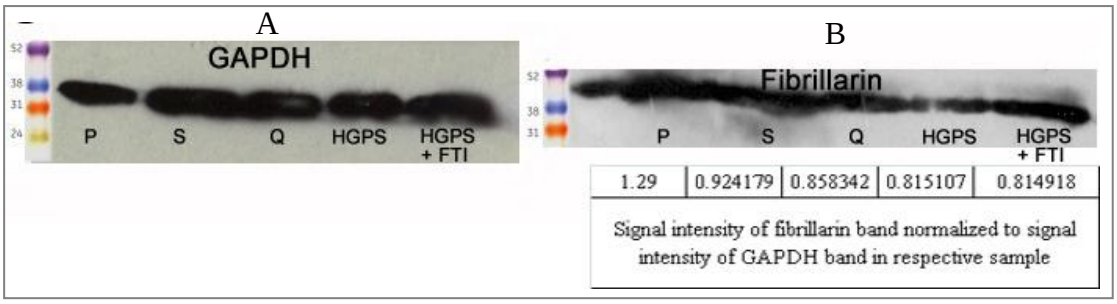


Figure 6.3. Western blot analysis for amount of fibrillarin protein in control and HGPS fibroblasts: Whole cell lysates from control (proliferating, quiescent and senescent) fibroblasts and HGPS fibroblasts before and after FTI treatment were prepared and proteins were separated on 8% SDS PAGE gel. All samples were loaded equally, 5 x 10^4 cells per lane. Images for GAPDH (A) and fibrillarin (B) are shown. {P – Proliferating control HDFs, Q – Quiescent control HDFs, S – Senescent control HDFs, HGPS – AG11498 p19 HDFs, HGPS +FTI – AG11498 p19 HDFs treated with FTI}. The table under B displays the signal intensity of fibrillarin band normalised by the signal intensity of GAPDH band for respective samples.

The amount of fibrillarin protein present in HGPS fibroblast is similar to that in control quiescent fibroblasts (Table in figure 6.3.B), which is slightly different to the amount of protein in proliferating and senescent control HDFs (Figure 6.3.B).

Thus, these data suggest that there are negligible differences in distribution and amount of fibrillarin in HGPS fibroblasts not corrected by FTI treatment.

6.3.3. Distribution and amount of nucleolin in HGPS fibroblasts:

Distribution of nucleolin was compared between control and HGPS fibroblasts by performing indirect immunofluorescence using anti-nucleolin antibodies. Nucleolin distribution was evaluated in both proliferating and non-proliferating HDFs for each sample. In normal fibroblasts, nucleolin is found in the DFC of the nucleolus (Kill, 1996) and hence is mostly localised within the nucleolus

and not at the nucleolar periphery (Figure 6.4. i and ii). In HGPS cells, typical (Figure 6.4. iii and iv) as well as atypical nucleolin distribution was observed, including no nucleolin distributed in DFC, presence of a prominent rim and only an homogenous nucleoplasmic distribution for nucleolin with no nucleolar staining (Figure 6.4. v and vi).

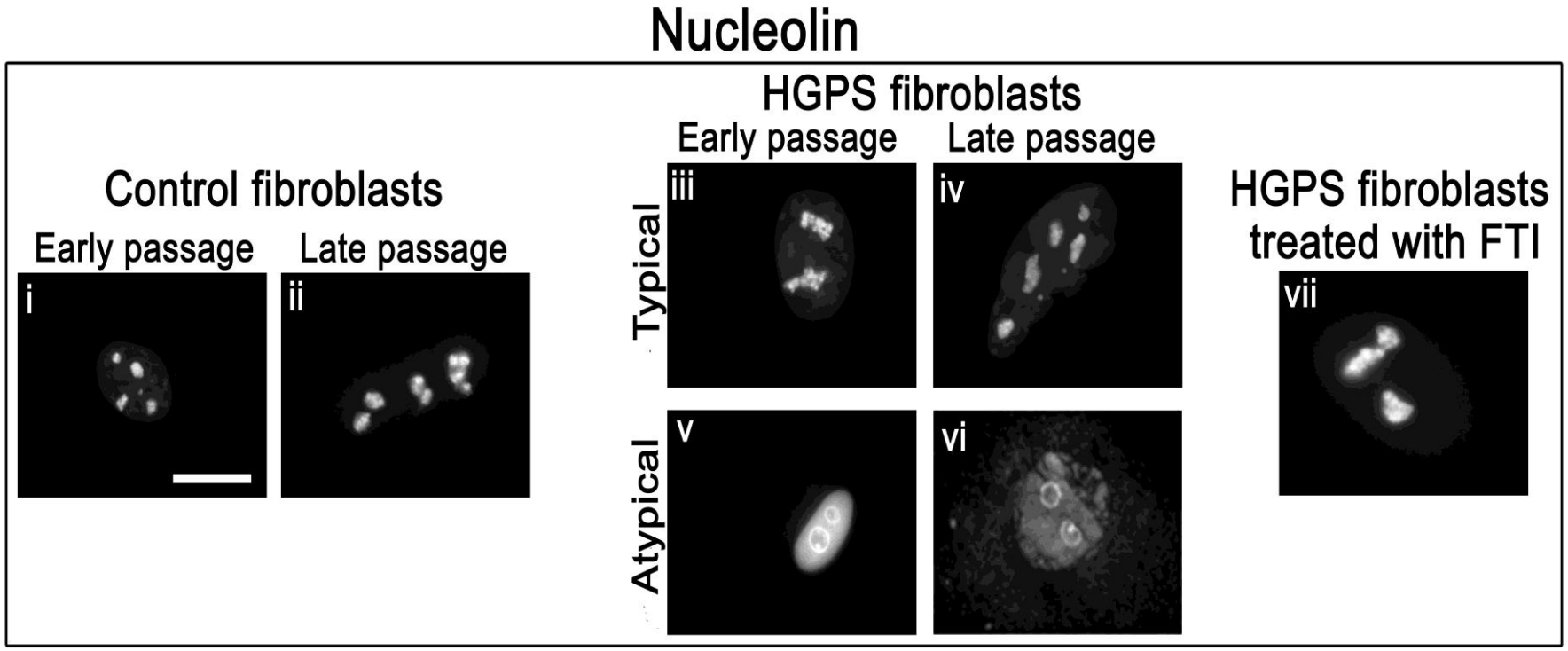


Figure 6.4. Representative images showing the distribution of nucleolin in control and HGPS HDFs: Indirect immunofluorescence assay for nucleolin was performed whereby early and late passage fibroblasts from control and HGPS patients were stained for nucleolin. i and ii represent typical nucleolin distribution in control early and late passage HDFs respectively while typical nucleolin distribution in early and late passage HGPS fibroblasts is displayed in iii and iv. Atypical nucleolin distribution patterns observed in HGPS fibroblasts are represented in v and vi. Distribution of nucleolin in HGPS fibroblasts after treatment with FTIs is represented in vii. Scale bar = 10µM.

Quantification of percentage of HDFs displaying atypical distribution was performed and further comparison for fraction of nuclei displaying atypical distribution was made between control and HGPS fibroblasts as well as between proliferating and non-proliferating HDFs.

Figure 6.5. Graphs displaying the fraction of control and HGPS HDFs that have atypical nucleolin distribution.

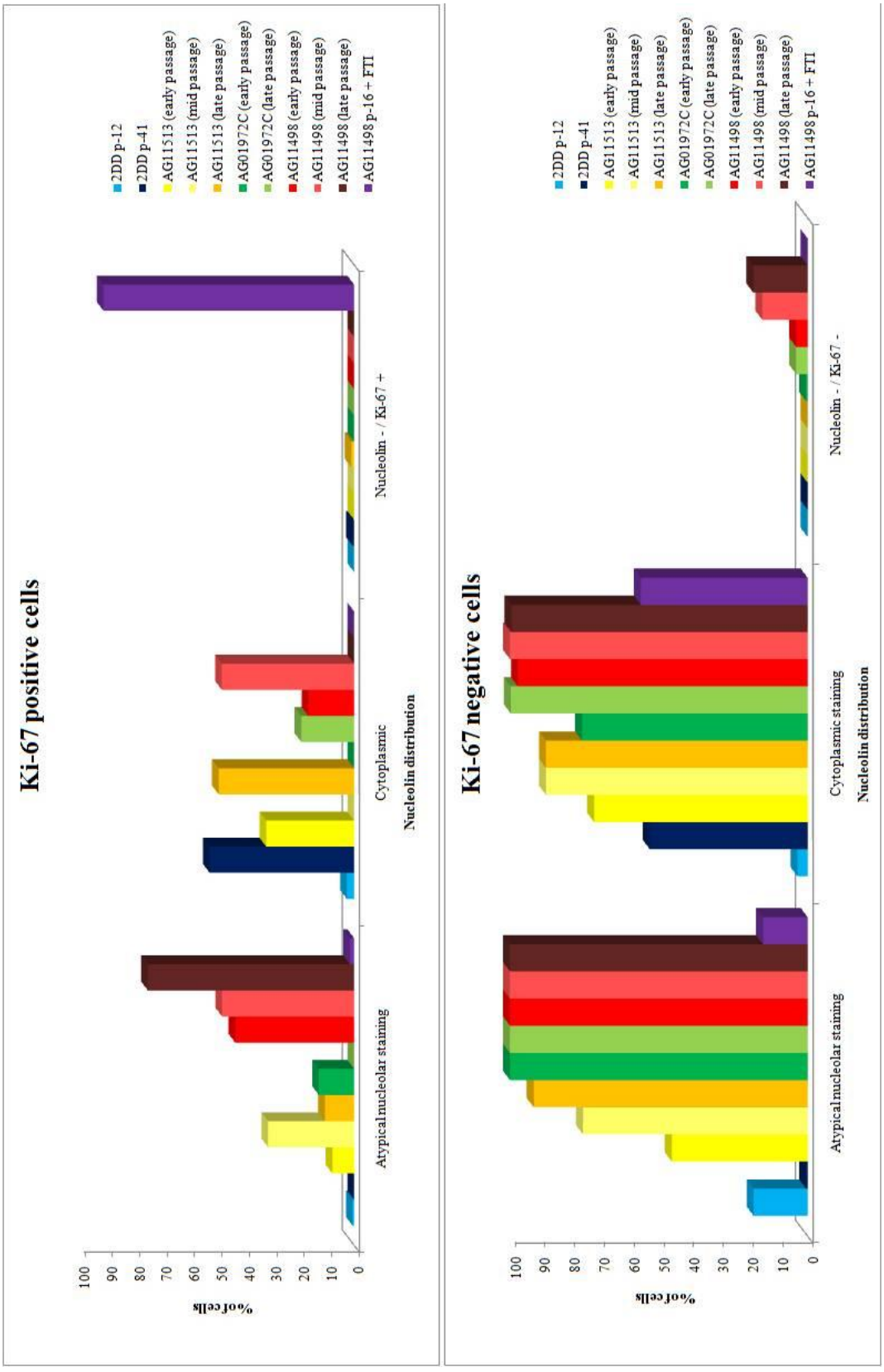


Figure 6.5. Graphs displaying the fraction of control and HGPS HDFs that have atypical nucleolin distribution: Nucleolin distribution in control and HGPS HDFs was assessed by categorising cells in terms of presence of nucleolin in nucleoplasm, nucleoli, at the nucleolar periphery and in the cytoplasm. Presence of nucleolin in the DFC domain of the nucleolus was classified as a typical nucleolar distribution while presence of nucleolin at the nucleolar rim, no nucleolar staining or a nucleolar rim in addition of nucleolar staining was termed as an atypical nucleolar distribution. % of cells showing cytoplasmic staining for nucleolin and % of cells showing no nucleolin staining at all was also quantified for each sample. % of cells showing atypical nucleolar distribution, cytoplasmic distribution and no nucleolin in proliferating fibroblasts is displayed in A and in non-proliferating fibroblasts is displayed in B. Proliferation status of the cells was assessed by the presence or absence of pKi-67 staining.

Table 6.3. Distribution of nucleolin in HGPS HDFs:

Staining / Cells	Ki67 positive				Ki67 negative				Total % typical nucleolar staining
	Typical Nucleolar (%)	Atypical Nucleolar (%)	Cytoplasmic (%)	Negative (%)	Typical Nucleolar (%)	Atypical Nucleolar (%)	Cytoplasmic (%)	Negative (%)	
2DD Early passage	44.1	0.2	1.2	0	45.5	10.1	1.9	0	89.6
2DD Late passage	4.1	0	0.7	0	95.2	0.5	50.8	0	99.3
AG11513 Early passage	68.4	5.9	39.2	0.5	11.8	9.0	15.6	0	80.2
AG11513 Late passage	17.3	2.1	7.0	0	5.8	58.0	64.2	8.2	23.1
AG01972 C Early passage	43.9	6.5	24.8	1.0	0	48.1	36.5	0.5	43.9
AG01972 C Late passage	0.5	0.7	0.7	0	0	93.0	92.8	5.6	0.5
AG11498 Early passage	34.1	26.0	36.5	0	1.9	34.1	35.1	3.8	45.8
AG1148 Late passage	0.6	1.8	0.4	0	0	79.4	79.0	18.3	0.6
AG11498 + FTI	30.2	0.4	14.7	0	58.8	10.3	38.7	0	89.0

In two (AG11513 and AG11498) of the three cell lines, the fraction of proliferating cells showing atypical nucleolin distribution increased in late passage cells as compared to early passage fibroblasts while in AG01972C the % of proliferating cells showing atypical nucleolin distribution decreased as compared to early passage cells (Figure 6.5A and table 6.4). Importantly, there were few proliferating late passage AG11513 cells that were negative for nucleolin (Figure

6.5A and table 6.4). ~50% of control proliferating fibroblasts derived from a late passage culture appear to accumulate nucleolin in their cytoplasm as compared to less than 5% control proliferating fibroblasts derived from early passage cultures. The fraction of cells with cytoplasmic nucleolin distribution in late passage proliferating HGPS fibroblasts is similar to that of control fibroblasts but that in young passage proliferating HGPS fibroblasts is significantly higher than in young passage control proliferating fibroblasts (Figure 6.5.A and table 6.4.). In non-proliferating cells, most of the HGPS fibroblasts have an atypical nucleolin distribution and significantly higher fraction of cells have cytoplasmic nucleolin (Figure 6.5.B and table 6.4.). Also, the number of nucleolin negative cells increased in AG01972C and AG11498 cells with their age in culture (Figure 6.5B and table 6.4.). In both proliferating and non-proliferating HGPS fibroblasts, FTI treatment restores the nucleolar distribution of nucleolin and the fraction of cells positive for cytoplasmic distribution of the protein following treatment is negligible in proliferating HGPS fibroblasts while the number is reduced to approximately half in non-proliferating HGPS fibroblasts (Figure 6.5.A and B and table 6.4).

The presence of an atypical distribution of nucleolin suggested that the protein may be present in higher or lower amounts. Western blots for nucleolin in proliferating, quiescent, senescent control cells and in HGPS fibroblasts before and after FTI treatment are displayed in figure 6.6.

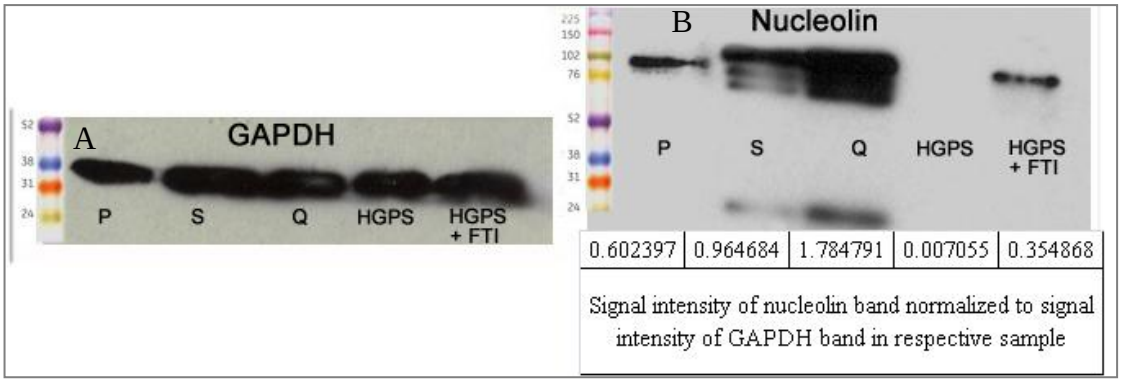


Figure 6.6. Western blots for nucleolin: Whole cell lysates from control (proliferating, quiescent and senescent) fibroblasts and HGPS fibroblasts before and after FTI treatment were prepared, proteins separated on 8% SDS PAGE gel and transferred to a nitrocellulose membrane. Amount of nucleolin protein was then determined by staining membranes with anti-nucleolin antibody. All samples were loaded equally, 5 x 10^4 cells per lane. Images for GAPDH (A) and fibrillarin (B) are shown. {P – Proliferating control HDFs, Q – Quiescent control HDFs, S – Senescent control HDFs, HGPS – AG11498 p19 HDFs, HGPS +FTI – AG11498 p19 HDFs treated with FTI}. The table under B displays the signal intensity of nucleolin band normalised by the signal intensity of GAPDH band for respective samples.

A 100 kDa band for nucleolin was observed for nucleolin in all samples except in HGPS fibroblasts (Figure 6.6. B).With the amount of cells loaded equal in each well and presence of GAPDH in the HGPS fibroblasts (Figure 6.6. A), suggest that HGPS fibroblasts have a very low amount of nucleolin. This amount of nucleolin is below the threshold that can be detected with a Western blot and hence no band was observed for HGPS fibroblasts. When the band intensities were measured, indeed the amount of nucleolin in lysates prepared from AG11498 fibroblasts is negligible - normalised area of band to GAPDH = 0.0071 as compared to 0.6024 in proliferating controls, 1.7847 in quiescent controls and 0.9646 in senescent control fibroblasts (Figure 6.6.B). FTI treatment restores nucleolin levels

closer to normal cells in HGPS fibroblasts (normalised area of band to GAPDH =0.3548) (Figure 6.6.B).

Interestingly, control senescent and quiescent fibroblasts not only have higher amount of nucleolin as compared to proliferating fibroblasts but also show presence of at least three extra bands with MW 24, 70 and 90 KDa.

6.3.4. Distribution of NPM in control and HGPS fibroblasts:

NPM distribution in control and HGPS fibroblasts was assessed by indirect immunofluorescence. Comparisons for distribution of NPM were made between control and HGPS fibroblasts and dual staining for pKi-67 with NPM allowed comparison between proliferating and non-proliferating cells within each of the same sample. Figure 6.7. displays representative images of NPM distribution in control and HGPS patient cells. In control cells, NPM is found at the nucleolar periphery in the GC domain and since it functions as a shuttling protein, cytoplasmic staining for this protein is also observed (Figure 6.7.i and ii) (Kill, 1996). Various atypical distributions of this protein including no nucleolar rim staining, no nucleolar staining at all, staining in the DFC and only cytoplasmic staining were observed in HGPS cells (Figure 6.7v and vi). A subset of HGPS fibroblasts also shows a typical distribution of NPM similar to control fibroblasts (Figure 6.7.iii and iv).

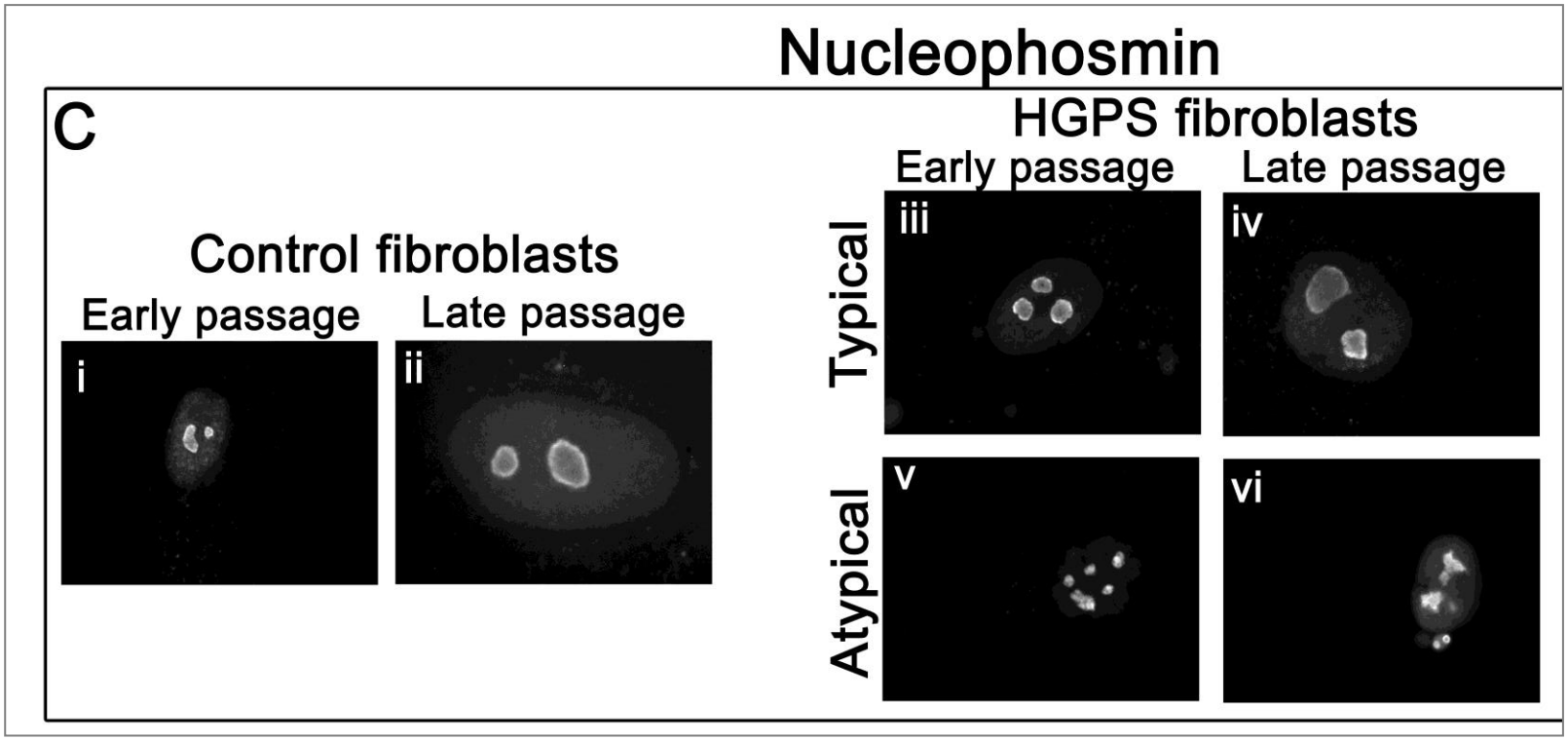


Figure 6.7. Representative images displaying the distribution of NPM in control and HGPS fibroblasts: NPM distribution in early and late passage control and HGPS fibroblasts was assessed by performing indirect immunofluorescence for NPM. i and ii represent typical NPM distribution in control early and late passage HDFs respectively while typical NPM distribution in early and late passage HGPS fibroblasts is displayed in iii and iv. Atypical NPM distribution patterns observed in HGPS fibroblasts are represented in v and vi. Scale bar = 10µM.

Microscopic analysis, counting and classification of NPM distribution in terms of typical and atypical nucleolar patterns and presence / absence of cytoplasmic staining was then performed for two independent sets of 200 NPM positive nuclei. Percentage of HDFs displaying atypical nucleolar distribution of NPM and presence or absence of cytoplasmic NPM staining in HGPS patient fibroblasts was then compared with that in control fibroblasts. NPM distribution was also compared between proliferating and non-proliferating HDFs, for both controls and HGPS fibroblasts.

Figure 6.8. Graphs representing the distribution of NPM in control and HGPS fibroblasts.

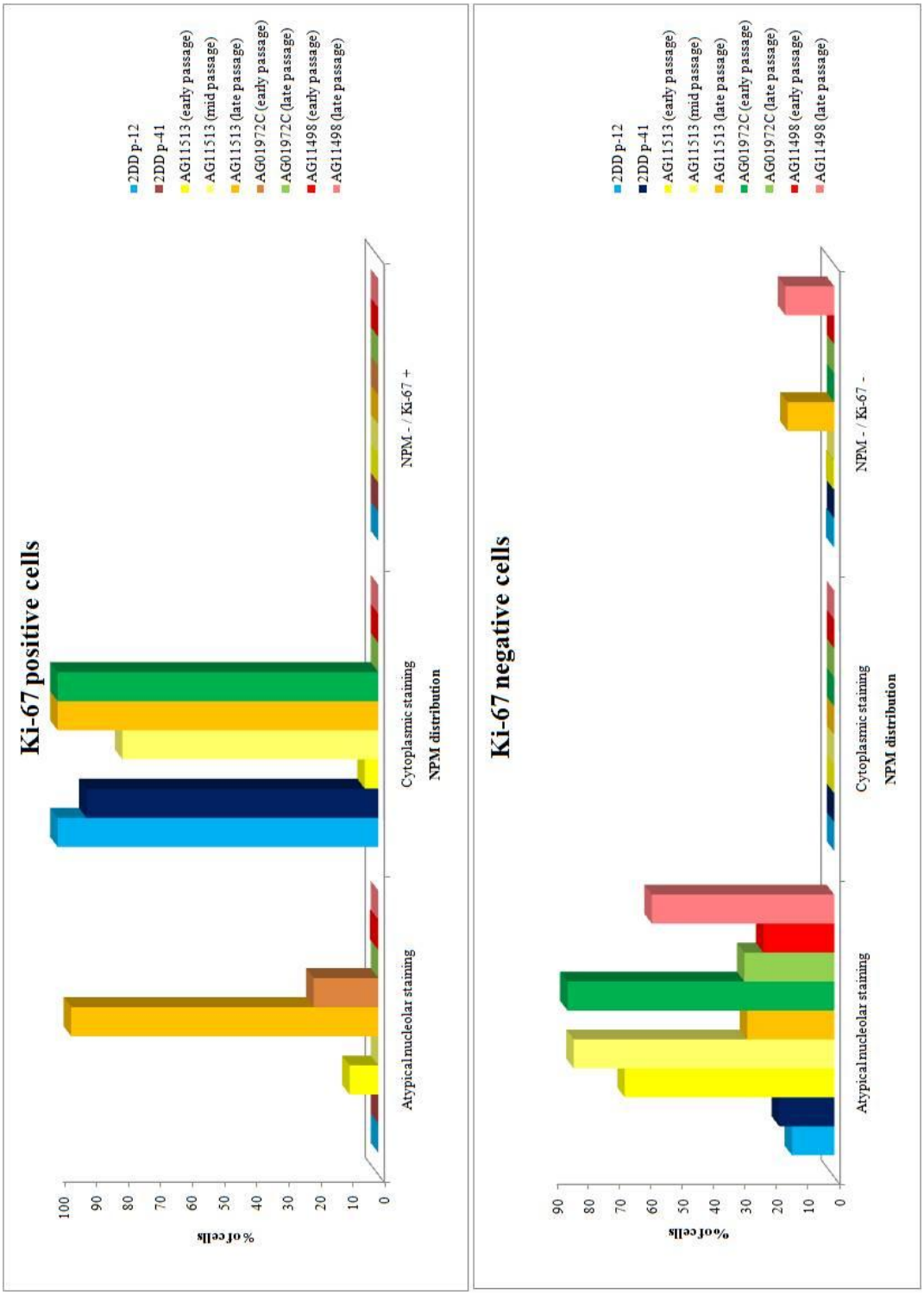


Figure 6.8. Graphs representing the distribution of NPM in control and HGPS fibroblasts: NPM distribution in control and HGPS HDFs was assessed by categorising cells in terms of presence of nucleolin in nucleoplasm, nucleoli, at the nucleolar periphery and in the cytoplasm. Presence of NPM in the GC of the nucleolus allows us to classify nuclear periphery distribution of this protein as typical nucleolar distribution while cells that had staining in the DFC of the nucleolus, no nucleolar staining or a nucleolar rim in addition of nucleolar staining was classed as an atypical nucleolar distribution. Fraction of cells showing cytoplasmic staining for NPM and fraction of cells showing no NPM staining at all was also quantified for each sample. Fraction of cells showing atypical nucleolar distribution, cytoplasmic distribution and no NPM in proliferating fibroblasts is displayed in A and in non-proliferating fibroblasts is displayed in B. Proliferation status of the cells was assessed by the presence or absence of pKi-67 staining.

Table 6.4. Distribution of NPM in HGPS HDFs:

Staining / Cells	Ki67 positive				Ki67 negative				Total % typical nucleolar staining
	Typical Nucleolar (%)	Atypical Nucleolar (%)	Cytoplasmic (%)	Negative (%)	Typical Nucleolar (%)	Atypical Nucleolar (%)	Cytoplasmic (%)	Negative (%)	
2DD Early passage	33.8	0	0.9	0	56.8	8.8	2.86	0.4	90.6
2DD Late passage	4.9	0	0	0	79.8	15.2	55.3	0	84.7
AG11513 Early passage	66.5	6.5	73.3	0	9.3	17.3	26.6	0.2	75.8
AG11513 Late passage	0.5	12.2	6.8	0	45.7	17.5	41.7	14.8	46.2
AG01972 C Early passage	18.7	4.7	23.5	0	11.5	64.9	76.2	0	30.2
AG01972 C Late passage	1.9	0	1.9	0	70.0	28.0	98.0	0	71.9
AG1148 Early passage	71.7	0.2	40.2	0	19.9	6.71	24.9	1.4	91.6
AG1148 Late passage	3.3	0	3.3	0	63.7	32.8	88.2	15.5	67

Most of the proliferating HGPS fibroblasts had a typical nucleolar distribution for NPM except fibroblasts from late passage AG11513 cell line which

had 90% of cells with atypical nucleolar distribution and fibroblasts from early passage AG01972C cell line had about 20% cells with atypical nucleolar distribution of NPM (Figure 6.8.A and table 6.5). Again, the cytoplasmic staining was close to normal in most of the proliferating HGPS fibroblasts except in early passage AG11513, late passage AG01972C and early and late passage AG11498 cells, very few cells showed presence of cytoplasmic staining (Figure 6.8.A and table 6.5). This suggests that although the nucleolar functions of NPM might not be affected, the shuttling function of NPM may be impaired or compromised. In non-proliferating HGPS fibroblasts, all the three cell lines had significantly higher numbers of cells that had atypical nucleolar distribution of NPM and in two of the three cell lines (AG11513 and AG01972C) the fraction of cells having an atypical nucleolar distribution decreasing with age in culture (Figure 6.8.B and table 6.5.). There was no difference in the fraction of cells displaying cytoplasmic staining of NPM in HGPS fibroblasts as compared to control fibroblasts (Figure 6.8.B and table 6.5.). Importantly, a fraction of non-proliferating fibroblasts from late passage AG11513 and AG11498 cell lines showed no staining for NPM at all (Figure 6.8.B and table 6.5.).

6.3.5. Number of nucleoli in control and HGPS fibroblasts:

The number of nucleoli in early and late passage control HDFs as well as control quiescent HDFs was compared with the number of nucleoli in early, mid and late passage HDFs from HGPS patient cells (AG11513).

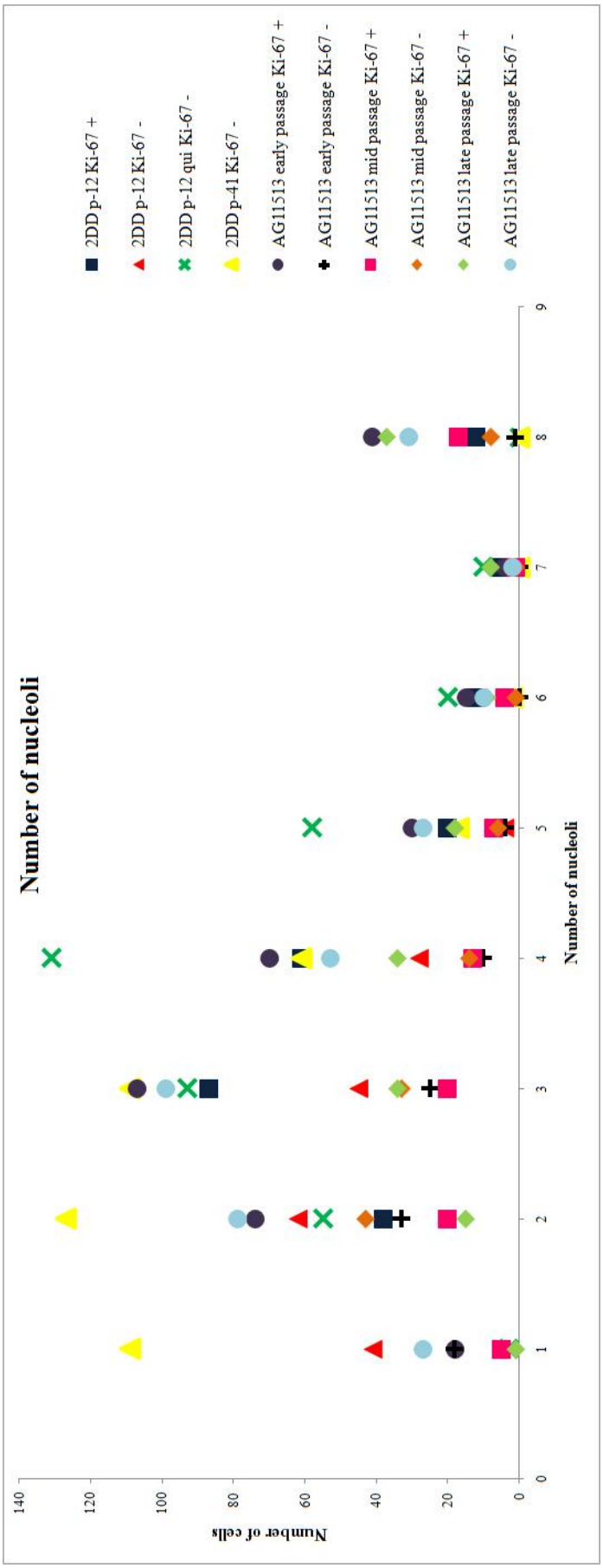


Figure 6.9. Graph displaying the number of nucleoli in control and HGPS fibroblasts: Early and late passage control and HGPS fibroblasts were fixed and subjected to indirect immunofluorescence for either anti-NPM or anti-nucleolin. The number of nucleoli per cell was documented by performing counts of at least 200 cells per sample. 4 sets of counts were performed for each sample from two independently treated coverslips. {F = number of fragmented nucleoli}

Indeed, in concurrence with a previous study (Bemiller and Lee, 1978), the fraction of cells having 1 or 2 nucleoli increased in senescent fibroblasts as compared to pre-senescent fibroblasts. This is true in both, control and HGPS fibroblasts. Interestingly, the mode value for number of nucleoli in quiescent cells is four as compared to 3 in proliferating cell nuclei (Figure 6.9.).

It should also be noted that the number of fragmented nucleoli was significantly higher in HGPS fibroblasts (Figure 6.9.). A reduction in number of fragmented nucleoli is observed in HGPS fibroblasts with length in culture.

6.4. Discussion:

Cells from HGPS patients have numerous nuclear functions impaired including chromosome organisation, DNA repair, cell cycle regulation, cell growth and differentiation, replicative senescence and apoptosis. Most of these functions involve not only the nuclear lamina, but also other components of the nuclear architecture. Understanding how an abnormal lamina affects other nuclear and cellular entities would assist to bridge the gap between mutation in a structural protein, such as lamin A and the drastic phenotypes observed in the patients.

By assessing differences in the distribution of four major nucleolar proteins between control and HGPS patient fibroblasts, the study in this chapter elucidates a possible link between the disease pathology and abnormal nucleolar domain. Three out of the four nucleolar proteins assessed, including nucleolin, fibrillarin and NPM demonstrated substantial differences, in HGPS fibroblasts as compared to control fibroblasts.

Although fibrillarin was distributed in the DFC domain in control as well as HGPS fibroblasts, the punctate foci distribution of this protein was absent in HGPS HDFs. The lack of structured distribution of fibrillarin together with compromised lamina may affect translation, protein modification and may further lead to increased nuclear abnormalities and deregulated cell cycle growth observed in HGPS patient cells. The signal intensities of fibrillarin staining in HGPS fibroblasts appeared to be lower than that in control cells, but Western blot analysis showed negligible difference in the amount of this protein in HGPS fibroblasts as compared to control fibroblasts. This suggests that fibrillarin epitopes may be masked in HGPS fibroblasts. Also, nucleolar proteins are attached to the nuclear matrix and it is highly

likely that in HGPS fibroblasts, these attachments are perturbed resulting in lower amount of bound protein that can be detected by indirect immunofluorescence. More quantification and analysis as well as improved techniques such as determining the presence of fibrillarin after extraction of the nuclear matrix would be required to confirm these data.

HGPS cells have a normal pKi-67 distribution similar to control fibroblasts but the fraction of cells displaying a type Ia distribution of the protein is lower as compared to control HDFs. Type Ia distribution of Ki-67 protein is seen in very early G_1 phase (upto first 2 hours) of the cell cycle and hence lower number of cells in this phase signifies that HGPS cells may traverse through G_1 faster as compared to control cells. Early G_1 phase is an important stage of the cell cycle when the nuclear compartments broken down during mitosis including nuclear envelope and nucleolus reassemble. Thus a shorter duration in this stage of the cell cycle may cause compromised rebuilding of nuclear components which then manifests into impaired cellular functions. Also, the influence that this rapid passage of cells through G_1 stage has on hyperproliferation observed in HGPS fibroblasts can be questioned. In addition to this, pKi-67 interacts with heterochromatin (Traut et al., 2002) and hence decrease in fraction of cells displaying Type Ia distribution of this protein, may signify loss of heterochromatin usually associated with this disease (Goldman et al., 2004).

The relative content of nucleolin is abnormally low in cells derived from HGPS patients. HGPS s display atypical nucleolar distribution as well as cytoplasmic distribution of this protein. Presence of this atypical distribution and lower amount of nucleolin in these cells may affect the downstream processes that involve this protein including chromatin remodelling, transcription (Borer et al.,

1989), regulation of apoptosis (Hirano et al., 2005) and cell cycle, cell growth (Ginisty et al., 1999, Shimono et al., 2005, Ma et al., 2007) and nucleolus formation (Ma et al., 2007). Also, the fraction of cells containing no nucleolin may have a very compromised or a dysfunctional nucleolus.

Atypical nucleolar and cytoplasmic distribution of NPM observed in HGPS HDFs, might interfere with normal NPM functions such as nucleic acid binding (Brady et al., 2004), ATP binding (Hingorani et al., 2000), and stimulation of DNA polymerase activity (Umekawa et al., 2001), stabilization of p53 in the nucleus when DNA is damaged (Colombo et al., 2002, Kurki et al., 2004a, Kurki et al., 2004b), protection of mitotic chromosomes (Amin et al., 2008) and cell cycle regulation by sequestering the tumor-suppressor gene *ARF* in the cytoplasm (Brady et al., 2004); and thereby resulting into some of the phenotypes associated with this disease including abnormal nuclear shape, higher fraction of micronucleation (Meaburn et al., 2007), hyperproliferation (Bridger and Kill, 2004) as well as a abnormal DNA damage response (Liu et al., 2006). NPM is also involved in maintenance of normal nuclear structure by maintaining the cytoskeleton structure through actin and tubulin fibres (Amin et al., 2008). The nucleic acid binding capacity of NPM (Brady et al., 2004) suggests an interaction of this protein with chromatin within the nucleus and thus there is a possibility of NPM being involved in chromosomal organisation. With presence of nuclear actin and its importance in repositioning nuclear entities such as whole chromosomes across the nucleus (Chapter 3), atypical distribution of NPM may also interfere with chromosome repositioning in these diseased cells. Also, the effect of aberrant distribution of these nucleolar proteins on organisation of chromatin that associates with the nucleoli, i.e. chromosome containing NOR regions, still needs to be identified.

Along with their specific cellular roles, the nucleolar proteins studied here are all involved in an ubiquitous process namely ribosome biogenesis. Studies on patients suffering from Diamond-Blackfan anaemia, a disease caused due to defective ribosomal protein gene, has shown that interference with ribosomal biogenesis alters not only translation and protein modification but also transcription, apoptosis and oncogenic pathways (Gazda et al., 2006). Thus, aberrant nucleolar distribution and relative content of nucleolar proteins in HGPS fibroblasts may result in compromised ribosome biogenesis and as a result affecting some of the downstream pathways mentioned, consequentially leading to the pathology observed in HGPS patient cells.

The relative content and distribution of nucleolar components is known to be modified by NMIβ along with ATP synthesis and actin (Cisterna et al., 2009). Thus, it might be possible that the protein complex involving lamin A, emerin, nuclear actin and NMIβ (Mehta et al., 2008) may be dysfunctional in HGPS HDFs due to presence of mutated lamin A, leading to low levels of NMIβ in the diseased cells (chapter 6), further leading to atypical distribution and lower levels of nucleolar proteins studied. This is further supported by the evidence that treatment with FTIs restores lamin A localisation in the nuclear lamina, relative content and amount of NMIβ and re-establishes the distribution and the amount of nucleolar proteins, i.e. nucleolin and fibrillarin in HGPS HDFs.

In summary, the results in this chapter demonstrate that nuclear lamina is not the only nuclear compartment affected in HGPS fibroblasts and mutation in *LMNA* gene results in atypical distribution of nucleolar proteins. Whether or not this is a direct or an indirect effect of the mutation still remains unclear. Both nuclear lamina and nucleolus are known to interact with several nuclear regions/compartments and

thus although there is no evidence of these two domains interacting or influencing each others functions, a direct or indirect cross-talk between these two compartments within the nuclear environment is not unlikely. Thus, this study suggests that the pathology observed in HGPS patients may not only be a result of abnormal lamina, but a combined effect of various components of nuclear architecture affected.

Chapter 7: Expression profiles of genes on chromosome 10 in proliferating, G_0, G_S and HGPS HDFs

7.1. Introduction:

Each chromosome territory in an interphase nucleus has its own preferred space with respect to the nuclear periphery and the interior (Meaburn and Misteli, 2007, Bolzer et al., 2005, Parada and Misteli, 2002, Boyle et al., 2001, Cremer et al., 2006). The proximity of chromosome territories to each other has been correlated with the number of translocations two or more chromosomes can undergo, such that there is a higher rate of possible chromosomal translocations in chromosome territories located close to each other rather than between chromosomes whose territories are further apart (Bickmore and Teague, 2002, Parada et al., 2004). Thus this implies that each chromosome territory has its preferred neighbouring territories which might differ in different cell types. Thus, the organisation of chromosome territories in an interphase nucleus is far from random and is instead highly specific within the environment of an interphase nucleus of a particular cell type. This systematic organisation prompts the question of the functional significance of positioning chromosome territories in nuclei, what effects, if any; does it have on genome function and what happens if this organisation is perturbed?

Studies involving gene rich chromosome 19 and gene poor chromosome 18 (Bridger et al., 2000, Croft et al., 1999) as well as active and inactive chromosome X (Eils et al., 1996) have demonstrated that the morphology of chromosome territories is linked to their transcriptional status which then regulates the organisation of chromosomal regions within the interphase nuclei. Various studies have now demonstrated distribution of chromatin regions with respect to the local gene density whereby chromosomes with higher gene density localise in the nuclear interior and those with lower gene density are positioned in the nuclear interior (Boyle et al.,

2001, Croft et al., 1999, Foster and Bridger, 2005, Meaburn and Misteli, 2007, Eils et al., 1996) (Chapter 2). In addition to this, recent studies have also demonstrated that chromosome territories may be organised in interphase nuclei according to their GC content with nuclear interior mainly comprising of GC-rich chromatin regions while GC-poor chromatin regions localising nearer to the nuclear periphery (Hepperger et al., 2008, Saccone et al., 2002). Similarly, early replicating chromatin regions have been shown to associate with the nuclear interior while late replicating regions do so with the nuclear periphery (Grasser et al., 2008).

Individual genes as well as sub-chromosomal regions also occupy preferential, distinct radial locations within interphase nuclei. Transcriptionally active genes can be found at the periphery of the chromosome territory (Kurz et al., 1996), within the chromosome territory (Mahy et al., 2002) and can also loop out of the chromosome territory (Tolhuis et al., 2002, Branco and Pombo, 2006, Scheuermann et al., 2004, Osborne et al., 2004). Clusters of certain genes are found to co-localize with transcription factories at all times (Chakalova et al., 2005, Sproul et al., 2005) while there are other genes that are more dynamic and localize differently within the chromosome territory depending on their transcriptional status (Osborne et al., 2004). To further assess the significance of this ordered organisation of gene loci within the intra-nuclear space in an interphase nucleus, various studies discuss the movement of specific regions genes within nuclei and consequence on gene expression. For example genes such as *IgH* (Kosak et al., 2002), *CD4* (Kim et al., 2004), *c-maf* (Hewitt et al., 2004) and *β-globin* (Ragoczy et al., 2006), *GATA2* (Szczerbal et al., 2009) involved in B-cell differentiation, T-lymphocyte differentiation, T-cell differentiation, erythroid differentiation and porcine adipogenesis differentiation respectively, preferential localise in the nuclear interior

when transcriptionally active and at the nuclear periphery when transcription is switched off. Same is true for *ANT2* required for inactivation of chromosome X (Dietzel et al., 1999), *HoxB* during development (Chambeyron and Bickmore, 2004) and *Cftr* in adeno-carcinoma cells (Zink et al., 2004). Activation of certain genes has been accompanied by their movement away from constitutive heterochromatin regions (Delaire et al., 2004, Hewitt et al., 2004, Brown et al., 1999). A few recent studies have also proposed that related genes or genes belonging to the certain pathways have preferential location with respect to specific transcription factories or interchromatin granules (Osborne et al., 2004, Osborne et al., 2007, Hu et al., 2008). DNA modifications such as acetylation have also been shown to influence nuclear positioning of chromatin domains (Strasak et al., 2009).

Contradictory to the above data, the positions of some genes have been demonstrated to be independent of their transcriptional status (Lanctot et al., 2007). For example, no relocalisation of the CD8 locus during T-cell differentiation (Kim et al., 2004), gene locus or interferon γ (Hewitt et al., 2004) or genes during early tumorigenesis (Meaburn and Misteli, 2008) was observed irrespective of their transcriptional activity. This, thus suggests that the model correlating nuclear localization and repositioning of a particular gene locus to the transcriptional status of that gene is more complex than anticipated and may apply only on a sub-set of mammalian genes.

Chromosome 10 occupies differential positions in proliferating, quiescent and senescent HDFs (Chapter 2). The functional significance of these distinct locations of chromosome 10 with respect to its proliferative status may be due to changes in its gene expression profile accompanied with cells entry into quiescence or senescence from a proliferative state. Indeed distinct gene expression profiles with

respect to entry of cells into either quiescence (Chechlinska et al., 2009, Kipling et al., 2009, Coller et al., 2006, Liu et al., 2007, Glynne et al., 2000, Venezia et al., 2004, Gos et al., 2005, Mizuno et al., 2009, Viatour et al., 2008) or senescence (Kipling et al., 2004, Shelton et al., 1999, Zhang et al., 2003, Schwarze et al., 2005) have been observed. The study in this chapter is an initiative towards examining if a correlation between the change in gene expression profile specifically for chromosome 10 and its distinct position within the interphase nucleus in proliferating, quiescent and senescent cells exists. In addition to this, in proliferating HGPS HDFs, chromosome 10 is located at the nuclear periphery similar to that in control quiescent HDFs (Chapter 4). Gene expression profiles of this prematurely ageing disorder is different from that of replicative senescent HDFs (Park et al., 2001). With its position in HGPS HDFs similar to quiescent HDFs (Chapter 4), an attempt to assess whether or not the gene expression profile of chromosome 10 in cells from these diseased patients resembles that of quiescent HDFs has been made in this chapter. Thus, studying gene expression profile HGPS HDFs would serve as a model system in order to reveal links, if any, between perturbed location of a chromosome within a cell nucleus and its influence on levels of expression of genes on that chromosome.

7.2. Methods and Materials:

7.2.1. Gene search:

All protein coding genes present on chromosome 10 were listed (NCBI database) along with their known functions (NCBI and Ensembl databases), pathways they are involved in (Kegg pathways database) and diseases linked to these genes (OMIM and Ensembl databases as well as available literature) (See appendix). Genes involved in similar pathways and performing analogous functions within the cell were then grouped together. 8 genes involved in cell-cycle related and apoptosis related functions were then short listed. For an internal positive control, *PFKF* gene, which is ubiquitously expressed in fibroblasts, was selected; while *FGF8* gene, whose expression in adults is limited to ovaries and testis, was selected as an internal negative control gene.

7.2.2. Cell culture:

Human dermal fibroblast cell lines derived from a normal individual (2DD) and a patient suffering from HGPS patient carrying a classical G608G mutation in *LMNA* gene (AG11498) were cultured in 10% NCS-DMEM and 15% FBS-DMEM respectively as in section 2.2.1. and 4.2.1. Cells were passaged twice every week until they enter a state of replicative senescence.

Cells were induced to enter a state of quiescence by incubation in medium containing low serum (0.5% NCS/FBS-DMEM) for a period of 7 days as in section 2.2.1.

7.2.3. RNA isolation:

RNA isolation was performed using GenElute™ Mammalian Total RNA kit {Sigma Aldrich}. Both control and HGPS patient fibroblasts were cultured in 10 cm^2 dishes, seeded at a density of 2 x 10^5. The cells were maintained in culture for at least 2 days after seeding, after which the HDFs were washed with sterile 1X PBS with three changes of the buffer. The cells were then lysed and RNases inactivated by incubation in a mixture containing lysis buffer and 2-mercaptoethanol for no more than 2 minutes with constant shaking. The lysate was filtered by centrifugation in GenElute filtration columns for 2 minutes at 14,000g. RNA was isolated from purified samples by binding to 70% ethanol solution in GenElute binding column. Isolated RNA is collected in the collection tube and taken through three washes with wash buffers provided in the kit in order to obtain ethanol-free RNA. The column is further dried completely before eluting the RNA using 35 µl of elution buffer in RNase free tube. Isolated RNA from each sample was maintained on ice at all times. Further, to avoid any contamination from DNA, isolated RNA was treated with DNase I {Sigma Aldrich}, whereby RNA was incubated with DNase I containing buffer for 15 minutes at RT. The action of DNase I was then stopped by addition of stop buffer and denaturation of the enzyme by incubation at 70ºC for 10 minutes. Amount of RNA in each sample was measured using nano-drop. Purified RNA isolated from each sample was stored at -80ºC until further use.

7.2.4. Reverse transcriptase PCR:

7.2.4.a. Primer design

Gene specific forward and reverse primers were designed using Primer 3.0 or invitrogen primer design software. mRNA sequence for each gene was exported from NCBI website and a BLAST search was performed in order to identify sequences that were unique to a particular gene. The unique system was then exported into Invitrogen primer design (http://tools.invitrogen.com/content.cfm?pageid=9716) or primer 3.0 software (http://frodo.wi.mit.edu/primer3/) and primers giving a product size between 100 – 600 bp were identified. In order to ensure high efficiency, primers with more than 50% GC content or presence of adenine or thymine bases at the ends or clusters of any nucleotide throughout the primer sequence; were rejected. Forward and reverse primers selected for all genes had similar annealing temperatures. In order to minimise risk of contamination by genomic DNA, it was ensured that primers selected were on two separate exons.

7.2.4.b. cDNA synthesis and PCR reaction

First strand cDNA synthesis was carried out using SuperScript^{TM} II reverse transcriptase kit {Invitrogen}. 400ng of RNA from each sample was incubated with 100ng random primers and 10mM dNTP mix at 65ºC for 5 minutes and the contents were then chilled on ice for 1 minute. Further, 5X first-strand buffer {250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM $MgCl_2$} and 0.1M DTT were then added to the above mixture before incubation at RT for 2 minutes. For each reaction, 200 units of SuperScript^{TM} II reverse transcriptase enzyme were added and the constituents were then incubated at 50ºC for 50 minutes. The enzyme was then inactivated by

incubation of the reaction tubes at 70ºC for 15 minutes. cDNA synthesised was then stored at -20ºC until use.

To further amplify the gene of interest, cDNA synthesised was subjected to a PCR procedure using enzyme KAPAHiFi DNA polymerase {KAPA biosystems}. For each reaction, PCR reaction mixture containing cDNA from the above reaction, 0.3mM of 5X KAPAHiFi reaction buffer, 0.3µM dNTP mix, 0.3µM final concentration each of gene specific forward and reverse primers (See table 7.1.) and 1U of KAPAHiFi DNA polymerase enzyme was prepared. The components were then mixed thoroughly and subjected to PCR amplification procedure whereby they were initially denatured at 95ºC for 2 minutes followed by 35 cycles at 98ºC for 20 seconds, primer specific annealing temperature (see table 7.1.) for 15 seconds and 68ºC for 30 seconds, the final cycle was followed by 68ºC extension for 5 minutes.

PCR products were then run on a 1% agarose gel along with the positive and negative control genes and the band size for each product was verified with the expected band size. The signal intensity of each band for the positive control gene as well as the experimental genes were measured and quantified.

Table 7.1. List of genes present on chromosome 10, whose expression has been studied by reverse transcriptase PCR: Forward and reverse primers as well as annealing temperature used for each set of primer are displayed in the table:

Genes and their isoforms	Forward primer	Reverse primer	Annealing Temperature used
PTEN	5'-ATGTGGCGGGACTCTTTATG-3'	5'-GCGGCTCAACTCTCAAACTT-3'	63.6°C
CDC2	5'-GCTGGCTCTTGGAAATTGAG-3'	5'-GGCCAAAATCAGCCAGTTTA-3'	63.8 °C
UNC5B	5'-TCCAGCTGCATACCACTCTG-3'	5'-AGCCACCAGCATCTCACACA-3'	64.1 °C
CCAR1	5'-AGTGCTCCGTGAATCTTGCT-3'	5'-TGAGGCCAACATTTTCTTCC-3'	63.9 °C
TLX1	5-CAAGGGAGGTAAGGGAGGAG-3'	5'-GTGACACCAGGAGGGACAGT-3'	64.0 °C
NOLC1	5'- GCAAAGAAGGCTGCTGTACC-3'	5'-CCTCTTCCTCGTCACTGGAG-3'	63.9 °C
PTF1A	5'-CCTGAGAAGTCCCAGACTCG-3'	5'-GCCAGAGTTCTCCCAACAAC-3'	63.8 °C
Caspase 7 4 isoforms α, β, and δ	5'-GAGCGACGGAGAGAGACTGT-3' (For isoforms α and δ) 5'-CTGGGTGGGTACTTCCTTCA-3' (For isoform β)	5'-CAACCCCCTGCTCTTCAATA-3' (For isoforms α and δ) 5'-GAACGCCCATACCTGTCACT-3' (For isoform β)	63.8 °C
PFKP	5'-ACAGGATCATCGAGGTCGTC-3'	5'-CGGTTCTCCGAGAGTTTGAC-3	64.0°C
FGF8	5'-TCATCCGGACCTACCAACTC-3'	5'-AATCTCCGTGAAGACGCAGT-3'	63.8 °C

To measure the signal intensity of PCR product, digital images of gels were opened in ImageJ. Each band was outlined individually and the signal intensity in pixels occupied by the band was quantified using the gel analysis tool in ImageJ menu. As a control, this was also repeated for an equivalent area of background for each gel. The mean pixel intensity of the background was subtracted from the mean intensity of each PCR product. The signal intensity of each for each PCR product, after background subtraction, was then normalised with signal intensity of PFKF, the

positive control gene (after background subtraction), so as to minimise errors caused during loading samples.

7.3. Results:

Positioning chromosome territories of all human chromosomes in quiescent and senescent fibroblasts using 2D and 3D FISH has led to the revelation that chromosome 10 was one of the two unique chromosomes, along with chromosome 15, to occupy completely different locations in proliferating, quiescent and senescent HDFs (Chapter 2) (Mehta, 2005).

7.3.1. Identification of genes of interest on chromosome 10:

To understand the functional relevance of differential position of chromosome 10, it would be a logical step to examine the expression profiles of genes on this chromosome at different stages of the cell cycle.

Chromosome 10 comprises of 131,666,441 base pairs which consists of 1357 genes, out of which 816 are protein coding genes while 430 are pseudogenes (Deloukas et al., 2004). To date, 166 diseases including various types of cancers, leukaemias, Charcot-Marie-Tooth disease (dominant intermediate 2), Cockayne syndrome, Alzhimers syndrome, Type I diabetes mellitus, Cowden syndrome and Crohns disease (http://www.genecards.org/cgi-bin/listdiseasecards.pl?type=chrom&search=10); have been linked to genes on chromosome 10. To perform a systematic study to identify genes that are most relevant for this study, all genes and diseases linked to chromosome 10 were listed. The genes were then categorised according to their broad function or the pathway they are involved in.

Genes involved in apoptosis and Wnt signalling pathway, has several growth factor genes, cell adhesion molecules and some cell cycle dependent genes are

present on chromosome 10. Since the difference in position of this chromosome was observed between proliferating, quiescent and senescent HDFs, this study focuses on 4 genes representing a small subset of genes involved in cell cycle regulation (Table 7.2.). Results from chapter 4 demonstrated aberrant nuclear positioning for chromosome 10 territories in cells derived from HGPS patients. Since these patient cells undergo higher rates of apoptosis with their increasing age in culture (Bridger and Kill, 2004), 3 genes that are implicated in apoptosis present on chromosome 10 were studied in addition to the cell cycle regulation genes.

Table 7.2.Genes on chromosome 10 on which reverse transcriptase PCR would be performed and the pathway they are involved in:

Genes and their isoforms	Pathway that it is involved in	Location of chromosome 10
pTEN	Tumor suppressor gene	10q23.31
CDC2	Cell cycle	10q21.1
TLX1	Cell cycle	10q24
NOLC1	Cell cycle	
UNC5B	Apoptosis	10q21-q22
CCAR1	Apoptosis	10q21.3
Caspase 7 (4 isoforms – α, β, γ and δ)	Apoptosis	10q25.1-q25.2
PTF1A	Pancreatic development	10p12.3
PFKF	Positive control	10p15.3-p15.2
FGF8	Negative control	10q24

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a well studied gene on chromosome 10 and mutations in this gene have been linked to various tumours including renal, gliomas, melanoma, endometrial, breast, prostrate,

lung, bladder and thyroid (Li and Ross, 2007), thus explaining its status as a tumour suppressor protein (Li and Sun, 1998). *PTEN* is an important regulator of cellular phosphoinositide 3-kinase signalling pathway which in turn controls various cellular processes including cellular proliferation, survival, growth and mobility (Katso et al., 2001, Cantley, 2002, Cantrell, 2001). *PTEN* has also been implicated in processes such as apoptosis, maintenance of cell size (Huang et al., 1999), maintaining chromosome integrity and stability (Shen et al., 2007) and differentiation (Zheng et al., 2008). *Cell division cycle 2* (*CDC2*) is a catalytic subunit of a protein kinase complex called the M-phase promoting factor present in eukaryotes and necessary for transition into mitosis from the G2 phase of the cell cycle (Lee et al., 1988). *CDC2* is vital for spindle formation, chromosome segregation (Li and Zheng, 2004), double strand break induced homologous recombination (Ira et al., 2004), regulation of cell cycle and cell division (Santamaria et al., 2007), initiation of sister chromatid separation (Holt et al., 2008) and degeneration of post mitotic neurons (Yuan et al., 2008). *TLX1* is a homeobox gene that controls cell survival (Dear et al., 1995) and is deregulated in T-cell leukemia (Hatano et al., 1991, Kennedy et al., 1991, Lu et al., 1991, Zutter et al., 1990). *TLX1*, an oncogene (Kennedy et al., 1991) is also known to be involved in global pattering of the human skeleton, particularly the sacral vertebrate (Wellik and Capecchi, 2003). Nucleolar and coiled body phosphoprotein 1 (*NOLC1*) is localised in the DFC domain within the nucleoli of cells and is known to interact with CDC2 kinase during mitosis (Pai et al., 1995). The major function of *NOLC1* is related to nucleogenesis (Pai et al., 1995), whereby it is implicated in RNA polymerase I dependent transcription and maintenance of nucleolar shape (Chen et al., 1999). Mislocalisation of *NOLC1* results in aberrant localisation of other nucleolar proteins including fibrillarin and RNA polymerase I, thus suggesting

that *NOLC1* is important for nucleolar integrity and organisation of proteins within the DFC domain of the nucleolus (Chen et al., 1999).

Cell cycle and apoptosis regulator 1 (CCAR1) is important for cell cycle regulation, controlling apoptosis (Rishi et al., 2003) and is involved in the process that recruits RNA polymerase II to protein coding genes (Kim et al., 2008). In addition to *CCAR1, UNC5B* is also involved in regulation of apoptosis and has been implicated in colorectal cancers whereby this genes acts as a tumour suppressor (Thiebault et al., 2003). Caspase 7, another protein regulating apoptosis, is one of the executioner caspases that cleave distinct intracellular proteins involved in promoting the apoptotic phenotype (Tiso et al., 1996). Loss of *caspase 7* leads to inhibition of apoptosis resulting in a tumourogenic phenotype (Soung et al., 2003). *Pancreatic transcription factor1A (PTF1A)* is implicated in pancreatic development (Kawaguchi et al., 2002) and has been linked to neonatal diabetes mellitus (Sellick et al., 2003).

Phosphofructokinase, fibroblast type (PFKF) gene encodes for an enzyme phosphofructokinase that catalyzes the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate and is a key regulatory enzyme in glycolysis (Schwartz et al., 1984). Since it is ubiquitously expressed in fibroblasts at all times (Weil et al., 1980), *PFKF* gene was chosen as a positive control gene for this study. *Fibroblast growth factor 8 (FGF8)* is an embryonic epithelial factor and has been implicated in gastrulation, organogenesis of face and limb and regionalisation of the brain (Payson et al., 1996). The adult expression of this gene is restricted to testis, ovaries (Payson et al., 1996), epithelial cells in colon and uterus, lactating human breast (Zammit et al., 2002), although an increased expression of this gene has also been observed in human prostrate (Tanaka et al., 1995) and breast cancer cell lines (Zammit et al., 2002, Tanaka et al., 1995). Since this gene is expressed only in testis

and ovaries in adult humans, it serves as a good candidate for a negative control for this study.

7.3.2. Reverse transcriptase PCR analyses for selected genes in control proliferating, quiescent, senescent HDFs and in HGPS HDFs:

To assess the expression of 8 genes including *PTEN, CDC2, CCAR1, TLX1, caspase 7*(α, β and δ isoforms), *PTF1A, UNC5B* and *NOLC1*, gene specific primers were designed. RNA was extracted from young proliferating cultures (passage 17), fibroblast cultures made quiescent by serum starvation for 7 days, aged fibroblast cultures (passage 42) representing senescent cells and from fibroblast cultures derived from HGPS patients (passage 18). cDNA was then synthesised from the RNA extracted and reverse transcriptase PCR was performed using gene specific primers.

In order to make sure the PCR for negative control gene (FGF8) worked for each reaction, the expressin of this gene was analysed in RNA extracted from cell line derived from a prostrate cancer patient (PC3) {A kind gift from Rana Hassan, Division of Biosciences, Brunel University, London}.

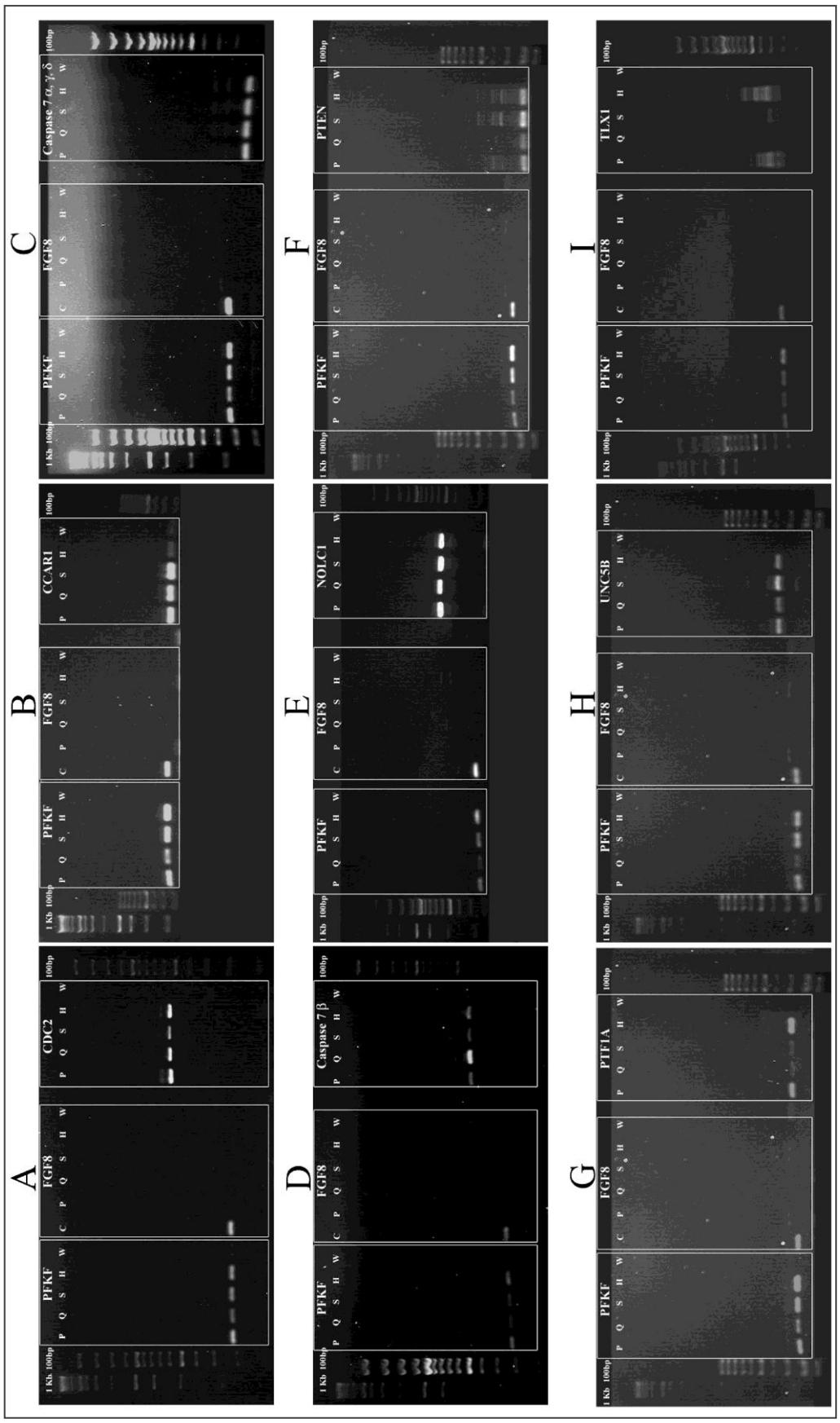


Figure 7.1. Representative images of gels showing the expression of 8 selected genes in control proliferating, quiescent and senescent as well as in HGPS HDFs

Figure 7.1. Representative images of gels showing the expression of 8 selected genes in control proliferating, quiescent and senescent as well as in HGPS HDFs: cDNA was synthesised from RNA samples obtained from control proliferating, quiescent and senescent HDFS and from HDFs derived from HGPS patients. A RT-PCR reaction was performed using gene specific primers for each of the 8 genes (including three isoforms of caspase 7). For amplification of each experimental gene, PCR for positive and negative control genes was also performed at the same time. To ensure that the PCR reaction for negative control has worked, cDNA sample obtained from prostrate cancer cell line expressing FGF8 was amplified using primers specific for this gene. A 100 bp and a 1Kb ladder were used to detect the size of the band for each product. {P= proliferating cells, Q = quiescent cells, S = senescent cells, H = HGPS HDFs, C = Prostrate cancer cell line, W = water (negative control)}.

In order to quantify the amount of RNA amplified for each sample for, the signal intensity in the area of the band was measured using ImageJ, background subtracted and then normalised with the area of positive control (*PFKF*) band for that particular sample. Thus normalised signal intensity for RNA amplified for each of the genes, from control proliferating, quiescent and senescent HDFs and in HGPS HDFs, are displayed in table 7.3. It should be noted that each sample had the same amount of cDNA that was subjected to PCR amplification for each gene.

Table 7.3.Normalised signal intensities representing the expression levels of 8 experimental genes that are involved in cell cycle regulation or apoptosis, present on chromosome 10:

Cells / Genes	Expected Band size	Control proliferating HDFs	Control quiescent HDFs	Control senescent HDFs	HGPS HDFs
PTEN	205 bp	2.68	2.89	2.11	1.0
CDC2	643 bp	1.86	2.33	1.06	1.74
CCAR1	170 bp	1.20	1.61	1.18	0.15
Caspase 7α	160 bp	0.13	0.18	0.09	0.06
Caspase 7β	392 bp	1.07	13.27	1.33	1.3
Caspase 7δ	200 bp	1.01	1.77	1.16	0.78
NOLCI	526 bp	3.94	4.79	2.63	1.87
UNC5B	350 bp	1.02	1.00	0.94	0.39
TLX1	319 bp	7.16	0.88	1.22	4.46
PTF1A	268 bp	7.61	0.03	1.12	4.37

Preliminary results, which would require further confirmation, suggests that expression levels of some genes such *PTEN*, *CCAR1*, *Caspase 7α* and *UNC5B* are very similar in control proliferating, quiescent and senescent HDFs (Figure 7.1. F, B, C and H respectively; Table 7.3.). Conversely, *TLX1* and *PTF1A* expression is higher in proliferating controls as compared to non-proliferating counterparts (Figure 7.1. I and G respectively; Table 7.3.). Levels of *CDC2*, *Caspase 7β*, *caspase 7δ* and *NOLC1* expression are highest in control quiescent HDFs; followed by control proliferating HDFs and the least in control senescent HDFs (Figure A, D, C and E respectively; Table 7.3.). The level of expression of all but three genes (*CDC2, Caspase 7β and Caspase 7δ*) studied, are reduced to half or less than half in HDFs derived from HGPS patients (Figure 7.1. A, D and C respectively; Table 7.3.). *CDC2* and *Caspase 7δ* expression in HGPS HDFs resemble that of control proliferating HDFs (Figure 7.1. A and C respectively; Table 7.3.), while that of *Caspase 7a* is close to that observed in control senescent fibroblasts (Figure 7.1. C; Table 7.3.).

Thus, it appears that movement of chromosome 10 to an interior location of the nucleus in senescent cells is not accompanied by increase in expression of the studied genes on the chromosomes and the same is true for repression of studied genes when chromosome 10 is localised at the nuclear periphery in quiescent HDFs. Although, in HGPS HDFs chromosome 10 is positioned in a similar nuclear compartment as quiescent HDFs, expression level of none of the above studied genes in these patient cells is similar to that in control quiescent HDFs.

7.4. Discussion:

Chromosomes, sub-chromosomal domains and individual genes are organised in a non-random and an ordered manner within interphase nuclei (Cremer and Cremer, 2001, Bridger et al., 2000, Croft et al., 1999, Foster and Bridger, 2005, Cremer and Cremer, 2006, Meaburn et al., 2007, Meaburn and Misteli, 2007). Various studies have shown that this organisation of genome within the nucleus, although being specific and non-random, is very dynamic, constantly changing during cells life span (Meaburn et al., 2007, Bridger et al., 2000, Solovei et al., 2004) (Chapter 2, 3 and 4). This high degree of dynamic spatial organisation of the genome within nuclei has been suggested to be a governing force behind nuclear processes (Misteli, 2007, Fraser and Bickmore, 2007, Lanctot et al., 2007).

In order to test the hypothesis if gene expression is related to spatial position of the genome, we studied genes on chromosome 10 that is differentially localised in proliferating, quiescent and senescent fibroblasts. Since alteration in the position of this chromosome is observed with cells exit from the cell cycle, the selection of genes was focussed on genes involved in proliferation, cell cycle regulation or apoptosis. In order to perform a first crude observation as to whether the expression levels of genes change in different cell states (proliferating, quiescence and senescence), only a small subset of 8 genes was chosen and the expression levels in proliferating, quiescent and senescent fibroblasts were measured. In senescent cells, transcriptional activity of none of the genes increased in correlation with the interior location of chromosome 10. Transcriptional activity of *Caspase 7β*, *NOLC1*, *PTEN* and *CDC2* increased in quiescent cells as compared to that in proliferating and senescent cells, thus suggesting that localisation of chromosome 10 at the nuclear

periphery does not result in repression of all the genes present on this chromosome. One of the problems associated with such analyses is that cultures may have a mixed population of cells, i.e. proliferating and quiescent cultures may have cells that have entered replicative senescence, while not all cells in senescent cultures are non-proliferating and their might be a small proportion of proliferating cells within these cultures. It might be also possible that in senescent cells, when chromosome 10 is located in the interior of an interphase nucleus, it might be interacting with components of the nuclear architecture including nucleoli or Cajal or PML bodies; and this association leads to repression of the genes present on this chromosome. Another mechanism by which this phenomenon can be explained is that, in quiescent cells when chromosome 10 is at the nuclear periphery, the genes might be present on the inner folds of the chromosome territory facing away from the nuclear periphery or on loops extending into the nuclear interior. To indeed prove that this is true, further experiments localising individual gene loci along with whole chromosome territory would be required. Other factors such as epigenetic modifications and local gene density may also work in cohort with the spatial organisation of the genome in order to regulate transcription. To further confirm these correlations, techniques such as microarray, 3C (chromosome, conformation and capture), 4C (circular chromosome conformation and capture) and 5C (carbon-copy chromosome conformation and capture) that allows simultaneous analyses for more than one gene on chromosome 10 in proliferating, quiescent and senescent HDFs, would be ideal to perform. This study would then highlight if there are any group of genes, like differentiation genes (Kosak et al., 2002, Ragoczy et al., 2006, Szczerbal et al., 2009) whose expression is correlated with differential position of the parent

chromosome. A possibility that change in spatial organisation is not a cause, but an effect of gene expression cannot be ignored.

Transcriptional activity of 7 genes studied is decreased at least to half the levels observed in HDFs derived from HGPS patients containing a mutant lamin A protein. This suggests that presence of progerin may interfere with transcription of certain or indeed most genes on chromosomes, thus highlighting the role of lamin A in regulation of transcription. Intriguingly, the transcriptional activity of the genes studied in HGPS HDFs were not similar to that in quiescent control cells, even if the location of chromosome 10 is similar between HGPS and control quiescent HDFs. This reinforces the idea that transcriptional activity of all genes may not be regulated just by the spatial distribution of genomic entities within an interphase nucleus.

Thus, although this study focuses only on a very small subset of genes on chromosome 10 and is not thorough, it indeed is suggestive of the idea that the relationship between spatial organisation and gene expression within an interphase nucleus may not be as straightforward as expected and that regulation of gene expression must be multi-factorial process. Thus, the link between spatial organisation of genomic regions and gene expression may be gene specific and may be affected by the neighbourhood the gene is present in, proximity to a transcription factory and association with other components of the nuclear architecture. Detailed analysis whereby the combination of factors proposed to influence gene expression including spatial organisation of the genome are studied together would provide a better understanding to regulation of gene expression.

Chapter 8:

General Discussion

Genomes are non-randomly organised, with chromosome territories, sub-chromosomal regions, as well as genes, occupying distinct and preferential locations within an interphase nucleus. Although genomic entities are arranged in this strict organisation, they are not rigid compartments and instead are dynamic structures that can be repositioned with respect to other nuclear structures and other genomic regions (Gasser, 2002). Most of the studies to date have focussed on repositioning of individual gene loci (Kosak et al., 2002, Ragoczy et al., 2006, Brown et al., 1999, Osborne et al., 2004, Williams et al., 2002, Volpi et al., 2000, Morey et al., 2007, Chambeyron and Bickmore, 2004, Fraser and Bickmore, 2007, Szczerbal et al., 2009) while some have observed reorganisation of sub-chromosomal regions (Bartova et al., 2001, Chuang et al., 2006, Dundr et al., 2007, Reddy et al., 2008, Kumaran and Spector, 2008, Chaly and Munro, 1996, Galiova et al., 2004, Manuelidis, 1985). In addition to these, dynamic repositioning of whole chromosome territories has also been observed during differentiation (Kim et al., 2004, Kuroda et al., 2004, Parada et al., 2004, Foster et al., 2005, Panning and Gilbert, 2005, Casolari et al., 2005) or when cells exit the proliferative cell cycle to become quiescent or senescent (Bridger et al., 2000, Mehta, 2005) (Chapter 3).

The main objective of this thesis is to establish a system whereby dynamic repositioning of whole chromosome regions is observed and then to identify components of the nuclear architecture that assist this re-localisation event. Organisation of chromosome territories differs between proliferating (Croft et al., 1999, Meaburn et al., 2007), quiescent (Chapter 2) (Figure 8.1.) and senescent HDFs (Mehta, 2005), with some chromosomes localising at similar locations while others occupy distinct positions within the interphase nuclei within all of these three states (Chapter 2). Indeed, in concurrence with the previous study (Bridger et al., 2000),

chromosome organisation in non-proliferating (both quiescent and senescent) HDFs changes to a size distribution as opposed to a gene density based distribution observed in proliferating HDFs (Chapter 2) (Figure 8.1.). Interestingly, two human chromosomes, i.e. HSA 10 and 15 territories are located in different nuclear compartment in proliferating, quiescent and senescent HDFs (Chapter 2) and chromosome territory organisation is perturbed in fibroblasts containing a mutant lamin A protein (derived from HGPS patients) (Chapter 4) (Figure 8.1.). These data reiterate that various nuclear components such as INM proteins including lamin A (Chapter 4) (Meaburn et al., 2007) and emerin (Meaburn et al., 2007), attachment to nuclear structures such as the nuclear matrix (Elcock and Bridger, 2008) (Elcock LS, Bridger JM in prep) and the nucleoli (Chubb et al., 2002) influence the positioning of chromosome territories within an interphase nucleus. Although HDFs derived from HGPS patients undergo accelerated senescence, the organisation of chromosome territories in these patient cells resembles that of control quiescent HDFs (Chapter 4) (Figure 8.1.). This observation is in agreement with previous studies that HGPS is a premature ageing disorder displaying some (Kipling et al., 2004) but not all features observed in normal ageing (Pereira et al., 2008).

Preliminary analysis suggest that repositioning of chromosome 10 territories following the exit of fibroblasts from the cell cycle may influence expression of some, but not all the genes present on this chromosome (Chapter 7). Hence the functional relevance of re-positioning of the genome following entry of fibroblasts into growth arrest states may not be just confined to gene expression. Intermingling between chromosome territories has been observed (Branco and Pombo, 2006). Entry of cells into a non-proliferating state may require interactions between different chromosomes as compared to that in proliferating HDFs and hence the re-

localisation of chromosome territory regions occurs in order to facilitate these interactions. How, when and why regions on one chromosome recognise the particular region on other chromosome that it needs to interact with, are unanswered questions. Previous studies have demonstrated repositioning of various genes to common sub-nuclear transcription factories in order to perform functions such as regulation of gene expression, imprinting and chromosome X inactivation (Osborne et al., 2004, Fraser and Bickmore, 2007). Chromosome territory organisation within an interphase nucleus may affect this clustering of genes. Entry and maintenance in non-proliferative growth arrest states potentially requires clustering of different genes in fibroblasts as compared to their proliferative counterparts which is assisted by repositioning of chromosome territories as cells exit the proliferative cell cycle. It might be also possible that differences in the radial distribution of chromosome territories following exit of fibroblasts from the proliferative cell cycle, may affect the interaction of chromatin domains with other nuclear structures. In other words, in addition to the radial chromosome territory organisation, the positioning of chromosomes with respect to other nuclear structures such as the nucleoli, PML bodies and Cajal bodies may differ in non-proliferating HDFs as compared to that observed in proliferating HDFs. Chromosome territory organisation in an interphase nucleus may be controlled by DNA modifications such as acetylation (Strasak et al., 2009). Whether or not differences in these modifications are observed between proliferating and non-proliferating fibroblasts; and if these manifest the differences in chromosome territory organisation is unknown.

Although, the functional significance of repositioning of genomic regions within an interphase nucleus is ambiguous, understanding of the process as to how this re-location of chromosome territories occurs is much clearer given the study

presented here. Upon induction of quiescence by serum starvation, repositioning of chromosome territories occurs within the fifteen minutes of serum withdrawal following which the chromosome territory locations are fixed throughout the cells existence in a quiescent state (Chapter 3). This rapid response to serum withdrawal is an active process, requiring energy and is dependent on the function of nuclear motors. NMIβ has been identified as one of the components of this nuclear motor that is involved in repositioning of chromosome territories within the nucleus (Chapter 3). The specific isoform of actin that is implicated in the nuclear motor is still unknown. Cells entry into the proliferative cell cycle following exit from quiescence also involves repositioning of chromosomes, but this re-localisation event occurs over a much longer duration of time and requires cells to traverse mitosis (Chapter 3). Intriguingly, redistribution of NMIβ from aggregates in quiescent HDFs to a nuclear rim and nucleolar distribution in proliferating HDFs occurs only after 24 hours post cells entry into the proliferative cell cycle (Chapter 5). This is suggestive of a plausible role of NMIβ in establishing chromosome positions following mitosis. The signalling cascade of factors that regulate the function of these nuclear motor proteins remains to be identified. Interaction between nuclear motor proteins and INM proteins and hence formation of a hypothetical complex between NMIβ, nuclear actin, emerin and lamin A has been suggested (Mehta et al., 2008). Accumulation of mutant lamin A protein observed in HGPS HDFs as well as in normal senescent cells (McClintock et al., 2006) compromises the ability of fibroblasts to reposition their chromosomes (Chapter 2 and 4), thus providing evidence towards the proposed link between lamin A and efficient functioning of nuclear motors. This is further supported by presence of atypical distribution of NMIβ in proliferating HGPS HDFs that resembles that of control

senescent fibroblasts (Chapter 5). The compromised function of the nuclear motor proteins can be further linked to accumulation of farnesylated form of lamin A in HGPS fibroblasts and senescent cells, as inhibition of farnesylation by treatment with FTIs restores NMIβ distribution as well as the chromosome repositioning response in HGPS fibroblasts (Chapters 4 and 5). No direct interaction between NMIβ and lamin A has been identified to date; hence the mechanism of cross-talk between these two proteins is also unknown. Whether farnesylated lamin A has an effect on other functions of NMIβ including transcription, chromatin remodelling and nucleo-cytoplasmic transport remains to be identified. It is also not known if the function of nuclear motors is compromised in all laminopathy patients or is confined to patients having a mutation in codon 608 of exon 11 of *LMNA* gene.

NMIβ localises in the nucleolus in proliferating HDFs (Chapter 5) (Figure 8.1.) (Nowak et al., 1997, Fomproix and Percipalle, 2004), whereby it is implicated in maintenance of the nucleolar domains including GC and DFC (Cisterna et al., 2009). No distinctive differences in distribution of proteins localised in DFC including nucleolin, fibrillarin and Ki-67 as well as NPM, a protein present in the FC compartment of the nucleolus have been observed in control proliferating and non-proliferating HDFs (Chapter 6) (Figure 8.1.), even if NMIβ is distributed differently in these cells (Chapter 5) (Figure 8.1.). Conversely, the distribution of nucleolar proteins such as nucleolin, fibrillarin and NPM is perturbed in HGPS fibroblasts containing farnesylated progerin (Chapter 6) (Figure 8.1.). Interestingly the relative amount of NMIβ (Chapter 5) as well as nucleolin (Chapter 6), a vital DFC protein, is much lower in HGPS fibroblasts as compared to control fibroblasts. Hence, functional lamin A may be required for maintenance and localisation of NMIβ which may then influence the distribution of nucleolar proteins. A regulatory mechanism

whereby lamin A directly maintains the distribution of nucleolar proteins cannot be ignored. Since control senescent fibroblasts do not display atypical distribution or content of nucleolar proteins, farnesylated lamin A may not be the only protein involved in regulation of nucleolar proteins. These data are suggestive of a possible interaction between lamin A and nucleolus which may be intact in senescent HDFs but are perturbed in HGPS patients fibroblasts. The atypical nucleolar protein distribution and amount may interfere with the downstream processes that these proteins are involved in, leading to disrupted RNA biogenesis and compromised transcription ability of cells, which then further would affect the organisation of genomic regions within these patient HDFs.

Figure 8.1. Genome organisation, nuclear motors and nucleolus in control proliferating, non-proliferating and HGPS fibroblasts.

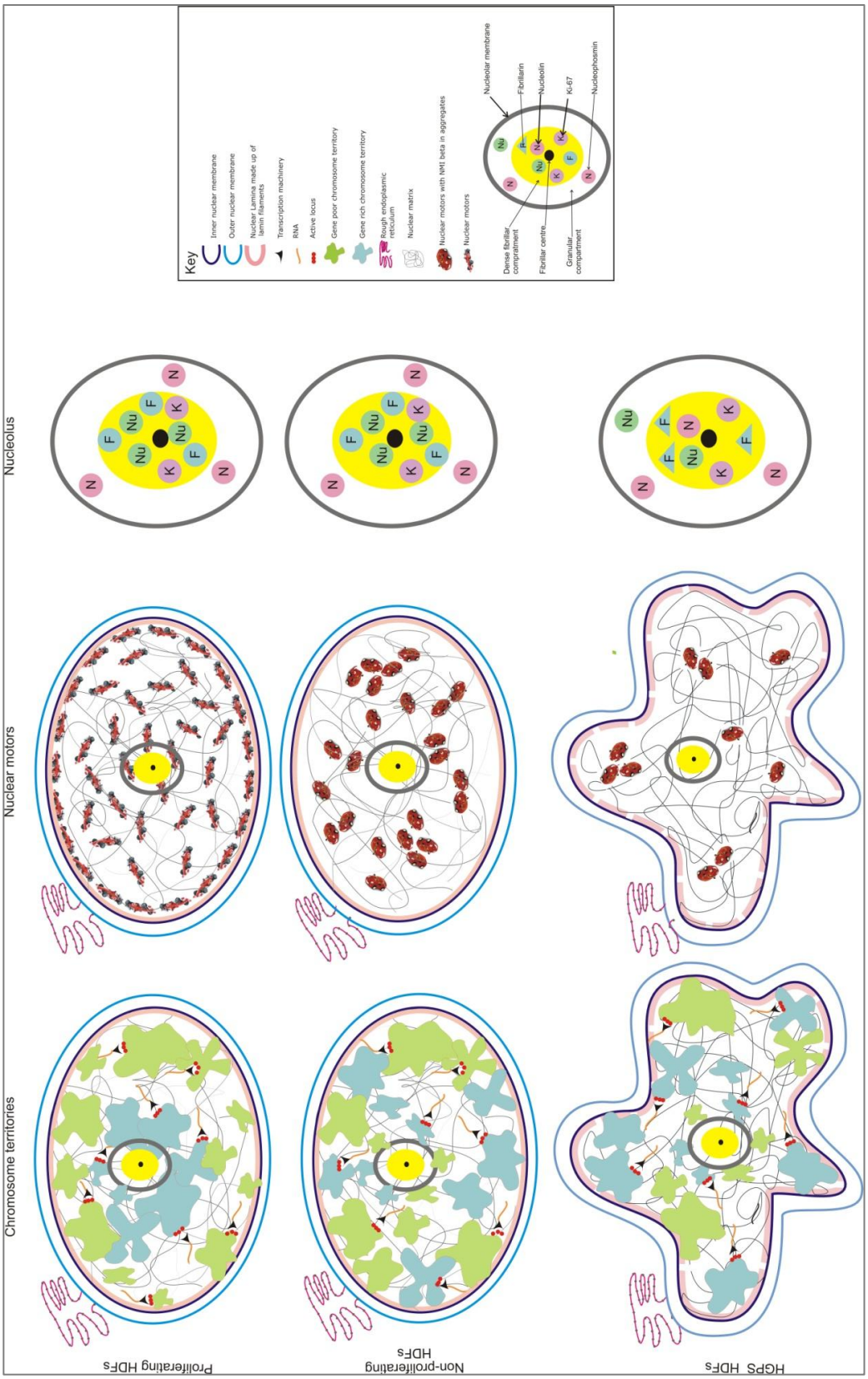


Figure 8.1. Genome organisation, nuclear motors and nucleolus in control proliferating, non-proliferating and HGPS fibroblasts: The spatial distribution of chromosome territories changes from a gene density based distribution in proliferating HDFs to a size dependent one in non-proliferating HDFs. The organisation of chromosomes in proliferating HGPS fibroblasts resembles that of non-proliferating HDFs. Nuclear motors, consisting of NMIβ are thought to support the movement of chromosomes within the interphase nuclei. NMIβ and thus possibly these motors are distributed at the nuclear periphery, within the nucleolus and throughout the nucleoplasm in proliferating HDFs while in non-proliferating and HGPS HDFs, NMIβ forms large aggregates and are only found in the nucleoplasm. Nucleolar proteins nucleolin, fibrillarin, Ki-67 and NPM display no difference in their location or structure between proliferating and non-proliferating HDFs, where nucleolin, fibrillarin and Ki-67 are found in the DFC domain while NPM is observed to be present in the FC of the nucleolus. Although, in cells derived from patients suffering from HGPS, nucleolin and NPM are distributed atypically in the FC and DFC respectively. Although, fibrillarin is still present in the DFC like in control proliferating HDFs, there appears to be a difference in the structural organisation of this protein within the nucleoli of HGPS HDFs. The distribution and localisation of pKi-67 in HGPS patient HDFs, is similar to that in control fibroblasts.

The array of nuclear functions and processes that occur within cells performing specialised functions such as differentiation or maintenance in states of growth arrest, are distinct as compared to when cells are cycling in the proliferative cell cycle. With changing requirements of the cells, regulation of nuclear processes may require co-ordinated function of architecturally associated nuclear components. One of these nuclear compartments includes chromosome territory organisation which responds to environmental cues and undergoes dynamic repositioning with the help of nuclear motors comprising of nuclear actin and NMIβ. Perturbation of one component comprising the nuclear architecture results in deregulation of many of these co-ordinated functions leading to a diseased condition. This is well demonstrated in case of HGPS patients, where it is clearly evident that accumulation

of progerin in cells may have even more widespread consequences than already discussed in the literature. The observations reported here; including alteration in chromosome territory organisation, repositioning of chromosomes, nuclear motor components as well as certain aspects of nucleolar structure in HGPS cells; may provide further insight into the cellular pathology of the disease as well as normal ageing. On a positive note, it appears that FTI treatment can effectively restore these chromosomal localisation and movement, NMIβ distribution as well as nucleolar abnormalities, a finding that provides more encouragement for the efficacy of FTI treatments currently undergoing clinical trials on HGPS patients.

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Appendix I

Figure A1: Comparison of DAPI

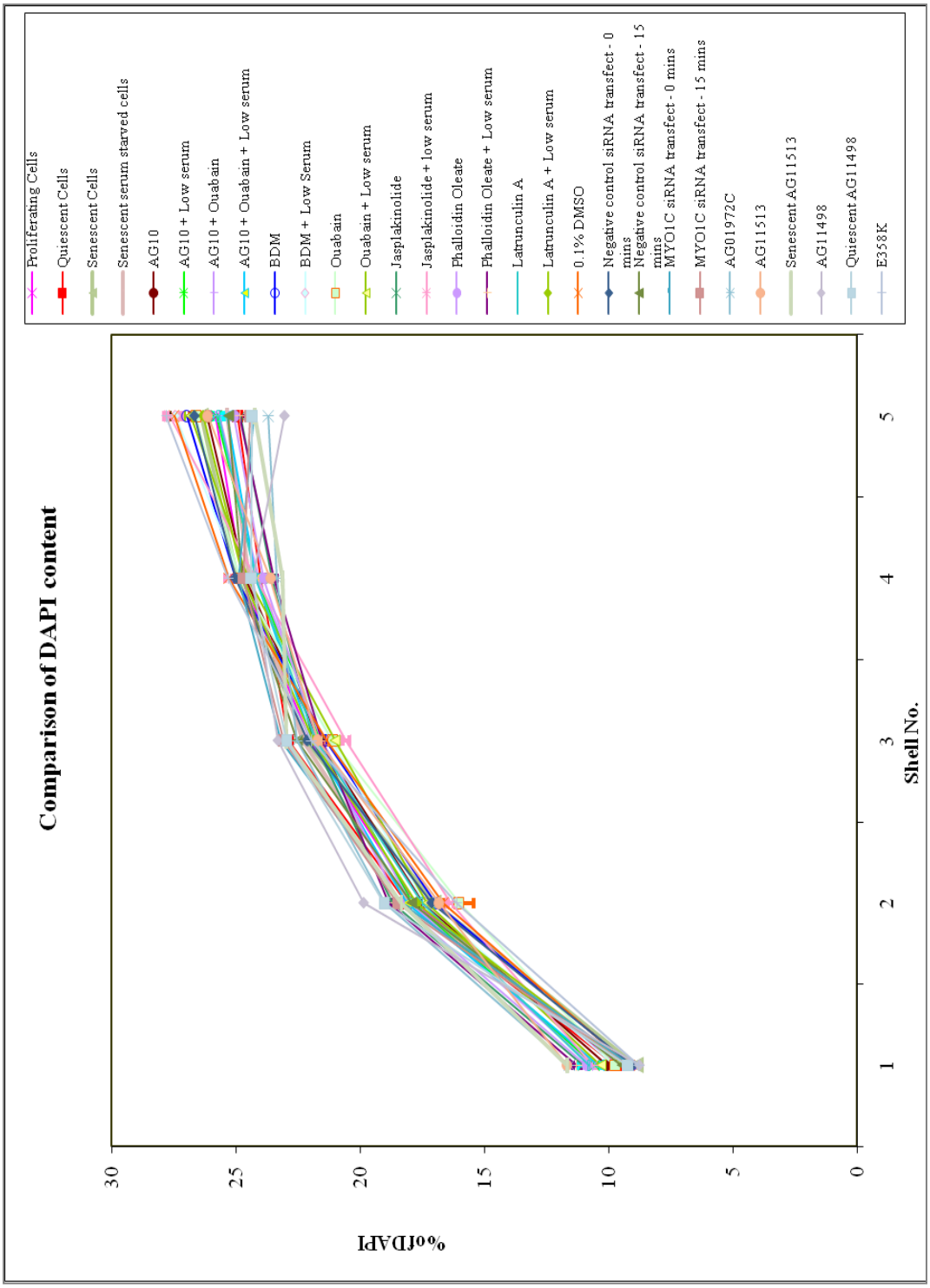

Figure A.1. Graph displaying the DAPI content: A line graph comparing the % of DAPI in each shell created by the erosion script for fibroblast nuclei under different situations analysed in this study.