CHARACTERISATION OF THE MOLECULAR LINKS BETWEEN THE NUCLEAR PORE COMPLEX AND THE NUCLEAR LAMINS AND RECONSTITUTION OF THE *XENOPUS* OOCYTE LAMIN ASSEMBLY *IN VITRO*

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TEIBA AL-HABOUBI
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auf Antrag von

Prof. Dr. Ueli Aebi
PD Dr. Birthe Fahrenkrog
Prof. Dr. Robert D Goldman

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Prof. Dr. Eberhard Parlow
Dekan

Abstract

Nuclear lamins and nuclear pore complexes (NPCs) are major components of the nuclear envelope in metazoan cells. The objectives of this thesis were first to study interactions between nuclear lamins and the nuclear pore protein Nup153 (Chapters 2 and 3) and second to determine lamin assembly conditions of the *Xenopus* oocytes LIII *in vitro* (Chapter 4). Nuclear lamins are major constituents of the nuclear lamina underlying the nuclear periphery along with inner nuclear membrane proteins. The nuclear lamina provides stability and determines the nuclear architecture and spacing of the NPCs. NPCs form supramolecular assemblies that regulate nucleocytoplasmic transport. An overview of the functional aspects associated with the nuclear lamina and NPCs in health and disease is provided in Chapter 1. In depth analysis of the interaction of nuclear lamins with the nucleoporin Nup153 is revealed in Chapters 2 and 3. Using *in vitro* solution binding assays as well as immunoprecipitation assays, in chapter 2 we show direct associations between Nup153 and nuclear lamins. This work is explored even further in chapter 3 using binding assays and immunofluorescence microscopy as well as immunoprecipitation assays; we examined the interaction in the presence of lamin related mutations. Finally, in chapter 4 we established buffer conditions for LIII assembly *in vitro*, analysed by electron microscopy (EM) using glycerol spraying/low-angle rotary metal shadowing and negative staining. Our results presented in this thesis contribute to expand our current knowledge of the interactions of the NPCs with the nuclear lamins, as well as to increase our understanding of the impact of mutations in lamins that can cause laminopathies. In addition, the studies on the assembly conditions of LIII provide a vehicle for further characterisation of the influence of binding partners and the importance of lamin sub-fragments on the formation of higher order assemblies.

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The nuclear lamina and the nuclear pore complex - implications in health and disease

Teiba Al-Haboubi and Birthe Fahrenkrog

M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland

1.1 Abstract

The nuclear envelope (NE) of eukaryotic cells represents a physical barrier separating the nucleus from the cytoplasm. It is comprised of an outer (ONM) and an inner (INM) nuclear membrane, separated by a perinuclear space (PNS). The ONM is continuous with the endoplasmic reticulum (ER), linking the PNS to the ER lumen. The INM represents a site where the nuclear lamina and chromatin are anchored to the nuclear periphery by interacting with several integral membrane proteins. The ONM and the INM fuse at sites of the nuclear pore complexes (NPCs). Some of the functions assigned for the NE include chromatin organisation, transcriptional regulation, mechanical integrity and signalling pathways, as well as acting as a key component in the organisation and function of the cytoskeleton. At the nuclear face, the NPCs are in close proximity to the nuclear lamina and their incorporation into the NE and spacing depend on the nuclear lamina by means of interactions between distinct nucleoporins and the nuclear lamins. Defects in proteins of the NE, the nuclear lamina and the NPCs cause several inherited diseases and abnormalities. Here, we summarise the current state of knowledge of the interactions between the NPC with the nuclear lamins and other associated nuclear proteins and their influence in viral infections and disease.

1.2 Introduction

The nuclear envelope (NE) does not merely isolate the chromosomes from the cytoplasm, but recently has been shown to play multiple roles in cell division, and to provide a linker site to the cytoplasm (Gerace and Burke, 1988; Starr, 2009). The outer nuclear membrane (ONM) is continuous with the endoplasmic reticulum (ER) and contains distinct group of proteins that share a common protein domain first found in *Drosophila melanogaster* ONM protein, termed the KASH domain (Klarsicht, Anc-1, Syne Homology) as well as in *Caenorhabditis elegans*, and human, respectively (Figure 1.1) (Stewart-Hutchinson et al., 2008). Moreover, in mammalian cell nesprins (NE spectrin repeat proteins) constitute a large family of ONM proteins that contain a KASH domain at the C-terminus for their localisation to the ONM and multiple spectrin-repeats extending to the cytoplasm (Zhang et al., 2002; Zhang et al., 2001). Nesprins associate with cytoskeleton components such as actin and plectin and are involved in nuclear positioning and migration (Zhen et

al., 2002). In addition, nesprins interact with SUN domain (Sad1p, Unc-84) containing proteins, first found in *C.elegans* these are the mammalian homologue of the C-terminal domain of Sad1p, a spindle pole body protein in *Schizosaccharomyces pombe* (Hodzic et al., 2004). Unc-84 is localised at the inner nuclear membrane (INM) and has a SUN-domain that anchors the ONM protein Anc-1 (Starr and Han, 2003). In mammalian cells, SUN1 and SUN2 are required for tethering the ONM proteins nesprin 1 giant and 2, respectively (Haque et al., 2006; Hodzic et al., 2004; Padmakumar et al., 2005). SUN domains are also implicated in other nuclear related functions; SUN1 is clustered around the NPCs (Liu et al., 2007) and telomeres in meiosis (Ding et al., 2007). In addition, SUN-domains interact with A-type lamins (Haque et al., 2006). The nesprin-SUN interactions are involved in LINC complexes (linker of nucleoskeleton and cytoskeleton) effectively connecting the actin cytoskeleton and microtubules to the nuclear lamina and are also implicated in NE organisation and spacing between the INM and the ONM (Crisp et al., 2006; Stewart-Hutchinson et al., 2008). An interesting hypothesis is that the SUN/KASH system transfers cytoskeletal information such as signs of mechanical stress, to the nucleus by means of interactions with the nuclear lamina, and subsequently activating the appropriate cellular responses. Several INM proteins associate with nuclear lamins such as lamin B receptor (LBR), LAP2, MAN1, emerin as well as others (Schirmer and Foisner, 2007). LAP2, MAN1 and emerin share a common domain, termed LEM (LAP2, emerin, MAN1), whereby they associate with lamin and chromatin (Lin et al., 2000; Liu et al., 2003a). The nuclear lamina is a filamentous meshwork composed mainly of A-, and B-type lamins which assemble into a 10 nm filamentous network in mammalian cells (Aebi et al., 1986), and are anchored to the INM through interactions with proteins of the INM (Herrmann and Aebi, 2000; Stuurman et al., 1998). Residing between the ONM and INM are the nuclear pore complexes (NPCs), large protein assemblies, comprising 30 different proteins termed nucleoporins and involved mainly in nucleocytoplasmic transport (Rout and Wente, 1994). Both the nuclear lamina and the NPCs are disassembled during NE breakdown, an essential process that enables spindle microtubules that assemble in the cytoplasm to access mitotic chromatin, a process that involves a series of phosphorylation of NE related proteins (D'Angelo and Hetzer, 2006). Components of the nuclear lamina as well as the NPCs are implicated in mitotic spindle assembly and are recruited to chromatin during cell division (Nigg, 1992a; Salina et al., 2001; Tsai et al., 2006). The nuclear lamina is implicated in the maintenance of the nuclear architecture and mechanical stability of the nucleus (Dahl et al., 2008), and

involved in multiple nuclear processes including chromatin organisation, DNA replication and RNA transcription (Dechat et al., 2008b). In addition, it is involved in cellular differentiation and spacing of the NPCs by means of interactions with individual nuclear pore proteins (Maeshima et al., 2006). The distinct roles of the nuclear lamins and the NPCs in nuclear related functions are reviewed in the following sections.

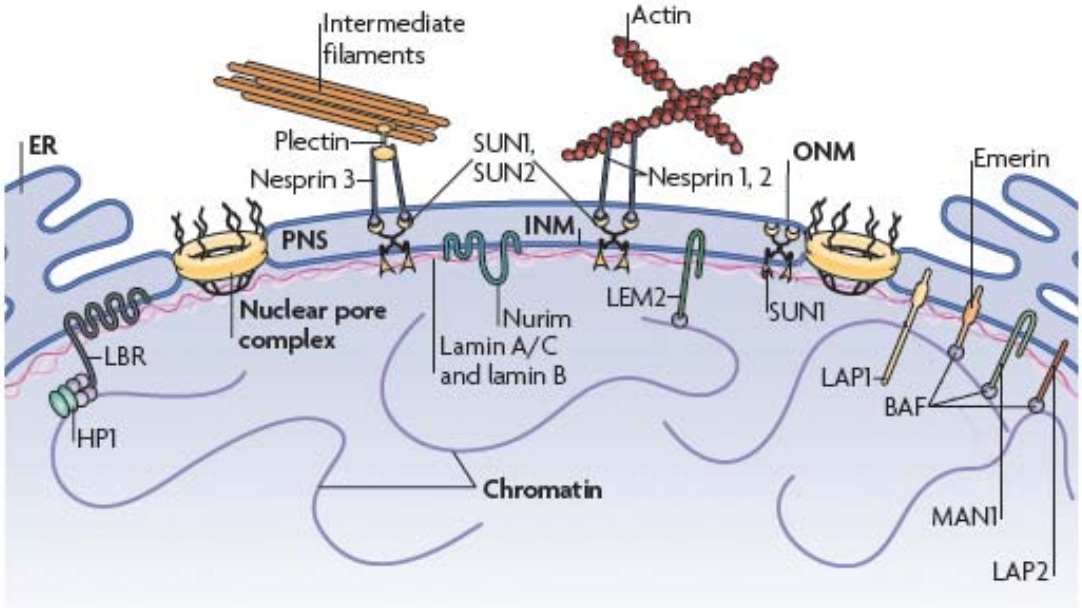

Figure 1.1: Structural organisation of the nuclear envelope - The nuclear envelope (NE) of metazoan cells is defined by an outer (ONM) and an inner nuclear membrane (INM) separated by a perinuclear space (PNS), and by a nuclear lamina and nuclear pore complexes (NPCs). Proteins of the INM interact both with the underlying nuclear lamina as well as with chromatin. Such INM associated proteins include lamin B receptor (LBR) which interacts with B-type lamins, and chromatin associated heterochromatin protein 1 (HP1). LEM domain containing integral proteins (LAP2, emerin and MAN1) form complexes with A- and/or B-type lamins. The INM SUN proteins interact with lamin A as well as nesprin proteins of the ONM which inturn interact with actin and plectin as illustrated, linking the nucleoskeleton with the cytoskeleton. Adapted from (Guttinger et al., 2009)

1.3 Nuclear lamins - domain organisation

Lamins are type V intermediate filament proteins (IFs). Like all IFs, lamins have a tripartite domain organisation with a central α-helical coiled-coil rod domain flanked by two non-helical short N-terminal (head) domain and a longer C-terminal (tail) domain (Figure

1.2). The rod domain consists of four coiled coil segments 1A, 1B, 2A and 2B separated by three linkers (Heins and Aebi, 1994). Within segment 1B of the rod domain an additional 42 amino acid residues are present which are shared only with IFs of invertebrates (Figure 1.2) (Herrmann and Aebi, 2004). The C-terminal domain has a nuclear localisation signal (NLS), an immunoglobulin-like motif (Ig-fold) domain (Dhe-Paganon et al., 2002; Krimm et al., 2002), and a -CAAX box domain that undergoes multiple posttranslational modifications (Ralle et al., 2004). Lamins have multiple phophorylation sites, which have several roles in the assembly and disassembly of lamins from the lamina during NE breakdown at the onset of mitosis (Heald and McKeon, 1990; Moir et al., 1995; Nigg, 1992a; Peter et al., 1991).

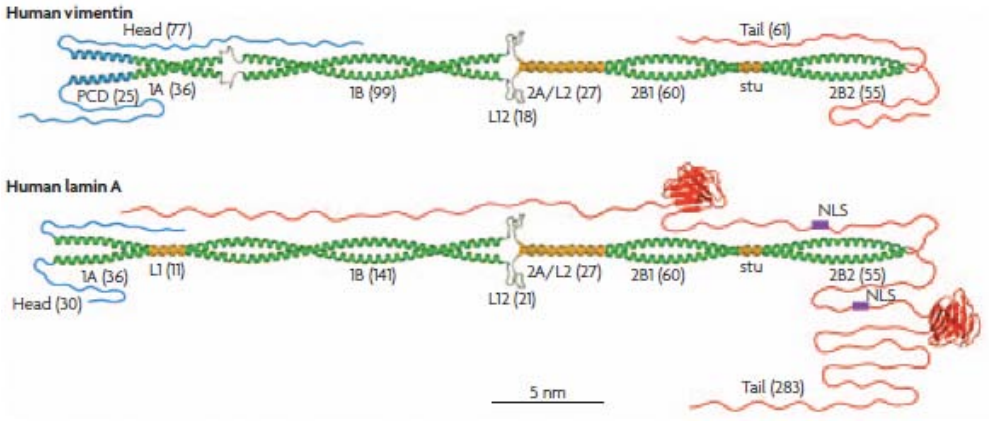

Figure 1.2: Model of domain organisation of nuclear lamin compared to cytoplasmic intermediate filament protein - Like all intermediate filament proteins (IFs), lamin structure is organised in three domains, a central α-helical coiled-coil rod domain flanked by two non helical N- and C-terminal domains. In comparison to the cytoplasmic IF vimentin, human lamin A has a shorter N-terminal (head) domain. The rod domain has similar heptad repeat segments designated 1A, 1B, 2A and 2B, separated by three linkers L1, L12 and L2. In addition the rod domain of lamins contains an extra 42 residues at segment 1B. The tail domain of lamins is larger and contains distinct structural features not shared with cytoplasmic IFs: a nuclear localisation signal (NLS), an immunoglobulin like fold (Ig-fold), in the tail domain (*red*, beta sheet structures) and a CAAX-box at the end of the tail domain (not shown, see Figure 1.3). The numbers in brackets correspond to the amino acid numbers in each respective domain. Adapted from (Herrmann et al., 2007)

1.3.1 Lamin isoforms in vertebrates

Two types of lamins exist in the mammalian cell, A- and B-type lamins which differ in their expression pattern, solubility properties and behaviour during mitosis (Schirmer and Gerace, 2004). Expressed only in differentiated cells, A-type lamins are present in four isoforms which are all transcribed from the same gene, *LMNA* (Constantinescu et al., 2006). Lamin A and C are the main isoforms expressed simultaneously in all differentiated somatic cells and generally termed lamin A/C. Other minor isoforms can be found in cells of the germ-line, lamin AΔ10 and lamin C2, which are specific to spermatogenic cells (Alsheimer and Benavente, 1996; Furukawa et al., 1994). A-type lamins are present at the nuclear periphery as part of the nuclear lamina meshwork and as a soluble pool in the nucleoplasm (Foisner, 2001; Goldman et al., 2002). B-type lamins are expressed in all cell types and are found in two major isoforms lamin B1 and B2, which are encoded by two separate genes *LMNB1* and *LMNB2*, respectively (Lin and Worman, 1995; Vorburger et al., 1989). Other minor isoforms of B-type lamins also exist, lamin B3 is a germ-line specific B-type lamin and lamin B4, which is found in sperm cells (Benavente et al., 1985; Furukawa and Hotta, 1993). Nuclear lamins are mainly present in the nuclear lamina, however, a small pool is also found in the nucleoplasm in an unknown structural form (Dorner et al., 2007). The incorporation of lamins into the nuclear lamina requires in addition to the NLS other segments including the head domain, part of the 42 residues at segment 1B of the rod domain as well as the CAAX-box domain at the C-terminus (Monteiro et al., 1994).

Lamins A, B1 and B2 undergo a series of posttranslational modifications at their C-terminal CAAX-box domain (where C is a cysteine, followed by two aliphatic residues AA and X can be any of several amino acid) (Mical and Monteiro, 1998). The sequence of this domain is CSIM and CAIM for lamins A and B1, respectively, while lamin C does not have this domain (Ralle et al., 2004). Three sequential modifications take place at the CAAX-box domain starting with farnesylation of the CAAX-box cysteine residue by a farnesyltransferase enzyme (see Figure 1.3), followed by endoproteolytic removal of the three remaining residues -AAX by the endoprotease RCE1 in lamin B and ZMPSTE24 in lamin A, respectively, and methylation on the new carboxyl-terminal cysteine by methyltransferase, ICMT (Rusinol and Sinensky, 2006; Sobotka-Briner and Chelsky, 1992). Prelamin A undergoes yet a second cleavage by ZMPSTE24 deleting 15 more residues at the C-terminus including the newly farnesylated and carboxy-methylated cysteine (Sinensky et al., 1994;

Young et al., 2005). Thus, while the mammalian B-type lamins are farnesylated proteins and associate tightly to the INM, the mature A-type lamins are not farnesylated.

Recently, lamins have been shown to undergo other posttranslational modifications such as sumoylation, where the inhibition of the latter by mutations in the lamin gene led to lamin aggregation and cell death (Zhang and Sarge, 2008).

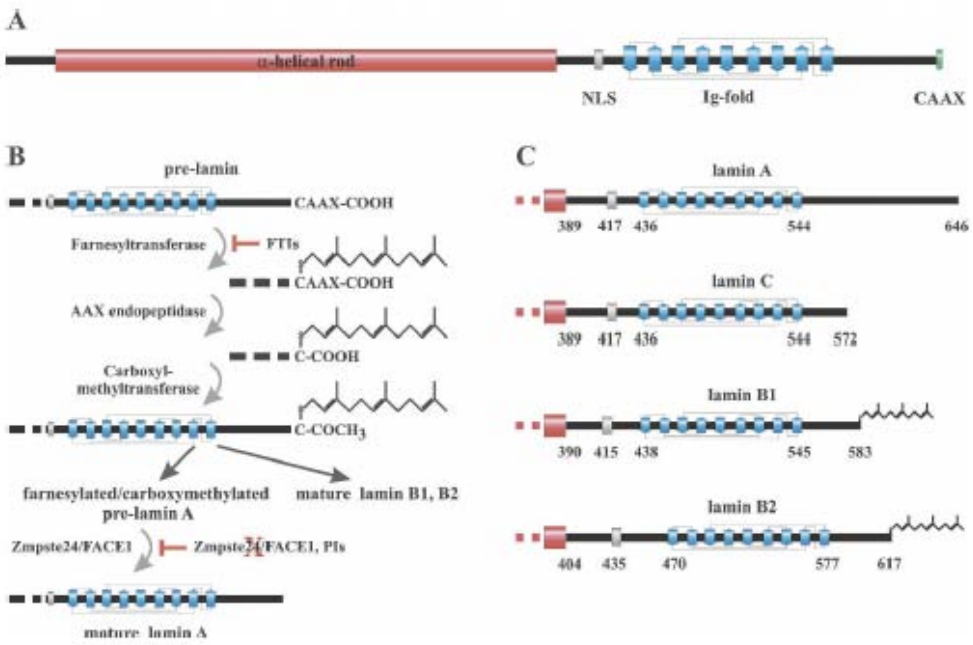

Figure 1.3: Post translational processing of nuclear lamins - Schematic representations of lamin domain organisation and post translational modifications. (**A**) Lamin molecule showing the tripartite domain organisation, note the -CAAX box in the end of the tail domain (*green*) is found in B type lamins and lamin A, but not in lamin C. (**B**) Three sequential modifications take place at the CAAX-box starting with farnesylation of the CAAX-box cysteine residue by a farnesyltransferase, followed by endoproteolytic removal of the three remaining residues -AAX by the endoproteases RCE1 in lamin B and ZMPSTE24 in lamin A, respectively, and methylation of the new carboxyl-terminal cysteine by a methyltransferase. Prelamin A undergoes yet a second cleavage by ZMPSTE24 deleting 15 more residues at the C-terminus including the newly farnesylated and carboxy-methylated cysteine. (**C**) Processed B-type lamins are permanently farnesylated, A-type lamins are not farnesylated. Adapted from (Dechat et al., 2008)

1.3.2 Lamin assembly

The nuclear lamina form 10 nm filamentous meshwork as visualised in NE extracted from amphibian oocytes (Aebi et al., 1986). *In vitro*, lamin assembly begins with the formation of lamin dimers by the association of two lamin molecules by their α-helical coiled coil domain, which is followed by protofilament formation by head to tail association of unstaggered dimers (Figure 1.4). Unlike cytoplasmic IFs which form filaments *in vitro*, except for lamin B of the *C.elegans* (Karabinos et al., 2003), lamin polypeptides form filamentous bundles and paracrystalline arrays, assemblies overextend laterally giving rise to thickened structure greater than 13 nm in diameter with striated appearance throughout their length (Stuurman et al., 1998).

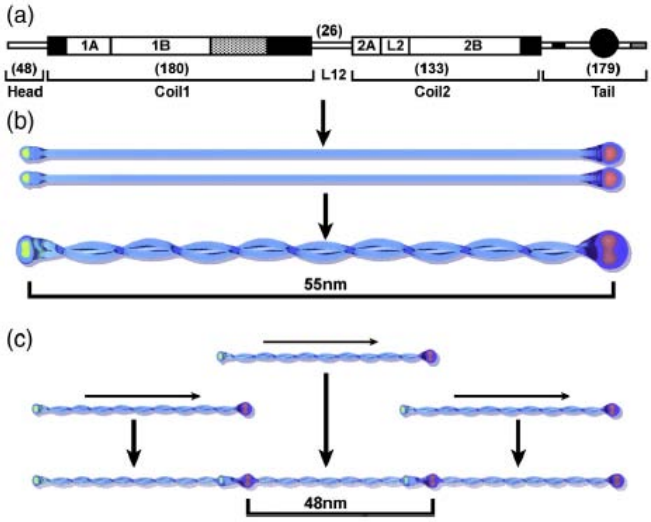

Figure 1.4: *In vitro* assembly of lamins - (**A**) Schematic representation of the domains of the *C.elegans* lamin. (**B**) Lamin dimer formation. Two lamin monomers associate at their α-helical coiled-coil rod domains, resulting in a long tail (*cyan*) with two globular heads at one end, which contain the Ig-fold motif (*red*). (**C**) Lamin dimers further associate longitudinally in polar head-to-tail polymers (the black arrows indicate the polarity). Adapted from (Ben-Harush et al., 2009)

In vivo, A- and B-type lamins homo-polymers assemble from distinct layers at the nuclear periphery and lamins do not co-polymerise to heteropolymers in *Drosophila* cells expressing exogenous lamin C (Furukawa et al., 2009). This is in agreement with yet recent studies where it was shown that A- and B-type lamins form different filamentous

structures in the *Xenopus laevis* oocyte, where B-type lamins were shown to form thin filaments closely associated to the INM and A-type lamins formed thinker bundles (Goldberg et al., 2008). Another recent study, demonstrated that A- and B-type lamins form separate but interconnecting microdomains (Shimi et al., 2008). HeLa cells depleted of lamin B1 showed an increase in the lamina meshwork size and changes in the nucleoplasmic distribution of lamin A. Moreover, B-type lamins formed separate but interconnected networks with distinct interaction properties with A-type lamins (Shimi et al., 2008). During interphase, lamin B is primarily associated with the nuclear lamina at the nuclear periphery of vertebrate cells as well as in the nucleoplasm, however during mitosis, the nuclear lamina disassemble, a process mediated by phosphorylation of nuclear lamins where lamins become dispersed in the cytoplasm (Luscher et al., 1991; Peter et al., 1990). However recent studies suggest that a fraction of lamin B remains associated with chromosome and mitotic spindle formation during mitosis (Beaudouin et al., 2002; Maison et al., 1997; Tsai et al., 2006). Most of nuclear functions related to lamins became obvious in defects of nuclear processes in conjunction with alteration in lamin expression or the presence of lamin mutations.

1.3.3 The nuclear lamina: lamin association with proteins of the NE

Within the NE, lamins interact with proteins of the INM including LEM-containing proteins first recognised in integral membrane proteins lamin associated peptide-2 (LAP2), emerin and MAN1 (Figure 1.1) (Laguri et al., 2001). These share a conserved two helices domain of approximately 42 residues (LEM-domain) which is also found in otefin and Lem-3, and required for the interaction with lamins and the chromatin-binding protein BAF (barrier to autoantigen factor) (Holmer and Worman, 2001; Lin et al., 2000; Senior and Gerace, 1988; Wagner and Krohne, 2007). Multiple isoforms of LAPs exist in vertebrates, which interact differently with A- and B-type lamins, and are implicated in nuclear assembly by anchoring lamins into the NE as well as mediating lamin polymerisation (Schirmer and Foisner, 2007). One LAP, LAP1 is present in three sub-isoforms; LAP1-A, -B and -C, where LAP1-A and -B interact with both lamin A and B *in vitro*, and LAP1C interacts specifically with lamin B (Foisner and Gerace, 1993; Maison et al., 1997). Another LAP, LAP2 is present in six isoforms α, β, γ, δ, ε and ζ. Except for LAP2α and ζ, all the other isoforms contain a single C-terminal transmembrane domain and a long nucleoplasmic N-terminal domain (Dechat et al., 2000). LAP2β binds to lamin B1 (Foisner and Gerace, 1993; Furukawa et

al., 1998). LAP2α lacks the transmembrane domain present in the other LAP2 isoforms instead it comprises a large C-terminal domain and unlike the other isoforms which are found at the INM, LAP2α is found in stable complexes with A-type lamins in the nucleoplasm (Dechat et al., 2000).

Emerin is another LEM-domain containing integral membrane protein that binds specifically to lamin A and its association at the INM depends on the presence of lamin A (Clements et al., 2000; Ostlund et al., 2001; Raharjo et al., 2001; Sullivan et al., 1999; Vaughan et al., 2001). Emerin also interacts with MAN1, another INM protein as well as the ONM proteins nesprin 1 and 2 (Mansharamani and Wilson, 2005; Wheeler et al., 2007). MAN1 is implicated in cellular signalling and it associates with lamin A at interphase and to lamin B at mitosis and like emerin, it requires lamin A for localisation at the INM (Lin et al., 2000; Ostlund et al., 2006; Paulin-Levasseur et al., 1996).

LBR is multiple transmembrane protein of the NE, which associates, with lamin B and its structure defines three DNA binding domains (Worman et al., 1990; Worman et al., 1988). LBR additionally interacts with heterochromatin-specific chromosomal protein (HP1)-type chromo-domain proteins assisting in heterochromatin attachment to the INM (Ye et al., 1997; Ye and Worman, 1994; Ye and Worman, 1996).

Lamin A also associates with the INM integral proteins, SUN1 and SUN2 which are expressed in somatic cells and extend to the nuclear perispace (NPS) where they interact with KASH domain proteins of the ONM proteins including members of the nesprin family of spectrin-repeat proteins in the ONM (also known as Syne proteins), Anc-1(Crisp et al., 2006; Haque et al., 2006; Tzur et al., 2006). Nesprins bind to the cytoskeletal components, actin and microtubules, effectively connecting the cytoskeleton to the nucleus and help in nuclear positioning (Starr, 2009). In addition, lamin A is found to interact directly with nesprin 2 at the INM in muscle cells (Zhang et al., 2005) and *in vitro* interacting with nesprin-1a (Libotte et al., 2005) as well as actin in the nucleus (Sasseville and Langelier, 1998), thus directly linking the nuclear lamina to the cytoskeleton.

1.3.4 Lamins association with the NPCs

The nuclear lamina interacts with the NPCs which was already documented in 1976 (Dwyer and Blobel, 1976). Lamins interfere with the assembly and distribution of NPCs and clustering of NPCs is associated with lamin mutations (Schirmer et al., 2001; Smythe et al., 2000). In addition, more recent data suggested a direct active role of lamin A in interacting

with NPCs at initiation lamina assembly regions (Furukawa et al., 2009). Lamins interact with protein subunits (nucleoporins or Nups) of the NPCs, mainly Nup153 interacts with both A- and B-type lamins directly *in vitro* as well as *in vivo* (Al-Haboubi et al., submitted; Smythe et al., 2000). Lamin B has also been shown to interact yet with another nucleoporin Nup53 (Hawryluk-Gara et al., 2005). In addition, the phosphorylation of gp210, another nucleoporin, is required for subsequent phosphorylation of lamin polymers disassembly prior to NE breakdown (Galy et al., 2008).

1.3.5 Lamin associations in the nuclear interior

In the nuclear interior, both nucleoplasmic lamin A as well as its associated polypeptides regulate gene expression and signalling through binding with multiple gene regulatory factors such as pRb and BAF (Holaska et al., 2003; Mancini et al., 1994; Shan et al., 1992), histones (Glass et al., 1993; Taniura et al., 1995), SREBP1 (Capanni et al., 2005; Lloyd et al., 2002), and c-Fos (Ivorra et al., 2006). Moreover, lamin A has been found to associate with RNA splicing speckles (Muralikrishna et al., 2001), implying nuclear related functional roles for lamin A (Dechat et al., 2008a). Lamins provide spatial organisation for DNA synthesis, transcription and repair as well as RNA synthesis where lamin B1 is required for RNA synthesis (Goldman et al., 2002; Tang et al., 2008). Heterochromatin association with the nuclear lamina maintains a transcriptionally inactive state at the nuclear periphery (Shimi et al., 2008; Shumaker et al., 2006). In addition, lamin B interacts directly with the proliferating cell nuclear antigen (PCNA) (Moir et al., 1994; Shumaker et al., 2008), also it was shown that DNA synthesis foci are at overlapping regions with the nucleoplasmic lamin A (Kennedy et al., 2000).

1.4 Nuclear lamins associated diseases

More than 24 inherited diseases are associated with the NE (Cohen et al., 2008). Mutations in lamins disrupt the nuclear lamina which as a result affect normal nuclear processes carried out by nuclear lamins (Burke and Stewart, 2006). Mutations in lamins can cause a group of inherited diseases termed laminopathies (Liu and Zhou, 2008). Due to the large number of lamin association with other proteins and DNA, abnormalities in lamins can have subsequent effects on nuclear and cellular processes. Nuclear integrity and architecture are affected in the presence of lamin A related mutations which result in fragile nuclei,

membrane invaginations and nuclear blebbing (Houben et al., 2007; Lammerding et al., 2006; Lammerding et al., 2004; Sullivan et al., 1999). Perturbed nuclear morphology is also associated with elevated expression levels of B-type lamins (Prufert et al., 2004; Ralle et al., 2004). Hence, altered expression of lamins can result in disruption of nuclear and cellular processes.

1.4.1 Lamin A associated laminopathies

More than 200 mutations have been identified in *LMNA* accounting for more than 11 distinct inherited diseases (Figure 1.5) (Sylvius et al., 2008). Lamin A associated diseases can

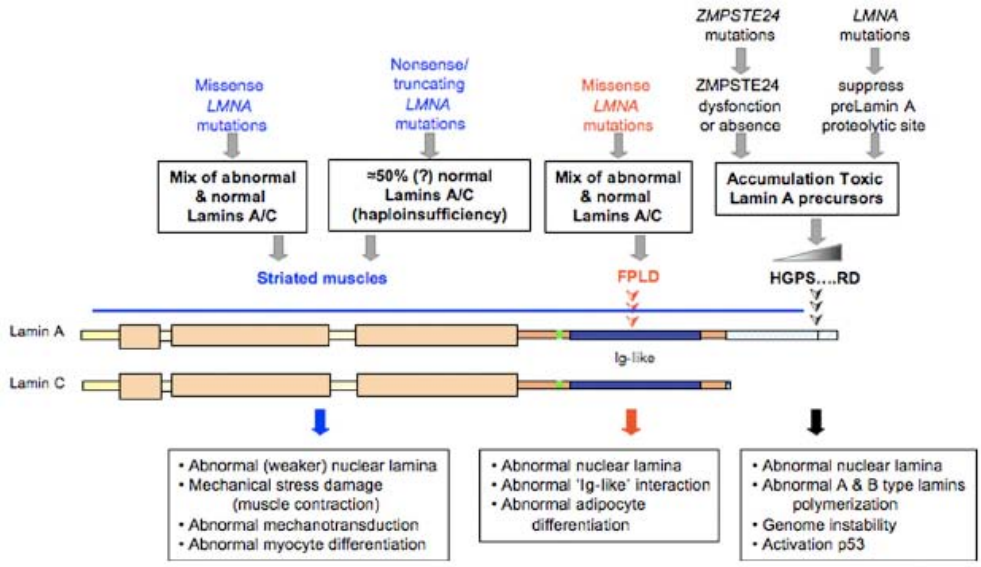

Figure 1.5: Lamin A associated mutations causing laminopathies - Laminopathies causing mutations in Lamin A/C and potential phenotypes. Missense mutations in *LMNA*, that result in muscular dystrophies, can be spread throughout the whole sequence, whereas missense mutations causing lipodystrophies are located in the Ig-fold. Substitution of a C to T at position 1824 in *LMNA*, as well as mutations in ZMPSTE24 (responsible for the second cleavage of the carboxy-methylated cystein at the tail domain, (see Figure 1.3), result in a protein that lacks 50 amino acids at the C-terminal domain (progerin), and are the underlying cause of Hutchison-Gilford progeria syndrome (HGPS). Adapted from (Worman and Bonne, 2007)

either affect striated and heart muscle tissue such as autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD), dilated cardiomyopathy (DCM) and limb-girdle muscular dystrophy 1B (LMG1B) characterised by skeletal muscle weakness and wasting and striated and smooth muscle related defects and subsequently heart failure (Bonne et al.,

1999). Or can affect fat tissue such as Dunnigan type-familial partial lipodystrophy (FPLD) and mandibuloacral dysplasia (MAD), characterised by aberrant adipose tissue distribution (Boguslavsky et al., 2006). Or can affect several tissues leading to premature aging, such as Hutchinson-Gilford progeria syndrome (HGPS) as well as atypical Werner's syndrome, which are characterised by loss of subcutaneous fat, decreased bone density, osteoporosis, abnormal dentition and growth retardation (Worman and Bonne, 2007).

Apart from HGPS, most mutations of lamin A are products from an amino acid substitution in *LMNA* (Worman and Bonne, 2007). Mutations resulting in AD-EDMD and DCM are spread through out the lamin molecule (Bonne et al., 1999; Mounkes et al., 2005; Speckman et al., 2000). In contrast, mutations in FPLD are clustered at the C-terminus of lamin A, where the rod domain is not affected and therefore not disrupting lamin assembly, but rather affecting interaction with binding proteins (Speckman et al., 2000; Stierle et al., 2003). Moreover, mutations resulting in lipodystrophies result in elevated levels of pre-lamin A (Capanni et al., 2005; Favreau et al., 2003).

Several mouse models have been generated to study disease heterogeneity resulting from mutations in *LMNA* (Stewart et al., 2007a). Four mouse models for muscular dystrophies have been created, either lacking *LMNA* ($LMNA^{-/-}$) or carrying a related mutation such as, H222P and N195K (Arimura et al., 2005; Mounkes et al., 2005; Stewart et al., 2007a). In such a model, it was shown that immortalised $LMNA^{-/-}$ myoblasts are impaired in their differentiation to myotubes, leading to EDMD (Frock et al., 2006; Sullivan et al., 1999). Other mouse models carrying missense mutations, H222P or N195K led to AD-EDMD and DCM, respectively (Arimura et al., 2005; Mounkes et al., 2005). Cellular analysis from these mice showed abnormal nuclear morphology that led to skeletal and striated muscle specific degeneration, which suggest that *LMNA* mutations cause cardiomyopathy by disrupting the internal organisation of the cardiomyocyte and/or altering the expression of transcription factors essential to normal cardiac development, aging and function (Arimura et al., 2005; Mounkes et al., 2005). In addition, a forth mouse model with targeted expression of *LMNA* mutation M371K in the heart with a heart-specific α-myosin heavy chain promoter, revealed extensive pathology with disruption of the cardiomyocytes and abnormal nuclei (Wang et al., 2006). Hence, the expression of *LMNA* mutant that alters nuclear morphology can cause tissue and organ damage in mice expressing the normal complement of endogenous lamins (Wang et al., 2006).

Four mouse models have also been established for HGPS (Maraldi and Lattanzi, 2007). One model shows a deletion of exon 9 in *LMNA* ($LMNA^{\Delta 9/\Delta 9}$) resulting in the expression of farnesylated lamin A (Mounkes et al., 2003). Other mouse models include those that express either homozygous ($LMNA^{HG/HG}$) or heterozygous ($LMNA^{HG/+}$) progerin, respectively (Yang et al., 2005; Yang et al., 2006). In addition, mice were also created that lack the enzyme ZMPSTE24 that cleaves pre-lamin A to mature lamin A (Bergo et al., 2002; Pendas et al., 2002; Shackleton et al., 2005). These mice retain farnesylated pre-lamin A that accumulated at the nuclear rim and showed osteoporosis and muscle weakening. Both homo- and heterozygous expression of farnesylated pre-lamin A can lead to MAD and progeria like syndromes (Shackleton et al., 2005).

Lamin A associated mutations can affect signalling pathways such as the Notch signalling pathway which is activated in human mesenchymal stem cells in HGPS and shows aberrant signalling in osteogenesis, and adipogenesis (Lowry and Richter, 2007; Scaffidi and Misteli, 2008). It was also demonstrated that pre-lamin A mutant lead to decreased proliferation and accumulated hair bulge in stem cells with pre-lamin A as a result of complete loss of the Wnt/B-catenin signalling pathway which regulates hair follicle stem cells (Espada et al., 2008). Overall, mutations in *LMNA* either disrupt the assembly of lamins into the nuclear lamina, resulting in mechanical stress and defects in nuclear architecture. Or can alter gene expression by disrupting lamin mediated interactions nuclear regulatory factors (Cohen et al., 2008).

1.4.2 Lamin B related diseases

Mutations in *LMNB* were thought to be embryonic fatal until recently when two mutations have been identified in *LMNB2* in patients with acquired partial lipodystrophy (APL) (Capanni et al., 2003). Additionally, duplications in *LMNB1* were identified in patients with an autosomal-dominant leukodystrophy (ADLD) (Capell and Collins, 2006; Hegele et al., 2006; Padiath et al., 2006). A mouse model was created with a gene trap insertion in *LMNB1* ($LMNB^{-/-}$) that results in lamin B1 lacking the NLS, CAAX-box as well as a portion of the rod domain which results in sever phenotype with abnormally misshapen nuclei (Vergnes et al., 2004). Although this mutation was shown to cause local disturbances in NE structure without causing generalized defects in nuclear organization, stiffness, and shape stability, which was thought to be largely due to the functional redundancies of lamin B1 and B2, and the influence of lamin B1 deficiency is masked by lamin B2 (Vergnes et al.,

2004). In the future, this issue needs to be addressed by creating lamin B1/lamin B2 double knockout cells (Stewart et al., 2007a).

1.4.3 Lamin binding proteins associated diseases

Mutations in the lamin A binding protein, emerin were first recognised in X-linked EDMD (X-EDMD) (Bione et al., 1994). Mutations in the LBR gene can either lead to Pelger-Huet anomaly with abnormal nuclear shape and chromatin organisation in neutrophils (Hoffmann et al., 2002), or to the autosomal recessive Greenberg skeletal dysplasia, characterised by short-limb dwarfism, ectopic bone ossification (Waterham et al., 2003). Furthermore, a mutation in the MAN1 gene can cause Buschke-Ollendorf syndrome, which is characterised by increased bone density (Hellemans et al., 2004). A rare mutation in LAP2α Arg690Cys, which might interfere with interaction with lamin A, can cause DCM (Taylor et al., 2005). In addition lamin A binding proteins, nesprin-1 and -2 have also been affected in AD-EDMD as well as X-linked EDMD, in which the interactions of those proteins with lamin A and emerin are disrupted, revealing an additional role for nesprins in the pathology of EDMD (Wheeler et al., 2007; Zhang et al., 2007). Emerin null mouse model has been created ($Emd^{-/-}$) and demonstrated normal phenotype with slightly abnormal muscle regeneration, but not as severe as the phenotype associated with $LMNA^{-/-}$ mice (Melcon et al., 2006; Ozawa et al., 2006).

1.4.4 Lamins and cancer

With respect to cancer, lamins may serve as biomarker because the expression levels in particular of lamin A/C are often altered in cancer tissues (Prokocimer et al., 2006). For example, in small cell lung cancer, the levels of lamin A and C are strongly reduced, whereas lamin B levels were not changed (Broers et al., 1993). Similarly no or reduced expression of lamin A/C was described in gastrointestinal neoplasms, with at the same time reduced lamin B levels (Moss et al., 1999). On the other hand, elevated levels of lamin A/C were found in colorectal cancer patients (Willis et al., 2008) as well as in oesophageal squamous cell carcinoma cells (Qi et al., 2008). Contrary results were found in studies regarding skin cancer (Oguchi et al., 2002; Tilli et al., 2003). Lamin A/C levels in one basal cell carcinoma study were reduced and elevated in another study. However, in other solid tumours of the gastrointestinal tract such as pancreatic and hepatocellular carcinomas, lamin A/C

and B1 levels were found unchanged (Hytiroglou et al., 1993). Contrary results were also found in the levels of lamin expression in breast cancer (Hudson et al., 2007; Moss et al., 1999). Recently, it was also shown that alterations of lamin B1 staining was increased with the degree of malignancy in breast cancer tissue (Bussolati et al., 2008).

Overall, altered expression levels of lamins associated with cancer tissue demonstrate the necessity for an intact lamina and normal nuclear related functions that are disrupted in transformed tissue.

1.4.5 Lamins and viral infections

DNA viruses replicate in the nucleus where they assemble their viral caspids and because of their large size (120 nm in diameter), these cannot transverse through the NPCs and require other mechanisms to exit the nucleus (Cohen et al., 2006). These viruses utilise an envelopment mechanism by which they form enveloped caspids through interactions with the INM and PNS, but have to overcome the nuclear lamina meshwork, either during mitosis or by locally destabilising the nuclear lamina (Bukrinsky, 2004). At the onset of mitosis lamins undergo multiple phosphorylation events to depolymerise the nuclear lamina, and some viruses utilise kinases to phosphorylate lamins or form complexes with proteins of the NE at interphase (Heald and McKeon, 1990; Milbradt et al., 2009; Moir et al., 2000; Mou et al., 2008; Nigg, 1992b). For example, Herpes simplex virus-1 (HSV-1) viral caspids that are pre-assembled in the nucleus develop a budding mechanism through the nuclear lamina, the INM and the PNS resulting in the formation of enveloped virions that fuse into the ONM and release its caspids into the cytoplasm (Mettenleiter, 2006). The HSV-1 viral proteins UL31 and UL34 disrupt the nuclear lamina by inducing conformational changes in lamin A/C (Reynolds et al., 2004). In addition, UL34 directly binds to lamin A/C and lamin B1, and it was shown that lamin A interferes with viral infectivity and lamin B1 in addition is required for optimal viral replication (Mou et al., 2008). Moreover, a kinase, US3 that phosphorylates UL31 also phosphorylates lamin A and redistributes emerin along with the viral protein UL34 to exit the nucleus (Leach et al., 2007; Mou et al., 2007). However, a role for lamin A phosphorylation in HSV-1 viral infection has been ruled out very recently, as it was demonstrated that US3 mediated phosphorylation of UL31 directly regulates the enveloped caspids localisation within the INM, where it aggregates at the nuclear rim and lead the aberrant accumulation of virions in herniations of the nuclear membrane (Mou et

al., 2009). Moreover, recent data indicate that lamin A act as repressor for gene transcription as well enhancer for viral gene transcription and as such it is required for HSV-1 viral genome targeting as well as DNA replication (Lee et al., 2009; Silva et al., 2008).

Nuclear egress of the human cytomegalovirus (HCMV) is accomplished by destabilising components of the nuclear lamina using the viral proteins UL50 and UL53, respectively (Camozzi et al., 2008). These were found in overlapping regions with mislocalised lamin A/C and lamin B1 and several viral proteins form complexes with proteins of the NE including LBR (Camozzi et al., 2008; Milbradt et al., 2009). The human immunodeficiency virus-1 (HIV-1) does not require NE breakdown to enter the nucleus (Bukrinsky, 2004), the viral protein Vpr induces changes in the nuclear lamina architecture by locally disrupting A- and B-type lamins and NE herniations that led to cell cycle arrest (de Noronha et al., 2001). To overcome the nuclear lamina, pre-assembled viral caspids overcome the nuclear lamina either by directly interacting with lamins, which result in local disruption of the nuclear lamina or relying on phosphorylation mechanism to breakdown the nuclear lamina and enetually egress the cell.

1.4.6 Nuclear lamina components as targets for autoantibodies

Components of the nuclear lamina, lamin A/C and B as well as the INM associated proteins, LBR, LAP1/2 and MAN1 are targeted by autoantigens in autoimmune diseases, such as autoimmune liver disease, systemic lupus erythematosus, and other related conditions (Courvalin et al., 1990; Enarson et al., 2004; Konstantinov et al., 1996; Ye and Worman, 1994). The underlying mechanism for autoantigen recognition is unknown.

1.5 Nuclear pore complexes

The nuclear pore complexes (NPCs) are large assemblies of multiple proteins, forming channel like structures that permit the bi-directional transport of molecules in and out of the nucleus (Alber et al., 2007; Lim et al., 2008). Nucleocytoplamic transport through the NPCs is mediated either by free diffusion of ions and small molecules less than 40 kDa, or facilitated diffusion by receptor-mediated translocation of macromolecules such as RNA and protein cargo. Thus, NPCs provide a selective highly regulated transport system through the nucleus (Feldherr et al., 1984; Terry et al., 2007).

1.5.1 Structural organisation of the NPC

NPCs have massive molecular weight of roughly 60-125 MDa in mammalian cells and 40-60 MDa in yeast cells (Reichelt et al., 1990; Yang et al., 1998). Nonetheless the structural organisation is evolutionarily conserved from yeast to mammals (Alber et al., 2007; Fahrenkrog et al., 1998; Suntharalingam and Wente, 2003; Yang et al., 1998). The NPC is composed of multiple copies of approximately 30 distinct proteins termed nucleoporins (or Nups) arranged in sub-complexes to form the NPC (Lim and Fahrenkrog, 2006; Rout et al., 2000; Tran and Wente, 2006). Much of the structural information about the NPC comes from extensive structural work done on the localisation of individual Nups or sub-complexes within the NPCs of yeast and vertebrate cells, using immuno-gold labelling, electron microscopy (EM), cryo-EM, cryo-electron tomography (CET), X-ray crystallography and most recently atomic force microscopy (AFM) (Beck et al., 2004; Brohawn et al., 2008; Jarnik and Aebi, 1991; Maco et al., 2006; Stoffler et al., 1999a; Yang et al., 1998).

The 3D reconstitution of the NPC structure is obtained from EM-based studies using negatively stained and frozen-hydrated NPCs from *Xenopus* oocyte NEs or frozen hydrated yeast cells (Akey and Radermacher, 1993; Hinshaw et al., 1992; Yang et al., 1998). A central framework (or the spoke complex) of the NPC resides in the plane of the NE (between the INM and the ONM), which has an eight fold rotational symmetry, embracing a central pore and connected to eight cytoplasmic filaments at the cytoplamic periphery and a nuclear basket arranged by eight filaments joint into a distal ring at the nuclear periphery (Figure 1.6A) (Beck et al., 2004; Beck et al., 2007). The central pore has a diameter of 60-70 nm at the periphery and 45 nm in the midplane of the NPC and the NE (Beck et al., 2004; Beck et al., 2007; Pante and Kann, 2002; Stoffler et al., 2003). This central pore mediates the traffic of macromolecules of up to 39 nm in diameter between the cytoplasm and the nucleus (Pante and Kann, 2002; Stoffler et al., 1999a). The NPCs peripheral channels have a diameter of 8 nm and proposed to mediate free diffusion of ions and small molecules or even INM associated proteins (Feldherr and Akin, 1997; Soullam and Worman, 1995). In addition, these peripheral channels act as a hub for central pore related deformations (Fahrenkrog and Aebi, 2003). The total length of the NPC was calculated to be 150 nm with an outer diameter of 125 nm based on information obtained from *Dictyostelium* NPCs, where the cytoplamic filaments and the nuclear basket measure 35 nm and 60 nm long, respectively, as well as the additional 50 nm for the central framework

(Beck et al., 2004; Beck et al., 2007). A central plug which can sometime be seen in the middle of the central pore, was thought to be a transporter in the early day (Feldherr and Akin, 1997; Jaggi et al., 2003; Stoffler et al., 1999b), but more recently it became evident that it represents cargo in transit (Beck et al., 2004; Beck et al., 2007; Stoffler et al., 2003).

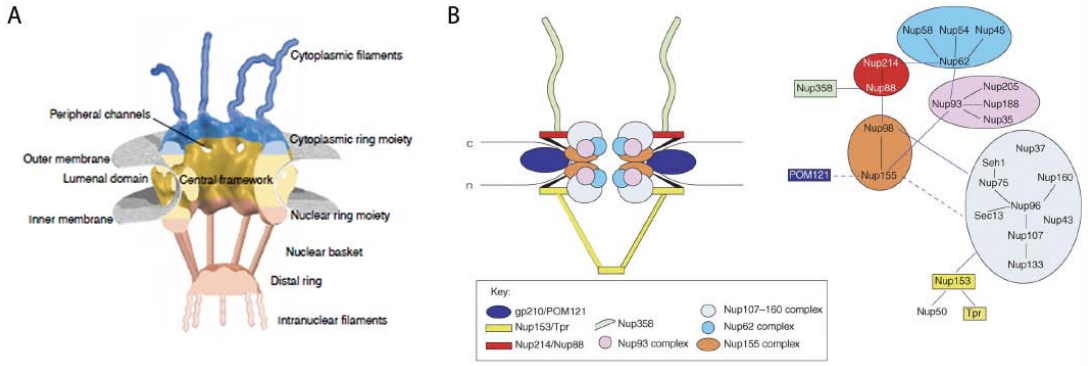

Figure 1.6: Architecture and composition of the nuclear pore complex - (**A**) A consensus model of the NPC based on a reconstruction of native NPCs embedded in thick amorphous ice. The main structural components include the central framework (*yellow*), the cytoplasmic ring moiety (*blue*) and attached cytoplasmic filaments (*blue*), and the nuclear ring moiety (*orange*) and the distal ring (*orange*) of the nuclear basket. Adapted from (Fahrenkrog and Aebi, 2003). (**B**) Schematic representation of the sub-complex localisation and possible interactions of nucleoporins of the mammalian NPC. Interactions confirmed by biochemical analysis and potential associations are indicated by connecting lines. Interactions between sub-complexes (*blue* lines), predicted interactions between subcomplexes based on homologues in *S. cerevisiae* (*dashed blue* line), interactions of nucleoporins within sub-complexes (*black* line). c, cytoplasm; n, nucleus. Adapted from (Lim et al., 2008)

1.5.2 Molecular composition of NPCs

Nups are found in multiple copies of 8 or higher with at least 456 Nups per yeast NPC, forming sub-complexes (Cronshaw et al., 2002; Rout et al., 2000). The complexity of the NPC architecture is however reduced by modularity (Alber et al., 2007). Recent work on the NPC organisation using integrative-based design combining biophysical and proteomic data have indicated simplicity in the composition and modularity of the NPC architecture in yeast cells (Alber et al., 2007; Devos et al., 2006). Based on putative structure motifs found in various Nups, the latter are classified into three groups: transmembrane, central scaffold and peripheral FG nucleoporins, respectively (see Figure 1.6B) (Alber et al., 2007). The

transmembrane group involves Nups with transmembrane α-helices and a cadherin fold and form the outermost layer of the NPC central framework, and are believed to anchor the NPC to the NE, such as gp210 (Mansfeld et al., 2006; Stavru et al., 2006). The central scaffold group contains α-solenoid and α-propeller folds, these domains coordinate multi-protein assemblies and are implicated in signal transduction, transcription regulation, cell cycle control and apoptosis (Devos et al., 2006). More than half of the of the NPC is made of scaffold proteins which resemble coated vesicles and these form a 16-fold repetition of columns at least in yeast (Alber et al., 2007). Such scaffold proteins include components of the Nup107-160 complex, Nup93 and Nup205 (Berke et al., 2004; Galy et al., 2003; Grandi et al., 1997). The peripheral FG group consists of nucleoporins mainly harbouring FG repeats and coiled-coil motifs. FG repeat are natively unfolded structures defined by long stretches of hydrophobic residues comprising phenyl-alanin- glycine/lysine repeats; FG, FXFG (x, any), GLFG (L, Leucine) usually separated by 5-50 residues of hydrophilic linkers (Denning et al., 2003; Fink, 2005; Rout and Wente, 1994). The FG repeats have multiple topological positions in the NPC and are involved in cargo translocation such as Nup50, Nup153, Nup214 and Nup358 (Bayliss et al., 2000; Bayliss et al., 2002; Denning et al., 2003; Fahrenkrog et al., 2002; Lim et al., 2006; Napetschnig et al., 2007). Other less frequent motifs include a zinc finger motif found in Nup153 and RanBP2/Nup358 (Higa et al., 2007) and an RNA binding motif found in Nup35 (Handa et al., 2006).

1.6 Nup153

Nup153 depicts its name from its molecular size of 153 kDa in human and Drosophila and share similar but not identical sequence to the *S. cerevisiae* yNup1/2, yNup60 and the *C. elegans*, npp-7, respectively (Dimaano et al., 2001; Galy et al., 2003; McMorrow et al., 1994; Pitt et al., 2000; Sukegawa and Blobel, 1993). Nup153 is a highly dynamic glyco-protein and has three distinct domains that represent a distinct topology along the NPC (Fahrenkrog et al., 2002; Krull et al., 2004; Rabut et al., 2004). The N-terminal domain (amino acids1-610) is located at the nuclear ring moiety of the NPC, and is thought to be responsible for initiating the incorporation into the NPC specifically within amino acid residues (NPAR) (39-339) and also implicated in the nuclear envelope targeting (NETC) between amino acid residues (1-144) (Enarson et al., 1998). An RNA binding domain is present within residues 250-400 (Dimaano et al., 2001). An M9-like NLS sequence is

thought to be located at the N-terminal domain (Nakielny et al., 1999). A central zinc finger domain is located between amino acid resides (650-880), which is comprised of four C_2-C_2 type zinc fingers (Sukegawa and Blobel, 1993). The C-terminal domain of Nup153 resides between amino acid residues (881-1475) and has approximately 28 unstructured FG- repeats (Denning et al., 2003; Lim et al., 2007; McMorrow et al., 1994). While the N-terminal, and the zinc finger domains have stationary localisation at the nuclear ring moiety and the distal ring, respectively, the C-terminal domain is flexible with multiple associations in the NPC (Figure 1.7) (Fahrenkrog et al., 2002). It can be mapped to the nuclear ring moiety, the distal ring and the cytoplasmic periphery of the central pore (Fahrenkrog et al., 2002; Paulillo et al., 2005; Walther et al., 2001). The C-terminus of Nup153 is highly implicated in nuclear transport, in an importin α/β-mediated transport, Nup153 binds to importin-β through the FG- repeat domain (Shah and Forbes, 1998). In addition, Nup153 also interacts with export receptors, exportins such as CRM-1 (Nakielny et al., 1999; Walther et al., 2003a). RanGTP is implicated in the regulation of Nup153 associations with import as well as export factors, with importin-β, mRNA export, as well as associations with other nucleoporins (Bastos et al., 1996; Higa et al., 2007; Ullman et al., 1999; Walther et al., 2003b).

Nup153 is a highly mobile protein, the NPC exchanging with a small population in the nucleoplasm (Daigle et al., 2001; Griffis et al., 2004). It associates with RNA polymerase I and II and interacts with RNA (Ball et al., 2007; Griffis et al., 2004; Ullman et al., 1999) and chromatin (Bodoor et al., 1999a; Sukegawa and Blobel, 1993). Nup153 regulates gene transcription by its roles in chromosomal translocation and dosage compensation (Heidenblad et al., 2008; Mendjan et al., 2006). The interaction of Nup153 with importin-β in addition to transport related function, is implicated in NPC assembly, NE breakdown (Harel and Forbes, 2004; Liu et al., 2003b; Prunuske et al., 2006). Moreover, Nup153 is one the early proteins recruited to the NE post mitosis, at late anaphase (Bodoor et al., 1999b; Haraguchi et al., 2000).

Nup153 undergoes several posttranslational modifications and during mitosis Nup153 is phosphorylated (Favreau et al., 1996). Recently, Nup153 is implicated both in early cell cycle progression and exit and demonstrated to have distinct roles during mitosis (Mackay et al., 2009). Manipulating the extent of Nup153 reduction led to defects either at an early stage of mitosis or delay at a later stage in lesser severe levels (Mackay et al., 2009). Its zinc finger domain is also involved in NE breakdown by interacting with COPI complex

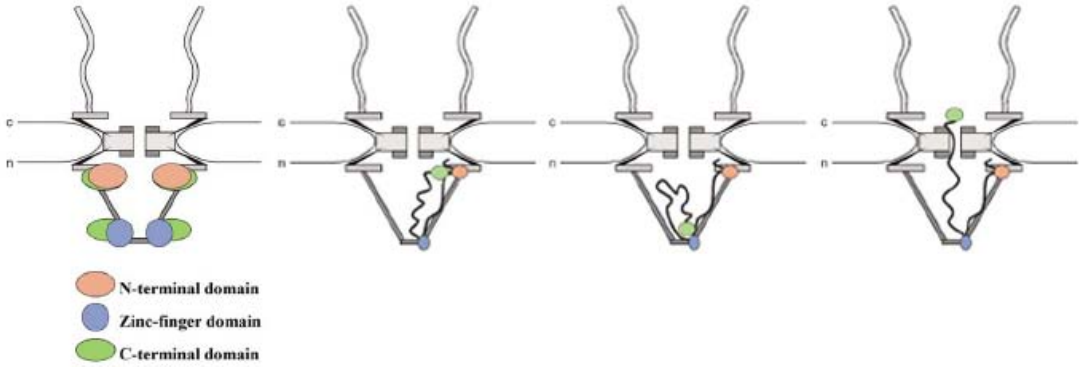

Figure 1.7: Domain localisation of Nup153 - A schematic representation of the NPC illustrating the localisation of Nup153 domains based on immunolocalization data obtained either with domain-specific antibodies or by using antibodies against epitope-tagged Nup153 incorporated into *Xenopus* NPCs antibodies. The N-terminal domain (*orange*) and the zinc finger domain (*blue*) are stationary at the nuclear ring moiety and the distal ring respectively, whereas the C-terminal FG-repeat domain (*green*) is highly mobile. It can be located at the nuclear ring moiety (left), at the distal ring (middle), or even at the cytoplasmic periphery of the central pore (right). Adapted from (Fahrenkrog et al., 2002)

that is recruited in NE disassembly (Liu et al., 2003b). In apoptosis, Nup153 is cleaved at the N-terminal domain between residues 36-391 by caspase 3 (Buendia et al., 1999; Ferrando-May et al., 2001). In addition, it is degraded during viral infection and oxidative stress (D'Angelo et al., 2009; Gustin and Sarnow, 2001; Kodiha et al., 2004).

Nup153 interacts with other nucleoporins, mainly Tpr at the nuclear basket, where Tpr requires Nup153 for its localisation (Hase and Cordes, 2003). However, Nup153 recruitment to the NPC does not require the nuclear basket protein Tpr for assembly (Hase and Cordes, 2003). Nup153 requires the Nup107/Nup160 complex for incorporation in the NPC, specifically its interaction with Nup160 at the N-terminal domain between residues 210-338 as well as the interaction with Nup96 and Nup133 (Boehmer et al., 2003; Krull et al., 2004; Vasu et al., 2001; Walther et al., 2003a). Nup153 also interacts with Nup62 at interphase (Stochaj et al., 2006). Nup153 is implicated in anchoring NPCs to the nuclear lamina due to an interaction with lamin B (Smythe et al., 2000; Walther et al., 2001) and lamin A (Al-Haboubi et al., submitted).

Nup153 is essential for cell viability (Galy et al., 2003; Harborth et al., 2001) and it is overexpressed in retinoblastoma with 6p genomic gain, compared with the normal adult human retina (Orlic et al., 2006).

1.7 Nucleocytoplasmic transport

The passive permeability of the NPCs is defined by the diameter of the pore measuring 70 nm at the periphery and 45 nm in the NE midplane (Keminer and Peters, 1999). Each NPC permits a flow of 100 MDa/s in highly selective manner (Ribbeck and Gorlich, 2001). The translocation of molecules greater than 40 kDa through the NPC is limited to cargo harbouring specific nuclear localisation signal (NLS) or nuclear export signal (NES) motifs that are recognised by transport receptors which mediate cargo passage through the NPC by means of hydrophobic interactions with FG domain containing Nups (FG-Nups) (Bayliss et al., 2000; Bayliss et al., 2002; Lange et al., 2007; Stewart, 2007; Strawn et al., 2004; Suntharalingam and Wente, 2003). FG-Nups form a diffusion barrier, whereby only cargo carrying transport-related signals could traverse though the NPCs (Lange et al., 2007; Macara, 2001). FG-Nups exist in multiple copies per NPC, and are comprised of up to 50 repeats, which yield in approximately 190 FG-repeat domains per FG-Nup and a total of 2700 FG motifs per NPC (Peters, 2009). However, half of the FG-repeats can be removed without affecting the overall protein transport (Strawn, 04).

Nuclear transport receptors involve a large group of structurally related members of karyopherins (importins, exportins, or transportins) (Figure 1.8) (Pemberton and Paschal, 2005). Most importin-β isoforms bind to cargo directly, while the importin-β1 isoform employs specific adaptors, such as importin-α, for cargo recognition (Gorlich et al., 1996). Importin-β contains 5 binding sites for FG-Nups based on X-ray crystallography and 10 binding sites based on molecular modelling (Bayliss et al., 2000; Bayliss et al., 2002; Isgro and Schulten, 2005; Isgro and Schulten, 2007). The directionality of the transport for importin-β isoforms is accomplished via the small guanosine triphosphatase (RanGTP) and also be influenced by the importin-β binding sites with FG-Nups (Stewart, 2007). Ran interacts with importin complexes, on the nuclear site RanGTP is found in a higher concentration where it destabilises import complexes and facilitates export complex formation, once in the cytoplasm it is hydrolysed to RanGDP along with RanBP1 and RanBP2/Nup358 (Fried and Kutay, 2003; Weis, 2003). Leucine rich NES are recognised by CAS and CRM1, where CAS helps in recycling importin-α from the nucleus, and CRM1 mediates the export of RNA as well as proteins (Fornerod et al., 1997; Kutay et al., 1997). Proteins with a NES motif form a complex with CRM1 and combined with RanGTP are exported from the nucleus by interacting with Nup98 FG-repeats, and are the transported protein is released

into the cytoplasm following the hydrolysis of RanGTP to RanGDP by RanGAP (Powers et al., 1997). Nup358/RanBP2 and Nup214 are also involved in CRM1 mediated export mechanism (Bernad et al., 2006; Bernad et al., 2004).

Several models have been proposed to explain the selectivity barrier of the NPC (Fahrenkrog and Aebi, 2003; Lim et al., 2008). The virtual gating model predicts an entropic barrier formed by fluctuations of the unfolded FG-Nups, which form filament-like structures on both sides of the NPCs (Rout et al., 2000). Nuclear transport receptors bind to FG-repeats and saturate transport complexes at the NPC, overcoming the entropic barrier and increasing the probability of diffusion through the NPC (Rout et al., 2003). This is in agreement with the oily-spaghetti model that proposed a repulsive gate formation in the NPC, repelling non-FG-binding molecules, hence prohibiting the translocation of non binding proteins (Macara, 2001).

Another model, the selective phase partitioning model predicts that intermolecular hydrophobic interactions between FG-Nups create a selective permeability barrier that prohibits free diffusion through the NPC (Ribbeck and Gorlich, 2001). In this model, the NPC acts as a physical barrier where the FG-Nups form a sieve-like meshwork that can be dissolved by a solubility diffusion process mediated by transport receptors that locally dissolve the barrier and permit passage of receptor-cargo complexes (Frey et al., 2006). FG-repeats of the yeast Nsp1p (GLFG) in saturated amounts form a gelatinous (hydro-gel) meshwork that reproduces the permeability properties of the NPC *in vitro* (Frey and Gorlich, 2007).

Another model, the reduction of dimensionality model refers to a two dimensional walk whereby transport receptors move through the NPC by interacting with the FG-repeats through out the innermost layer of central framework of the NPC to the nuclear basket. The model predicts the existence of a selectivity filter in the central channel, which is also generated by the FG-repeats with an unobstructed narrow tube in the centre that would enable the diffusion of small molecules (Peters, 2005). A two-gate model of NPC architecture featuring a central diffusion gate formed by a meshwork of cohesive GLFG nucleoporin filaments and a peripheral gate formed by repulsive FxFG nucleoporin filaments (Patel et al., 2007). This is supported by recent data which demonstrated that the FG-repeat domain of Nup153 form polymer brushes that undergo a reversible collapse upon the addition of nuclear transport receptors *in vitro* with entropic repulsion properties, the reversible FG domain collapse model (Lim et al., 2007; Lim et al., 2006).

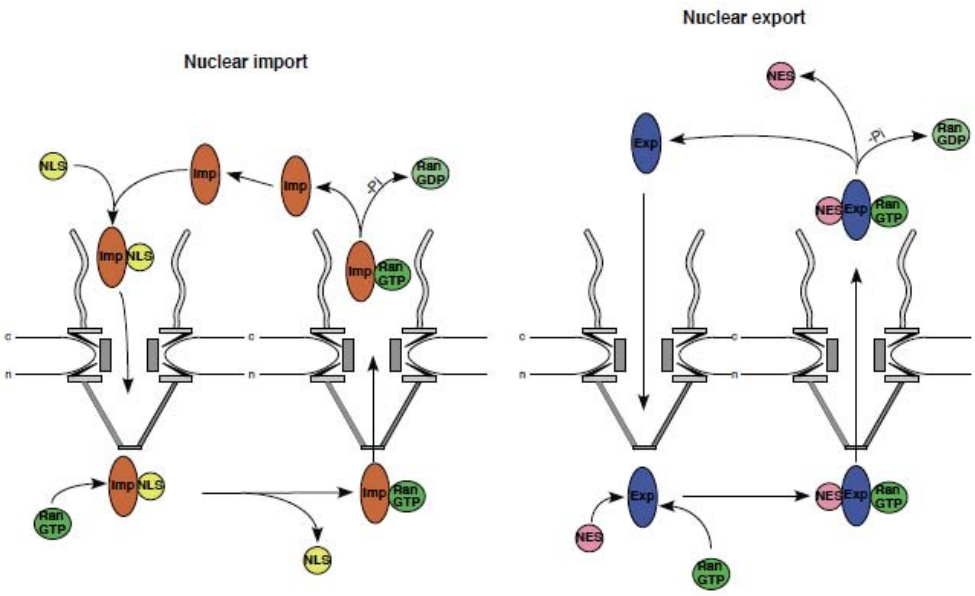

Figure 1.8: Nuclear import and export cycles mediated by Ran GTP - Nuclear import of cargo is initiated at the cytoplasmic importins (*red*), which interact with cargo carrying an NLS. The importin-cargo complex translocates the cargo into the nucleus via interactions with the NPC. Inside the nucleus, RanGTP dissociates the importin-cargo complex from the NPC. Importins are recycled back to the cytoplasm by means of RanGTP hydrolysis. Nuclear export is mediated by export receptors (*blue*) that recognise NES and require the presence of RanGTP. Once in the cytoplasm, the complex is dissociated upon RanGTP hydrolysis. c, cytoplasm; n, nucleus. Adapted from (Fahrenkrog, 2006)

1.8 NPC disassembly during NE breakdown

The NE of metazoan cells undergoes open mitosis to allow the spindle microtubules, which are assembled in the cytoplasm, to gain access to the mitotic chromosomes in the nucleus (Figure 1.9) (Stewart et al., 2007b). An increase of the NPCs permeability is an early sign of the NPC disassembly prior to NE breakdown (Lenart et al., 2003). Once the NPCs are disassembled, holes are formed in the nucleus, followed by redistribution of nuclear membranes into the ER and ultimately the disassembly of the nuclear lamina (Beaudouin et al., 2002). The process of NE disassembly is accompanied by the phosphorylation of multiple NE related components, such as the nuclear lamina (Gerace and Blobel, 1980), as well as components of the NPCs, including gp210 as well as Nup153 (Favreau et al., 1996; Galy et al., 2008). Once the NPCs are dismantled, some nucleoporins are recruited to the cytoplasm and others reassemble around the segregated chromosomes, or recruited to kinetochores mitotic spindle assembly, such as Nup358 and Nup107-160 complex (Dawlaty et al., 2008; Salina et al., 2003; Zuccolo et al., 2007). The latter is also implicated in correct spindle assembly possibly via interactions with spindle-associated proteins (Orjalo et al., 2006). For example, the mitotic spindle checkpoint protein complex MAD1-MAD2 is bound to the yeast homoloug of Nup35 (Nup53p in yeast) through interactions with MAD1 and MAD2 in interphase and is released from kinetochores upon phosphorlyation of MAD1 by Nup53p at the onset of NE breakdown and mitosis (Iouk et al., 2002). Recently, the nuclear basket protein Tpr was demonstrated to interact directly with the mitotic spindle checkpoint proteins MAD1 and 2 as well (Lee et al., 2008). In addition, Nup153 is required at early mitotic stages for cell cycle progression as well as at the end of mitosis (Mackay et al., 2009). The disassembly of Nups from the NPCs is thought to occur in stepwise manner. Nup98 and Nup50 are among the earliest Nups released, followed by Nup153, Nup358 and gp210 (Belgareh et al., 2001; Joseph et al., 2004; Prunuske et al., 2006; Salina et al., 2003). Ultimately, Tpr, Nup96 and another population of Nup153 and Nup107-160 sub-complex are recruited in mitosis (Hase and Cordes, 2003; Loiodice et al., 2004).

1.8.1 NPC assembly

NPCs undergo two assembly phases, one at mitosis and another during interphase (Hetzer et al., 2005). NE reassembly commences in anaphase, with stepwise recruitment of NE components, when the NE reforms around the segregated chromosomes where several

Nups are recruited to chromatin in a sequential manner starting in early anaphase (Bodoor et al., 1999b; Sheehan et al., 1988; Ulbert et al., 2006). The assembly of the NPCs has to be spatially organised and restricted to chromatin surface (Antonin et al., 2008). Distinct Nups bind to chromatin at an early mitotic stage; these are the pore forming Nups, ELYS/Mel28 as well as the Nup107-160 sub-complex (Fernandez and Piano, 2006; Franz et al., 2007; Glavy et al., 2007; Rasala et al., 2006). Recent data suggested a step wise recruitment initiated by AT-rich chromatin regions with ELYS, then the Nup107-160 complex followed by POM121 and NDC1 containing membrane vesicles and then other Nups including Nup93 and Nup62 complexes (Bodoor et al., 1999b; Rasala et al., 2008). This is followed by the appearance of INM proteins and the formation of a continuous double membrane. These membrane components originate in the peripheral ER network. NPC proteins also play a crucial role in membrane assembly. Interplay between Nup107-160 and the NPC membrane protein POM121 seems to function as a membrane assembly checkpoint, which ensures that a sealed NE does not form in the absence of functional NPCs (Antonin et al., 2005). This is followed by the recruitment of other Nups, Nup93, Nup62 subcomplex and later the peripheral Nups; Nup214, Nup50, Nup153 and Tpr and gp210 (Bodoor et al., 1999b; Burke and Ellenberg, 2002; Hase and Cordes, 2003). Although small pool of Nup153 as well as Nup50 is already seen at an earlier stage associated with chromatin (Dultz et al., 2008).

Recent studies showed that A- and B-type lamins do not co-polymerise in the nuclear lamina and have separate functions during NE assembly with lamin C is mainly involved in NPC distribution (Furukawa et al., 2009) which contradicts with previous studies that showed that lamin A is recruited to the NE after assembly of all the Nups and lamin B (Moir et al., 2000b). Assembly of Nup153 and POM121 occurred earlier than lamin B1 recruitment and followed by lamin import (Daigle et al., 2001; Hetzer et al., 2000). Overall, disruption in lamin assembly or levels did not interefer with NE and NPC assembly (Daigle et al., 2001; Newport et al., 1990; Steen and Collas, 2001). NPCs can also assemble *de novo* (D'Angelo and Hetzer, 2006). Furthermore, newly synthesised nucleoporins are inserted into the intact NE during S-phase, which results in the doubling of the number of NPCs prior to the next cell division and second during interphase in which cells double in their number of NPCs in preparation for the next round of division (Antonin et al., 2008).

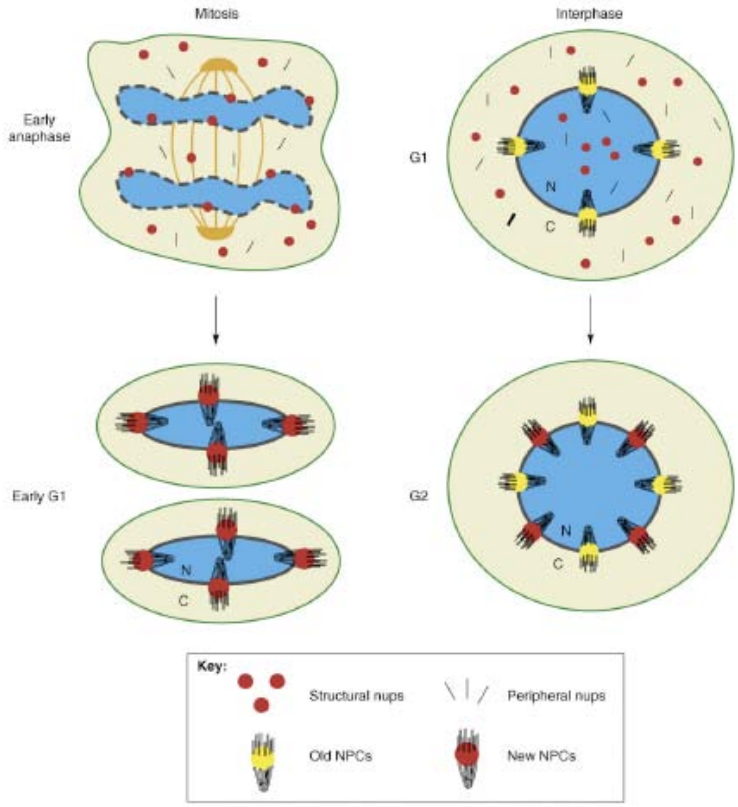

Figure 1.9: Assembly of nuclear pore complexes - Schematic drawings of the nuclear envelope (NE) and nuclear pore complexes (NPCs) during mitosis and interphase. During mitosis NE reassembly commences at early anaphase with sequential recruitment of structural NPC components, such as ELYS/Mel28 and the Nup107-160 sub-complex, to chromatin. NPCs assemble *de novo* from newly synthesised nucleoporins inserted into intact NEs during interphase. Adapted from (D'Angelo and Hetzer, 2008)

1.9 Nucleoporins and viral infections

DNA viruses utilise viral proteins to gain access to the host DNA machinery, either by directly interacting with distinct Nups or import mediated mechanisms to facilitate nuclear transport (Trotman et al., 2001). Adenoviruses have a diameter of 70-100 nm and associate with microtubules that direct the viral caspids to the ONM of the NE (Suomalainen et al., 2001; Suomalainen et al., 1999). At the NE, these viral caspid proteins either initiate interactions with distinct Nups, such as the direct association of adenovirus with Nup214 (Trotman et al., 2001). Or become import competent by having an NLS for their nuclear delivery and most viral caspid proteins of adenoviruses as well as paroviruses contain an NLS (Saphire et al., 2000; Vihinen-Ranta et al., 2002; Wodrich et al., 2006). Relying on an importin-mediated mechanism for their transport is also described for other DNA viruses such as, herpes viruses, hepadnaviruses and papoviruses (Kann et al., 1999; Nelson et al., 2000; Ojala et al., 2000). Most retroviruses have an RNA genome but employ DNA for their replication and hence require nuclear entry and rely on mitosis except for lentiviruses including HIV-1 (Lewis and Emerman, 1994). Once in the cytoplasm, the HIV-1 RNA genome is transcribed into DNA, which is in a complex with an integrase (IN), a reverse-transcriptase, a matrix protein (MA) and an accessory protein (Vpr), respectively (Stevenson, 1996). In addition to having both nuclear import as well as export in the MA (Dupont et al., 1999; Haffar et al., 2000), the HIV-1 Vpr is imported by Nup153 interactions with importin-β (Varadarajan et al., 2005). Recently, HIV-1 IN was shown to interact directly with the C-terminal domain of Nup153 (Woodward et al., 2009). In addition, HIV-1 Rev is an RNA binding protein which is also imported through Nup358- transportin mechanism and acts as an export factor as well, mediating transport of viral RNA in an NES/exportin-1 (or known as CRM-1) dependent transport to the cytoplasm, possibly through interactions with Nup98 and Nup214, respectively (Fischer et al., 1995; Fornerod et al., 1997; Hutten et al., 2009; Neville et al., 1997; Zolotukhin and Felber, 1999). RNA viruses usually replicate in the cytoplasm, except for influenza virus which requires the host DNA splicing machinery which is transported to the nucleus by importin-mediated mechanism through the NPC (Wu et al., 2007), whereby viral ribonucleoproteins contain non-conventional NLS motifs (Martin and Helenius, 1991). Another RNA virus, vesicular somatitis virus (VSV), which belongs to rhabdoviruses, replicates in the cytoplasm, and hijacks the nucleocytoplasmic transport system (Fontoura et al., 2005). It employs its own matrix protein M

to block cellular RNA export, by forming a ternary complex with RAE1, an mRNA export factor, and Nup98, respectively (Fontoura et al., 2005). Nup153 among other Nups are targeted in viral infections of enteroviruses most commonly poliovirus, rhinoviruses either by proteolysis of Nup153 and Nup62 (Belov et al., 2004; Gustin and Sarnow, 2001) or by phosphorylation of Nup62 and Nup98 which aborts transport via these Nups (Porter and Palmenberg, 2009). Other RNA viruses interfere with normal phosphorylation signaling, such as cardioviruses, which employ leader proteins that interfere with phosphorylation pathways (Bardina et al., 2009).

1.9.1 Nucleoporins related cancer and other diseases

The main function of the NPCs is nucleocytoplasmic transport and a tight regulation of transport is essential for cell homeostasis. Alterations in components of the NPC or the nuclear transport process have a strong impact on cell growth and survival and have been associated with several diseases including cancer (Kau et al., 2004; Poon and Jans, 2005) and the rare autosomal recessive disorder tripe A syndrome (Cronshaw and Matunis, 2004). Defects in nuclear transport linked to cancer and tumourgenesis can be through the unmasking of normally hindered NLS and NES of transcriptional activators. Abnormal nuclear localisation of the transcriptional activator NF-κB which is usually in complex in the cytoplasm where its NLS is hindered is prominent in cancer cells (Beg et al., 1992) and elevated levels of NF-κB in the nucleus is associated with multiple cancer types including Hodgkin's lymphoma and childhood acute lymphoblastic leukaemia (Rayet and Gelinas, 1999). NES unmasking is implicated in mutations in the tumour suppressor INI1/SN5 (integrase interactor 1) which is a member of the mammalian SWI/SNF chromatin-remodelling complex. Mutations affecting its NES, in particular, a dominant-negative form of INI1, which is mislocalised to the cytoplasm, can lead to malignant and atypical teratoid rhabdoid tumours (Craig et al., 2002). Distinct Nups are also implicated in cancer as fusion products of chromosomal translocations with several oncogenes that lead to transcriptional activation of cellular signalling such as Nup98 and Nup214 in acute and chronic myeloid leukaemia (De Keersmaecker et al., 2008; Fornerod et al., 1995; Moore et al., 2007). Fusion products led to aberrant transcription, decrease in differentiation and increase in proliferation related fusion proteins as these proteins retained their FG-repeat domain that initiate interactions with other transcription activating factors such as CREB (Kasper et al., 1999). A recently discovered component of the NPC is ALADIN and

mutation in its gene is associated with triple A syndrome (Cronshaw and Matunis, 2004). Another important component of the NPC, which is assigned as a tumour marker in cancer, is Nup88 (Emterling et al., 2003). The expression levels of Nup88 were demonstrated to correlate with severity of tumourgenesis in colon cancer and tumour aggressiveness in breast cancer, respectively (Agudo et al., 2004; Emterling et al., 2003). In addition, recent data suggested a possible direct interaction of Nup88 with NF-κB, supporting earlier interaction data from the *Drosophila* homolog (Takahashi et al., 2008; Uv et al., 2000; Xylourgidis et al., 2006).

1.9.2 NPCs components as targets for autoantibodies

As for the nuclear lamina, components of NPCs are targets for autoantibodies in human autoimmune diseases (Enarson et al., 2004). NPCs subunits gp210, Nup62, Nup358, Nup153 and Tpr are targeted in autoimmune liver diseases, systemic lupus erythematosus and other related diseases. The underlying mechanism for this is not clear but molecular mimicry has been suggested, in particular it was demonstrated that a region in the C-terminal domain of Nup153 shows similarity with a hepatitis B virus (HBV) DNA polymerase domain (Gregorio et al., 1999). In addition, gp210 also shows transmembrane domain similarity with a number of viral enveloped proteins (Greber et al., 1990).

1.10 Concluding remarks

Due to the large interconnected network of proteins associated with the NE, hence, complications arise in mutations associated with distinct protein components of the NE. Both the nuclear lamina and NPCs are major structural and functional components of the NE. On one hand, nuclear organisation and stability as well as bridging to the cytoskeleton is achieved by components of the nuclear lamina. On the other hand, the NPCs offer a highly regulated nucleocytoplamic transport. Essential nuclear functionality is perturbed in mutations associated with NE components, which are well demonstrated in laminopathies and other related diseases. The nuclear lamina and NPCs are hijacked during viral infections and cancer. Hence, understanding the molecular mechanism behind NE mediated interactions help to relate to disease outcome.

2

Direct association of the nuclear pore protein Nup153 with the Ig-fold domain of A- and B-type lamins

Teiba Al-Haboubi[1], *Joachim Köser*[1,*], *Dale K Shumaker*[2,3], *Robert D Goldman*[3] *and Birthe Fahrenkrog*[1]

[1]M.E. Mueller Institute for Structural Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland;
[2]Department of Urology, Feinberg School of Medicine, Northwestern University, 303 East Chicago Avenue, Chicago, IL 60611;
[3]Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, Illinois 60611, USA
[*]Present address: Institute of Chemistry and Bioanalytics, School of Life Sciences, University of Applied Sciences, 4132 Muttenz, Switzerland

2.1 Abstract

The nuclear envelope (NE) represents a physical barrier around the nucleus and it assembles from inner and outer nuclear membrane proteins, nuclear pore complexes (NPCs) and the nuclear lamins. The nuclear lamina, which is composed of lamins and inner nuclear membrane proteins, is crucial for maintaining the structural integrity of the nucleus and for positioning of NPCs within the NE. The nucleoporin Nup153 has previously been reported to bind to B-type lamins, the specificity of this interaction, however, is not well established. Here we have carried out solution-binding and immunoprecipitation assays to further characterise the interplay between Nup153 and nuclear lamins. Our *in vitro* binding assays revealed that Nup153 exhibits binding sites for both A-and B-type lamins. Moreover, we found that the N-terminal domain of Nup153 as well as its C-terminal domain associates with the Ig-fold motif in the tail domain of nuclear lamins. Similarly, antibodies against Nup153 co-precipitate A-and B-type lamins from HeLa nuclear extracts. In addition, we show that the binding of Nup153 to nuclear lamins requires distinct residues in the N-terminal domain of Nup153. Whereas lamin A exhibits multiple binding sites between residues 39 to 339, the so-called nuclear pore association region (NPAR) of Nup153, binding of B-type lamins requires residues 2 to 339. Our results indicate a far more intricate interplay between Nup153 and nuclear lamins than previously accepted.

2.2 Introduction

Embedded within the nuclear envelope (NE) of eukaryotic cells are the nuclear pore complexes (NPCs), 125 MDa supra-molecular structures which facilitate the passage of macromolecules in and out of the nucleus (Lim et al., 2008). NPCs are made up of about 30 different proteins termed nucleoporins (or Nups), which are typically found in multiple copies due to an eight-fold rotational symmetry of the NPC and which assemble into distinct sub-complexes (D'Angelo and Hetzer, 2008; Lim and Fahrenkrog, 2006). The nucleoporin Nup153 is a highly dynamic glycoprotein being involved in multiple nuclear processes, such as nuclear protein import, RNA export, nuclear assembly/disassembly and mitosis (Ball and Ullman, 2005; Daigle et al., 2001; Griffis et al., 2004; Liu et al., 2003; Mackay et al., 2009; McMorrow et al., 1994). Moreover, recent findings have demonstrated the importance of Nup153 in gene regulation as it is implicated in chromosomal

translocations as well as dosage compensation (Heidenblad et al., 2008; Mendjan et al., 2006; Orlic et al., 2006). Nup153 possesses a distinct domain topology along the NPC with the amino (N)-terminal domain (residues 1-610 of human Nup153) stationary localised to the nuclear ring moiety of the NPC (Fahrenkrog et al., 2002; Walther et al., 2001). This N-terminal domain harbours an M9-like nuclear localisation signal (Nakielny et al., 1999), a nuclear envelope targeting cassette (NETC; residues 1-144), the NPC association region (NPAR; residues 39-339) as well as a RNA binding domain (Bastos et al., 1996a; Enarson et al., 1998; Nakielny et al., 1999; Ullman et al., 1998). A central zinc finger domain (residues 650-880) is stationary anchored to the distal ring of the nuclear basket of the NPC (Fahrenkrog et al., 2002). The carboxyl (C)-terminal domain of Nup153 (residues 881-1475) is highly flexible and dominated by about 30 phenylalanine-glycine (FG)-repeat motifs, which have little secondary structure, i.e. natively unfolded, and mediate the interaction of Nup153 with soluble nuclear transport receptors (Ball and Ullman, 2005a; Lim et al., 2006; Lim et al., 2007). This C-terminal domain is highly flexible and has been demonstrated to shuffle between the nucleoplasmic and the cytoplasmic face of the NPC (Fahrenkrog et al., 2002; Paulillo et al., 2005). Nuclear lamins belong to the family of intermediate filament (IF) proteins (Szeverenyi et al., 2008) and are the main constituents of the nuclear lamina (Aebi et al., 1986). They play an important role in various nuclear processes, such as determining nuclear size and shape, NPC positioning, transcription, DNA replication and mitotic spindle assembly (Salina et al., 2001; Vaughan et al., 2000). In eukaryotes, lamins are subdivided into A and B types. The major A-type lamins, lamin A and C, originate from alternative splicing of a single gene LMNA gene, whereas lamin B1 and B2 are encoded by two genes LMNB1 and LMNB2, respectively (Fisher et al., 1986). While B-type lamins are ubiquitously expressed, A-type lamins are found only in differentiated cells. Like all IFs, lamins consist of α-helical coiled coil rod domain flanked by two globular non- helical N- (head) and C- (tail) terminal domains (Heins and Aebi, 1994). The tail domain encompasses (i) a nuclear localisation signal (NLS) that targets the lamins into the nucleus, (ii) a unique highly conserved immunoglobulin like fold (Ig-fold) that is involved in protein-protein interactions and chromatin binding (Moir et al., 1994; Shumaker et al., 2008; Stierle et al., 2003), and (iii) a -CAAX box required for posttranslational lipid modification (Schirmer et al., 2001). Mutations in the human LMNA gene account for at least 18 distinct heritable diseases, collectively termed laminopathies

(Worman and Bonne, 2007). These include muscle diseases, such as Emery-Dreifuss muscular dystrophy (EDMD) and dilated cardiomyopathy (DCM), metabolic diseases, such as familial partial lipodystrophy (FPLD) or premature aging syndromes as Hutchinson-Gilford progeria syndrome (HGPS). The disease causing mutations are distributed through out the LMNA gene, but most mutations known to date are located in the tail domain of the protein, in particular in the Ig-fold (Shumaker et al., 2005). Previous reports showed that Nup153 interacts with *Xenopus* lamin B3 (also known as LIII) in cell-free extracts from *Xenopus* eggs (Smythe et al., 2000). Blot overlay assays furthermore revealed that Nup153 binds directly via its C-terminal FG-repeat domain to LIII, but not to lamin A or the lamin-binding protein emerin (Smythe et al., 2000). Nup153 recruitment to the NPC is strictly lamin-dependent, as Nup153 incorporation into the NPC failed in cells lacking an intact lamina (Smythe et al., 2000). Moreover, Nup153 localisation is often perturbed in cells expressing disease-related lamin A mutants (Hubner et al., 2006b). Based on the recent findings that FG-repeat domains are natively unfolded (Denning et al., 2003; Denning et al., 2002), dynamic and flexible within the NPC (Fahrenkrog et al., 2002; Paulillo et al., 2005) and dispensable for viability (Strawn et al., 2004), we speculated that the FG-repeat domain of Nup153 is unlikely to present the only interaction site between Nup153 and the nuclear lamins. We demonstrate here that Nup153 binds to both A- and B-type lamins, in particular to the Ig-fold. Using solution-binding assays, we revealed that this binding is not part of a nuclear import complex. Furthermore, both the N- and the C-terminal domain of Nup153 interact with the Ig-fold of lamin A and B and the interaction is abolished when particular amino acid residues of Nup153 are deleted. Importantly, lamin A and lamin B association with the N-terminus of Nup153 necessitates differing amino acids. The experiments described here demonstrate that the Nup153-lamin interplay is extremely variegated and imply functional differences.

2.3 Experimental Procedures

All experimental procedures were carried out at room temperature, unless otherwise stated.

2.3.1 DNA constructs

Bacterial expression of recombinant GST-HA-Nup153-His fragments was from pGEX 4T-3 and pGEX 6P-1 vectors. HA-Nup153-His fragments were PCR amplified from the HA-

Nup153 encoding eukaryotic expression vectors (Enarson et al., 1998) and cloned into the BamHI site of the pGEX vectors. The 3' primers for these PCR reactions encoded a 6xHis tag, whereas the HA-tag was part of the eukaryotic expression constructs and is separated from the Nup153 aa3 (Ser) by a XhoI site. Certain fusion constructs were also obtained by first cloning PCR-amplified Nup153-fragments into pBluescript and from there into pGEX-HA-vectors or by direct ligation of the fragments cut out from the eukaryotic expression vectors into pGEX-HA-Nup153-His opened with XhoI and NotI. Transfer of the Nup153 encoding fragments from pGEX 4T-3 to pGEX 6P-1 was done using the BamHI cloning sites and allowed the expression of GST-Nup153 fusion fragments which can be cleaved with precission protease. pGEX-C1-Nup153 (aa 1001-1385) was obtained from Katie Ullman (Fahrenkrog et al., 2002).

2.3.2 Antibodies

The following antibodies were used in Western blot analysis. Mouse monoclonal SA1 (1:50 dilution; a gift of Brian Burke, University of Calgary, Calgary, Alberta, Canada (Bodoor et al., 1999)) or 7A8 (1:50; a gift from Volker Cordes, University of Heidelberg, Heidelberg, Germany (Cordes et al., 1993)) against the C-terminus and the N-terminus of human Nup153, respectively. Mouse monoclonal importin-α (l1784; 1:1000, Sigma-Aldrich, St.Louis, MO, USA) and -β (l2534; 1:1000, Sigma-Aldrich). Rabbit polyclonal anti-lamin B1 (ab16048; 1:3000; Abcam, Cambridge, MA, USA) recognises an epitope within residues 400-500, *Xenopus* lamin B2, *Xenopus* lamin A (monoclonal) and rabbit polyclonal 324 against human lamin A peptide cVTVTRSYRSVGGSG, which corresponds to residues 620-633; (1:1000). A rabbit polyclonal human lamin B2 antibody (328) against a specific peptide cTVKKSSVMRENENG, which corresponds to residues 576-589 (1:1000). All secondary antibodies were obtained from Sigma-Aldrich.

2.3.3 Recombinant protein expression and purification of glutathione S-transferase (GST) coupled Nup153 fragments

GST-fusion proteins of Nup153 were expressed in *E.coli* BL21-DE3 codon (+) cells. A 5ml over night culture was used to inoculate 1-3 litres of LB medium and grown at 37°C until an optical density at 595 nm of 0.6 was reached. Protein expression was next induced by the addition of 0.1 mM IPTG and cells were grown in culture for 3 hours at 37°C and

shaking at 180 rpm. Bacterial pellets were collected by centrifugation at 5000 rpm for 10 min at 4 °C (SLA3000, Sorvall). The pellet was resuspended in phosphate buffered saline (PBS) containing 0.5 mM PMSF, 5.0 μg/ml aprotinin and 5.0 μg/ml leupeptin, lysed by sonication and cleared centrifugation at 11000 rpm (20 000 g) for 45 min at 4 °C. The supernatant was next incubated with glutathione sepharose-4B (GE Healthcare Biosciences, Uppsala, Sweden) for 2 hours while shaking, transferred onto a ply-prep chromatography column (Bio-Rad laboratories, CA), washed by the addition of PBS (binding buffer) 3-4 volumes and eluted using elution buffer (20 mM glutathione (reduced form, AppliChem, Darmstadt, Germany), 100 mM Tris pH 8.0, 120 mM NaCl, 10 mM DDT). In a second purification step, proteins were loaded on a nickel column (Ni-NTA agarose, Qiagen GmbH, Hilden, Germany), washed and eluted using 500 mM Imidazole, collected fractions were dialysed against PBS and stored in aliquots at -80 °C.

2.3.4 Cell culture

HeLa cells were maintained in Dulbecco's modified Eagle medium (Gibco/Invitrogen, Life Technologies Corporation, CA), supplemented with 10% FBS (PAA), 100-units/ml penicillin/ 100 μg/ml streptomycin (Gibco/Invitrogen) and 2 mM glutamine (Gibco/Invitrogen). The cells were grown in a monolayer in a humidified 37 °C incubator supplied with 5% CO_2.

2.3.5 Coupled *in vitro* transcription and translation

Coupled *in vitro* transcription and translation of proteins was performed using the TNT® quick- translation system kit (Promega, Madison, WI, USA) following the manufactures protocol. In brief, 40 μl of quick master mix were incubated with 2 μg of plasmid DNA, 1-2 μg/μl of aprotinin and leupeptin, and 4 μl of [^{35}S]-methionine (either L-Methionine 500 μCi; GE-Amersham Biosciences, Buckinghamshire, UK, or Easytag Methionine-[^{35}S]; Perkin Elmer Life Sciences, Boston MA, USA) at 30 °C for 90 min. 1μl of RNase inhibitor (RNasin N251A; Promega, Madison WI, USA) was added just 5 min prior to terminating the reaction, as specified by the manufacturer's protocol. 5 μl of product was mixed with 10 μl of 3x sample buffer and boiled at 95 °C for 10 min and then separated by SDS page followed by autoradiography.

2.3.6 Solution binding assays

Solution binding assays were performed as previously described (Walter et al., 2006).

2.3.7 Immunoprecipitation assays

HeLa cell extracts either commercially available (HeLa (IP) cell lysate, sc-24785, Santa Cruz Biotechnology, Santa Cruz, CA) or from HeLa cells cultured in our laboratory were used. Cells were lysed using either RIPA buffer (sc-24948, Santa Cruz Biotechnology) or in 50 mM Tris-HCl, pH 7.2, 250 mM NaCl, 0.1% Igepal, 2 mM EDTA, 10% glycerol and protease inhibitor cocktail (Roche Diagnostics, Indianapolis IN, USA). The cell pellet was resuspended in 500 μl ice-cold lysis buffer and homogenised using a Dounce homogeniser. The lysate was then centrifuged at full speed using a tabletop centrifuge for 30 min. Next, the supernatant was pre-cleared by incubating with 20 μl of pre-equilibrated protein A-agarose (sc-2001, SantaCruz Biotechnology) for 1 hour at 4 °C rotating and the supernatant was subsequently incubated with 1μg of antibody and rotated for 2 hr at 4 °C. To immobilise the antibody, the mixture was incubated with 60 μl of protein A-agarose and rotating at 4 °C for 2 hours. The beads were washed 3-4 times with lysis buffer and 30 μl of 3x sample buffer was added to beads and boiled while mixing at the same time to elute bound fractions.

2.3.8 SDS-PAGE and immunoblotting

Protein samples were resolved by SDS-PAGE and either stained with Coomassie blue or transferred to PVDF membrane and probed with the appropriate antibodies and developed using the Western Lighting CDP-Star® Chemiluminescence Reagent (Tropix, Bedford MA, USA) and Fuji Super RX-film (FujiFilm Corporation, Tokyo, Japan).

2.4 Results

2.4.1 Nup153 interacts with both A- and B-type lamins *in vitro*

In previous reports it was demonstrated by blot overlay assays that residues 896-1475 of human Nup153 are binding exclusively to the *Xenopus* lamin LIII, but not to lamin A or emerin (Smythe et al., 2000). Immunoprecipitation of Nup153 from rabbit reticulocyte

lysate, however, co-precipitated both lamin B and lamin A (Smythe et al., 2000). To overcome these discrepancies, we performed solution-binding assays to more specifically analyse the interaction between Nup153 and nuclear lamins. To do so, we expressed distinct Nup153 fragments recombinantly in *E.coli* and human A- and B-type lamins by coupled *in vitro* transcription/translation using the rabbit reticulocyte lysate system. Recombinant GST-Nup153 fusion proteins were purified, immobilised on glutathione-sepharose beads and incubated with ^{35}S-labelled lamin. After incubation, bound and unbound protein fractions were analysed by SDS-PAGE. As shown in Figure 2.1A, GST-fusions of full-length human Nup153 (GST-Nup153), a N-terminal fragment expressing residues 2-339 (GST-N339), and a C-terminal fragment of *Xenopus* Nup153 (residues 1001-1382; GST-C1) bind to pre-lamin A (pre-LA), mature lamin A (LA) as well as lamin B1 (LB1). No binding of pre-LA, LA or LB1 was observed to GST alone, despite some non-specific background binding. To more precisely determine which domain of the lamins mediates the interaction with Nup153, we next produced the tail domains of lamin A and C (LAT and LCT, respectively) by coupled *in vitro* transcription/translation and analysed their ability to bind to our GST-Nup153 fusion proteins. As shown in Figure 2.1B, all Nup153 fragments bind to both LAT and LCT. Together these data indicate that the N-terminal as well as the C-terminal domain of Nup153 exhibits binding sites for the tail domain of both A- and B-type lamins.

2.4.2 Nup153 interacts with the Ig-fold of A- and B-type lamins

To further dissect the interactions of Nup153 with the tail domain of lamins, we examined the Ig-fold domain of the nuclear lamins for their potential to bind Nup153. The Ig-fold is embedded in the tail domain of lamins and is known to be involved in protein-protein interactions (Dechat et al., 2008). We employed ^{35}S-lamin fragments comprising the Ig-fold of lamins A, B1 and B2, respectively, and carried out solution binding assays using GST-Nup153 fusion proteins as described above. As shown in Figure 2.1C, the Ig-folds of LA, LB1 and LB2 bind to GST-Nup153, GST-N339 and GST-C1 fusion proteins, but not to GST alone, indicating that Nup153 specifically interacts with the Ig-fold of A- and B-type lamins. Similarly we found that Nup153 also interacts with *Xenopus* LIII, its tail and Ig-fold domain (Figure 2.2).

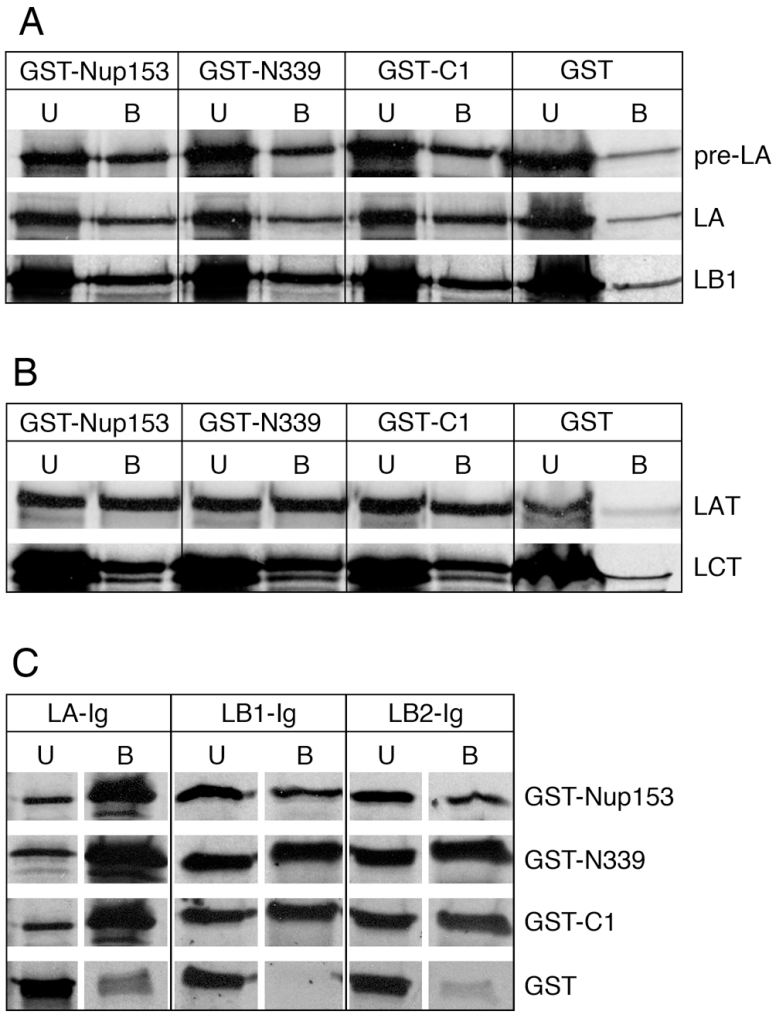

Figure 2.1: Nup153 binds directly with the Ig-fold of A- and B-type lamins *in vitro* - Solution binding assays were carried out using recombinant GST-Nup153 fragments comprising the full-length protein (GST-Nup153), a fragment of the N-terminal domain of human Nup153 (residues 2-339; GST-N339), a C-terminal domain fragment (residues 1001-1382 of *Xenopus* Nup153; GST-C1) and/or GST alone expressed in *E.coli*. Lamin fragments corresponding to (**A**) pre lamin A (pre-LA), mature lamin A (i.e. after cleavage of 18 amino acid residues at the end of the C-terminal domain; (LA)) and full length lamin B1 (LB1), (**B**) to the tail domain fragments corresponding to lamin A tail (LAT), lamin C tail (LCT), and (**C**) to the Ig-fold of lamin A (LA-Ig), lamin B1 (LB1-Ig), lamin B2 (LB2-Ig) were labelled with ^{35}S-methionine by coupled *in vitro* transcription and translation (see Experimental Procedures). GST-Nup153 fragments were immobilised on glutathione sepharose beads, incubated in solution with 35S-lamin fragments and unbound (U) and bound (B) fractions were resolved by SDS-PAGE and autoradiography.

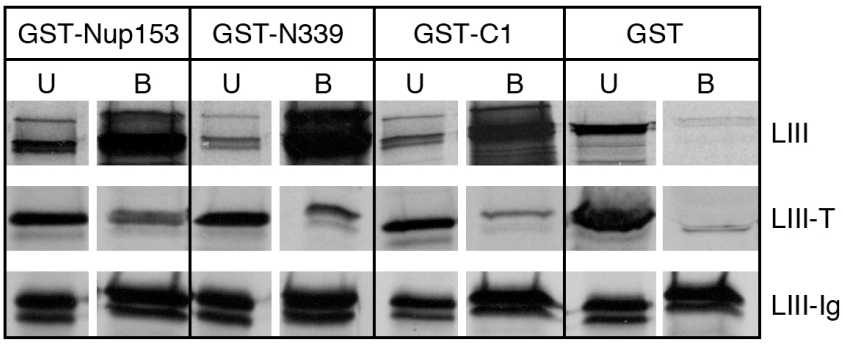

Figure 2.2: Nup153 binds to Xenopus LIII - Solution binding assays were carried out as summarised in Figure 2.1. GST-Nup153 fragments corresponding to the full length (Nup153), the N-terminal domain (N339), C-terminal domain (C1), or GST alone were expressed in *E.coli*. Purified fragments incubated with *Xenopus* LIII fragments labelled with ^{35}S-methionine by coupled transcription and translation (see Section 2.3) corresponding to the full length (LIII), the tail domain (LIII-T), the Ig-fold (LIII-Ig). GST-Nup153 fragments were immobilised with glutathione sepharose beads and then incubated in solution with ^{35}S-lamin fragments and unbound (U) and bound (B) fractions were eluted by the addition of sample buffer and resolved by SDS-PAGE and autoradiography.

2.4.3 The N-terminal and C-terminal domains of Nup153 form distinct complexes with nuclear lamins

Nup153 interacts with importin-β via its C-terminal FG-repeat domain (Moroianu et al., 1995; Shah et al., 1998) and lamins in turn are thought to be imported via the importin-α/β pathway (Adam et al., 2008; Loewinger and McKeon, 1988). The observed complexes between Nup153 and the lamins might therefore resemble a nuclear import complex that eventually would include importin-α and -β. Therefore we next analysed the association of importin-β with the Nup153-lamin complexes. To do so, the bound fractions of our solution binding assays were loaded onto SDS-PAGE, transferred to a PVDF membrane and probed with anti-importin-β antibodies. We found that importin-β is present in the complexes of the C-terminal domain of Nup153 with lamin A and lamin B, but is absent from complexes between lamins and the N-terminal domain of Nup153 (Figure 2.3A). These data indicate that the N-terminal domain of Nup153 binds directly to both A- and B-type lamins, whereas complexes between the C-terminal domain of Nup153 and lamins are at least heterotrimeric. Thereafter we analysed whether the heterotrimeric complex

of the C-terminal fragment of Nup153, lamins and importin-β resembles a nuclear import complex. If so, the complex would (i) depend on the presence of a NLS in lamin, (ii) contain importin-α that typically mediates the interaction between the cargo (i.e. lamin) and importin-β, and (iii) become dissociated by RanGTP. Alternatively, Nup153 may bind to lamin and importin β simultaneously via its FG-repeat domain. To distinguish between the two possibilities, we first performed solution-binding assays with the C-terminal domain of Nup153 and the Ig-fold of lamins A and B1. The Ig-fold is lacking the NLS and is therefore not able to bind to an importin-α/β complex. While importin-α and -β are detectable in the reticulocyte lysate (control), importin-β, but not importin-α, co-assembled with the complexes of Nup153-C and the Ig-fold of lamin A and lamin B1, respectively (Figure 2.3B). This indicates that importin-β is binding to the FG-repeats of Nup153 and not to either of the lamins, mediates by importin-α as part of an import complex. Similarly, importin-α is absent from complexes of Nup153-C with full-length LA or LB1 (data not shown). To confirm the validity of the results, we asked whether the heterotrimeric Nup153-C-lamin-importin-β complex gets dissociated in the presence of RanGTP. Complexes between importin-α/β and their cargo are dissociated by the binding of RanGTP to importin-β, resulting in decreased affinity of importin-α to the NLS (Kobe, 1999). We therefore employed RanQ69L-GTP, a constitutively active Ran mutant (Palacios et al., 1996), to our solution binding assay. As shown in Figure 2.3C, excess amount of RanQ69L-GTP did neither release the complex between the N-terminal or the C-terminal domain of Nup153 and lamins (Figure 2.3C). This provides further evidence that the complex between the C-terminal domain of Nup153 and the nuclear lamins does not resemble a nuclear protein import complex (see also (Smythe et al., 2000)). Together our data indicate that the C-terminal domain of Nup153, despite the presence of importin-β, is able to directly interact with the Ig-fold of A- and B-type lamins, analogue to the N-terminal domain.

2.4.4 Nup153 co-immunoprecipitated lamin A and lamin B from HeLa cell extracts

To validate our *in vitro* data, we examined whether Nup153 and lamins can be co-immunoprecipitated from HeLa cell extracts. To precipitate Nup153, a monoclonal antibody against the N-terminus (7A8; IP Nup153-N) or the C-terminus (SA1; IP Nup153-C) of human Nup153 were used. Immunoprecipitates were analysed for the presence of nuclear lamins

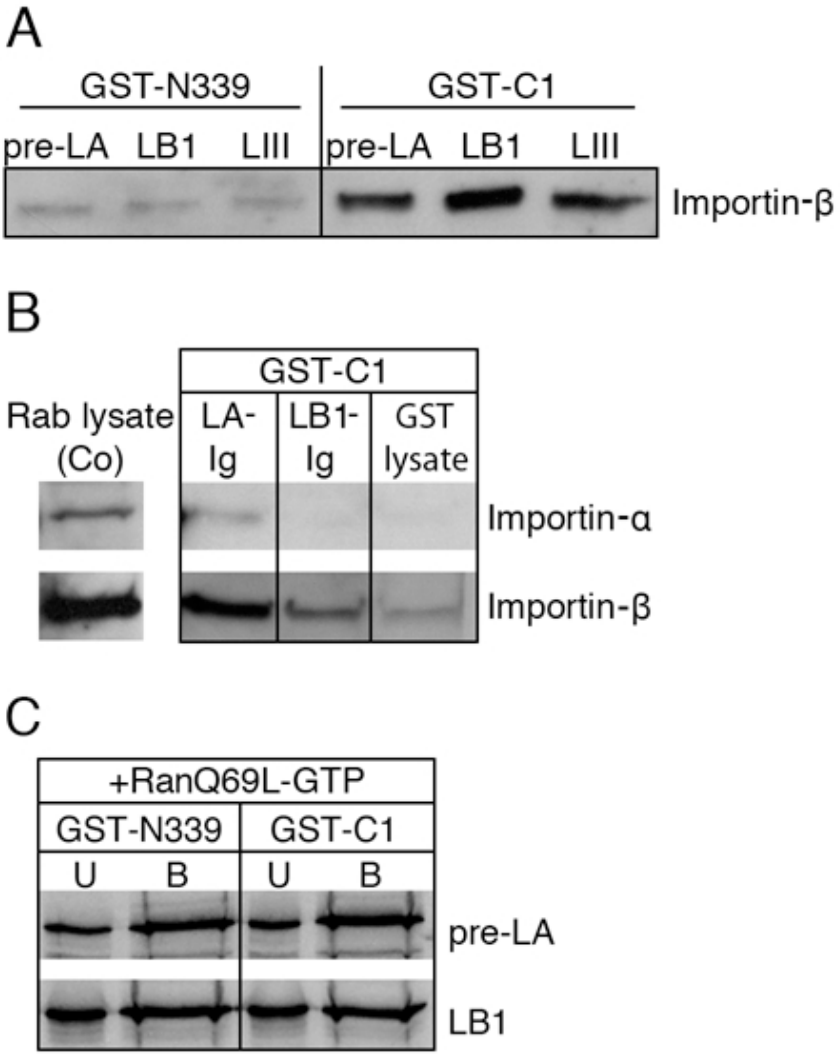

Figure 2.3: Nup153 association with lamins is not part of an import complex - (**A**) Solution binding assays were carried out using GST-Nup153 fragments corresponding to the N-terminal domain (GST-N339) or the C-terminal fragment of the *Xenopus* Nup153 (GST-C1) expressed in *E.coli*. ^{35}S-lamin fragments produced *in vitro* by transcription and translation (see Section 2.3) corresponding to pre-LA, LB1 and *Xenopus* LIII. GST-Nup153 fragments were immobilised on glutathione sepharose beads, incubated in solution with 35S-lamin fragments and unbound and bound fractions were resolved by SDS-PAGE and immunoblotted with an antibody that recognises human importin-β. Importin-β is co-assembling with the complex between Nup153-C and lamin Ig-folds, but not with the complex of Nup153-N and lamins. (**B**) Importin-α is not detectable in the complex between Nup153-C and the nuclear lamins. (**C**) RanQ69L-GTP does not dissociate the Nup153-C-lamin complex. ^{35}S-lamin fragments were first incubated with 20 μg RanQ69L-GTP for 10 min at 37°C prior to solution binding assay, then incubated with GST-Nup153 fragments, immobilised on glutathione sepharose beads, and unbound (U) and bound fractions (B) resolved by SDS-PAGE and subjected to autoradiography.

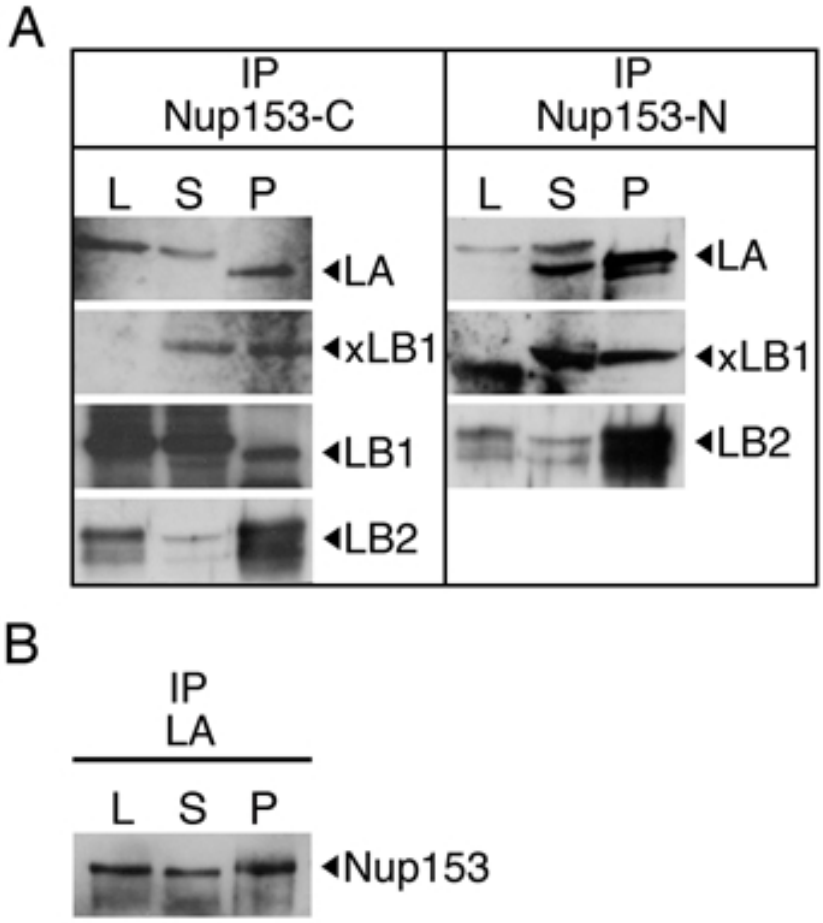

Figure 2.4: Co-immunoprecipitation of Nup153 and nuclear lamins from HeLa extracts - (**A**) Total HeLa extracts were immunoprecipitated using anti-Nup153 antibodies that recognises the C-terminal domain (SA1; Nup153-C) or the N-terminal domain of Nup153 (7A8; Nup153-N). Equivalent amounts of HeLa extracts (L), immune supernatants (S) and immune precipitate (P) were separated by SDS-PAGE and analysed by immunoblotting using anti-lamin A (LA), *Xenopus* (xLB1) or human (LB1) lamin B1 and lamin B2 (LB2) antibodies. (**B**) Immunoblotting using anti-Nup153 verified co-precipitation with lamin A.

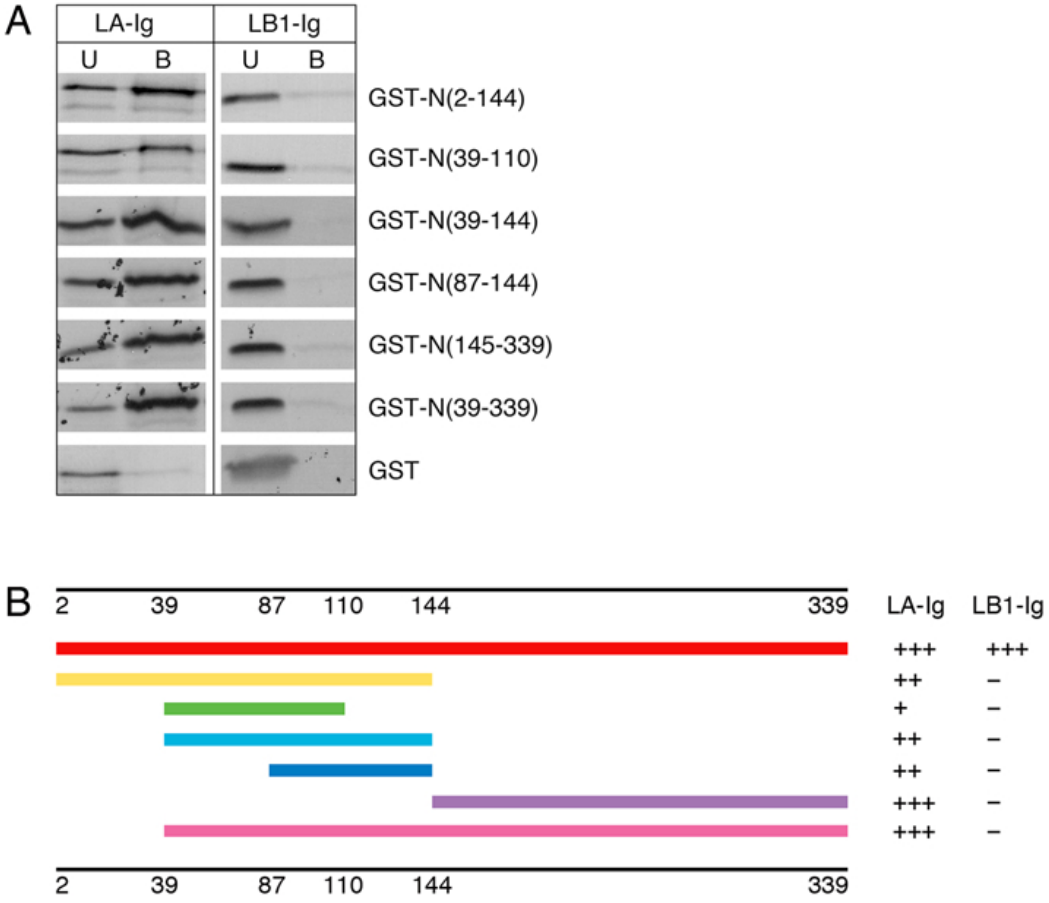

Figure 2.5: Nup153 binding to the Ig-fold of lamin A and lamin B1 requires distinct residues - (**A**) Solution binding assays were carried out using recombinant GST-Nup153 fragments comprising truncations of its N-terminal domain between residues 2-339, i.e. GST-N(2-144), GST-N(39-110), GST-N(39-144), GST-N(87-144), GST-N(145-339), GST-N(39-339) and GST alone expressed in *E.coli* as and lamin fragments comprising the Ig-fold of lamin A (LA-Ig) and lamin B1 (LB1-Ig) labelled with ^{35}S-methionine by coupled *in vitro* transcription and translation (see Section 2.3). GST-Nup153 fragments were immobilised on glutathione sepharose beads, incubated with lamin fragments, and unbound (U) and bound (B) fractions eluted by the addition of sample buffer and resolved by SDS-PAGE and autoradiography. (**B**) Schematic representation of the residues between 2-339 of human Nup153 that are required for a sufficient binding to the lamin A and the lamin B1 Ig-fold, respectively. +++, strongest binding; ++ strong binding; +, moderate binding; -, no binding.

by immunoblotting and we found that both antibodies against Nup153 co-precipitated lamin A, B1 and B2 (Figure 2.4A). Using the same procedure, antibodies raised against both human and *Xenopus* lamin A were found to co-precipitate Nup153 (Figure 2.4B), whereas lamin B1 and B2 did not co-immunoprecipitated Nup153, most likely due to the low solubility of B-type lamins. Taken together, our data show that Nup153 is bound to lamin A and lamin B in HeLa cell extracts.

2.4.5 Binding of the Ig-fold of lamins occurs at a specific region at the N-terminal domain of Nup153

We next aimed to further define the interaction between the N-terminal domain of Nup153 and the Ig-fold. To do so, we employed smaller fragments of the N-terminal domain of Nup153, specifically residues 2-339 (GST-N339; Figure 2.1C), to map the amino acid residues involved in the interaction site with the lamin Ig-folds. Nup153-fragments fused to GST comprising residues 2-144, 39-110, 39-144, 87-144, 145-339, and 39-339, respectively, were expressed in *E.coli*, immobilised on glutathione sepharose, and incubated with Ig-fold of lamin A and lamin B1 produced by coupled *in vitro* transcription and translation. Unbound (U) and bound (B) fractions were resolved by SDS-PAGE and subjected to autoradiography. We found that the lamin A Ig- fold bind to all Nup153 N-terminal truncations with strongest binding to residues 39-339 (GST-N(39-339); Figure 2.5A and 2.5B), and weakest binding to residues 39-110 (GST-N(39-110); Figure 2.5A and 2.5B). We conclude that multiple residues are contributing to the association of the N-terminus of Nup153 with the Ig-fold to nuclear lamins. The Ig-fold of lamin B1 as well binds to the N-terminal domain of Nup153 within residues 2-339 (Figure 2.1C), however in contrast to lamin A it did not interact with any of the above listed shorter fragments of Nup153 (Figure 2.5A). This demonstrates that residues 2-39 of human Nup153 are critical but not sufficient for binding of Nup153 to the Ig-fold of lamin B1.

2.5 Discussion

Previous studies have shown that Nup153 interacts with lamin B while the exact binding specificity had remained unclear. Here we further investigated the binding of Nup153 with lamins and characterised the domains that are involved in this interaction. Our data demonstrate that both the N- and the -C terminal domains of Nup153 bind to the Ig-fold

of A- and B-type lamins. The association of lamins with Nup153 appears to be direct, notwithstanding the presence of importin-β in complexes of the C-terminal domain of Nup153 and the Ig-fold of lamins. A closer examination of the interface between the N-terminal domain of Nup153 and the Ig-fold of lamin B1 revealed that residues 2-339 are essential for binding. In contrast, the lamin A Ig-fold exhibits multiple interaction sites within residues 39-339 of Nup153, i.e. the NPC association region (Bastos et al., 1996b; Enarson et al., 1998). These different binding premises indicate functional differences of the complexes between Nup153 and A- and B-type lamins, respectively.

The existence of distinct complexes between Nup153 and nuclear lamins can be explained by (i) the domain topology of Nup153 in the NPC and (ii) the multiple functions associated with both Nup153 and the nuclear lamins. Nup153 resides at the nuclear basket of the NPC with the N-terminal domain localising to the nuclear ring moiety of the basket (Fahrenkrog et al., 2002; Pante et al., 2000; Walther et al., 2001). The nuclear ring moiety is in close proximity to the inner side of the NE where the majority of the nuclear lamins reside. Nup153 recruitment to the NE and NPCs depends on an intact nuclear lamina (Smythe et al., 2000) and it is required for an even distribution of NPCs in the NE (Walther et al., 2001). Lamins on the other hand are also critical for NPC positioning in the NE, indicating that an interaction between Nup153 and the lamins is important to hold NPCs in place. Given the close proximity of the N-terminal domain of Nup153 and the nuclear lamina it is most likely the complex between the N-terminus of Nup153 and the Ig-fold of nuclear lamins functions in order to maintain a stable NE architecture.

The C-terminal domain of Nup153 contains multiple FG-repeats, which resemble natively unfolded structures that are mainly involved in nucleocytoplasmic transport (Shah et al., 1998). Nup153 is known to have high affinity binding site for importin-β (Ben-Efraim and Gerace, 2001) and lamins are in turn thought to be imported into the nucleus by an importin-α/β-dependent manner (Adam et al., 2008; Loewinger and McKeon, 1988), which implicated that the complex between Nup153 and the nuclear lamins may reflect the presence of both proteins in an import complex. Several lines of evidence presented here suggest that this is not the case. First, in solution binding assays importin-β was detected in complexes between the C-terminal domain of Nup153 and the nuclear lamins, but not so importin-α (Figure 2.3A and 2.3B). Secondly, Nup153 and the nuclear lamins remained associated in the presence of RanQ69L-GTP, which typically dissociates nuclear import complexes. Third, Nup153 associates with the Ig-fold of A-and B-type lamins, while

the NLS of lamins is located a few residues upstream of the Ig-fold. Together these data suggest that the observed association between the C-terminal domain of Nup153 and the nuclear lamins is functionally different a nuclear import complex, consistent with previous findings (Smythe et al., 2000).

Our data demonstrate that both the N-terminal and the C-terminal domain of Nup153 interact with the nuclear lamins. This gives rise to the question, whether two different complexes exist. Further studies are required to fully resolve this question, but two scenarios are plausible. The C-terminal domain of Nup153 is highly flexible and can shuffle between the nucleoplasmic and the cytoplasmic face of the NPC (Fahrenkrog et al., 2002; Paulillo et al., 2005). Therefore it is conceivable that the C-terminus of Nup153 folds back onto an intermediate complex between Nup153 and the respective lamin at the NE. This could help stabilising the complex and/or regulating the accessibility of both proteins for other binding partner. Alternatively, multiple populations of Nup153 may exist in the NPC or the nucleoplasm (Griffis et al., 2004), which may include different complexes between Nup153 and nuclear lamins. This is in particular interesting, as lamin A, but not lamin B, is present in a minor soluble pool within the nucleoplasm, which has been shown to participate in various nuclear processes (Dorner et al., 2007). It remains to be seen if Nup153 is able to associate with this soluble lamin A pool. The existence of multiple Nup153-lamin complexes with differing functions is further supported by our finding that lamin A binds to different sites within the N-terminal domain of Nup153 than lamin B. The Ig-fold of lamin A displayed multiple binding within residues 39-339 of human Nup153, while the lamin B1-Ig fold did not bind to fragments shorter than residues 2-339 (Figure 2.5, A and B).

As a summary, our data provide evidence for a complex interplay between Nup153 and A-type as well as B-type lamins, which appears far more sophisticated than previously assumed. Future studies will be required to expand our current understanding of the interactions of Nup153 with nuclear lamins, in particular regarding their importance in the context of lamin A-associated disease, the so-called laminopathies. Previous studies have indicated that Nup153 co-aggregated with lamin A in the presence of lamin A mutation causing HGPS, which led to clustering of NPCs (Goldman et al., 2004; Hubner et al., 2006b). Moreover, in particular the N-terminal domain of Nup153 was detected in some of these aggregations (Hubner et al., 2006a). Hence, more studies will be needed to examine

the role of Nup153 interactions with nuclear lamins in disease associated mutations of lamin A.

3

Characterisation of nuclear lamin binding to the nucleoporin Nup153 in the presence of lamin mutations

Teiba Al-Haboubi and Birthe Fahrenkrog

M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland

3.1 Abstract

Recently, we showed that the Ig-fold of A-, and B-type lamins interacts directly with the nucleoporin Nup153 and we mapped multiple binding sites within residues 39 to 339 of the N-terminal domain of Nup153 (Nup153-N) that are required for the interaction. Here, we further analysed the effect of altered expression of either lamin A or B on Nup153 binding and localisation. We carried out solution binding assays, immunofluorescence microscopy and co-immunoprecipitation. Our *in vitro* binding assays revealed that the interaction between Nup153 and lamin A is altered when two point mutations, R453W and R482W, respectively, were introduced into the Ig-fold of lamin A. Furthermore, a deletion of 50 residues at the C-terminal end of lamin A tail domain (LAΔ50) did not interfere with binding *in vitro*. Furthermore, Nup153 localisation depicts normal punctuated staining at the nuclear rim and more overlapping with nucleoplasmic lamin A in HeLa cells expressing wild-type or R453W and is not recruited into R482W intranuclear foci. Faint or lack of Nup153 staining is seen in perturbed cells expressing LAΔ50. Furthermore, Nup153 co-aggregates with lamin B in HeLa cells transiently expressing B-type lamin-GFP fusion constructs, but not with a mutant of lamin B1. Conversely, lamins are not recruited to Nup153 aggregates in cells expressing GFP-Nup153. Furthermore, GFP antibodies co-precipitate more Nup153 from HeLa cells transfected with GFP-lamin A and the R453W mutant than with the R482W or LAΔ50 mutants. Our results suggest distinct mutations in lamin can manipulate Nup153 binding to a certain extent, the degree of which can be related to the severity of the mutation-linked diseases.

3.2 Introduction

Nuclear lamins are the main components of the nuclear lamina; a filamentous meshwork residing at the nuclear periphery of the inner nuclear membrane (INM) of the nuclear envelope (NE) of vertebrate cells (Aebi et al., 1986). Lamins belong to type V intermediate filament proteins (IFs), and similar to all IFs, the lamin molecule has a tripartite structure with a large α-helical coiled coil rod domain flanked by two non-helical N- and C-terminal domains (Fisher et al., 1986). The rod domain contains an additional 42 residues not found in the cytoplasmic IFs of vertebrates (Monteiro et al., 1994). Compared to cytoplasmic IFs, the C-terminal domain of lamins contains three unique features; an nuclear

localization signal (NLS) for nuclear targeting, an Ig-fold like motif which is highly conserved among lamins and engaged in protein and chromatin interactions, and a CAAX-box, which undergoes post-translational modifications and constitutes a site for the addition of a farnesyl lipid group (Heitlinger et al., 1992; Mical and Monteiro, 1998; Ralle et al., 2004). In mammalian cells, lamins are divided into two groups; A-type and B-type lamins (Schirmer and Gerace, 2004). A-type lamins are encoded by a single gene *LMNA*, which by alternative splicing, gives rise to two major A-type lamins in somatic cells; lamin A and lamin C, respectively (Burke and Stewart, 2006). B-type lamins are encoded by two individual genes *LMNB1* and *LMNB2* giving rise to lamin B1 and lamin B2, respectively in somatic cells (Stuurman et al., 1998; Vorburger et al., 1989). While B-type lamins are ubiquitously expressed in all cell types, A-type lamins are only found in differentiated cells (Gerace and Burke, 1988). Biochemically, B-type lamins are permanently farnesylated at the CAAX domain, whereas, lamin A is only transiently farnesylated and loses its farnesyl characteristic once incorporated into the nuclear lamina (Mical and Monteiro, 1998; Rusinol and Sinensky, 2006). Nuclear lamins are implicated in nucleus-related functions including maintaining nuclear architecture, ordering the spacing between nuclear pore complexes (NPCs), regulating transcription, controlling differentiation and chromatin organisation (Dechat et al., 2008; Mattout et al., 2006; Shimi et al., 2008). Consequently, dysfunction of lamins is often associated with human diseases. To date, more than 200 mutations have been identified in *LMNA*, which cause heterogeneous disorders, termed laminopathies (Gruenbaum et al., 2005; Stewart et al., 2007; Worman and Bonne, 2007). Laminopathies can either affect striated muscle, such as in autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD), dilated cardiomypathy (DCM), and limb girdle muscular dystrophy (LMG1B) (Bonne et al., 1999). Or they can affect adipose tissue, such as in Dunnigan-type familial partial lipodystrophy (FPLD) and mandibuloacral dysplasia (MAD) (Mounkes et al., 2003). Moreover, mutation in *LMNA* can lead to premature aging syndromes, such as Hutchinson-Gilford progeria syndrome (HGPS) and atypical Werner's syndrome (Eriksson et al., 2003). In contrast, mutations in *LMNB* are thought to be embryonic lethal and only two mutations in *LMNB2* were identified recently in patients with acquired partial lipodystrophy (APL) (Capanni et al., 2003), as well as duplications in *LMNB1* which result in autosomal dominant leukodystrophy (ADLD) (Capell and Collins, 2006; Hegele et al., 2006; Padiath et al., 2006). More than 20% of the LMNA mutations are allocated to the Ig-fold in the tail domain. Two such mutations by which an arginine

(R) is mutated to a tryptophan (W) at codons 453 and 482, result in AD-EDMD and FPLD, respectively (Cao and Hegele, 2000; Speckman et al., 2000). R453 resides at the core of a β-sheet of the Ig-fold where it is involved in a salt bridge formation, whereas residue R482 resides at the core of the Ig-fold. When mutated, R453 destabilised the overall structure of the C-terminal domain of the lamin molecule, while R482 mutation affect binding sites to lamin A-binding proteins (Dhe-Paganon et al., 2002; Krimm et al., 2002). The most frequent mutation resulting in HGPS is a *de novo* C to T substitution at position 1824 of *LMNA*, which results in a protein that lacks 50 amino acids at the C-terminal domain, called LAΔ50 or progerin. Progerin is permanently farnesylated and irreversibly anchored to the nuclear membrane, thereby disrupting normal lamin function and localisation (Scaffidi and Misteli, 2005) (Amati et al., 2004; D'Apice et al., 2004; Eriksson et al., 2003). Within the NE, lamins interact with nuclear pore complexes (NPCs) that mediate the passage of molecules in and out of the nucleus. NPCs are made up of multiple copies of 30 different proteins, termed nucleoporins, one of which is Nup153. Nup153 is a highly dynamic glycoprotein comprised three distinct domains; an N-terminal domain (residues 1-610) required for its incorporation into the NPC and the NE stationary (Bastos et al., 1996; Enarson et al., 1998; Nakielny et al., 1999), a central zinc-finger domain (residues 650-880) as well as a C-terminal domain (residues 881-1475) implicated in nucleocytoplasmic transport (Ball and Ullman, 2005). Nup153 is long known to bind to B-type lamins (Smythe et al., 2000), and that Nup153 distribution is often affected in cells expressing lamin mutations (Bechert et al., 2003; Goldman et al., 2004; Hubner et al., 2006a; Matitioli et al., 2008; Morris, 2001; Muchir et al., 2003; Pan et al., 2007; Raharjo et al., 2001; Sagelius et al., 2008a; Sagelius et al., 2008b; Shackleton et al., 2000; Sullivan et al., 1999). We have previously showed that Nup153 exhibits binding sites for the Ig-fold of A- and B-type lamins in its N- and C-terminal domain (Al-Haboubi et al., submitted). Here we analysed the effect of alteration in lamin A and lamin B1, respectively, on the interaction with Nup153 by means of biochemistry, immunofluorescence microscopy and co-immunoprecipitation. Our *in vitro* binding assays reveal that mutations in the Ig-fold causing AD-EDMD and FPLD disrupt binding specificity of lamin A to Nup153. Immunoprecipitation assays further supported this in vitro data. Furthermore, we show that Nup153 is co-localising with lamin A in cells expressing wild-type and AD-EDMD mutation, but it its localisation is not affected in FPLD expressing cells. Furthermore, Nup153 is re-distributed in cells expressing lamin A carry-

ing HGPS mutation. Our findings suggest that mutation specific defects in lamin-Nup153 interactions associated with lamin related diseases.

3.3 Experimental Procedures

All experimental procedures were carried out at room temperature, unless otherwise stated.

3.3.1 DNA constructs

a) Lamin expression constructs and mutagenesis pEGFP-lamin A, pEGFP-lamin B1 and pET-lamin III were obtained from R.D. Goldman (Northwestern University, Chicago) and pEYFP-lamin B2, pET-lamin A/$delta$50 and pEGFP-lamin AΔ50 were generous gift of H Harald Herrmann (German Cancer Research Center, DKFZ, Heidelberg). pcDNA-lamin A-Ig (Ilona Huegi, former MSc-student). Site directed mutagenesis were carried out following manufacturer's protocol for single amino acid substitution using Pfu Ultra HF; High fidelity DNA polymerase (Stratagene, La Jolla CA, USA). A single amino acid from lysine (R) to a tryptophane (W) i) R453W in pEGFP-lamin A and pcDNA-Lamin A Ig using the following primers (5'-GAG GGC AAG TTT GTC TGG CTG CGC AAC AAC TCC-3' and 3'-GGA GTT GTT GCG CAG CCA GAC AAA CTT GCC CTC-5') ii) R482W in pEGFP-Human lamin A and pcDNA-Lamin A Ig using the following primers (5'-CCC TTG CTG ACT TAC TGG TTC CCA CCA AAG TTC-3' and 3'-GAA CTT TGG TGG GAA CCA GTA AGT CAG CAA GGG-5') iii) CAIM to SAIM; A cysteine (C) change to serine (S) in pEGFP-Lamin B1, using the following primers (5'-TCC AAT AGA AGC TCA GCA ATT ATG TAA TCT AGA G-3' and 3'-CTC TAG ATT ACA TAA TTG CTG AGC TTC TAT TGG A-5'). b) Nup153 expression constructs pGEX-4T-HA-Nup153-His and pGEX 4T-HA-Nup153-N (residues 3-600) are as described previously (Al-Haboubi et al., submitted). pGEX-6P-C-hNup153 (residues 874-1475) was previously described (Lim et al., 2006), pGEX-C1-Nup153 (Xenopus residues 1001-1385) and pET-C2-Nup153 (Xenopus residues 1375-1603), were obtained from Katie Ullman (Fahrenkrog et al., 2002).

3.3.2 Cell culture and transfection

HeLa cells were maintained in Dulbecco's modified Eagle medium (Gibco/Invitrogen, Life Technologies Corporation, CA), supplemented with 10% FBS (PAA), 100-units/ml penicillin/ 100 μg/ml streptomycin (Gibco/Invitrogen) and 2 mM glutamine (Gibco/Invitrogen).

HeLa cells were grown in a monolayer in a humidified 37 °C incubator supplied with 5% CO_2. Cells seeded in either 6- or 24-well plates one day prior to transfection 30% density. Cells were transiently transfected using either TurboFect (Fermentas Life Sciences, St. Leon-Rot, Germany) or Lipofectamine2000 (Invitrogen Life Sciences, CA), following manufacturer's protocol. Briefly 1 μg of DNA and 2 μl of transfection reagent were used and cells were incubated for 24-48 hours.

3.3.3 Immunofluorescence microscopy

HeLa cells grown on glass coverslips were fixed and stained as described earlier (Spector and Smith, 1986). Briefly, cells were fixed in 2% paraformaldehyde for 15 min, followed by three steps of washing in 1xPBS with 5 min intervals. Fixed cells were permeabilised in PBS containing 0.2% Triton X-100 and 1% BSA for 10 min on ice. Cells were then washed three times in PBS containing 1% BSA and incubated with primary and secondary antibodies for 1 hr, respectively. After washing 3 times in PBS, cells were mounted on glass slides using Mowiol. The nuclei were stained with blue-fluorescent DAPI. Images were recorded on a Leica TCS SP5 microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) using 100x or 63x oil objectives and processed using the Leica imaging software, ImageJ 1.41o and Adobe Photoshop CS4.

3.3.4 Antibodies

The following antibodies were used in Western blot analysis and immunofluorescence labelling, respectively. Mouse monoclonal SA1 (1:50 dilution; a gift of Brian Burke, University of Calgary, Calgary, Alberta, Canada, (Bodoor et al., 1999)) against the C-terminus of human Nup153. Rabbit polyclonal anti-lamin B1 (ab16048; 1:3000; Abcam, Cambridge, MA, USA) recognises an epitope within residues 400-500. A rabbit polyclonal (no. 324, gift of R.D. Goldman, (Dechat et al., 2007)) against human lamin A peptide cVTVTRSYRSVGGSG, which corresponds to residues 620-633; (1:1000). A rabbit polyclonal human lamin B2 antibody (no. 328, gift of R.D. Goldman) against a specific peptide cTVKKSSVMRENENG, which corresponds to residues 576-589 (1:1000). All secondary antibodies for immunoblotting coupled to alkaline phosphatase were obtained from Sigma-Aldrich. A monoclonal antibody, mAb414 recognises FG repeat-containing Nups, predominantly Nup62 but also Nup153 (Davis and Blobel, 1986). Two mouse monoclonal GFP antibodies

(1:1000, MAB0043, Dianova GmbH, Heidelberg, Germany), and (118144600001, 1:1000, Roche Diagnostics, Indianapolis IN, USA) were used in immunoprecipitation assays. Indirect immunofluorescence was visualized using red-orangeÐfluorescent Alexa Fluor® 568 goat antiÐmouse IgG antibody (A11004, Invitrogen Molecular Probes, CA) and far-red-fluorescent Alexa Fluor® 647 goat anti rabbit IgG (A21245, Invitrogen Molecular Probes, CA).

3.3.5 Recombinant protein expression and purification of glutathione S-transferase (GST) coupled Nup153 fragments

GST-fusion proteins of Nup153 were expressed in *E.coli* BL21-DE3 codon (+) cells. A 5ml over night culture was used to inoculate 1-3 litres of LB medium and grown at 37°C until an optical density at 600 nm of 0.6 was reached. Protein expression was next induced by the addition of 0.1 mM IPTG and cells were grown in culture for 3 hours at 37°C and shaking at 180 rpm. Bacterial pellets were collected by centrifugation at 5000 rpm for 10 min at 4°C (SLA3000, Sorvall). The pellet was resuspended in phosphate buffered saline (PBS) containing 0.5 mM PMSF, 5.0 μg/ml aprotinin and 5.0 μg/ml leupeptin, lysed by sonication and cleared centrifugation at 20,000 g for 45 min at 4°C. The supernatant was next incubated with glutathione sepharose-4B (GE Healthcare Biosciences, Uppsala, Sweden) for 2 hours while shaking, transferred onto a poly-prep chromatography column (Bio-Rad laboratories, CA), washed by the addition of PBS (binding buffer) 3-4 volumes and eluted using elution buffer (20 mM glutathione (reduced form, AppliChem, Darmstadt, Germany), 100 mM Tris pH 8.0, 120 mM NaCl, 10 mM DDT). In a second purification step, proteins were loaded on a nickel column (Ni-NTA agarose, Qiagen GmbH, Hilden, Germany), washed and eluted using 500 mM Imidazole, collected fractions were dialysed against PBS and stored in aliquots at -80°C.

3.3.6 Coupled *in vitro* transcription and translation

Coupled *in vitro* transcription and translation of proteins was performed using the TNT® quick- translation system kit (Promega, Madison, WI, USA) following the manufacturer's protocol. In brief, 40 μl of quick master mix were incubated with 2 μg of plasmid DNA, 1-2 μg/μl of aprotinin and leupeptin, and 4 μl of [^{35}S]-methionine (either L-Methionine 500 μCi; GE-Amersham Biosciences, Buckinghamshire, UK, or Easytag Methionine-[^{35}S];

Perkin Elmer Life Sciences, Boston MA, USA) at 30 °C for 90 min. 1μl of RNase inhibitor (RNasin N251A; Promega, Madison WI, USA) was added just 5 min prior to terminating the reaction, as specified by the manufacturer's protocol. 5 μl of product was mixed with 10 μl of 3x sample buffer and boiled at 95 °C for 10 min and then separated by SDS page followed by autoradiography.

3.3.7 Blot overlay assays

His-tagged lamin fragments were expressed and purified as previously described (Shumaker et al., 2005). Equal amount of lamin fragments were then resolved by SDS-PAGE, transferred onto a PVDF membrane and blocked for 1 hr in PBS containing 0.1% Tween-20 and 5% milk. Next, the membranes were incubated at 4 °C overnight with [^{35}S]-Nup153 fragments produced using a coupled transcription and translation system, washed in PBS containing 0.1% Tween-20, air-dried and subjected to autoradiography.

3.3.8 Solution binding assays

Solution binding assays were performed as previously described (Walter et al., 2006).

3.3.9 Immunoprecipitation assays

HeLa cell extracts were prepared from cultured cells grown in 6-well plates (approximately 95% confluence) post transfection with GFP-coupled lamin constructs. Cells were lysed in 500 μl ice-cold RIPA buffer (sc-24948, Santa Cruz Biotechnology) and homogenised with 20 strokes in a Dounce homogeniser. The lysate was then centrifuged at full speed using a tabletop centrifuge for 30 min. Next, the supernatant was pre-cleared by incubating with 20 μl of pre-equilibrated protein A-agarose (sc-2001, SantaCruz Biotechnology) for 1 hour at 4 °C rotating and the supernatant was subsequently incubated with 1μg of GFP-antibody and rotated for 2 hr at 4 °C. To immobilise the antibody, the mixture was incubated with 60 μl of protein A-agarose and rotating at 4 °C for 2 hours. The beads were washed 3-4 times with lysis buffer and 30 μl of 3x sample buffer was added to beads and boiled while mixing at the same time to elute bound fractions.

3.3.10 SDS-PAGE and immunoblotting

Protein samples were resolved by SDS-PAGE and either stained with Coomassie blue or transferred to PVDF membrane and probed with the appropriate antibodies and developed using the Western Lighting CDP-Star® Chemiluminescence Reagent (Tropix, Bedford MA, USA) and Fuji Super RX-film (FujiFilm Corporation, Tokyo, Japan).

3.4 Results

3.4.1 Binding of Nup153 with the Ig-fold is diminished in the presence of EDMD-causing mutant R453W *in vitro*

By solution binding assays we have recently shown Nup153 interacts with the Ig-fold of lamins A, B1, B2 and *Xenopus* LIII (Al-Haboubi et al., submitted). To further analyse the interaction between Nup153 and the Ig-fold of lamins we introduced a mutation in the Ig-fold of lamin LIII and carried out blot-overlay assays. We mutated R454 to W by site-directed mutagenesis (Shumaker et al., 2005). The corresponding mutation in lamin A (R453W) is associated with EDMD (Krimm et al., 2002). We employed [^{35}S]-methionine labelled Nup153 fragments corresponding to the full N-terminal domain (^{35}S-hNup153N), residues 2-339 N339 (^{35}S-hNup153N339), and one fragment corresponding to the C-terminal domain of Xenopus Nup153 ((^{35}S-xNup153C), residues 1375-1603)). Equal amounts of full-length human lamin A, Xenopus LIII, LIII-Ig, and LIII-IgR454W fragments were separated by SDS-PAGE 3.1A, blotted on a PVDF membrane and incubated overnight at 4 °C with ^{35}S-Nup153 fragments. The membrane was then air-dried and subjected to autoradiography. Consistent with our previous studies (Al-Haboubi et al., submitted), we observed binding of full-length Nup153 to LA and to LIII (see Figure 3.1B). This interaction appears enhanced to the Ig-fold of LIII, but is weakened in the R454W mutant 3.1B. Similarly, the N-terminal domain of Nup153 binds to LA, LIII and enhanced to the Ig-fold with of LIII 3.1C. This interaction of the Ig-fold with Nup153-N was not affected by the R454W mutation 3.1C, in contrast to full-length Nup153. The C-terminal domain of Nup153, as shown in Figure 3.1D, binds to hLA and even stronger to LIII and the LIII-Ig-fold, whereas the interaction appears lost in the presence of the R454W mutation. Together these data indicate different binding sites of the N-terminal and C-terminal domain of Nup153 for the Ig-fold of nuclear lamins.

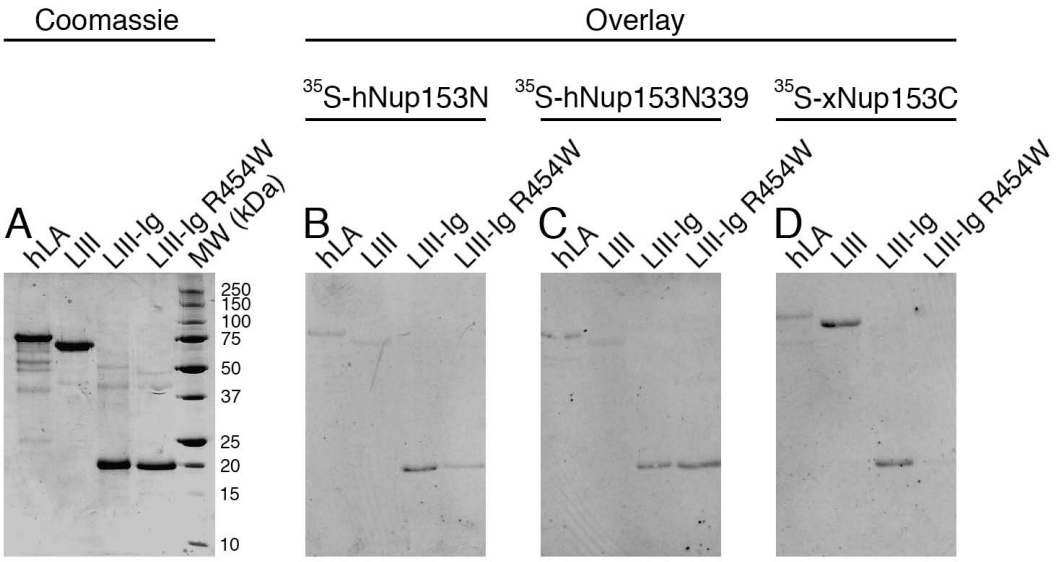

Figure 3.1: Nup153 binds weaker to the Ig-fold carrying the AD-EDMD mutation R453W - Blot overlay assays were carried out as described in Experimental Procedure. (**A**) Full-length human lamin A, full-length *Xenopus* LIII, the LIII Ig-fold (LIII-Ig), and the Ig-fold carrying AD-EDMD mutation R454W (LIII-IgR454W) fragments were separated by SDS-PAGE and incubated with ^{35}S-Nup153 fragments produced by *in vitro* coupled transcription and translation. The membrane was incubated with ^{35}S-Nup153 fragments corresponding to either (**B**) the human full N-terminal domain (^{35}S-hNup153N, residues 2-601), (**C**) the human N-terminal fragment between residues 3-339 (^{35}S-hNup153N339) or (**D**) the *Xenopus* C-terminal fragment residues (1375-1603) (^{35}S-xNup153C). Post incubation, the blots were air dried and subjected to autoradiography.

3.4.2 Nup153 interaction with the Ig-fold mutants of human lamin A

We next carried out solution binding assays to study the effect of the corresponding R453W mutation in human lamin A and the ability to bind Nup153. We recombinantly expressed N- and C- terminal fragments of human Nup153 fused to a GST motif in *E.coli*, and we expressed human lamin A Ig-fold fragments by a coupled *in vitro* transcription/translation system using rabbit reticulocyte lysate. Recombinant GST-Nup153 fusion proteins were purified, immobilised on glutathione-sepharose beads and incubated with ^{35}S-labelled lamins. Bound and unbound protein fractions were analysed by SDS-PAGE and subjected to autoradiography. As shown in Figure 3.2A, GST-fusions comprising an N-terminal fragment of human Nup153 (residues 2-339; GST-N339), and the C-terminal domain of human Nup153 (residues 874-1475; GST-hC) interact with the Ig-fold of wild-type lamin A (LA-Ig). Consistent with our blot overlay data, we observed that the R453W mutant binds to the N-terminal domain of Nup153, but weaker binding to the C-terminus (Figure 3.2B). Similarly, when we mutated R482 to W, a mutation related to FPLD (Hegele et al., 2000), the binding of the C-terminal domain of Nup153 was weaker as compared to wild-type LA the AD-EDMD mutant, whereas the association with the N-terminal domain was not affected by this mutation (Figure 3.2C). No banding of either wild-type or mutant Ig-fold fragments to GST alone was observed. Together these data support that binding of the N-terminal and C-terminal domain of to lamins involves distinct residues in the Ig-fold.

3.4.3 The N-terminal sub-fragments of Nup153 between residues 3-339 reveal specific interaction with the mutant containing Ig-fold

We next aimed to more precisely analyse which residues in the N-terminal domain of Nup153 are implicated in the interaction with the Ig-fold of human LA and subsequent effect of the Ig-fold mutants on binding. To do so, we employed sub-fragments of the N-terminal domain of human Nup153 fused to GST comprising residues 2-144, 39-110, 39-144, 87-144, 145-339, and 39-339, respectively. These were immobilised on glutathione sepharose, and incubated with the Ig-fold of lamin A mutants produced by coupled *in vitro* transcription/translation using rabbit reticulocyte lysate. Unbound (U) and bound (B) fractions were resolved by SDS-PAGE and subjected to autoradiography. As shown in Figure 3.3B, the wild-type LA Ig-fold binds strongly to Nup153 fragments 39-339, 39-144, 87-144, 145-339 and weaker to residues 2-144 and 39-110. The R453W mutant binds

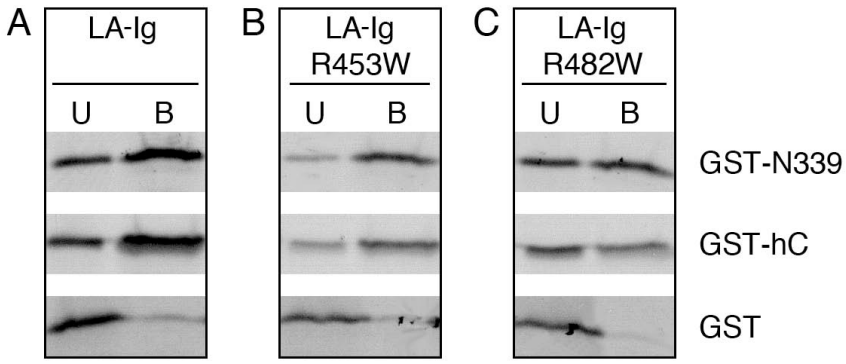

Figure 3.2: Nup153 binds to the Ig-fold mutants of lamin A - (**A**) Solution-binding assays were performed using recombinant GST-Nup153 fragments comprising a fragment of the N-terminal domain of human Nup153 (residues 2-339; GST-N339) and the C-terminal domain of human Nup153 (residues 874-1475; GST-hC). The Ig-fold of lamin A; (**A**) wild-type (LA-Ig), (**B**) the AD-EDMD mutant, (**C**) R453W (LA-IgR453W) and the FPLD mutant, R482W (LA-IgR482W) were labelled with ^{35}S-methionine. GST-Nup153 fragments were immobilised on glutathione-sepharose beads, incubated in solution with ^{35}S-lamin fragments and unbound (U) and bound (B) fractions were resolved by SDS-PAGE and autoradiography.

strongly to 39-144 and shows weaker interaction to all other N-terminal truncations of Nup153 (Figure 3.3B). In contrast, the R482W mutant exhibits strong binding to residues 39-339, 87-144, 145-339, but only weak binding to 2-144, 38-110 and 39-144 (Figure 3.3C). A mutation in the tail domain downstream of the Ig-fold (LAΔ50) does not affect binding of the Nup153 fragments to lamin A (Figure 3.3D). Together these data indicate that the Ig-fold of LA exhibits multiple binding sites for the N-terminal domain of Nup153, with residues 39-87 being critical for binding to R453 and residues 2-39 for interaction with R482.

3.4.4 Expression of distinct GFP-lamin A constructs in HeLa cells

After our biochemical characterisation of the interaction between Nup153 and lamin A, we aimed to study the interplay between Nup153 and nuclear lamins in a cellular contex. To do so, we performed transient transfections in HeLa cells. We expressed, GFP-lamin A, GFP-lamin A R453W, GFP-lamin A R482W and GFP-lamin AΔ50 in HeLa cells and cells were fixed and stained with appropriate antibodies 24-48 hour post transfection. As shown in

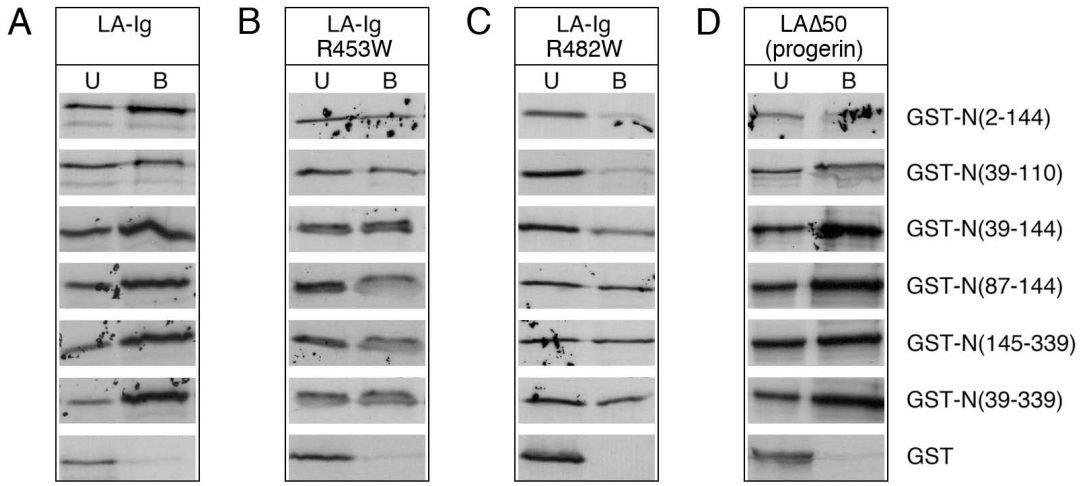

Figure 3.3: FPLD mutant in the Ig-fold destroys the binding site with Nup153 - Solution binding assays were carried out using recombinant GST-Nup153 sub-fragments of the N-terminal domain within residues 2-339, i.e. GST-N (2-144), GST-N (39-110), GST-N (39-144), GST-N (87-144), GST-N (145-339), GST-N (39-339) and GST alone. GST-Nup153 fragments were immobilised on glutathione-sepharose beads and incubated with fragments comprising the Ig-fold of lamin A; (**A**) wild-type (LA-Ig), (**B**) AD-EDMD mutant (LA-IgR453W), (**C**) FPLD mutant (LA-IgR482W) and (**D**) the HGPS causing mutant (LAΔ50, progerin) labelled with ^{35}S-methionine. Unbound (U) and bound (B) fractions were eluted by the addition of sample buffer and resolved by SDS-PAGE and autoradiography.

Figure 3.4A, expression of GFP-LA did not alter Nup153 localisation at nuclear pore complex and also lamin B1 localisation remained unchanged as compared to untransfected cells. Similarly, expression of GFP-LAR453W in HeLa cells led to the formation of intranuclear aggregates and nuclear lobulation (Figure 3.4C). While Nup153 did not co-localise with the aggregates of GFP-LAR482W, it was absent from nuclear blebs. Similarly, other FG-repeat nucleoporins as detected by the antibody mAb414, lamin B2 as well as endogenous LA did not co-localise with the R482W aggregates, although more lamin A was detected in the nucleoplasm. Together these data indicate that mutations in the Ig-fold of lamin A did not extensively alter the localisation of Nup153 in the context of HeLa cells.

3.4.5 Expression of lamin AΔ50 in HeLa cells disrupts the localisation of Nup153

We next expressed GFP-lamin AΔ50 in HeLa cells and as shown in Figure 3.4D, 24 h post transfection cells expressing lamin AΔ50 have misshapen nuclei and show speckle like nucleoplasmic aggregates of lamin A. Nup153 staining is partially co-localising with some of these aggregates and is absent from nuclear blebs (Figure 3.4D). In addition 48 hour post transfection, Nup153 staining is mislocalised and endogenous lamin A staining is missing in cells expressing lamin AΔ50. FG-nucleoporins detected by mAb414 and lamin B1 localisation were not affected in transfected cells. Our data indicate that the localisation of Nup153 is particularly altered in the presence of lamin AΔ50 mutant in HeLa cells.

3.4.6 HeLa cells expressing lamin B variants show overlapping regions with Nup153

Next we studied the effect of altered B-type lamin expression on Nup153 localisation in HeLa cells. We expressed wild-type human lamin B1 and lamin B2 fused to GFP and YFP, respectively, or *Xenopus* LIII in HeLa cells and cells were fixed and stained with appropriate antibodies 24-48 hour post transfection. As illustrated in Figure 3.5A, cells overexpressing any B-type lamin have misshapen nuclei including aggregation and nuclear blebbing of lamin B. Nup153 co-aggregates with lamin B and is also recruited in nuclear blebs (Figure 3.5A). Moreover, Nup153 is absent from the nuclear rim in cells overexpressing lamin B2. Staining with the mAb414, revealed that other FG-nucleoporins partially co-localise with lamin B1 aggregates (Figure 3.5A). Next, we introduced a mutation in the lamin B1

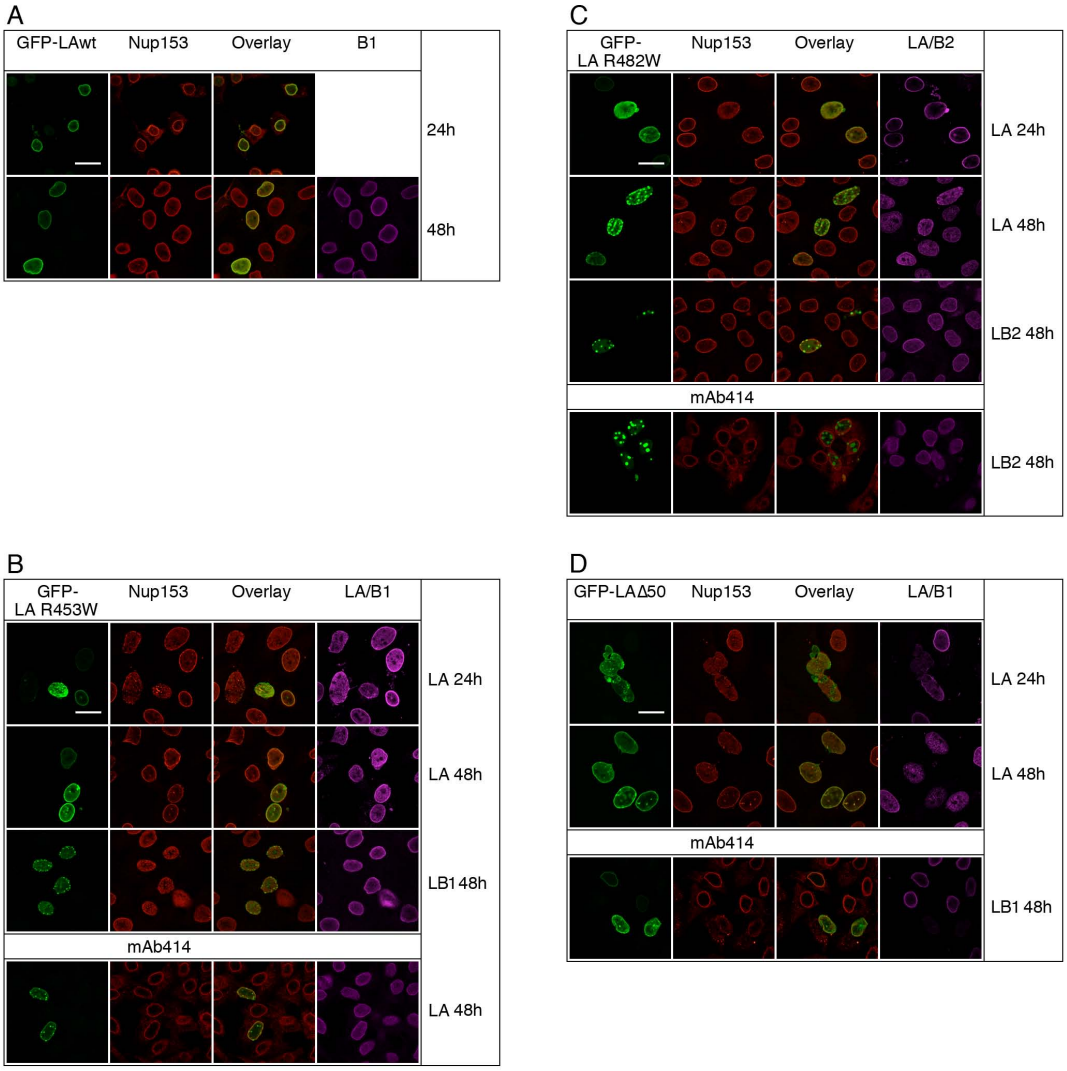

Figure 3.4: Nuclear Nup153 localisation in cells expressing GFP-lamin A with Ig-fold and tail domain mutant - Confocal images of HeLa nuclei transiently expressing wild-type GFP-human lamin A and mutations (*green*). (**A**) GFP-lamin A wild-type, (**B**) GFP-lamin A with R453W mutation (GFP-R453W), (**C**) GFP-lamin A with R482W (GFP-R482W), and (**D**) GFP-lamin A with a deletion of 50 residues at the C-terminal domain (GFP-LAΔ50). 24-48 post transfection, cells were fixed and co-stained with appropriate antibodies against Nup153, mAb414 (*red*), lamin A (LA), B1 (LB1) and B2 (LB2) (*magenta*). Scale bar, 20 μm.

CAAX-box motif (i.e. CAIM) by substituting the cysteine (C) by a serine (S). This mutation prevents the lipid modification of lamin B1 and its incorporation into the nuclear lamina as well as lamin B1 accumulation into intranuclear aggregates in cells transfected with this mutation (Mical and Monteiro, 1998). We transiently expressed GFP-LB1 construct with this mutation (GFP-LB1 SAIM) in HeLa cells and examined the influence of this mutant on the localisation of Nup153, mAb414 and lamins. Figure 3.5B shows that expression of the CAAX-box mutant causes nuclear abnormalities and butterfly- shaped nuclei, lamin B1 aggregation in the nucleoplasm and its absence from the nuclear rim. Nup153 and mAb414 exhibit minor co-localisation with lamin B1-SAIM aggregates and showed largely normal rim staining in these cells 24 h and 48 h post transfection. Endogenous lamin B1 co-localises with the lamin B1-SAIM aggregates. These data indicate that Nup153 binding to lamin B1 requires an intact CAAX-box motif.

3.4.7 Lamins are not recruited to nuclear foci in HeLa overexpressing human Nup153

We have previously shown that overexpression of GFP-Nup153 in HeLa cells leads to its accumulation in intranuclear foci (Lussi et al., submitted). Hence, we next transiently overexpressed Nup153 in HeLa cells and examined for the presence of nuclear lamins in Nup153 aggregates. Figure 3.6 shows that Nup153 when overexpressed accumulates in foci in nucleoplasmic aggregates, however, these aggregates do not contain lamin A or lamin B1. Moreover, some nuclei expressing GFP-Nup153 show slight nuclear blebbing and lamin B1 is not recruited to these nuclear blebs (Figure 3.6). These data indicate that Nup153 only associates with membrane-bound nuclear lamins and that soluble Nup153 does not disrupt an intact nuclear lamina.

3.4.8 A- and B-type lamins fused to GFP do not bind equally to Nup153 in HeLa cells

Finally, we examined whether Nup153 and lamins over-expressed in HeLa cells can be co-immunoprecipitated from cell extracts. Cells were first transfected with GFP-lamin A constructs, with wild-type GFP-LA as well as R453W, R482W and LAΔ50 constructs, respectively. 24-48 hr post transfection, cells were harvested and lysed. A monoclonal antibody against GFP was used to precipitate the GFP-LA fusion proteins. The immunoprecipitates were analysed for the presence of Nup153 by immunoblotting and it was found

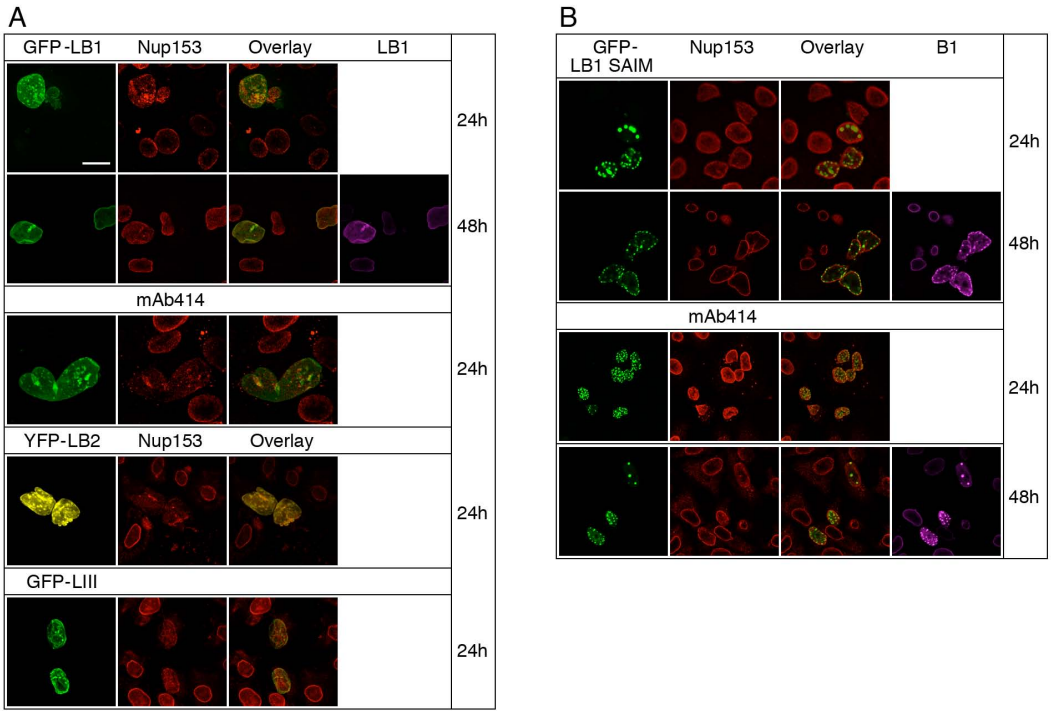

Figure 3.5: Nup153 co-aggregates with GFP-lamin B variants - (**A**) Confocal images of HeLa cells transiently expressing GFP-human lamin B and *Xenopus* LIII constructs (*green*). (**A**) GFP-human lamin B1 (GFP LB1), YFP-human lamin B2 (YFP LB2, *yellow*), or GFP-Xenopus LIII (GFP LIII). (**B**) GFP-lamin B1 with an amino acid substitution in the CAAX box domain, SAIM (GFP LB1 SAIM). 24-48 h post transfection, cells were fixed and co-stained with appropriate antibodies against Nup153, mAb414 (*red*), lamin A (LA), B1 (LB1) and B2 (LB2) (*magenta*). Scale bar, 20 μm.

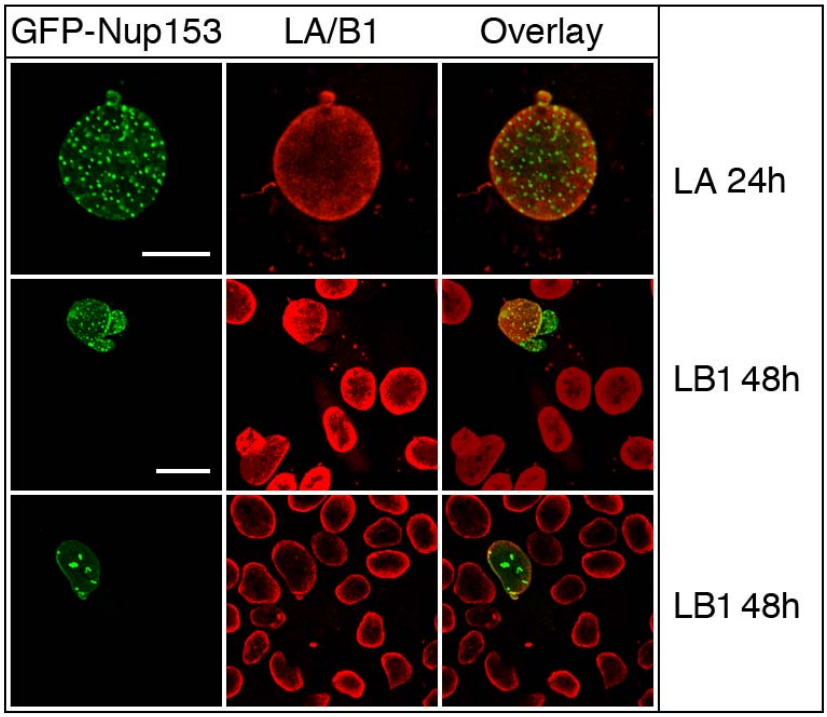

Figure 3.6: Distribution of A- and B-type lamins in HeLa nuclei overexpressing Nup153 - HeLa cells transfected with GFP-human Nup153 (GFP-Nup153) (*green*) were fixed 24-48 h post transfection and were co-stained with antibodies against lamin A (LA), lamin B1 (LB1) or mAb414 (*red*). Scale bar: top left, 10 μm; middle left, 20 μm.

that Nup153 co-precipitated with GFP-LA, GFP-LA(R453W) and GFP-LA(R482W), but not with GFP-LAΔ50 (Figure 3.7). When GFP-lamin B1, lamin B1-SAIM, lamin B2 and LIII were expressed in HeLa cells and immunoprecipitated, neither amounts were found to co-precipitate Nup153, consistent with previous data (Al-Haboubi et al., submitted).

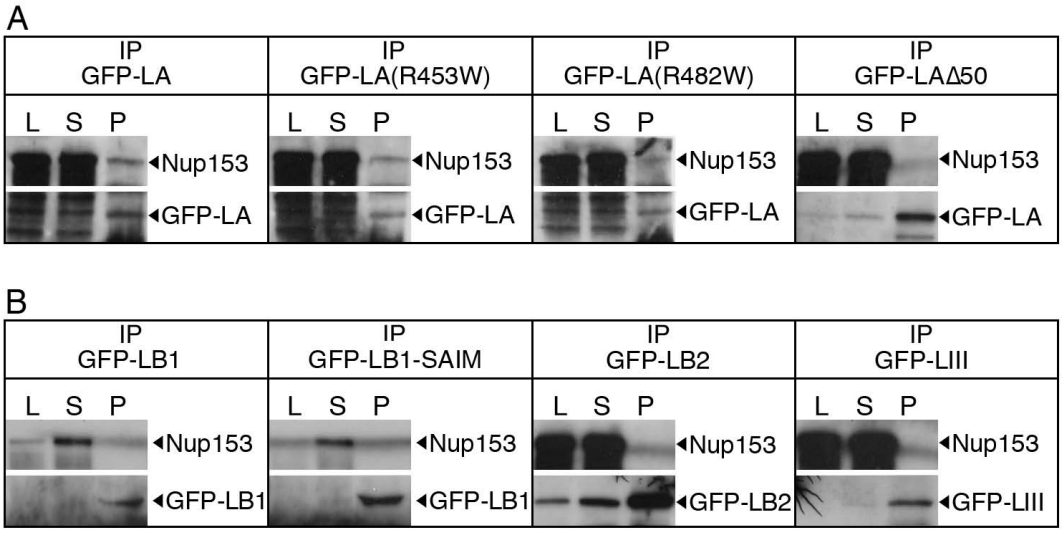

Figure 3.7: Co-immunoprecipitation of Nup153 from HeLa transfected with lamins - (**A**) Wild-type GFP-lamin A (GFP-LA) and mutants; R453W (GFP-LA(R453W)), R482W (GFP-LA(R482W)), and lamin AΔ50 (GFP-LAΔ50), overexpressed in HeLa were immunoprecipitated using anti-GFP antibodies. Equivalent amounts of HeLa extracts (L), immune supernatants (S) and immune precipitate (P) were separated by SDS-PAGE and analysed by immunoblotting using anti-Nup153 that recognise the C-terminal domain (SA1; Nup153), anti-lamin A (LA) and anti-GFP antibodies, respectively. (**B**) Immunoblots of GFP fused fragments containing human; lamin B1 (GFP-LB1), B1-SAIM (GFP-LB1-SAIM), B2 (GFP-LB2) and the *Xenopus* LIII (GFP-LIII) overexpressed in HeLa and analysed by immunoblotting using anti-Nup153 and anti-GFP antibodies, respectively.

3.5 Discussion

Our recent data showed that Nup153 interacts with lamin A and lamin B both *in vitro* from solution binding assays and from immunoprecipitates of HeLa cell extracts. Mutually the N-and the C-terminal domains of Nup153 were found to bind specifically to the Ig-fold domain of the lamins. In addition we mapped the interaction at a specific site in the NPAR

of the N-terminal domain of Nup153 between residues 39-339 to be involved in binding to the Ig-fold of lamin A. Our findings led us to predict a role for Nup153 binding to lamin A which could be relevant in lamin disease related mutations. Here we examined this interaction in the presence of three mutations in lamin A. Our binding assays reveal that the strength of interaction between Nup153 and the Ig-fold is altered in the presence of Ig-fold related mutations R453W and R482W, respectively, nut not disrupted (see Figure 3.1, 3.2B and 3.2C). Nevertheless, our data document that Nup153 binding to lamins is perturbed in the presence of mutations in the Ig-fold. We show that overall both mutants of the Ig-fold R453W and R482W do not lose their tendency to bind to the fragments comprising the residues 2-339 and the C-terminal fragments of Nup153 *in vitro* (Figure 3.2B and 3.2C). However, it became more obvious when sub-fragment of the N-terminal binding domain of Nup153 were analysed for binding specificity that the FPLD mutant R482W interfered dramatically with the Ig-fold binding strength with Nup153. Very weak to no binding is prominent when Nup153 fragment comprising residues 39-144 was tested (Figure 3.3C). This is the binding site that presented a strong binding with the Ig-fold of wild-type lamin A, which is in agreement with our recent binding data (Al-Haboubi et al., submitted). This implicates that the binding site influenced by the R482W mutation is critical for binding with Nup153. While the R453W mutation that was shown to affect the overall lamin molecule structure (Krimm et al., 2002) retained some binding with Nup153 from our solution binding assays. In addition, our data show that a deletion of 50 residues in the tail domain of lamin A causing HPGS (LAΔ50) did not disrupt the binding between lamin A and Nup153 *in vitro* (Figure 3.3D). Moreover, LAΔ50 binding with Nup153 demonstrated a similar pattern seen with Ig-fold of wild-type lamin A when distinct Nup153 sub-fragments of the N-terminal binding domain were examined for binding, i.e. strong binding within residues 39-144 and 39-339, respectively. We further analysed the interaction between lamin A and Nup153 *in vivo* using HeLa cells transiently expressing lamin A mutants. Clearly, cells expressing AD-EDMD (R453W) and FPLD (482) mutations show accumulation of lamin A into intranuclear foci (see Figure 3.4B and 3.4C). Cells transiently expressing R453W show some lamin A aggregates with minor re-distribution of Nup153 in mutated cells (Figure 3.4B), whereas cells expressing R482W showed an increase of nuclear aggregates and some blebbing to which Nup153 is not recruited. Furthermore, cells transiently expressing lamin AΔ50 displayed misshapen nuclei with nuclear blebs and some lobulation (Figure 3.4D). Staining of Nup153 in these nuclei was either very faint to no rim staining as well as

some gaps or hole-like regions in Nup153 localisation (Figure 3.4D). Our cell based studies showed that Nup153 is mislocalised in cells overexpressing the HGPS causing mutant, lamin AΔ50 which is supported by earlier finding where Nup153 was mislocalised and co-aggregated with lamin A in the presence of lamin A mutation (Goldman et al., 2004; Hubner et al., 2006b), although both proteins still interact *in vitro* (see Figure 3.3D).

Furthermore, R482W immunoprecipitated a lesser amount of Nup153 than R453W or wild-type lamin A which suggest a stronger binding of Nup153 with the Ig-fold at that particular site (i.e. residue 482). Our immunoprecipitation assays revealed binding of Nup153 to lamin AΔ50, again in contrast to our *in vitro* studies (Figure 3.3D).

When analysing the interaction between Nup153 and B-type lamins, co-localisation of Nup153 with lamin B in cells transiently expressing human lamin B1, B2 and the *Xenopus* LIII was found (Figure 3.5A). In particular, Nup153 is present in nuclear blebs seen in cells overexpressing lamin B1 (Figure 3.5A). We then questioned whether removal of the membrane tethering domain of lamin B could influence its nuclear lamina localisation and further re-distribute Nup153 into intanuclear foci. Cells expressing lamin B1 with a mutation at the CAAX-box motif (lamin B1-SAIM) show huge intranuclear foci but Nup153 is not associated with these aggregates (Figure 3.5B). We also did not detect Nup153 in lamin B1-SAIM immunoprecipitates, and our data show no Nup153 detected in the bound as well as when GFP lamin B1 wild-type and lamin B2. Our data presented here show that the association of the Ig-fold of lamin A with Nup153 is perturbed in the presence of two missense mutations in the Ig-fold. A mutation responsible for AD-EDMD showed weaker binding but overall the interaction is preserved. Whereas a mutation causing FPLD showed loss of binding with Nup153 both *in vitro* and in cells expressing the mutation, indicating the importance of this site for binding to Nup153. The interaction of Nup153 with lamin A is unaffected in the presence of LAΔ50 *in vitro* further supporting a direct interaction of Nup153 with nuclear lamins to be with the Ig-fold. Our data implicate the importance of a mutation in the Ig-fold that is responsible for FPLD as a mutation at this site interferes dramatically with binding to Nup153 and to a lesser extent a mutation causing AD-EDMD. The exact influence of Nup153 on the disease severity and phenotype connected with lamin A mutations upon binding to lamins is yet to be defined.

4

Characterisation of the assembly of the *Xenopus* oocytes lamin LIII *in vitro*

Teiba Al-Haboubi and Ueli Aebi

M.E. Mueller Institute for Structural Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland;

4.1 Abstract

LIII is the major B-type lamin of the *Xenopus* oocytes, which was previously demonstrated to form 10 nm IF-like filament structures in extracted nuclear envelopes. *In vitro*, LIII was shown to form paracrystal like assemblies, however, defining earlier steps of ultrastructures from LIII self-assembly are not yet determined. Here, we characterised assembly conditions of LIII *in vitro*. We first expressed and purified recombinant LIII in *E. coli*, followed by ultra-structural studies of LIII assembly pattern by electron microscopy (EM) using glycerol spraying/low-angle rotary metal shadowing and negative staining. LIII samples were dialysed against different assembly buffer conditions to study the effect of varied pH and ionic strength on lamin assembly. Our EM of glycerol sprayed/rotary metal shadowed samples show that at high salt (300 mM NaCl), high pH (8.6), LIII forms dimers. These dimers comprise a long tail measuring ∼52 nm and two globular heads at one end. The tail represents two lamin α-helical coiled-coil rod domains and the globular head represents the C-terminal domain, predominantly the globular Ig-fold structure. Furthermore, LIII dimers associate longitudinally into polar head-to-tail polymers at 300 mM NaCl, pH 6.5, which tend to associate laterally in a partially staggered fashion, when the NaCl concentration is lowered to 100 mM. In addition, LIII dimers form paracrystal-like structures with ∼24 nm axial repeats at pH 6.5 in the absence of $CaCl_2$. More tightly packed paracrystal repeats were seen when $CaCl_2$ was added at pH 8.6 revealing axial repeats of ∼17 nm length. Moreover, we reveal that LIII head-to-tail polymers are disrupted in the presence of Nudel, an LIII-interacting protein, which may play a role in recruiting LIII to microtubules, and subsequently in spindle matrix specific LIII assembly.

4.2 Introduction

The nuclear lamina is a filamentous meshwork comprising mainly the nuclear lamins as well as lamin-associated polypeptides at the nuclear periphery of all metazoan cells. Nuclear lamins are type V intermediate filament proteins (IFs) and like all IFs, lamins have a tripartite domain organisation, comprising a central α-helical coiled-coil rod domain flanked by two non-helical short N- terminal (head) domain and a longer C- terminal (tail) domain (Parry et al., 1987). The long rod domain consists of four coiled-coil heptad repeat

segments; 1A, 1B, 2A and 2B separated by three non-helical linkers; L1, L12 and L2, respectively (Heins and Aebi, 1994). The N- and C-terminal end segments of the rod domain are highly conserved among all IFs and influence lamin assembly into higher order structures (Fraser and MacRae, 1985; Stuurman et al., 1998). Additionally, the rod domain of lamins contains an extra six heptad repeats (42 residues) in segment 1B, which are shared by the rod domain of invertebrate IFs, but not present in the cytoplamsic IFs (Herrmann and Aebi, 2004; Weber et al., 1989). The tail domain is characterised by distinct domains that are found only in lamins but not in cytoplasmic IFs: i) a nuclear localisation signal (NLS), ii) an immunoglobulin-like motif (Ig-fold) domain and iii) a -CAAX box domain that undergoes multiple posttranslational modifications (Kitten and Nigg, 1991; Monteiro et al., 1994; Ralle et al., 2004). Lamins have multiple phophorylation sites located at the rod domain, which have several roles in the assembly and disassembly of lamins from the lamina during NE breakdown at the onset of mitosis (Heald and McKeon, 1990; Moir et al., 1995; Nigg, 1992; Peter et al., 1991). Lamins are divided into two major types, A- and B-type lamins, which can be distinguished by their expression pattern, amino acid sequence and biochemical properties. A-type lamins are splicing products encoded by a single gene, *LMNA* which give rise to lamin A and lamin C in somatic cells, as well as lamin AΔ10 and lamin C2 in germ-line specific cells, respectively (Alsheimer and Benavente, 1996; Furukawa et al., 1994). A-type lamins are only found in differentiated cells, they have a neutral iso-electric point, and are soluble during mitosis (Fisher et al., 1986; Furukawa et al., 1994; Gerace and Burke, 1988). B-type lamins are encoded by two separate genes, *LMNB1* and *LMNB2*, giving rise to lamin B1 and B2 in somatic cells as well as lamin B3 and B4 in sperm cells, respectively (Furukawa and Hotta, 1993; Lin and Worman, 1995; Lourim et al., 1996; Vorburger et al., 1989). B-type lamins are present in all cell types, have an acidic isoelectric point and remain membrane-bound during mitosis (Georgatos et al., 1994). Nuclear lamins of the *Xenopus* oocytes form 10 nm diameter IF-like filaments, which are organised in a lattice meshwork of near tetragonally oriented filaments (Aebi et al., 1986). The *Xenopus* oocytes contain one major unique B-type lamin termed LIII or LB3 sharing a closer sequence homology with lamin A and primary sequence similarities with invertebrate IF genes as well (Doring and Stick, 1990; Stick, 1988; Stuurman et al., 1998). Two isoforms are found in *Xenopus* oocytes, which differ by 12 residues at the C-terminal domain in their CAAX-encoding exons. LIIIb is a minor variant that carries an extra cysteine and a cluster of six basic residues in proximity of the CAAX-box domain

(Doring and Stick, 1990). Other lamins in the *Xenopus* include three somatic lamins LI, LII and LA equivalent to the mammalian lamin B1, B2 and A, respectively as well as a minor germ-line specific lamin LIV (Benavente et al., 1985; Stick and Hausen, 1985). Characterisation of the self-assembly properties of lamins have been extensively studied *in vitro* using transmission electron microscopy (EM) using recombinant lamin expressed in bacteria. *In vitro*, lamin assembly begins with lamin monomers association in their rod domain in a parallel unstaggered fashion giving rise to two-stranded α-helical coiled-coil dimers, measuring $\sim$50 nm long in the rod domain and flanked by two globular heads at the C-terminal domain (Heitlinger et al., 1991; Heitlinger et al., 1992). Lamin dimers elongate longitudinally head-to-tail forming $\sim$2 nm wide head-to-tail polymers with an axial repeat of 48-50 nm, thus differing from cytoplasmic IFs that form lateral association into anti-parallel unstaggered or half staggered tetramers (Heins and Aebi, 1994; Heitlinger et al., 1991). Eventually, lamins form filamentous bundles and paracrystalline arrays, assemblies that overextend laterally with a diameter of more than 13 nm and 24-25 nm axial repeats (Heitlinger et al., 1991; Stuurman et al., 1998). Unlike cytoplasmic IFs which form filaments *in vitro*, only *C.elegans* lamin B has been form filaments in vitro that are composed of 3 and 4 tetrameric protofilaments, each of which contains two partially staggered anti-parallel head-to-tail polymers (Ben-Harush et al., 2009; Karabinos et al., 2003). In addition, the tunicate *C. intestinalis* lamin also assembles into filaments, but with a smaller average diameter of 5.4 nm (Karabinos et al., 2003). Paracrystal- like structures are also seen when *Xenopus* lamin A or *Drosophila* lamin C are expressed in insect cells, whereas *Xenopus* LI and *Drosophila* Dm0, a B-type lamin, assemble into filamentous fibrous in insect cells (Klapper et al., 1997). In addition, mutations studies have show that the head and tail domains of lamins play major roles in the assembly steps of the polypeptides. Headless chicken lamin A and lamin B2 do not form head-to-tail assemblies, whereas a tailless mutant forms long filaments and enhanced paracrystalline fibers assembly with normal axial repeats (Heitlinger et al., 1992). Tailless lamin *Xenopus* lamin A, C. elegans lamin B, or the *Drosophila* lamin Dm0, on the other hand, form filament bundles lacking the 24-nm axial repeats seen in normal paracrystals, and tailless human lamin C forms 15-30 nm filaments instead of paracrystals (Ben-Harush et al., 2009; Gieffers and Krohne, 1991; Izumi et al., 2000; Moir et al., 1991; Sasse et al., 1998). These studies demonstrated that these mutations have a dominant negative effect when expressed in mammalian cells, leading to aggregation of the endogenously expressed lamin isoforms (Izumi et al., 2000; Moir et

al., 2000; Spann et al., 1997). Other studies have shown that the Ig-fold motif as well as the tail domain of the *Xenopus* LIII is sufficient to inhibit lamin polymerisation in vitro and nuclear assembly in *Xenopus* egg extracts (Lopez-Soler et al., 2001; Shumaker et al., 2005). More recently, it was shown that LIII polymerisation *in vitro* into paracrystals is prevented by importin-α (Adam et al., 2008). Here, we have established buffer conditions required for distinct assembly steps of recombinant LIII *in vitro*. Furthermore, we show that Nudel, a recently identified LIII-binding protein (Ma et al., 2009), which is predicted to induce filamentous structures of LIII, disrupts LIII head-to-tail associations.

4.3 Experimental Procedures

All experimental procedures were carried out at room temperature, unless otherwise stated.

4.3.1 Bacterial expression and purification of LIII

Xenopus LIII in pET vector was a generous gift of Prof. R.D. Goldman. pET-LIII was expressed in *E.coli* BL21-DE3 codon (+) strain. 10 ml overnight culture was diluted into 1 litre of LB-medium with appropriate antibiotics and grown at 37°C until an OD_{600} of 0.6 was reached. LIII expression was then induced by the addition of 0.4 mM IPTG and cells were grown in culture for 3 hours at 37°C and shaking at 180 rpm. Bacterial pellets were collected by centrifugation at 5000 rpm for 15 min at 4°C (SLA3000, Sorvall). The pellet was resuspended in 10 ml of lysis buffer containing 50 mM Tris-HCl pH 8.0, 25% sucrose, 1mM EDTA, 1mM PMSF, 1mM leupeptin, 1mM aprotinin, and 1mM DTT on ice. The pellet was then homogenised by 10 sec sonication bursts and 20 ml of detergent buffer containing 25 mM Tris-HCl pH 8.0, 200 mM NaCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 1mM PMSF, 1mM leupeptin, 1mM aprotinin, and 1mM DTT was added to the resuspended bacterial cells on ice and was then homogenised using a sonicator. Lysed cells were then pelleted by centrifugation at 9000 g for 45 min at 4°C. Insoluble lamin pellet was then washed 3 times by resuspending in wash buffer containing 25 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.5% NP-40, 1mM EDTA, 1mM PMSF, 1mM leupeptin, 1mM aprotinin, and 1mM DTT on ice, and centrifugation at 9000 g for 15 min at 4°C. Inclusion bodies were then dissolved in 8 ml of column buffer containing 10 mM Tris-HCl pH 8.0, 8 M urea, 2 mM EDTA, 1mM PMSF, 1mM leupeptin, 1mM aprotinin, 10 mM DTT, using a Dounce homogeniser and centrifuged at 200,000 g for 45 min at room temperature.

4.3.2 LIII protein purification

Solubilised inclusion bodies were loaded onto 3 ml of Q-sepharose pre-equilibrated column, which was eluted with a linear 0-600 mM NaCl gradient in column buffer. Eluted fractions were separated by SDS-PAGE and desired fractions were undergone a second step of purification onto a Superdex 200 FPLC size exclusion chromatography column (GE healthcare- Amersham Biosciences, Uppsala, Sweden) following manufacture's protocol. LIII fractions were collected in 10 mM Tris-HCl pH 8.6, 8 M urea, 300 mM $NaCl_2$, 1mM leupeptin, 1mM aprotinin, 10 mM DTT. Aliquots were stored at -80 °C.

4.3.3 SDS-PAGE and immunoblotting

Protein samples were resolved by SDS-PAGE and either stained with Coomassie blue or transferred to PVDF membrane and probed with the appropriate antibodies and developed using the Western Lighting CDP-Star® Chemiluminescence Reagent (Tropix, Bedford MA, USA) and Fuji Super RX-film (FujiFilm Corporation, Tokyo, Japan).

4.3.4 Recombinant Nudel protein

Recombinant NudeI protein fused to with His-tag was kindly supplied by Dr Y Zheng (Carnegie Institution of Washington, Department of Embryology, Baltimore).

4.3.5 Sample preparation for electron microscopy

For glycerol spraying/low-angle rotary metal shadowing, 20 μl lamin sample was mixed with glycerol to a final concentration of 30% and ∼10 μl was sprayed onto freshly cleaved mica which was then mounted face up onto a rotary table of a Balzers apparatus (BAE 120T; Balzers Pfeiffer GmbH, Asslar, Germany) and dried at room temperature in vacuo. Dried samples were rotary shadowed with platinum/carbon at an elevation angle of 3.5 ° and coated with a carbon film apparatus for metal evaporation, followed by rotary shadowing with platinum/carbon at an angle of 3-5 ° as described previously (Aebi and Baschong, 2006; Fowler and Aebi, 1983). For negative staining, a sample aliquot of 5 μl was adsorbed for 1 min to a glow-discharged carbon-coated collodion film on a copper grid (Aebi and Pollard, 1987). Excess sample was drained with filter paper and grid was washed on two drops of Milli-Q water, mounted with 1-2 μl of 2% uranyl acetate for 1 min and excess liquid was drained with filter paper and the grid was air-dried. Samples were examined

in either a Hitachi H-7000 transmission electron microscope (Hitachi, Japan) operated at voltage 100 kV or Philips CM-100 transmission electron microscope (Philips, Eindhoven, The Netherlands) operated at 100 kV. Electron micrographs were recorded on Kodak SO-163 electron image film at a magnification of 50 000x. EM negative films were scanned using PrimeScan D7100 Drum scanner (PrimScan, Heidelberg) and were further analysed using Adoobe Photoshop CS4.

4.4 Results

4.4.1 Bacterial expression and purification of recombinant LIII

Xenopus LIII in a pET vector was first expressed in *E.coli* DE3 codon (+) cells in inclusion bodies, followed by two steps of purifications of the solubilised LIII in 8 M urea containing buffer. LIII was loaded onto a Q-anion exchange chromatography column, which resulted in partial purification of LIII. These partially purified LIII fractions were next loaded onto a Superdex 200 size exclusion chromatography column and fully purified protein fractions were resolved by SDS-PAGE as shown in Figure 4.1A. The identification of the 67-kDa proteins was further verified by immunoblotting with LIII specific antibody 299 (a kind gift of Prof R.D. Goldman), as well as by mass spectrometry (Dr Paul Jenö, Mass spectrometry, Biozentrum, University of Basel). Protein concentrations were measured at λ595 to be $\sim$ 4 mg/ml, and protein aliquots in dimer buffer (10 mM Tris, 300 mM NaCl, 8 M urea, 5 mM DTT, pH 8.6) were stored at -80 °C. For LIII assembly analysis, the protein was first renatured stepwise by dialysing against dimer buffer with the decreasing urea concentrations ranging from 8-0 M. A protein concentration of 0.2-0.4 mg/ml was measured for the fully renatured protein and was resolved by SDS-PAGE as shown in Figure 4.1B. For *in vitro* assembly studies a protein concentration of 0.2 mg/ml was used.

4.4.2 LIII dimer formation

LIII aliquots in 8 M urea containing dimer buffer were first renatured stepwise dialysis from 8, 6, 4, 2, 1, and 0 M urea containing 10 mM Tris-HCl pH 8.6, 300 mM NaCl, 2 mM DTT for 4 hours at room temperature. Figure 4.2C shows EM micrographs of glycerol sprayed/low-angle rotary metal shadowed LIII dimers. These dimers are represented by a long tail with a length of 51.9 $\pm$ 1.3 nm and two globular heads at one end, similarly to previously

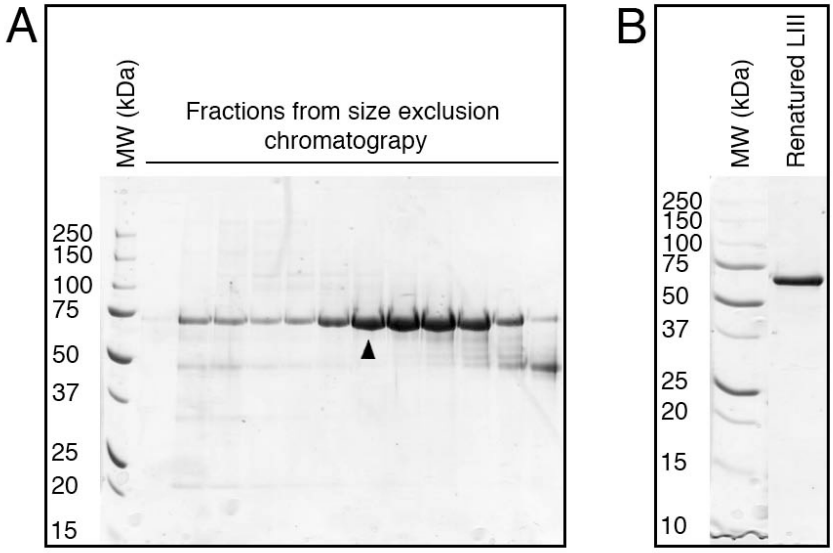

Figure 4.1: Purification of recombinant LIII - LIII was expressed in *E.coli* and purified by Q-anion exchange chromatography followed by (**A**) size exclusion chromatography as described in the experimental procedures. Collected fractions were resolved by SDS-PAGE. (**B**) Renatured LIII following sequential dialysis in dimer buffer lowering urea concentration from 8, 6, 4, 2,1 and 0 M.

documented ultrastructures seen in chicken lamin B2 and other lamins (Heitlinger et al., 1991; Sasse et al., 1997). The tail represents two lamin molecules associating at the α-helical coiled-coil of rod domain. The two globular head structures correspond to the Ig-fold of the tail domain of LIII.

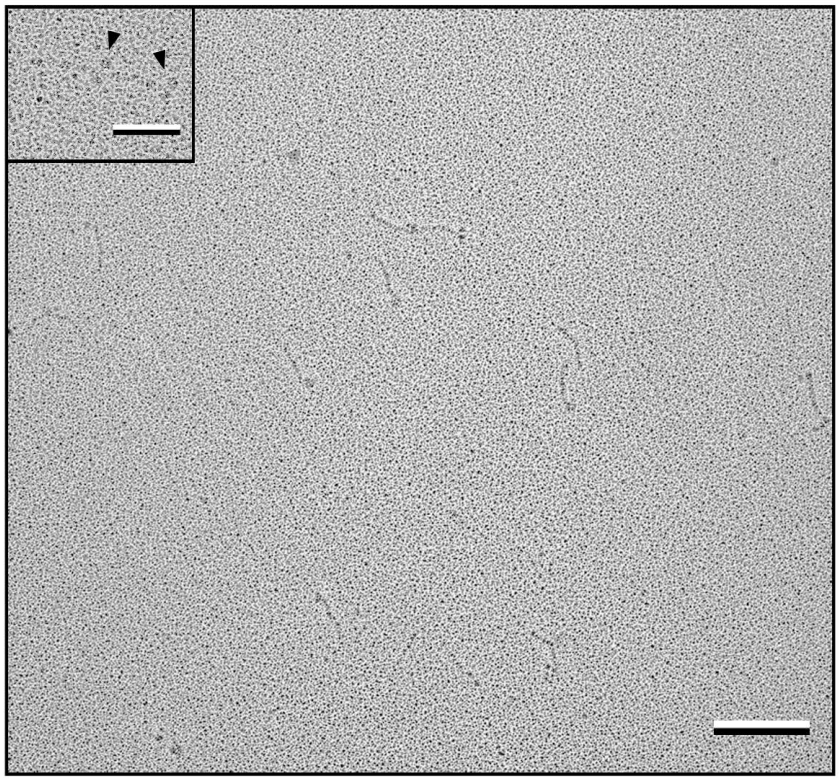

Figure 4.2: LIII dimers - 0.2 mg/ml recombinant LIII was dialysed stepwise against 8 M to 0 M urea in dimer buffer containing 10 mM Tris-HCl pH 8.6, 300 mM NaCl, 8 M urea, 5 mM DTT and protease inhibitors for 4 hours. Dialysed LIII samples were glycerol sprayed/rotary metal shadowed as described in the experimental procedures. LIII dimers have a long tail of 51.9 $\pm$ 1.3 nm representing the rod domain and two globular heads at one end representing the Ig-fold of the tail domain. Scale bar, 100 nm and 50 nm in the insert.

4.4.3 "Head-to-tail" assemblies of LIII dimers *in vitro*

LIII samples renatured by stepwise dialysing into dimer buffer were then dialysed against different buffer conditions with varying pH from 9-6 and salt concentration from 0.5-0 M NaCl. Longitudinal polar head-to-tail polymer associations were observed when LIII dimers

were dialysed against 25 mM MES pH 6.5, 300 mM NaCl, 1 mM DTT for 2-3 hours as shown on EM micrographs of glycerol sprayed/low-angle rotary shadowed LIII assembled head-to-tail polymers in Figure 4.3A. Maintaining the pH at 6.5 but lowering the salt concentration to 200 mM NaCl resulted in shorter head-to-tail association as shown in Figure 4.3B. However, in the presence of only 100 mM NaCl lateral association of half staggered head-to-tail polymers were becoming more obvious as seen in Figure 4.3C.

4.4.4 Paracrystal assemblies of LIII

LIII samples were first renatured in dimer buffer and then dialysed against 25 mM MES pH 6.5, 100 mM NaCl, 1 mM DTT for 2 hours, which resulted in some filament- like structures when examined by negative staining (see Figure 4.4A). However, Figure 4.4B shows an EM micrograph of negatively stained LIII dimers dialysed against 25 mM MES pH 6.5, 1 mM DTT which induced paracrystal-like structures, revealing 24.7±1.3 nm axial repeats. Furthemore, dialysing LIII dimers in other established conditions for paracrystal formation resulted in more loosely packed paracrystals when dialysed against 25 mM MES pH 6.5, 20 mM $CaCl_2$ for 1-2 hours, as shown in Figure 4.4C. However, Figure 4.4D, shows negatively stained LIII that the presence of 20 mM $CaCl_2$ in more alkaline pH (8.6) led to the formation of a tightly packed paracrystalline arrays with 17.2±1.4 nm axial repeats.

4.4.5 LIII head-to-tail assemblies are affected by the presence of Nudel

We next examined whether the addition of Nudel have an influence on the polymerisation of LIII into higher order assemblies. LIII samples were pre-assembled into either dimers or head-to-tail polymers, mixed 1:1 with Nudel full length and then dialysed against head-to-tail buffer containing 25 mM MES pH 6.5, 300 mM NaCl, 1 mM DTT, 1 mM PMSF for 2-3 hours at room temperature. Figure 4.5 shows EM micrographs of glycerol sprayed/ rotary metal shadowed LIII in head-to-tail assembly condition in the presence of Nudel. Nudel was added either to pre-assembled LIII dimer as represented in Figure 4.5A, or with pre-polymerised head-to-tail assemblies in Figure 4.5B. The addition of Nudel disrupts the tendency of LIII dimers to form polar head-to-tail association (Figure 4.5A; see also Figure 4.3A). In addition, when added to pre- assembled head-to-tail LIII, Nudel disrupted these polymers as demonstrated by the absence of longitudinal polar head-to-tail associa-

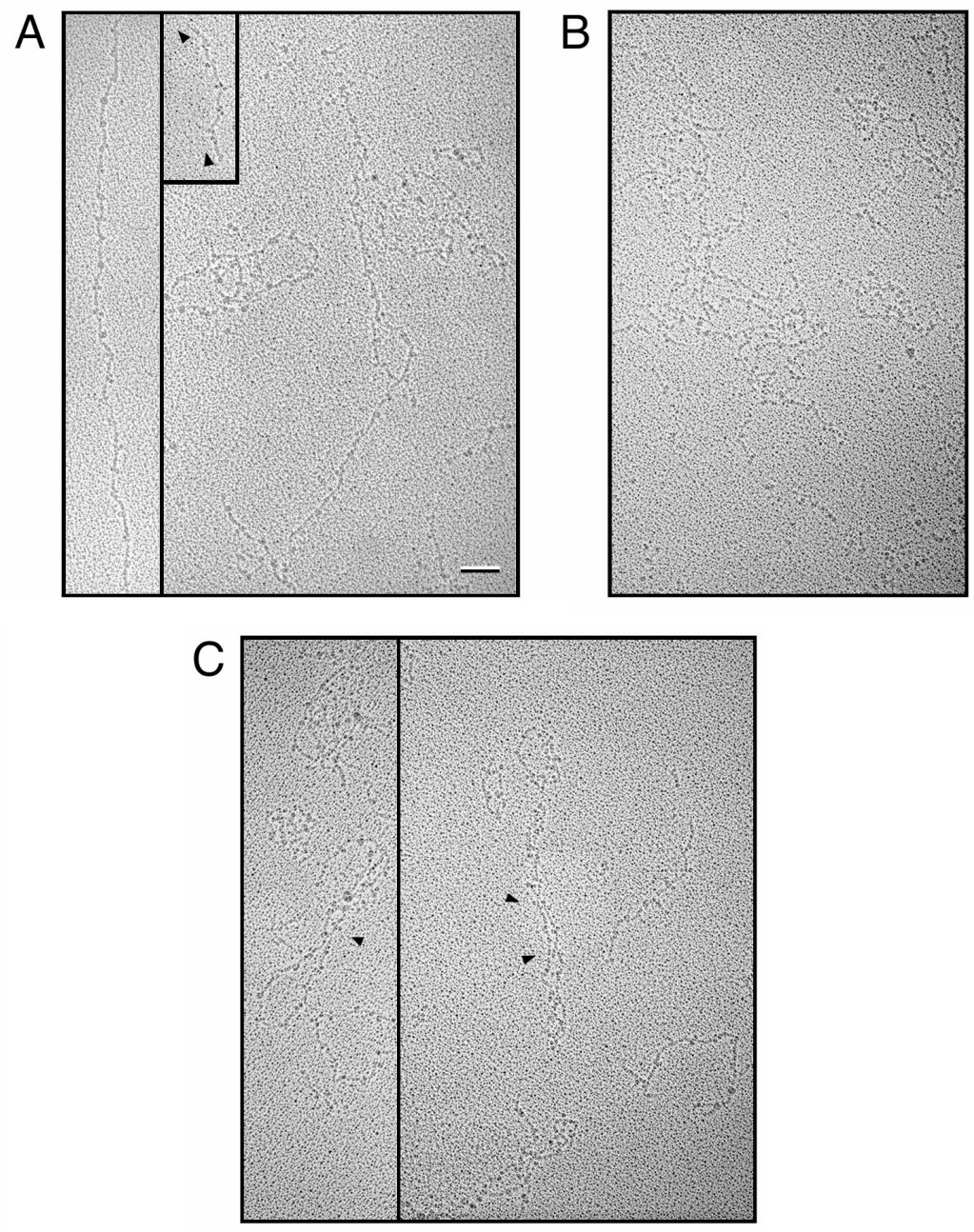

Figure 4.3: LIII dimers form head-to-tail polymers that extend laterally by lowering ionic strength - EM of glycerol sprayed/low-angle rotary metal shadowed LIII samples. LIII samples in 8 M urea were dialysed stepwise against dimer buffer (10 mM Tris-HCl pH 6.5, 300 mM NaCl, 8 M urea, 5 mM DTT) for 4 hours and were then dialysed into low pH buffer of varying salt concentration. (**A**) LIII dimers dialysed against 25 mM MES pH 6.5, 300 mM NaCl, 1 mM DTT, 1 mM PMSF and protease inhibitors for 2-3 hours resulted into polar head-to-tail associations. (**B**) Lowering salt concentration to 200 mM NaCl resulted into head-to-tail aggregates. (**C**) Lowering salt concentration even further to100 mM NaCl resulted in head-to-tail polymers to associate laterally in a staggered fashion. Scale bar (A-C), 100 nm.

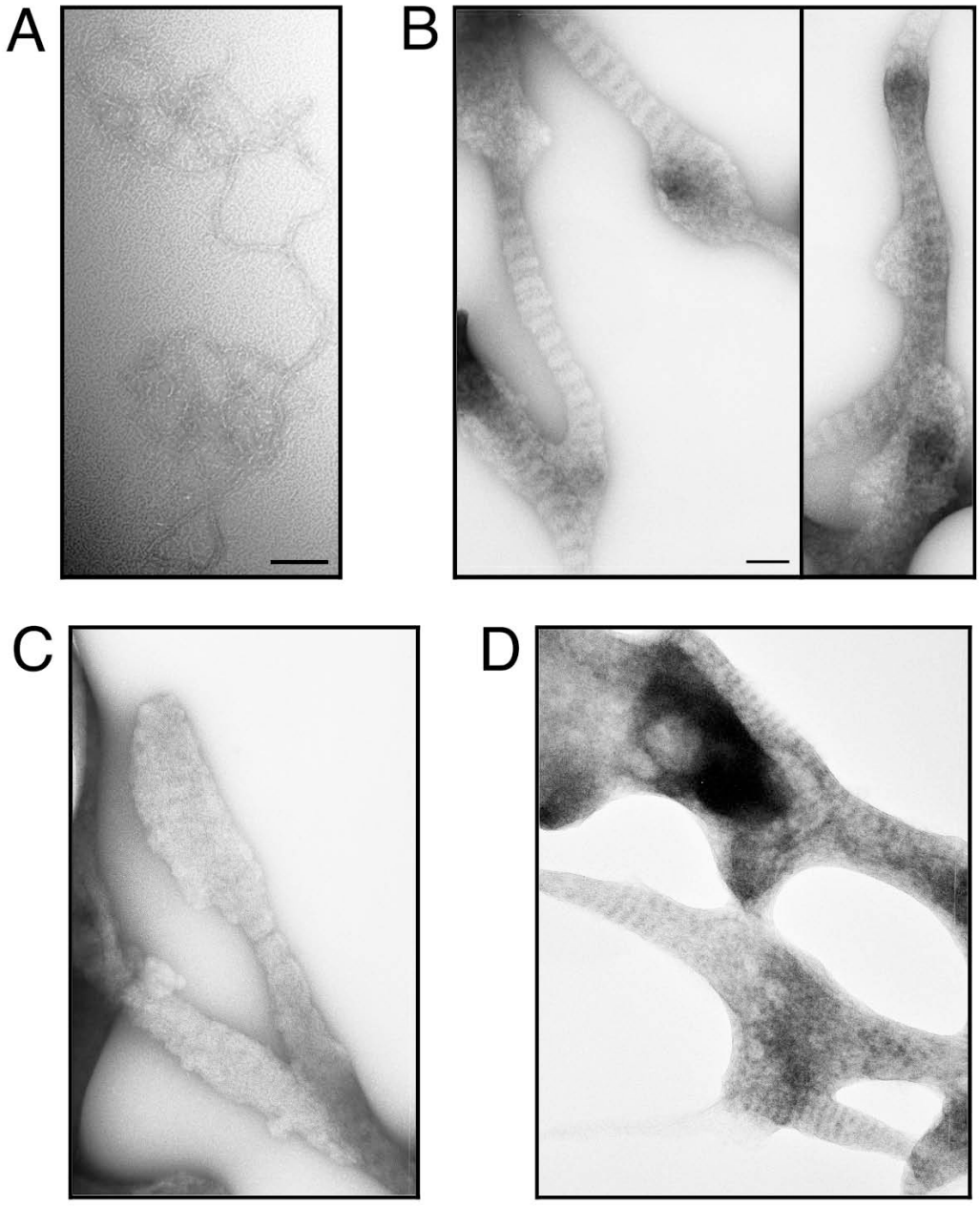

Figure 4.4: LIII paracrystal assemblies - EM of negatively stained LIII higher order structures formed in different buffer conditions. Renatured LIII dimer samples were dialysed for 2 hours against (**A**) 25 mM MES pH 6.5, 100 mM NaCl, 1 mM DTT to form filament like structures. Paracrystals were formed when dialysed against three different buffers: (**B**) 25 mM MES pH 6.5, 1 mM DTT. (**C**) 25 mM MES pH 6.5, 20 mM $CaCl_2$, 1 mM DTT and (**D**) 10 mM Tris-HCl pH 8.5, 20 mM $CaCl_2$, 1 mM DTT. Scale bar, 100 nm.

tions seen in Figure 4.5B and resulted in protein aggregation when examined by negative staining (data not shown).

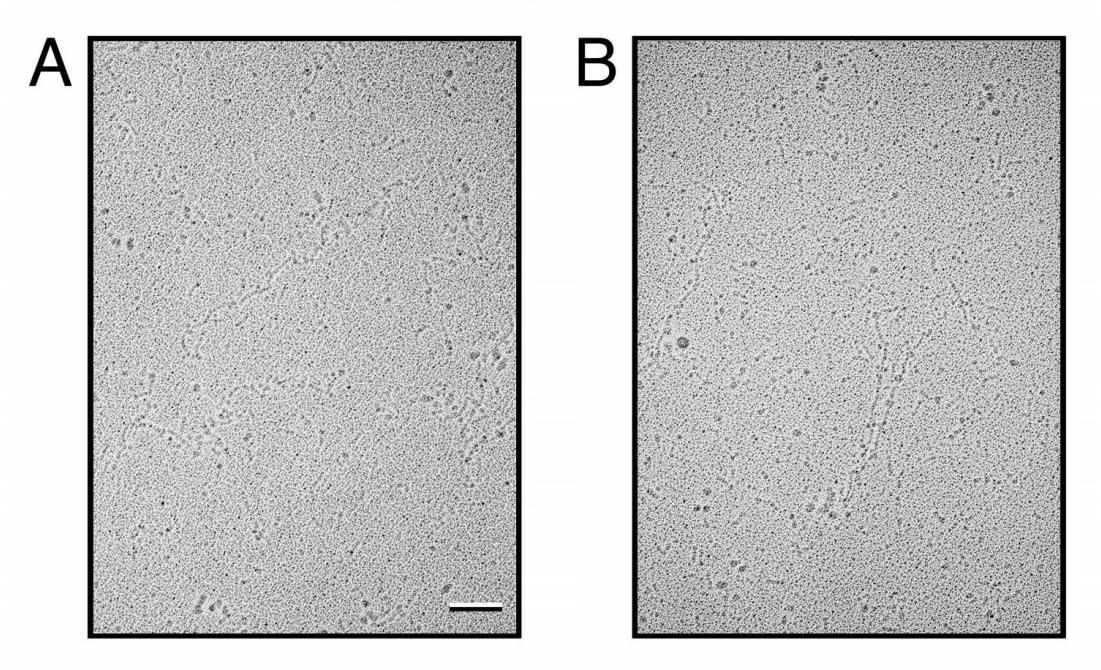

Figure 4.5: Nudel interferes with LIII assembly into head-to-tail polymers - LIII samples dialysed in dimer buffer as described in Section 4.3 were (**A**) pre-assembled onto head-to-tail polymers by dialysing in 25 mM MES, 300 mM NaCl, 1 mM DTT, 1 mM PMSF, pH 6.5 for 3 hours and then were mixed 1:1 with Nudel and incubated for another 2-3 hours at room temperature. (**B**) LIII dimers were first mixed with Nudel and then dialysed against head-to-tail buffer condition. Samples were then subjected to glycerol spraying/low-angle rotary metal shadowing as described in Section 4.3. Scale bar, 100 nm.

4.5 Discussion

Xenopus LIII is the major B-type lamin found in the oocytes and its structural assembly of 10 nm IF-like filaments meshwork at the nuclear envelope was one of the earliest documented (Aebi et al., 1986). However, following the assembly of LIII *in vitro* was not determined yet. Here we sought to examine the ultrastructural assembly pattern of LIII *in vitro*. We expressed recombinant untagged LIII in *E.coli* to avoid any tag related influence on the biology of the expressed protein and assembly. LIII accumulated in inclusion bodies and therefore was first solubilised in urea. The resulting denatured protein was purified by anion exchange and size exclusion chromatography. Upon LIII renaturation in dimer buffer containing 300 mM NaCl, pH 8.6, LIII molecules formed dimer-like structures with a tail measuring 51.9 ± 1.3 nm in length and two globular heads at one end. The long tail represents two α-helical coiled-coil that result from the association of two lamin molecules at their rod domain and the globular heads represent the C-terminal domain with its globular Ig-fold domain (Aebi et al., 1986; Heitlinger et al., 1991). LIII dimers associated longitudinally upon lowering pH conditions (pH 6.5, 300 mM NaCl), leading to polar head-to-tail assemblies, similar to previous studies (Heitlinger et al., 1991). These head-to-tail polymers tend to become less longitudinally associated when lowering the ionic strength (200 mM NaCl, pH 6.5) resulting in shorter assemblies, which start to associate more laterally at an even lower salt condition (100 mM NaCl, pH 6.5) resulting in a partially staggered association. Furthermore, LIII dimers formed paracrystal like structures, with striated repeats when dialysed in buffers that favour paracrystal formation (Heitlinger et al., 1991). However, the packing of these paracrystal structures deduced from LIII varied depending on the pH as well as the presence of $CaCl_2$. LIII formed large paracrystals with 24.7± 1.3 nm axial repeats in pH 6.5 and with no salt. However, when LIII was dialysed in the addition of 20 mM $CaCl_2$, pH 6.5, more loosely packed striated repeats were observed. Moreover, when 20 mM $CaCl_2$ was added at pH 8.6, this resulted in paracrystal which were more tightly packed than either of the other paracrystal conditions with 17.2± 1.4 nm axial repeats. This is in agreement with previously published assembly studies of chicken lamin B2, where it was suggested that the pH influences the strength of lateral inter-subunit associations without affecting overall strength of the longitudinal inter-subunit bond (Heitlinger et al., 1991). Furthermore, we examined the effect of Nudel on LIII head-to-tail assemblies, which has recently been demonstrated to recruit and aid in

the assembly of LIII at the mitotic spindle (Ma et al., 2009). The exact influence of Nudel on LIII assembly is not established. It is known however that Nudel associates with the cytoplasmic IF protein vimentin, and interacts directly with LIII and neurofilaments (NFs) (Helfand et al., 2002; Ma et al., 2009; Nguyen et al., 2004). Interaction of Nudel with NFs induces filament formation (Nguyen et al., 2004). Our data show that unlike NFs, Nudel did not induce LIII polymerisation into filaments; to the contrary, it disrupted head-to-tail associations of LIII and resulted in aggregations of the protein observed by negative staining. The mixture of Nudel with LIII dimers prevented head-to-tail association and further disrupted pre-assembled head-to-tail polymers and led to aggregation. Recently, it was shown that Nudel interacts specifically with coil 2 of the LIII rod domain, which is highly conserved between all IFs and involved in lamin assembly to higher order structures (Herrmann et al., 2007; Ma et al., 2009; Stuurman et al., 1998). However, how Nudel interaction with coil 2 interferes with the head-to-tail assemblies that involve the very ends of the rod domain is not clear. We speculate that Nudel function in the recruitment of LIII in mitosis is mediated by further dissolving the small pool of non-solubilised LIII nuclear envelope remnants which were not solubilised prior to nuclear envelope breakdown and eventually transform LIII into a mitotic state specific meshwork which possibly result in a distinct assembly pattern different than the normally assembled LIII on the nuclear periphery in interphase cells and which is yet to be defined. In summary we have established buffer conditions required for the assembly of recombinant LIII from dimers to head-to-tail and further lateral associations as well as paracrystal formation. Certainly looking at the effects of mutations in the head and tail domains of LIII on the assembly as well as the influence of lamin-associated proteins on forming higher order filamentous meshwork seen in the living cell is of future interest.

5

Conclusions and future directions

Already in 1976, it was documented that the nuclear lamina interacts with NPCs (Dwyer and Blobel, 1976), and in the following that proper assembly of the NPCs into the nuclear envelope requires an intact nuclear lamina (Enarson et al., 2001; Enarson et al., 1998). More specifically, it was shown that the nucleoporin Nup153 recruitment to the nuclear envelope also requires association with the lamina possibly through binding with lamin B (Smythe et al., 2000). Direct association with nuclear lamins was thought to occur through the C-terminal domain of Nup153 interacting with lamin B, but not lamin A (Smythe et al., 2000). However, the localisation of Nup153 was also shown to be altered in cells expressing mutations in lamin A, although no direct binding of Nup153 and lamin A has been reported (Bechert et al., 2003; Goldman et al., 2004; Hubner et al., 2006; Muchir et al., 2003).

5.1 Nup153 presents multiple binding sits for nuclear lamins

In order to analyse the interaction between Nup153 and nuclear lamins more systematically we performed solution binding assays and co-immunoprecipitation experiments. Our studies revealed that the interaction between Nup153 and nuclear lamins is more complex than anticipated (Al-Haboubi et al., submitted). Nup153 exhibits multiple binding sites not only to lamin B but also to lamin A (Figure 2.1). Both the N-terminal and the C-terminal domains of Nup153 interact with both A- and B-type lamins. In addition, we showed that the interaction of the C-terminal, FG-repeat domain is not part of a nuclear import complex (Figure 2.3). The C-terminal FG-repeat domain of Nup153 appears to be

an unfolded structure, which has multiple topology at the NPCs (Fahrenkrog et al., 2002), suggesting that the C-terminal domain of Nup153 folds back onto an intermediate complex between Nup153 and the respective lamin at the NE. However, Nup153 also exists in multiple populations at the nuclear interior as well as within the NPC (Griffis et al., 2004), which indicates distinct association with lamin A in the nucleoplasm. However, it remains to be seen whether potential nucleoplasmic Nup153-lamin A complexes can be correlated to distinct functions, e.g. a structural role at the periphery of the NE or in complex with chromatin associated proteins in the nucleoplasm. We further revealed that Nup153 binds particularly to the Ig-fold of lamins and that differing binding sites exist for lamin A and B, supporting the notion of multiple functions of the distinct Nup153-lamin complexes. We mapped the specific region in the N-terminal domain of Nup153 within residues 39-339 which are necessary for binding to lamin A and residues 2-339 critical for interaction with lamin B (Figure 2.5). Our immunoprecipitation data revealed that Nup153 interact with lamin A, as well as B-type lamins in HeLa cell extracts providing in vivo evidence that further support our *in vitro* data.

5.2 Nup153 interaction with lamins in the presence of lamin mutation

In order to assign potential roles to the different kinds of interactions between Nup153 and nuclear lamins, we examined these interactions in the presence of lamin-associated mutations. We employed further *in vitro* binding assays, immunofluorescence microscopy and co-immunoprecipitation assays. Our studies revealed that a mutation in the Ig-fold, responsible for FPLD (i.e. R482W), which results in disrupting a specific binding site of lamin A, interferes dramatically with Nup153 binding. More specifically, the R482W mutation caused loss of binding with Nup153 (Figure 3.3 and 3.4) not only *in vitro* but also *in vivo*, further indicating the importance of this site for binding to Nup153. Another mutation within the Ig-fold domain that destabilises the 3D-architecture of the lamin molecule (i.e. R453W), and results in AD-EDMD, also proved to affect binding between Nup153 and lamin A *in vitro*, however, to a lesser extent. In contrast, the interaction of Nup153 with lamin A remained unaffected by the presence of the LAΔ50 mutation (responsible for HGPS) *in vitro*, thus further supporting a direct interaction of Nup153 with nuclear lamins to be with the Ig-fold, which is not affected by the LAΔ50 mutation. A correlation between

the position of laminopathy-causing mutations and their upstream effect on binding with Nup153 is plausible, and may account more explicitly for the specific tissue damages observed with the different laminopathies. We hypothesise that the multiple lamin binding sites within Nup153 exhibit multiple functional roles in conjunction with other nuclear proteins, depending on the specific Nup153 domain binding (i.e. N-terminal or C-terminal) as a result of complex formation with lamins. Altered interaction between Nup153 and laminopathy-causing mutants of the tail domain (Ig-fold) may influence the functionality or viability of Nup153 (see Section 1.6) and consequently subsequent pathologies, depending on the mutation in question. We propose several possible roles for Nup153 in laminopathies. Nup153 may have a structural role in the architecture of the nucleus. Loss of binding with lamins as a result of lamin associated mutations could interfere with subnuclear localisation of Nup153 at the nuclear periphery and with subsequent functions of Nup153 in NE assembly and disassembly. Active genes are anchored to the NPC and silenced to lamins at the periphery, thus the disruption of the Nup153-lamin complex could interfere with the chromatin organisation at the periphery. It is also plausible that Nup153-lamin complexes exist in the nucleoplasm. Whether these could act as genomic insulators blocking the action of enhancer elements or as genomic activators is yet to be determined. Moreover, altered binding of Nup153 with lamin A may alter the level of expression of Nup153, which in turn could lead to severe effects in cytokinesis and cell cycle exit as seen previously (Mackay et al., 2009). Altered domain functionality can lead to aberrant nuclear morphology seen in cells with reduced levels of Nup153 at a early stage of mitosis with multi-lobed nuclei, these where partially rescued in the addition of NPAR region, which support an additional role for this region in cell cycle progression. Moreover, recently it was shown that Nup153 mediated nuclear import is reduced in laminopathic cells (expressing LAΔ50 mutation) (Busch et al., 2009). Mislocalised Nup153 can impair normal trafficking of mRNA and proteins and possibly the transport of regulatory proteins required for cell cycle progression. Nup153 may have a role in the pathology of the disease through gene regulation, e.g. regarding expression of adipocyte-specific genes or the provision of binding sites at the nuclear periphery that protect the cells against apoptosis, and changes in chromosomal positioning due to defects in the cell cycle (Kalverda et al., 2008).

5.3 Lamin LIII assembly

The *Xenopus* oocytes lamin LIII was shown to from 10 nm IF-like filaments that arrange in a regular meshwork (Aebi et al., 1986). Previously, *in vitro* assembly studies of other mammalian lamins were documented (Heitlinger et al., 1991; Klapper et al., 1997; Sasse et al., 1997). However the conditions for *in vitro* reconstitution of lamin LIII have not yet been extensively studied. We dialysed recombinantly expressed LIII in various buffer conditions with differing pH and ionic strength and examined LIII ultrastructures by electron microscopy (EM) using glycerol spraying/low-angle rotary metal shadowing and negative staining. LIII dimers were found to exhibit a typical ∼52 nm long tail and two globular heads at one end (Figure 4.2). These dimers associated longitudinally into polar head-to-tail polymers at high NaCl concentration and low pH (Figure 4.3). Without NaCl, also at low pH paracrystal-like structures with ∼24 nm axial repeats were observed. These paracystal-like structures were also formed in the presence of $CaCl_2$ at high or low pH. However, more tightly packed paracrystals with axial repeats of ∼17 nm were seen when $CaCl_2$ was added at high pH (Figure 4.4). Establishing the conditions required for head-to-tail assemblies was important for future examination of the influence of the presence of LIII binding proteins on the subsequent assembly into higher order structures. We showed that LIII head-to-tail polymers are disrupted in the presence of Nudel, a protein that interacts with LIII (Ma et al., 2009), and which may play a role in recruiting LIII to the microtubules and subsequently in the assembly of mitotic spindle LIII specific meshwork (Tsai et al., 2006).

5.4 Future directions

Future work should address the roles of distinct lamin A mutations affecting Nup153 binding in relation to type and severity of the diseases associated with these mutations. For example, future studies should be directed toward better understanding of the role of Nup153 in cell cycle progression in cells expressing lamin A mutations. The use of stable cell lines with disease-causing lamin A mutants would allow for monitoring of the levels of Nup153 and study of the cell cycle progression by the use of live-imaging techniques. In addition, studies may include examination of the effects of up-regulation or down-regulation of Nup153 in cells with disease causing lamin A mutations. In addition, fibroblast derived

from *LMNA* null mice could be used to investigate the effect that the transiently expressed or microinjected Ig-fold of lamin A may have on the localisation of Nup153 in these cells.

To continue the work on LIII assembly, future in vitro studies should be directed at investigating the effects of mutations in the head and tail domains of LIII as well as the influence of LIII-associated proteins on the formation of higher order filamentous meshwork, as seen in the living amphibian cells.

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Curriculum Vitae - Teiba Al-Haboubi

Personal Details

Name: Al-Haboubi, Teiba
Date of Birth: 27 August 1979

Tertiary Education

2004-2009

PhD thesis at the Müller Institute for Structural Biology, Biozentrum and University of Basel, Switzerland. Supervised by Prof. Ueli Aebi

2002-2004

Master of Science in Biological Science, The University of Auckland, New Zealand. Supervised by Prof. Garth Cooper

1999-2001

Bachelor of Science (Biological Science), The University of Auckland, NZ

Publications

Teiba Al-Haboubi, Joachim Köser, Dale K Shumaker, Robert D Goldman, Birthe Fahrenkrog (2009) Direct association of the nuclear pore protein Nup153 with the Ig-fold domain of A- and B-type lamins. **Submitted to Journal of Biological Chemistry**

Teiba Al-Haboubi and Birthe Fahrenkrog (2009) Characterisation of nuclear lamin binding to the nucleoporin Nup153 in the presence of lamin mutations. **Manuscript in preparation**

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Teiba Al-Haboubi, Dale Shumaker, Robert D Goldman, Ueli Aebi, Birthe Fahrenkrog (2007) Characterisation of lamin binding to the nuclear pore protein Nup153. **Published abstract, American Society of Cell Biology annual meeting 2007**

Poster Presentations

The Ig-fold of the nuclear lamins interacts directly with the nuclear pore protein Nup153- implications in Emery Dreifuss muscular dystrophy
Teiba Al-Haboubi, Dale Shumaker, Robert D Goldman, Ueli Aebi, Birthe Fahrenkrog
Gordon Research Conference - Intermediate Filaments, September 2008, Oxford, United Kingdom

Characterisation of lamin binding to the nuclear pore protein Nup153
Teiba Al-Haboubi, Dale Shumaker, Robert D Goldman, Ueli Aebi, Birthe Fahrenkrog
American Society of Cell Biology annual meeting, December 2007, Washington DC, USA