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1 Abstract

1.1 Summary

Lamins are Type V intermediate filament proteins and the major components of the nuclear lamina, a stable filamentous meshwork underlying the inner nuclear membrane of metazoan cells. They are grouped into A- and B-type lamins. Particularly A-type lamins (lamin A and C) are also found in a dynamic and mobile state within the nuclear interior and may serve important functions in chromatin organization and regulation of cellular processes. LAP2 α , a protein of the LEM family, was identified as a specific binding partner of this lamina-independent pool of lamins. Studies revealed an essential role of LAP2 α in keeping lamin A/C soluble in the nucleoplasm most likely by forming a mobile lamin A/C-LAP2 α complex.

As the possible stoichiometric composition of such complexes was unknown the first aim of my work was to determine the respective abundances and the molecular ratio of lamin A/C versus LAP2 α within a cell. I found that, depending on the cell type, the amounts of both proteins in the nuclear interior of cells suggest a potential molecular stoichiometry within the lamin A/C- LAP2 α complex from 15:1 in BJ1 cells to 2:1 in HeLa and 1:1 in U2OS cells.

The second part of my work investigates the potential interfering effect of LAP2 α on lamin A assembly *in vitro*. In initial experiments I defined the conditions under which both lamin A assembly and lamin A-LAP2 α interactions can be analyzed. Sucrose density gradient centrifugation analysis provided evidence for a lamin A-LAP2 α interaction at high salt non-assembly conditions and revealed a negative impact of LAP2 α on the formation of larger lamin structures at assembly conditions. These findings were supported by negative staining electron microscopy depicting structural changes in lamin paracrystal structures in the presence versus absence of LAP2 α . Furthermore, sucrose density gradient centrifugation confirmed a potential molecular ratio of lamin A to LAP2 α of 1:1 within the complex *in vitro*. This finding supports the hypothesis that lamin A and LAP2 α may form an equimolar complex. Finally my data indicate that LAP2 α not only impairs lamin A assembly *in vitro* but may also prevent lamin A aggregation and vice versa lamin A increases solubility of LAP2 α . Altogether the data lend support for the existence of a lamin A-LAP2 α complex in the nucleus and show for the first time a negative impact of LAP2 α on lamin A assembly.

1.2 Zusammenfassung

Lamine gehören zu den Typ V Intermediärfilamenten und sind die Hauptkomponenten der nukleären Lamina, einem stabilen filamentösen Netzwerk an der inneren Kernmembran von Metazoa-Zellkernen. Sie werden in A- und B-Typ Lamine eingeteilt. A-Type Lamine (Lamin A und C) lassen sich, in einem dynamischeren und mobileren Zustand, auch im Kerninneren nachweisen, wo sie wichtige Funktionen in Chromatin-Organisation und der Regulation zellulärer Prozesse erfüllen. LAP2 α , ein Protein der LEM-Familie, wurde als spezifischer Bindungspartner dieser löslichen nukleoplasmatischen Lamine identifiziert. Studien zeigten, dass LAP2 α eine essentielle Rolle in der Aufrechterhaltung dieses löslichen Lamin A/C Pools im Nukleoplasma spielt. Dies geschieht vermutlich durch Lamin A/C-LAP2 α Komplexbildung.

Da die mögliche stöchiometrische Zusammensetzung dieser Komplexe bis jetzt nicht geklärt ist, war ein erster Ansatz meiner Arbeit die jeweiligen Mengen und das molekulare Verhältnis von Lamin A/C zu LAP2 α innerhalb einer Zelle zu bestimmen. Es zeigte sich, dass abhängig vom Zelltyp, die Mengen beider Proteine im Kerninneren auf eine mögliche molekulare Stöchiometrie innerhalb eines Lamin A/C-LAP2 α Komplexes von 15:1 in BJ1 Zellen, 2:1 in HeLa und 1:1 in U2OS Zellen hinweisen.

Der zweite Teil meiner Arbeit untersuchte den möglichen Einfluss von LAP2 α auf die Assemblierung von Lamin A *in vitro*. In ersten Experimenten legte ich die Bedingungen fest unter welchen Lamin A-Polymerisierung und Lamin A-LAP2 α -Interaktion analysiert werden konnten. Analyse durch Sucrose-Dichtegradientenzentrifugation lieferte den Nachweis für eine Lamin A-LAP2 α Interaktion in Hochsalz-Puffern in denen Lamin A noch gelöst vorliegt. Unter Assemblierungsbedingungen zeigte sich bereits ein negativer Einfluss von LAP2 α auf die Bildung größerer Lamin-Strukturen. Bestätigt wurde dies durch Untersuchungen von Lamin-Parakristallen im Elektronenmikroskop. Diese zeigten Veränderungen der Kristallstruktur in der Gegenwart von LAP2 α . Weiters ergab die Sucrose-Dichtegradientenzentrifugation ein mögliches molekulares Verhältnis von Lamin A zu LAP2 α in einem Komplex von 1:1. Somit unterstützen *in vitro* und *in vivo* Ergebnisse die Hypothese, dass Lamin A und LAP2 α einen äquimolekularen Komplex bilden. Zusätzlich konnte ich Hinweise darauf finden, dass nicht nur LAP2 α Einfluss auf die Polymerisierung von Lamin A hat, sondern auch Lamin A die Löslichkeit von LAP2 α steigern kann.

Zusammengefasst weisen meine Daten auf die Existenz von Lamin A-LAP2 α Komplexen im Zellkern hin und stellen, zum ersten Mal, einen negativen Einfluss von LAP2 α auf Lamin A-Assemblierung dar.

2 Introduction

2.1 The Nuclear Lamina

The nucleus of metazoan cells is enclosed by the nuclear envelope (NE), which is comprised of two membrane systems, the inner and the outer nuclear membrane, separating the nucleoplasm from the cytoplasm (Figure 2.1). The NE is perforated by nuclear pore complexes that facilitate the transport of macromolecules between cytoplasm and the nuclear interior (Stuurman et al., 1998). Underlying the inner nuclear membrane is a complex filamentous network, referred to as nuclear lamina (Dechat et al., 2010a; Fawcett, 1966; Gerace and Huber, 2012). This nuclear lamina is a proteinaceous scaffold structure that not only provides shape and mechanical stability to the nucleus, but additionally fulfils functions in chromatin organization, gene regulation, cell proliferation and differentiation (Dechat et al., 2009; Goldman et al., 2002; Gruenbaum et al., 2005; Peric-Hupkes and van Steensel, 2010). The major components of the nuclear lamina are type V intermediate filaments (IFs), the nuclear lamins (Dechat et al., 2008; Dittmer and Misteli, 2011; Stuurman et al., 1998), along with a large number of lamin-binding proteins (Schirmer and Foisner, 2007; Vlcek and Foisner, 2007; Wilson and Foisner, 2010).

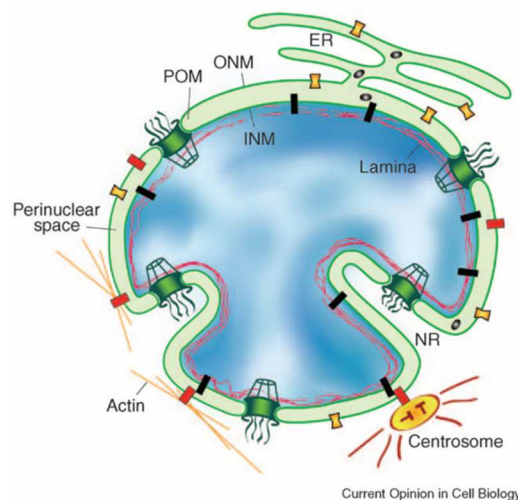


Figure 2.1: Schematic diagram of the nucleus, the nuclear envelope and associated structures. The NE consists of the outer nuclear membrane (ONM), the inner nuclear membrane (INM) and the pore membrane (POM). The ONM is connected to the endoplasmic reticulum (ER). The INM contains distinct proteins (black) that contact the underlying lamina and chromatin. Modified from (Prunuske and Ullman, 2006).

2.2 The Nuclear Lamins

Nuclear lamins are the evolutionary most conserved IF proteins and the main building blocks of the nuclear lamina. Based on sequence homologies, expression patterns, structural

and functional properties, lamins are grouped into A- and B-type lamins (Burke and Stewart, 2013; Butin-Israeli et al., 2012). The two major isoforms of A-type lamins, lamin A and the alternative splice variant lamin C, are encoded by one gene, *LMNA*, whereas the B-type lamins, lamin B1 and B2, derive from different genes, *LMNB1* and *LMNB2*, respectively (Adam and Goldman, 2012; Peter et al., 1989). Concerning their expression pattern, B-type lamins are constitutively expressed during cell development and are suggested to be important for cellular differentiation, proliferation and cell survival. The expression of lamins A/C is suggested to take place at later stages during development in a tissue-specific manner (Broers et al., 2006; Dechat et al., 2010a).

2.2.1 Structure of Nuclear Lamins

Lamins share a highly conserved structure with all intermediate filament proteins. They consist of an α -helical central rod domain flanked by globular amino-terminal head and carboxy-terminal tail domains (Figure 2.2) (Herrmann and Aeibi, 2004; Stuurman et al., 1998). The rod itself is composed of four coiled-coil forming sub-domains termed 1A, 1B, 2A, and 2B, which are connected by the variable linker motifs L1, L2 and L12. Each lamin coiled-coil segment contains patterns of heptad repeats with conserved numbers of amino acids (Herrmann and Strelkov, 2011; Worman, 2012), with coil 1B containing 42 additional amino acids (six heptad repeats) compared to cytoplasmic IFs (Worman, 2012). Within their C-terminal tail domain all lamins have a nuclear localization signal (NLS), which mediates import into the nucleus, and an immunoglobulin like motif (Ig-fold), that is likely involved in protein-protein interactions (Dechat et al., 2000a; Shumaker et al., 2008).

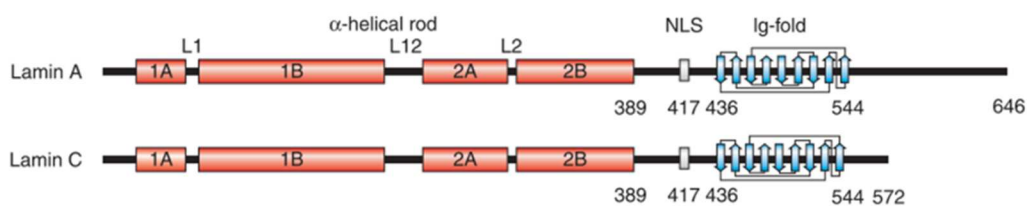


Figure 2.2: Structure of nuclear lamins. A schematic model of mature lamin A and lamin C polypeptide chains. Lamins consist of a short amino terminal head domain, a central α -helical rod domain (red), and the carboxy-terminal domain that contains the nuclear localization signal and the Ig-fold (blue). Modified from (Dechat et al., 2010a)

All lamin proteins, except lamin C, become post-translationally modified in several steps, as they are expressed as pre-lamins containing a carboxy-terminal –CAAX box. These modifications include the farnesylation of the cysteine residue by a farnesyltransferase, followed by the cleavage of the last three amino acids (–AAX) by the endoproteases Rce1 and/or

Zmpste24/FACE1. Subsequently, the remaining cysteine residue becomes carboxy-methylated. Lamin A but not B-type lamins is further processed by a second proteolytic cleavage that removes the last 15 C-terminal amino acids, including the farnesylated and carboxy-methylated cysteine residue, facilitated by Zmpste24/FACE1 (Figure 2.3) (Dechat et al., 2008; Zwerger and Medalia, 2013). Therefore, lamin A is less tightly associated with the inner nuclear membrane and also present within the nuclear interior in more soluble and dynamic structures compared to the lamina-associated lamin A at the nuclear periphery. In contrast, the permanently farnesylated and carboxy-methylated B-type lamins are stably associated with membranes throughout the cell cycle (see 2.2.4) (Dechat et al., 2010b; Gruenbaum and Medalia, 2015).

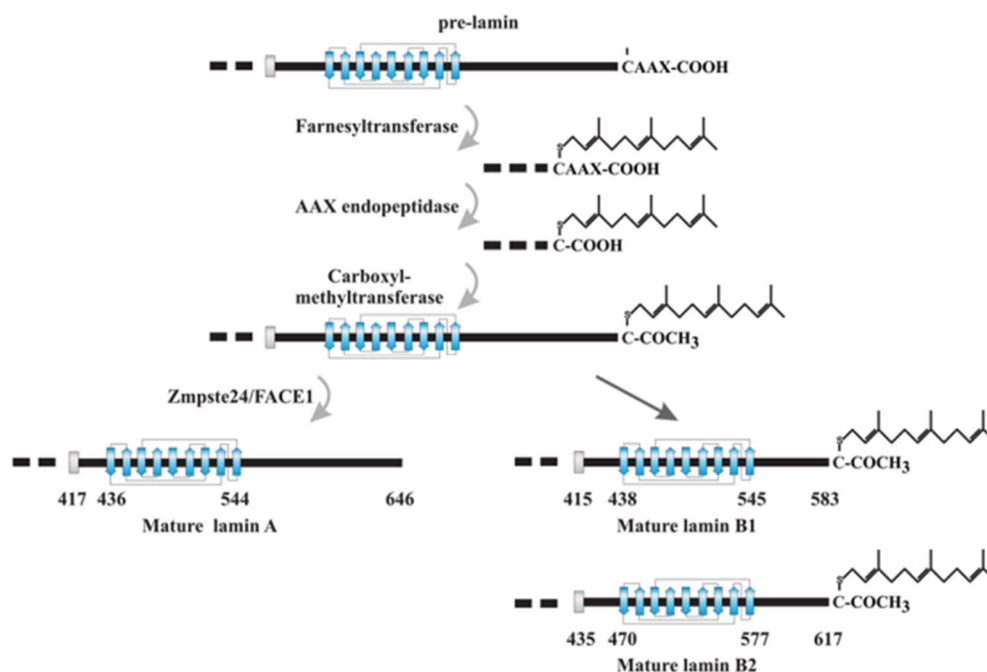


Figure 2.3: Posttranslational processing of pre-lamin A, B1 and B2. The pre-proteins are modified by farnesylation of the carboxyl terminus. These steps occur sequentially beginning with the addition of farnesyl to the cysteine residue of the –CAAX sequence, followed by removal of the three terminal amino acids and methylation of the terminal carboxylic acid group. In lamin A, additional 15 amino acids are removed by protease cleavage along with the attached farnesyl motif. B-type lamins remain permanently farnesylated (Adam and Goldman, 2012).

Upon nuclear envelope breakdown at the onset of mitosis the lamina has to disassemble. Therefore, A- and B-type lamins become phosphorylated by cyclin-dependent kinases and dissociate sequentially from the lamina (Dechat et al., 2010a; Heald and McKeon, 1990). Recent studies revealed that in a mitotic cell farnesylated B-type lamins mostly remain associated with nuclear membrane fragments, while A-type lamins become soluble and

subsequently disperse within the whole cell (Dechat et al., 2007). During nuclear envelope reassembly at the end of mitosis lamins become dephosphorylated and start to relocate to the newly forming nuclei. While B-type lamins exclusively relocate to the nuclear periphery, A-type lamins accumulate first in the nucleoplasm and subsequently reincorporate into the lamina meshwork (Dechat et al., 2010b; Moir et al., 2000).

2.2.2 Mechanisms of Lamin Assembly

A characteristic feature of all lamins and necessary for most of their functions is their ability to assemble into higher order structures. Although the lamin assembly process has been extensively studied *in vitro*, still very little is known regarding lamin assembly properties and mechanisms *in vivo* (Osmanagic-Myers et al., 2015; Zwerger and Medalia, 2013). Previous studies reveal a dimer formed by parallel association of the α -helical rod domains of two lamin monomers as the basic subunit of all higher order lamin structures (Figure 2.4A) (Heitlinger et al., 1991; Stuurman et al., 1998). In the next assembly step the lamin dimers associate longitudinally in a “head to tail” fashion forming polar proto-filaments (Figure 2.4B,D), that can further laterally assemble in an antiparallel orientation forming either ~10 nm thick filamentous structures or paracrystals (Figure 2.4C) (Ben-Harush et al., 2009; Karabinos et al., 2003; Stuurman et al., 1998). Whereas lamin filaments form under more physiological conditions (i.e. pH 6.0, 300 mM NaCl), high-pH conditions (i.e. pH 9.0) lead to the formation of paracrystalline fibers exhibiting a pronounced axial repeat pattern of 24-25 nm (Figure 2.4E,F) (Herrmann and Aebi, 2004). The repeating pattern presumably occurs through the lateral association of the paired globular tail domains, forming the high-density band (Ben-Harush et al., 2009).

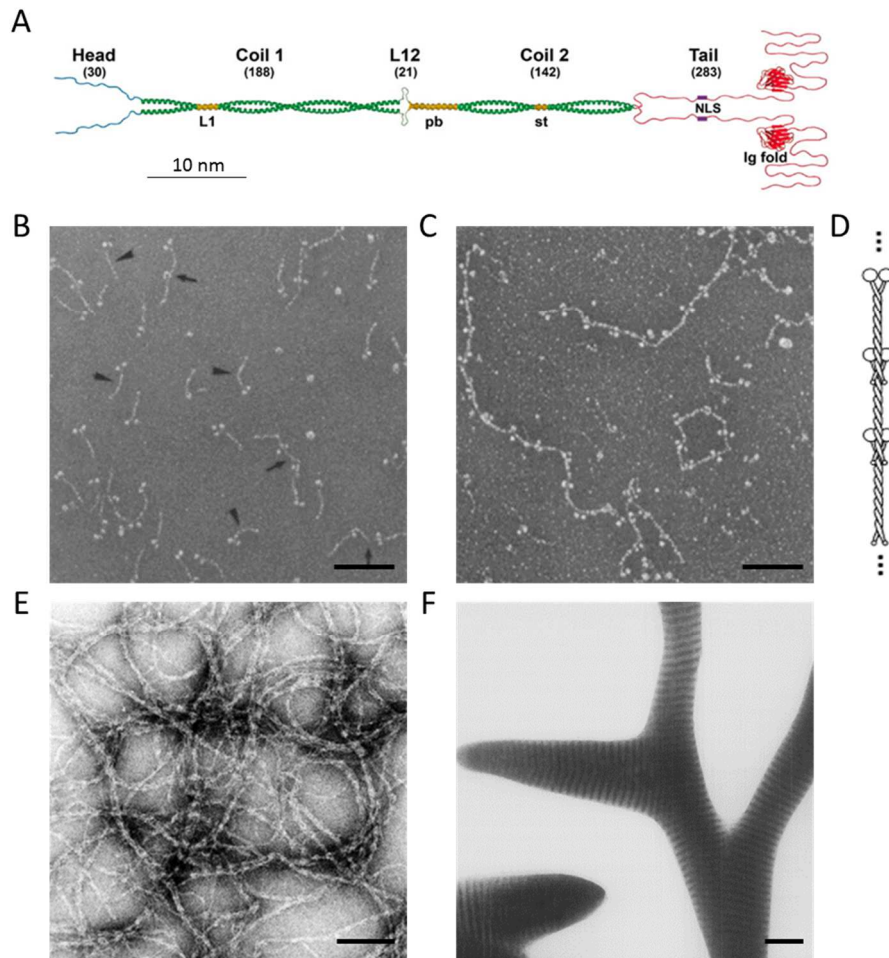


Figure 2.4: Schematic representation and *in vitro* studies of lamin assemblies. A) Structural model of the lamin dimer formed via lateral association of two rod domains (green). α -helical amino-terminal domain (head) shown in blue and carboxy-terminal domain (tail) shown in red. L: Linker; pb: paired α -helices; stu: stutter; NLS: nuclear localization signal; (Kapinos et al., 2010). B) As a first step of structural organization bacterially expressed chicken lamin B2 forms “myosin-like” dimers (arrowheads), short head-to-tail polymers (arrows) and further linear head-to-tail fibers (C, D); Modified from (Heitlinger et al., 1991; Stuurman et al., 1998). Higher order structures include beaded filaments or fibers (E) and eventually paracrystalline arrays (F) dependent on dialyzing conditions (Heitlinger et al., 1992; Stuurman et al., 1998). For electron microscopy, samples were prepared by either glycerol spraying/low-angle rotary metal shadowing (B, C) or negative staining (E, F). Scale bar (B-F) 100 nm.

Contrary to the lamin assembly *in vitro*, the *in vivo* situation remains mostly unclear. Even though the *in vitro* studies reveal important informations, direct conclusions on the assembly of lamins within a living cell have to be made with care (Zwerger and Medalia, 2013). Furthermore in most *in vitro* studies on assembly lamin proteins were purified from tissue or bacteria, which required denaturing conditions, for example high molar concentrations of urea or guanidinium hydrochloride (Herrmann and Aebi, 2004). It is still not entirely clear whether the reconstitution conditions enable lamins to re-establish their native structure. Additional modifications, the interaction with lamin binding partners and possible chaperone activity within a living cell presumably influence the assembly process *in vivo* and need consideration (Zwerger and Medalia, 2013).

2.2.3 Disease-associated Lamin Mutations: Laminopathies

Given the important structural and functional role of lamins in the functional organization of the nucleus, mutations in lamin genes resulting in aberrant expression patterns, structure, assembly properties, binding activities and/or processing of lamins, in particular A-type lamins, cause a broad spectrum of diseases, collectively termed “laminopathies” (Worman, 2012). These diseases can be roughly classified into four distinct groups, considering possible overlaps between them: 1. Diseases of striated muscles such as Emery-Dreifuss muscular dystrophy or limb-girdle muscular dystrophy; 2. Peripheral Neuropathy (Charcot-Marie-Tooth type 2B1); 3. Lipodystrophy Syndromes, e.g. Dunnigan-type Familial Partial Lipodystrophy; and 4. Accelerated Aging Disorders including Hutchinson-Gilford Progeria Syndrome (HGPS) and Atypical Werner Syndrome (Worman and Bonne, 2007). Most mutations occurring in *LMNA* are single point mutations leading to mutated lamin A and lamin C proteins with a single amino acid change (Gotzmann and Foisner, 2006). Hence, these mutations can affect protein structure and localization and impair interaction with other proteins (Bollati et al., 2012; Capanni et al., 2005; Eriksson et al., 2003). Regarding the effect of laminopathies causing mutations on lamin A/C filament and paracrystal assembly very little is known. So far it has been shown that some mutations interfere with the assembly of *Caenorhabditis elegans* lamin (Ce-lamin) and lead to impaired filament and paracrystal structures *in vitro* (Bank et al., 2012; Wiesel et al., 2008). Although the underlying pathogenic mechanisms of laminopathies still need more investigations, the variety and tissue-specificity of these diseases already enable to connect some potential functions of lamins with laminopathy phenotypes (Gruenbaum et al., 2005).

2.2.4 Lamins in the Nuclear Interior

Traditionally lamins have been regarded as the main constituents of the nuclear lamina and it was assumed that lamins are present only at the nuclear periphery. However, recent data clearly revealed the presence of lamins in the nuclear interior (Dechat et al., 2010b). In case of A-type lamins, this nucleoplasmic pool can make up to 10% of the total lamin A/C amount (Gruenbaum and Medalia, 2015; Kolb et al., 2011). Fluorescence correlation spectroscopy (FCS) (Shimi et al., 2008) and fluorescence recovery after photobleaching (FRAP) (Broers et al., 1999; Moir et al., 2000) studies with GFP-tagged A-type lamins revealed a very fast recovery throughout the nucleoplasm but a low recovery within the lamina, indicating that nucleoplasmic A-type lamins are more dynamic most likely due to their presence in a lower assembly state. In contrast, nucleoplasmic B-type lamins are present as more immobile and stable structures (Moir et al., 2000; Shimi et al., 2008).

Recent studies suggest that both peripheral and nucleoplasmic lamins may serve important functions in chromatin organization, gene transcription, cell cycle progression and differentiation but the detailed mechanisms still have to be revealed (Dechat et al., 2010b; Gesson et al., 2014). Further it is not completely clear how the nucleoplasmic fraction of lamins is regulated. One possibility is that A-type lamins are kept in the nuclear interior and prevented from incorporation into the nuclear lamina through interaction with specific binding partners (Dorner et al., 2007). Among many lamin binding proteins the lamina-associated polypeptide (LAP) 2 α seems to be a key factor in the establishment and stabilization of the nucleoplasmic lamin A/C pool (Dechat et al., 2010b; Dechat et al., 2000a). Evidence for this comes from immunofluorescence studies which revealed a substantial loss of nucleoplasmic A-type lamins in fibroblast cells derived from LAP2 α knock-out mice compared to wild type fibroblasts (Naetar et al., 2008). Interestingly, the expression of full length LAP2 α , but not of a LAP2 α mutant lacking the lamin A/C binding domain (Dechat et al., 2000a), can restore the nucleoplasmic pool of A-type lamins in the LAP2 α ^{-/-} fibroblasts. In addition, biochemical extraction studies showed that the soluble fraction of lamins A/C disappeared completely in the LAP2 α ^{-/-} cells (Naetar et al., 2008). In agreement with the data obtained from LAP2 α deficient mouse fibroblasts the knock-down of LAP2 α by RNAi in human fibroblasts reduces the amount of nucleoplasmic lamins (Pekovic et al., 2007; Pilat et al., 2013). In addition to the regulation of nucleoplasmic lamins by protein-interactions the phosphorylation of lamins, especially at serine residues 22 (Ser22) and 392 (Ser392), both flanking the rod domain of A-type lamins, could contribute to the solubilization of lamins A/C and their localization in the nuclear interior (Gruenbaum and Medalia, 2015; Kochin et al., 2014; Swift et al., 2013).

2.3 Lamina-Associated Polypeptide 2, isoform α (LAP2 α)

The mammalian LAP2 proteins (originally termed thymopoietines (TMPO)) belong to the LEM protein family, such as Emerin, MAN1, LEM2, LEMD1, Ankle 1 and Ankle2 (Brachner and Foisner, 2014). Six isoforms (α , β , γ , δ , ϵ and ζ) derive from the LAP2 gene by alternative splicing and all of them share a common N-terminal 187 amino acid region (Figure 2.5A) (Berger et al., 1996; Dechat et al., 2000b; Furukawa et al., 1995; Pekovic et al., 2007). This common region contains the conserved LEM (LAP2-Emerin-MAN1) domain, a structural motif of about 40 amino acids and mainly composed of two α -helices, that mediates the interaction with chromatin via binding to the DNA-bridging protein barrier-to-autointegration factor (BAF). Up-stream of the LEM domain LAP2 α proteins contain an additional LEM-like region that has been shown to interact with DNA directly (Cai et al., 2001; Segura-Totten and

Wilson, 2004; Snyers et al., 2007). In contrast to the other LAP2 isoforms, LAP2 α contains a unique and functionally divergent C-terminal domain (CTD) lacking a transmembrane domain. Therefore LAP2 α localizes exclusively within the nucleoplasm while most of the other proteins are integral proteins of the inner nuclear membrane (Dechat et al., 2004; Dechat et al., 1998). LAP2 α 's CTD further contains a chromosome association site (Vlcek et al., 1999), a region supposed to interact with the tumor suppressor and major cell cycle regulator retinoblastoma protein (pRb) (Markiewicz et al., 2002), and at its very C-terminus an interaction site for lamins A/C (Dechat et al., 2000a). In addition, the LAP2 α specific domain has been shown to bind to the LAP2 α interactor-25 (LINT-25) and to the high mobility group protein N5 (HMGN5) (Naetar et al., 2007; Zhang et al., 2013). The crystal structure of the residues between 459 and 693 of LAP2 α reveals an extensive four-stranded coiled-coil (Figure 2.5B) and is suggested to form an oligomer, most likely a dimeric (Bradley et al., 2007) or trimeric structure (Snyers et al., 2007).

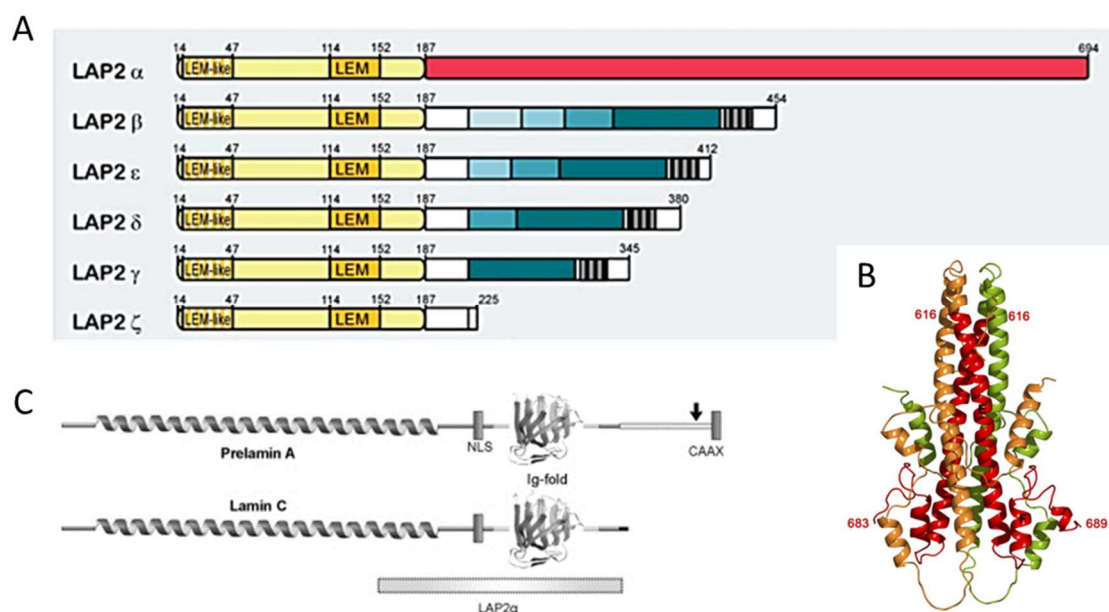


Figure 2.5: Structural organization of the LAP2 isoforms and schematic model for lamin A/C binding. A) Common amino-terminal region containing LEM-like and LEM domains for DNA binding is depicted in yellow. LAP2 α has a unique and functionally different carboxy-terminal domain (red) lacking a transmembrane region. Modified from (Wagner and Krohne, 2007) B) The crystal structure of the LAP2 α C-terminal domain reveals an extensive four-stranded coiled-coil and is suggested to form a dimer. One monomer is shown in yellow, one in green. Red displays the last 78 residues that are reported to bind lamin A/C (Bradley et al., 2007). C) Depicted is the proposed binding region of LAP2 α to lamin A/C (Dechat et al., 2010b).

2.4 The Nucleoplasmic Lamin A/C-LAP2 α complex

A number of studies examined the interaction between A-type lamins and LAP2 α , using various assays, such as co-immunoprecipitation, co-immunofluorescence microscopy, solid-phase overlay, pull-down experiments and *in vivo* proximity analysis (BioID) (Dechat et al., 2004; Dechat et al., 2000a; Gesson et al., 2014; Roux et al., 2012). It has been shown that LAP2 α not only co-localizes with intranuclear lamin A/C structures but also seems to be both essential and sufficient to maintain the nucleoplasmic pool of A-type lamins in G1 cells (Dechat et al., 2000a; Naetar and Foisner, 2009). As mentioned in the previous chapter, LAP2 α binds to A-type lamins via its C-terminal end, presumably amino acids 615-693. The corresponding binding region was mapped to amino acids 319-572 on lamin A/C and can be most likely narrowed down to the Ig-fold motif (Figure 2.5C) [(Dechat et al., 2000a), and unpublished data]. The LAP2 α -lamin A/C complexes may contribute to various specific cellular functions, such as chromatin organization and/or gene regulation, transcriptional control, cell cycle regulation and even maintenance of mechanical stability, but the detailed mechanisms remain in many cases elusive (Andres and Gonzalez, 2009; Dechat et al., 2010a; Dechat et al., 2010b; Dorner et al., 2007; Kind and van Steensel, 2010; Markiewicz et al., 2002). Since LAP2 α binds to a region on A-type lamins where many disease associated mutations are located, it has been suggested that an impaired lamin A/C-LAP2 α interaction might contribute to distinct laminopathy-phenotypes (Goldman et al., 2004; Pekovic et al., 2007). Further evidence for this suggestion comes from a mutation in the C-terminal domain of LAP2 α , which has been associated with dilated cardiomyopathy and leads to an impaired lamin A binding *in vitro* (Snyers et al., 2007; Taylor et al., 2005).

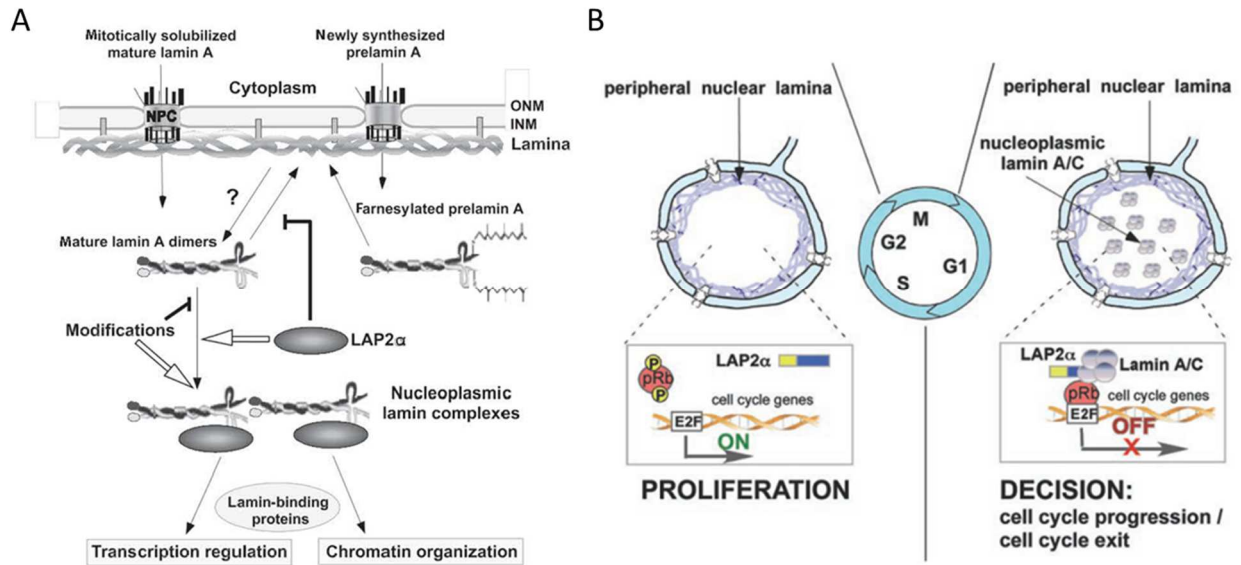


Figure 2.6: Potential regulation of nucleoplasmic lamins and model of the function of nucleoplasmic lamins A/C-LAP2α-pRB complexes in cell cycle progression. A) Mitotically solubilized, mature lamin and newly synthesized prelamin A are imported into the nucleus through nuclear pore complexes (NPC). Prelamin A is processed and finally integrated into the lamina. Mature lamin either polymerizes into the lamina or is retained in the nucleoplasm with the help of LAP2α and, likely, specific modifications. Nucleoplasmic LAP2α-lamin complexes serve important functions in gene expression and chromatin organization. (ONM) Outer nuclear membrane, (INM) inner nuclear membrane (Dechat et al., 2010b). B) While in S phase cells A-type lamins are found exclusively at the nuclear periphery, LAP2α stabilizes a pool of A-type lamins in the nuclear interior during G1. The nucleoplasmic lamins A/C-LAP2α complex binds to pRb at E2F dependent promoters G1 phase and helps to inhibit E2F-dependent transcription, which is essential for cell cycle exit and differentiation. In S phase, pRb is phosphorylated, causing dissociation of the complex, loss of nucleoplasmic lamins A and C and activation of E2F-dependent transcription (Naetar and Foisner, 2009).

There are still many open questions regarding the mechanisms how LAP2α regulates nucleoplasmic lamins and prevents their integration into the nuclear lamina. A possible scenario suggests a dynamic exchange of A-type lamin subunits between the peripheral lamina and the nuclear interior (Figure 2.6A). Thereby, LAP2α binding stabilizes the nucleoplasmic pool by preventing mature lamin A/C translocation to the nuclear periphery and assembly into the lamina. Such nucleoplasmic A-type lamin-LAP2α complexes, presumably together with other lamin binding proteins, are suggested to be involved in various cellular processes (Dechat et al., 2010b; Gesson et al., 2014; Naetar and Foisner, 2009). For example, several studies revealed a role of the lamin A/C-LAP2α complex in cell cycle progression or exit, via the pRb pathway (Figure 2.6B) (Markiewicz et al., 2005; Naetar and Foisner, 2009; Pekovic et al., 2007). Both, lamins and LAP2α, bind to the hypophosphorylated form of pRb (Mancini et al., 1994; Markiewicz et al., 2002; Ozaki et al., 1994), presumably causing the stabilization and nuclear retention of the active form of the suppressor protein and thereby facilitating the inhibition of the E2F-dependent transcription in G1 phase that is essential for cell cycle exit and

differentiation (Mittnacht and Weinberg, 1991; Naetar and Foisner, 2009). At the G1/S phase transition, pRb becomes phosphorylated and dissociates from the complex. Hence, reactivation of E2F transcription factors leads to cell proliferation (Naetar and Foisner, 2009).

2.5 Aim of this Study

Both peripheral and nucleoplasmic lamins play an important role in providing mechanical stability to the nucleus but also fulfill essential functions in diverse cellular processes (Dechat et al., 2010b). The regulation of these lamin pools by potential interaction partners, such as LAP2 α , is the basis of continuous investigations. A number of studies have shown that LAP2 α interacts directly with nuclear A-type lamins (Dechat et al., 2004; Dechat et al., 2000a; Naetar and Foisner, 2009; Roux et al., 2012) and it is assumed that LAP2 α keeps lamin A/C soluble in the nucleoplasm by preventing their assembly into the nuclear lamina through complex formation (Dechat et al., 2010b; Gruenbaum and Medalia, 2015; Naetar and Foisner, 2009). However, the mechanisms underlying the regulation of the nucleoplasmic pool of A-type lamins remain mostly unclear.

The aim of this work was first to determine the abundance of LAP2 α , lamin A and lamin C in human cells to gain information about the potential lamin A/C to LAP2 α ratio per cell for both, total protein and soluble nucleoplasmic protein amounts. These data can provide important information on the possible composition/stoichiometry of LAP2 α -lamin A/C complexes. Therefore, cell lysates of HeLa, hTERT-BJ1 and U2OS cells were prepared and the amounts of lamin A, C and LAP2 α were estimated by quantitative western blot analyses.

The second part of my thesis focuses on the impact of LAP2 α on the assembly and formation of lamin structures *in vitro* and on the stoichiometric ratio of the lamin A and LAP2 α interaction. Depending on the conditions recombinant lamin A assembles *in vitro* into filamentous or paracrystalline structures. Alterations in the lamin assembly process as well as in the solubility of recombinant lamin A in the presence of LAP2 α were studied by electron microscopy and various sedimentation assays.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

Name	Company
Acrylamide 30% (29:1)	Serva
Ammonium persulfate (APS)	Loba
Ampicillin Sodium Salt	Sigma
Benzonase (25 U/ μ l)	Novagen
Bovine Serum Albumin (BSA)	Sigma
Bromophenol blue	Sigma/Merck
Carbenicillin disodium salt	Sigma
Coomassie® Brilliant Blue G250	Serva
D(+)-Saccharose	Roth
Dithiotreitol (DTT)	Sigma
DNaseI (10 mg/ml)	Roche
Ethanol 100%	Merck
Ethylene-diamine-tetraacetic acid (EDTA)	Loba
Ethylene-glycol-tetraacetic acid (EGTA)	Sigma
Fetal Calf Serum (FCS)	Sigma
Glycerol	Sigma
Glycine	Merck
Hepes	Sigma
Hydrochlorid acid (HCl)	Appli. Chem.
Imidazole	Appli. Chem.
Isopropyl β -D-1-thiogalactopyranoside (IPTG)	Peqlab
KH ₂ PO ₄	Sigma
LB broth base	Roth
L-Glutamine	PAA/Pan-Biotech
Magnesium chloride (MgCl ₂ x 6 H ₂ O)	Sigma
Magnesium sulfate (MgSO ₄ x 7 H ₂ O)	Loba
MES	Sigma
Milk powder	Roth
Morpholinoethane sulfonic acid (MES)	Serva
Na ₂ HPO ₄ x 2 H ₂ O	Merck
Nonessential amino acids (NEAA)	GE/Pan-Biotech
Ponceau S	Sigma
Potassium chloride KCl	Merck
RNase 10 mg/ml	Thermo Scientific
Sodium chloride (NaCl)	Sigma/VWR
Sodium dodecyl sulfate (SDS) 20% in H ₂ O	Sigma
Sodium hydroxide (NaOH)	Merck
β -Mercaptoethanol	Merck

TEMED
 Tris-(hydroxymethyl)-aminomethane
 Triton X-100
 Tween-20
 Urea

Sigma
 Sigma/Appli. Chem.
 Sigma
 Merck
 Merck

3.1.2 General Equipment

Name	Company
Airfuge High-speed centrifuge	Beckman Coulter
Bio Photometer	Eppendorf
CASY Cell Counter and Analyzer	Roche
Centrifuge Avanti J-26 XP	Beckman Coulter
Centrifuge Avanti™ J-25	Beckman Coulter
Centrifuge Varifuge 3.0R	Heraeus
CO ₂ Incubator	Binder
Dry block thermostat	Biosan
EM910 transmission electron microscope	Zeiss
End-over-end Rotator	Labor Brand
Gel electrophoresis power supply	Bio-Rad
Gel electrophoresis power supply Phero-stab. 500	Biotec-Fischer
Gradient-Mixer	Hoefer
Hot plate magnetic stirrer MR 3001	Heidolph
Incubator Shaker Innova 40	New Brunswick Scientific
Incubator Shaker Innova 43	New Brunswick Scientific
Incubator SM-30 Control	Edmund Bühler
Megafuge 1.0	Heraeus
Multiskan RC	Labsystems
Nanodrop Spectrophotometer ND-1000	Peqlab
Odyssey	LI-COR
pH-Meter	Schott
Pipetboy 2	Integra
Pipettes (P2, P20, P200, P1000)	Gilson
Precision balance PJ6000	Mettler Toledo
Precision balance PM460	Mettler Toledo
Precision balance PR503	Mettler Toledo
SDS-Gel apparatus	Bio-Rad
Shaker Duomax 1030	Heidolph
Shaker KL2	Edmund Bühler
Sputter Coater SCD 005	Bal-Tec
Table Centrifuge 5417R	Eppendorf
Table centrifuge Biofuge pico	Heraeus
Table Centrifuge Perfect Spin 24 Plus	Peqlab
Thermomixer compact	Eppendorf

Ultracentrifuge L-60	Beckman Coulter
Ultracentrifuge Optima L-80 XP	Beckman Coulter
Ultracentrifuge Optima-XLA	Beckman Coulter
UV-Vis Spectrophotometer 4E	Varian Cary
UV-Vis Spectrophotometer 6315	Jenway
Vortex Genie 2	Scientific Industries
Waterbath	Memmert
Western blot transfer chamber	Bio-Rad

3.1.3 Disposables

Name	Company
Cuvettes (1.5 ml)	Sarstedt
Dialysis clamps	Sigma
Dialysis tubing cellulose membrane (12-14 kDa)	Sigma
Eppendorf reaction tubes (1.5 ml, 2 ml)	Eppendorf
Falcon tubes (15 ml, 50 ml)	Falcon/VWR
Filter tips	Starlab
Folded Filters	Peqlab
Inoculation loops	VWR
Low bind reaction tubes (1.5 ml)	Eppendorf
Nitrocellulose membrane	GE Healthcare
Parafilm	Pechiney
Pasteur capillary glass pipettes	Marienfeld
Petri dishes (Ø 6 cm, Ø 10 cm)	Thermo Scientific
Pipettes (5 ml, 10 ml, 25 ml)	Falcon
Polyallomer Centrifugation tubes #331374	Beckman
Syringe Filters (0.2 µm)	Roth

3.1.4 Media, Buffers and Solutions

10 x SDS Laemmli Running Buffer	0.25 M Tris 1.92 M Glycine 1% SDS pH 8.3
10% Resolving gel (30ml)	11.9 ml dH ₂ O 10.0 ml 30% Acrylamid/Bisacrylamid 7.5 ml 1 M Tris/HCl, pH 8.8 0.3 ml 10% SDS 0.3 ml 10% APS 12 µl TEMED

10x PBS	15 mM KH_2PO_4 80 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 26 mM KCl 1.37 M NaCl pH 7.4
10x Western Blot Transfer Buffer	0.25 M Tris 1.92 M Glycine pH 8.3
1x PBST	1x PBS + 0.05% Tween 20
3x Laemmli Sample Buffer (SB)	30% Glycerol 9% SDS 187.5 mM Tris.HCl 150 mM DTT 0.01% Bromophenol blue pH 6.8
5% Stacking gel (10ml)	6.8 ml dH_2O 1.7 ml 30% Acrylamid/Bisacrylamid 1.25 ml 1M Tris/HCl, pH 6.8 0.1 ml 10% SDS 0.1 ml 10% APS 10 μl TEMED
50 x Protease Inhibitor Roche	1 Tablet in 50 ml dH_2O
Blocking Buffer (5% milk)	2.5 g milk powder in 50 ml PBST
Dulbecco's Modified Eagle Medium (DMEM) GE/Healthcare	High Glucose (4.5 g/l) Supplemented with: 10% fetal calf serum (FCS) 1% Penicillin/Streptomycin 1% L-Glutamine 1% NEAA
Dulbecco's Phosphate Buffered Saline (PBS) Gibco	

Filament-Assembly Buffer (high pH)	250 mM NaCl 25 mM Tris pH 8.0 1 mM DTT
Filament-Assembly Buffer (low pH)	250 mM NaCl 25 mM MES pH 6.5 1 mM DTT
Fractionation Buffer	50 mM Hepes, pH 7,4 10 mM MgCl ₂ 5 mM EGTA 100 mM NaCl 1 mM DTT 1 x complete protease inhibitor cocktail (Roche) 0.5% Triton TX-100 (keep at 4°C)
LB Medium	2% LB-Broth-Base 0.25% MgSO ₄ x 7 H ₂ O pH 7.5
Paracrystal-Assembly Buffer	300 mM / 200 mM / 100 mM / 50 mM NaCl 10 mM Tris.HCl pH 7.5 1 mM DTT
Elution-Buffer	10 mM Tris.HCl pH 8.0 300 mM NaCl 8 M Urea 1 mM β-Mercaptoethanol 250 mM Imidazole
Purification Buffer	10 mM Tris.HCl pH 8.0 300 mM NaCl 8 M Urea 1 mM β-Mercaptoethanol 10 mM Imidazole
Wash-Buffer	10 mM Tris.HCl pH 8.0 300 mM NaCl 8 M Urea 1 mM β-Mercaptoethanol 20 mM Imidazole

Trypsin
Pan-Biotech

Supplemented with:
50 mM EDTA

3.1.5 Antibodies

Primary Antibody

Anti-Lap2alpha 245.2 (rabbit polyclonal):

Dilution 1:5000 in 2% BSA-PBST

Anti Lamin A/C 3A64C11 (mouse monoclonal):

Dilution 1:1000 in 2% BSA-PBST

Secondary Antibody

Goat anti rabbit (IRDye 800):

Dilution 1:15000 in PBST

Goat anti mouse (IRDye 800):

Dilution 1:15000 in PBST

3.1.6 Recombinant protein constructs

Presented in Figure 3.1 are all lamin A and LAP2 α constructs used in this study and Table 3.1 and 3.2 summarize the properties [for details see chapter 3.2.1 and (Vlcek et al., 1999; Zwerger et al., 2015)].

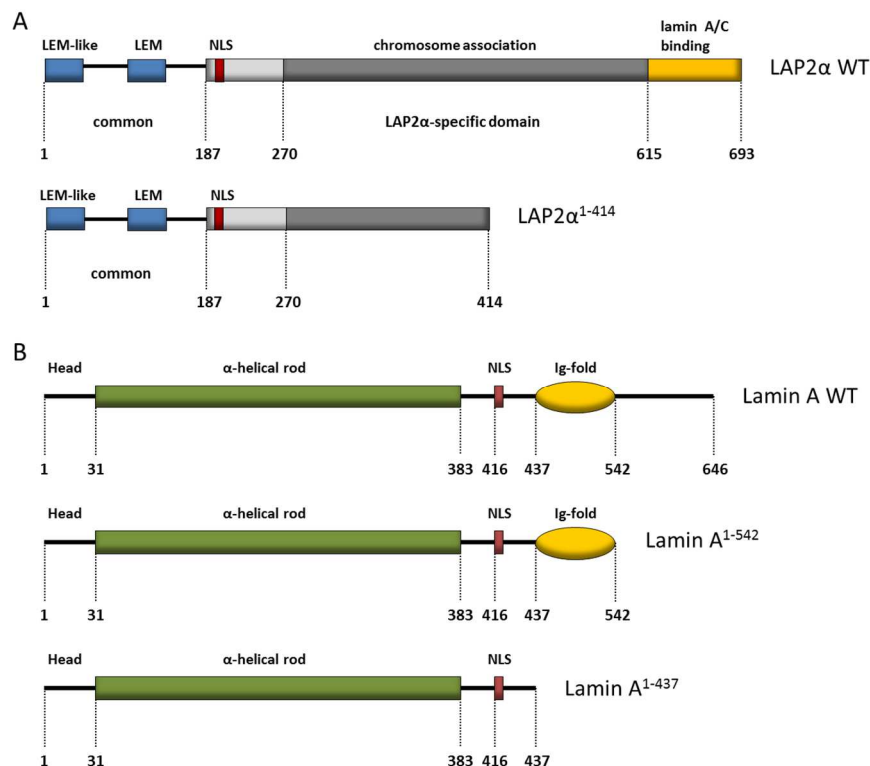


Figure 3.1: Schematic representation of all protein constructs used in this study. A) Full length LAP2 α WT and LAP2 α ¹⁻⁴¹⁴. Depicted in grey is the α -isoform specific carboxyl terminal domain and in yellow the lamin A/C binding domain. B) Full length lamin A WT, lamin A¹⁻⁵⁴², lamin A¹⁻⁴³⁷. The green rod domain is flanked N-terminal by a head region and C-terminal by a domain containing nuclear localization signal (NLS) and Ig-fold.

Protein	Amino acids	Molecular weight [kDa]	Number of Cys
LAP2 α WT	693	75.50	10
LAP2 α ¹⁻⁴¹⁴	414	45.02	4

Table 3.1: LAP2 α wild type and mutant constructs

Protein	Amino acids	Molecular weight [kDa]	Ig-fold
Lamin A WT	646	72.23	Yes
Lamin A ¹⁻⁵⁴²	542	61.74	Yes
Lamin A ¹⁻⁴³⁷	437	50.11	No

Table 3.2: Lamin A wild type and mutant constructs

3.1.7 Others

Name	Company
Coomassie Plus (Bradford) Assay Kit	Thermo Scientific
Gel Code TM Blue Safe Protein Stain	Thermo Scientific
Mixed Bed Resin	Sigma
Ni-NTA Agarose beads	Quiagen
Precision Plus Protein TM Standards All Blue	BioRad
Protease Inhibitor complete, EDTA-free	Roche
Unstained Protein Marker, Broad Range	New England BioLabs

3.2 Experimental Procedures

3.2.1 Protein Preparation

For this project human full length LAP2 α and LAP2 α ¹⁻⁴¹⁴ had to be expressed to obtain enough protein for further analysis. The expression of the recombinant proteins was carried out in *Escherichia coli* strain BL21 (DE3). The plasmids pSV5 and pJH12 were carrying the coding sequence for full length LAP2 α and LAP2 α ¹⁻⁴¹⁴, respectively, under an inducible T7-promoter in a pET23a(+) vector as described in (Vlcek et al., 1999). This vector system added a 6x His-tag to the C-Terminus of the expressed proteins for further affinity purification. Frozen bacterial stocks were provided by the lab of Prof. Roland Foisner, MFPL, Vienna.

Purified recombinant human wild type lamin A, lamin A¹⁻⁴³⁷ and lamin A¹⁻⁵⁴² used in this work were a kind gift of Prof. Harald Herrmann-Lerdon, DKFZ, Heidelberg. Lamin A and lamin A truncated mutants in a pET24a(+) vector were expressed and purified from BL21-CodonPlus(DE3) as described in (Zwerger et al., 2015).

3.2.1.1 Expression of Recombinant Protein

For protein expression, an overnight culture of the respective bacterial strain was diluted 1:50 into fresh LB-Media supplemented with 200 µg/ml Ampicillin and grown at 37°C until an OD₆₀₀ of 0.6 – 0.7. Protein expression was induced with 0.5 mM Isopropyl-β-D-thiogalactopyranosid (IPTG) for 3 h. Cells were harvested by centrifugation at 4.000 rpm for 10 min at 4°C and the obtained bacterial pellets were resuspended in 10 mM Tris.HCl pH 8.0, 100 mM NaCl, and 1 mM DTT. Bacteria were lysed by freezing overnight, thawing and a 3 x 20 sec sonication step in the presence of 170 µg/ml protease inhibitor cocktail. Then 5 µg/ml DNase I and 10 µg/ml RNase were added. After incubation for 15 min at room temperature the suspension was centrifuged at 14.000 rpm for 10 min at 4°C. The pellet contained inclusion bodies of the respective recombinant proteins and bacterial cell components.

3.2.1.2 Protein Purification

For purification of the poly-histidine-tagged recombinant proteins a Ni-NTA-Agarose column was used. 250 µl Ni-NTA-resin (capacity 50 mg/ml) per 500 ml bacterial culture were washed with dH₂O and equilibrated in two volumes Purification Buffer. The protein pellets were resuspended in Purification Buffer and the suspension was cleared from insoluble components by centrifugation. The resulting supernatant was incubated with the washed Ni-NTA-Agarose beads for 30 min at room temperature by end-over-end rotation to induce binding of the His-tagged proteins to the beads. The lysate-bead mix was filled into a column and washed with three column volumes of Wash-Buffer. The recombinant protein was eluted with 250 mM Imidazole (contained in the Elution-Buffer), 0.5-1.0 ml fractions were collected and stored at -20°C. The collected fractions were analyzed by SDS-PAGE regarding the presence and purity of the recombinant proteins. The best fractions were pooled and dialyzed twice against 500 ml of 8 M Urea, 10 mM Tris.HCl pH 8.0, 300 mM NaCl, 1 mM DTT using a cut off of 12-14 kDa to remove Imidazole and β-Mercaptoethanol.

3.2.1.3 Determination of Protein Concentration

Protein concentrations were determined with the Coomassie Plus (Bradford) Assay Kit according to the manufacturer's instructions. Therefore, BSA standards and the recombinant protein samples were mixed with Bradford reagent and the absorbance at 595 nm was measured after 10 min incubation. The concentration of the recombinant protein samples were calculated according to the standard curve.

3.2.2 Cell Culture Maintenance

HeLa, hTERT-BJ1 and U2OS cells were provided as frozen stocks from Dr. Thomas Dechat and Dr. Andreas Brachner (MFPL, Vienna). Cells were thawed, cultivated in DMEM supplemented with 10% FCS, 1% Penicillin/Streptomycin, 1% L-Glutamine and 1% NEAA at 37°C in a humidified 5% CO₂ atmosphere and passaged every three to four days. For passaging the old medium was removed from the petri dish and the cells were washed once with Dulbecco's PBS. Subsequently, the cells were incubated in Trypsin/EDTA at 37°C for 2-3 min to detach, resuspended and diluted 1:5 (hTERT-BJ1) or 1:10 (HeLa and U2OS) in fresh DMEM.

3.2.3 Cell Fractionation

Two 10 cm dishes of approx. 90% confluent HeLa, hTERT-BJ1 or U2OS cells, respectively, were used per experiment. One dish was used to determine the cell number and the other for cell fractionation and subsequent quantitative immunoblot analysis.

For all fractionations the medium was removed, cells washed twice with Dulbecco's PBS and resuspended in 1 ml ice cold Fractionation Buffer (50 mM Hepes pH 7.4, 10 mM MgCl₂, 5 mM EGTA, 100 mM NaCl, 1 mM DTT, 1x complete protease inhibitor, 0.5% Triton TX-100). After a 5 min incubation step on ice a 200 µl aliquot was taken and mixed with 3x Sample Buffer (total sample). The rest of the sample was centrifuged at 14 000 rpm for 10 min at 4°C. The supernatant (800 µl) was mixed with 3x SB and the pellet was resuspended in 1.2 ml 1x SB. To obtain samples lacking mitotic cells, the petri dishes with the adherent cells in PBS were gently tapped a few times to mechanically shake-off the mitotic cells and washed with medium to remove them before lysis.

3.2.4 Quantitative Western Blot

3.2.4.1 Calibration Curve

To quantify the amounts of lamin A, C and LAP2α present in the fractionated cell samples, 60 ng, 40 ng, 20 ng, 10 ng and 5 ng of the purified recombinant lamin A or LAP2α, respectively, were mixed with 1x SB, separated by SDS-PAGE, immunoblotted and quantified (for details see below).

3.2.4.2 Immunoblot

The recombinant proteins and the cell fractionation samples (total, supernatant and pellet) were incubated for 5 min at 95°C and separated on a 10% polyacrylamide gel using a constant current of 25 mA/gel. As protein standard 4 µl Precision Plus Protein™ Standards All Blue

(BioRad) per gel were used. Subsequently, the polyacrylamide gels were rinsed briefly with dH₂O, equilibrated with Western Blot Transfer Buffer and assembled in a wet chamber according to the following scheme: (+) pad–filter–nitrocellulose membrane–gel–filter–pad (–). Here it was important to place the nitrocellulose on the side of the gel facing the cathode, so that the proteins which became negatively charged by SDS, were transferred onto the membrane. The transfer was performed at 60V for 2 hours. To detect the respective proteins the membrane was probed with antibodies. Therefore, unspecific binding sites on the nitrocellulose membrane had first to be blocked by incubation in 30 ml Blocking Buffer for 60 min. Next, the membrane was incubated for 2 hours at room temperature in 15 ml PBST containing 2% BSA and the respective primary antibodies. The blot was then washed with PBST (3 x 5 min) and incubated for another 2 hours in 15 ml PBST containing the respective IRDye-coupled secondary antibodies (protected from light). Finally, the blot was washed again 3x with PBST and protein bands were detected using a LI-COR Odyssey Scanner.

3.2.4.3 Quantification

To quantify the amount of LAP2 α and lamin A present within a single HeLa, hTERT-BJ1 or U2OS cell, respectively, the intensities of the individual Western blot bands (derived from the fractionated cell samples as well as from the recombinant proteins) were measured using *ImageJ* software (see <http://howtowesternblot.net/data-analysis-3/quantification/> and (Taylor and Posch, 2014)). Using the intensities of the bands, representing known amounts of recombinant lamin A and LAP2 α , a standard curve was calculated. Based on this, the number of lamin A, C and LAP2 α molecules per cell was calculated taking into account the number of cells loaded per lane.

3.2.5 In vitro Lamin A Assembly

Assembly of purified recombinant lamin A, LAP2 α and the respective mutants was done according to the following protocols. The denatured proteins dissolved in 8 M Urea, 10 mM Tris.HCl pH 8.0, 300 mM NaCl, 1 mM DTT, were used at a concentration of 0.3 g/l – 0.7 g/l depending on the subsequent experiments performed. As a first step the protein solutions were centrifuged for 75 min in a Beckmann Airfuge at 30 psi to remove aggregated molecules that could interfere with the following dialysis. The proteins were dialyzed either alone or in the presence of their potential binding partner or the respective mutant in a 1:1 ratio against 1 l of the various assembly buffers depending on the used protocol (see below). Therefore, the protein samples were diluted to a concentration of 0.2 g/l to 0.4 g/l in the respective buffer, mixed in

8 M Urea, 10 mM Tris.HCl pH 8.0, 300 mM NaCl, 1 mM DTT, and filled into the dialysis tubes, which were washed 5 times in dH₂O and equilibrated in the first dialysis buffer before.

3.2.5.1 *Paracrystal Assembly of Lamin A*

To slowly induce paracrystal assembly the dialysis was performed stepwise in a 10 mM Tris.HCl pH 7.5, 1 mM DTT buffer containing decreasing salt concentrations, starting at 300 mM NaCl, followed by 200 mM NaCl, and ending at either 100 or 50 mM NaCl. The formation of paracrystals usually starts at a NaCl concentration between 200 and 100 mM. Each step was done at room temperature for 30 min. After the dialysis was completed, samples were taken and analyzed by centrifugation in an Eppendorf centrifuge, sucrose density gradient centrifugation, analytical ultracentrifugation and/or electron microscopy.

To increase the solubility of LAP2 α and thereby facilitate the influence on lamin assembly, in some experiments buffers supplemented with 50 mM DTT instead of 1 mM DTT were used to prevent aggregation of the C-terminal domain (CTD), which contains many cysteine residues, by oxidation induced formation of disulfide bonds.

3.2.5.2 *Filament Assembly of Lamin A*

Lamin A filaments were formed by a two-step dialysis each 1 h at room temperature. In the first step a high pH start buffer (250 mM NaCl, 25 mM Tris.HCl pH 8.0, 1 mM DTT) was used and in the second step a low pH assembly buffer (250 mM NaCl, 25 mM MES pH 6.5, 1 mM DTT). Samples were analyzed by centrifugation in an Eppendorf centrifuge, sucrose density gradient centrifugation and/or electron microscopy.

3.2.6 Negative Staining Electron Microscopy

A 10 μ l aliquot of the dialyzed sample was transferred to a carbon coated electron microscopy grid, which was prior exposed to glow discharge to maintain a hydrophilic surface and thereby increase the affinity for proteins, and left there for 1 min. Then the grid was washed on a drop of distilled water before it was blotted on a drop of 2% uranyl acetate for 15 sec, briefly washed again in water and carefully dried on filter paper.

Samples were examined in a Zeiss EM910 transmission electron microscope in cooperation with Dorothea Möllner and Monika Mauermann (DKFZ, Heidelberg).

3.2.7 Sedimentation Assays

3.2.7.1 *Eppendorf Centrifugation Assay*

For the total sample a 50 μ l aliquot from all paracrystal and filament assembly reactions was taken and mixed with 3x SB. Another 50 μ l sample was spun in an Eppendorf table centrifuge at 13.000 rpm for 10 min at room temperature. The supernatant was collected, mixed with 3x SB and the pellet was resuspended in 75 μ l 1x SB. All protein samples were analyzed by SDS-PAGE. Therefore, the samples were incubated at 95°C for 5 min and loaded on a 10% polyacrylamide gel. To visualize the proteins, the gels were stained with Gel Code™ Blue Safe Protein Stain. For quantitative analysis density measurements of the protein bands were done using *ImageJ* software and the ratio of lamins and LAP2 α , respectively, present in the supernatant fraction versus the total sample was calculated to estimate the percentage of soluble proteins.

In order to investigate the influence of the centrifugation speed on filament assembly dialyzed samples were spun in a table centrifuge for 10 min at 4.000 rpm, 7.000 rpm, 9.000 rpm, 11.000 rpm or 13.000 rpm, after an aliquot (total sample) was taken. The supernatant and the pellet fractions were analyzed as described above.

3.2.7.2 *Analytical Ultracentrifugation (AUC)*

Sedimentation velocity (SV) analytical ultracentrifugation was used to characterize some of the physical properties of LAP2 α , such as its shape, molar mass and assembly state, and to verify whether the sample is homogeneous or if aggregates are present. Furthermore, the method was used to study the potential interaction between LAP2 α and lamin A and the stoichiometry of possible complexes. Measurements were done on an Optima XL-A analytical ultracentrifuge equipped with an An-60 Ti Rotor. Since DTT absorbs at the same range as proteins (280 nm), its concentration was reduced to 0.2 mM. To prevent oxidation the last step of the paracrystal assembly dialysis was performed in degassed buffers.

SV-analysis of LAP2 α were done after the recombinant protein has been dialyzed into paracrystal assembly buffer supplemented with either 300 mM or 100 mM NaCl to monitor the solubility of LAP2 α at lamin non-assembly and lamin assembly conditions, respectively. The dialyzed samples were diluted to a final concentration of 0.1 g/l in a total volume of 400 μ l. The sedimentation velocity run was carried out in a double-chamber cell at 130 000 x g over night at 4°C.

In order to investigate the lamin A-LAP2 α interaction all samples were dialyzed according to the paracrystal assembly protocol and subsequently centrifuged at 13.000 rpm for

10 min to remove aggregates that could disturb the AUC data. Then the collected supernatants were diluted to a final concentration of 0.1 g/l for individual samples and 0.2 g/l for mixed LAP2 α and lamin A samples. The sedimentation velocity run was carried out as described above. All measurements were done and analyzed in collaboration with Dr. Norbert Mücke (DKFZ, Heidelberg).

3.2.7.3 Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation was used to analyze the potential size and composition of lamin A and LAP2 α paracrystal and filament assembly intermediates. Depending on their molecular weight and their shape molecules and protein complexes show varying sedimentation patterns in a density gradient (Brown et al., 2008; Schuck, 2013). A gradient mixer was used to establish a continuous 10 ml gradient ranging from 10% to 30% sucrose in the corresponding assembly buffer layered on top of 1 ml 70% sucrose cushion in a centrifuge tube. For the dialysis paracrystal and filament assembly buffers were complemented with 10% sucrose to maintain a required protein concentration of 0.2 g/l. The dialyzed samples (500 μ l) were then loaded on top of the gradient and centrifuged for 20 h at 190.000 x g in a SW-40 Rotor (Beckman Optima L-70 or Beckman Optima L-80 XP) at 4 °C. Collection of the sedimented protein layers was done by pipetting off 1 ml fractions from the top to the bottom of the gradient, starting with the applied dialyzed protein sample (SN). Aliquots of all gradient fractions were mixed with 3x SB and separated on a 10% polyacrylamide gel. For quantification gels were stained with Gel Code™ Blue Safe Protein Stain and density measurement of the protein bands was done with *ImageJ* software. The amount of protein per fraction was calculated as percentage of the total protein quantity applied onto the gradient.

In order to calculate the amount of lamin A and LAP2 α within each sucrose gradient fraction and hence the stoichiometry of the two proteins within a potential assembly complex, a calibration curve ranging from 2 μ g to 0.4 μ g of the respective recombinant protein was generated (for details see chapter 3.2.4.3). Taking into account the molecular weight of a lamin A or LAP2 α molecule, it was possible to estimate the amount of molecules per fraction for each protein and further the ratio of lamin A to LAP2 α within a potentially formed complex.

4 Results

In 2000, Dechat *et al.* revealed that LAP2 α and A-type lamins specifically interact with each other in the nuclear interior via their C-terminal binding domains (Dechat *et al.*, 2000a). This interaction appears to influence the polymerization state and solubility of lamins A/C and prevent their integration into the nuclear lamina *in vivo* (Naetar *et al.*, 2008). The complex formed by LAP2 α and A-type lamins within the nucleoplasm together with other lamin binding proteins is involved in the regulation of various cellular processes (Dechat *et al.*, 2000b; Dorner *et al.*, 2007; Markiewicz *et al.*, 2005; Naetar and Foisner, 2009). A step towards a better understanding of the relation between nuclear lamins and LAP2 α is to determine the respective quantities of both proteins within a single cell. These data will provide an insight into the potential stoichiometric composition of LAP2 α -lamin A/C complexes within a cell and, most importantly, will allow estimating if there is enough LAP2 α present in the nucleus to regulate the solubility and assembly properties of the nucleoplasmic A-type lamin pool.

To study the influence of LAP2 α on lamin assembly directly, *in vitro* experiments were performed in which the formation of lamin A filaments and paracrystals was investigated in the presence and absence of LAP2 α . In parallel, control experiments were performed using binding-deficient mutant lamin A and LAP2 α . Possible interference of LAP2 α on lamin A polymerization was observed by electron microscopy and various sedimentation assays. While the prior revealed alterations in lamin structures through LAP2 α , the latter (eppendorf centrifugation assay, sucrose density gradient centrifugation and analytical ultracentrifugation) provided information about solubility, altered polymerization and potential stoichiometry of the complex. Overall the results point to an influence of LAP2 α on lamin polymerization and solubility.

4.1 Preparation of recombinant wild type LAP2 α and LAP2 α ¹⁻⁴¹⁴

To construct a standard curve for the quantification of LAP2 α within a cell and study the influence of LAP2 α on the assembly properties of A-type lamins recombinant LAP2 α protein had to be generated. Therefore, full length LAP2 α and LAP2 α ¹⁻⁴¹⁴, a binding-deficient LAP2 α C-terminal truncation mutant, were expressed in *E. coli* and purified via their poly-histidine tag by affinity chromatography. Bound protein was eluted with increasing imidazole concentrations and fractions were collected, which were subsequently analyzed by SDS-PAGE regarding purity and size of the protein products (Figure 4.1). Both, full length LAP2 α as well as LAP2 α ¹⁻⁴¹⁴ had the expected molecular masses (75 kDa and 45 kDa, respectively). In addition to the band of the main product, some smaller fragments were detectable in fractions with high

concentration of eluted protein, possibly degradation products generated during the protein preparation process. Recombinant lamin A was provided by Prof. Harald Herrmann-Lerdon, DKFZ, Heidelberg.

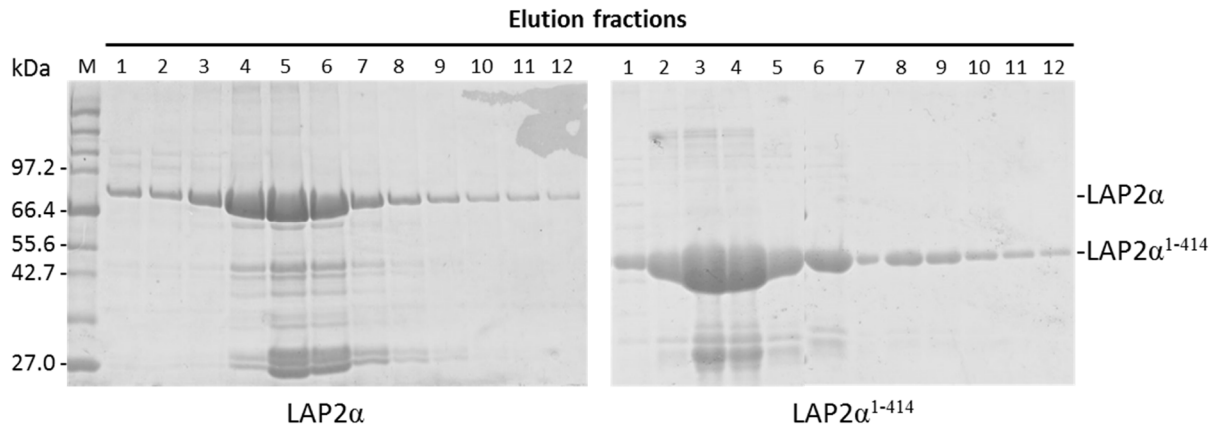


Figure 4.1: SDS-PAGE of elution fractions from affinity chromatography. Lane M shows the molecular weight standard. The numbered lanes represent elution fractions for LAP2 α (left panel) and LAP2 α^{1-414} (right panel).

4.2 Abundance and stoichiometry of lamin A/C and LAP2 α within a cell

4.2.1 Determination of lamin A/C and LAP2 α quantities in human cells

For the quantification of lamin A/C and LAP2 α amounts in cell extracts of HeLa, hTERT-BJ1 and U2OS cells a calibration curve, using recombinant full length lamin A and LAP2 α , was produced. Total, supernatant and pellet fractions of the respective cell lysates, following cell lysis in 1 ml Fractionation buffer, and specific amounts of recombinant proteins ranging from 60 ng to 5 ng were separated by SDS-PAGE, transferred to a nitrocellulose membrane and detected by immunoblotting using antibodies against lamin A/C and LAP2 α . Subsequently, the intensities of the protein bands were evaluated using *ImageJ* software (see Materials and Methods). Different sample quantities had to be loaded to correct for large differences in the expression levels of LAP2 α and lamin A/C between the cell lines and hence to prevent too high or too low signal intensities outside the linear range. These differences were of course taken into account in the subsequent calculations. Figure 4.2 shows representative exemplary blots of the recombinant proteins and the corresponding calibration curves and blots of total, supernatant and pellet fractions of the respective cell lines.

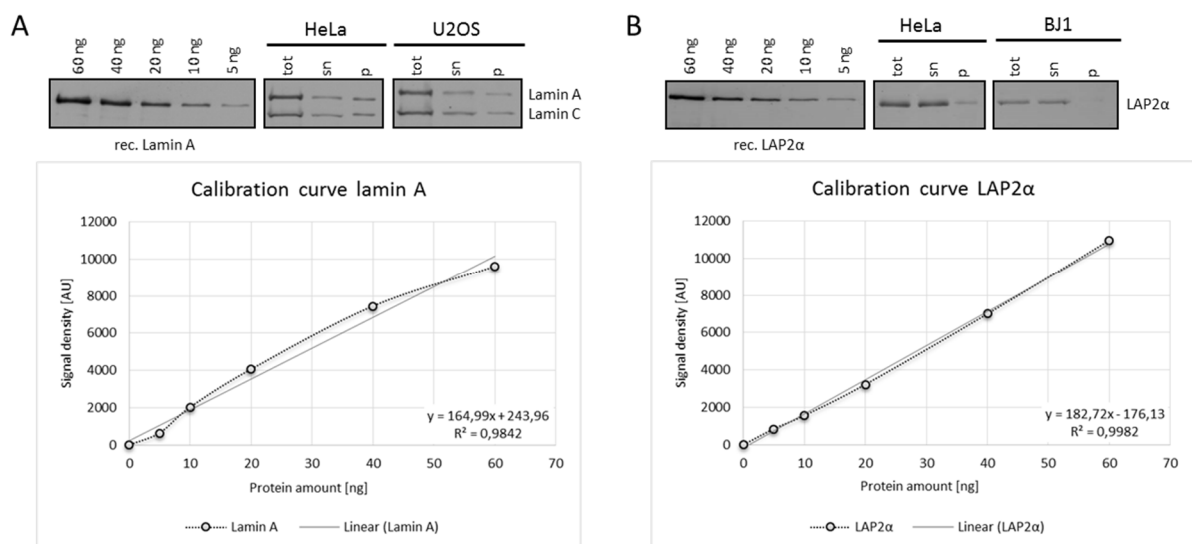


Figure 4.2: Western blot analyses and calibration curves for the standard proteins and cell lysate fractions. 60 ng, 40 ng, 20 ng, 10 ng, 5 ng of recombinant lamin A (A), LAP2α (B), and total (tot), supernatant (sn) and pellet (p) fractions of cell lysates were analyzed by SDS-PAGE and Western blotting. Fluorescence intensities were detected using LI-COR Odyssey and optical density of the blotted bands were measured with ImageJ software.

Both recombinant proteins, lamin A and LAP2α, display a linear signal to protein amount ratio. Therefore the calibration curves could be used to calculate the amounts of LAP2α, lamin A and C present in the cell lysates. While lamin A and lamin C are present in both, the supernatant and pellet fraction (Figure 4.2A), LAP2α is highly enriched in the supernatant, suggesting that it is mostly soluble within cells (Figure 4.2B). More detailed results based on these analyses are depicted in chapter 4.2.2 and 4.2.3.

4.2.2 The lamin A/C to LAP2α ratio depends on the cell line

A step towards better understanding the relation between nuclear lamins and LAP2α is to determine their respective quantities within a cell. Therefore, in this part of my thesis, I quantified the amounts of LAP2α, lamin A and lamin C present within a single HeLa, hTERT-BJ1 and U2OS cell. The obtained data provide an insight into the stoichiometric relation between the respective proteins and therefore may help to understand the mechanisms by which LAP2α influences the solubility of A-type lamins. The number of protein molecules per cell was calculated as described in Materials and Methods. The absolute numbers for all cell lines used (with mitotic cells or after removal of them) are shown in table 4.1. For a better overview the data are also presented as a graph in figure 4.3A. In summary, a HeLa cell and a U2OS cell contain 9.3×10^6 molecules of LAP2α which is twice as much as a BJ1 cell. On the other hand, there is twice as much lamin A (3.1×10^7) present in a BJ1 cell than in a HeLa cell and three times as much than in a U2OS cell. Similarly, BJ1 cells contain three times more lamin C than

HeLa or U2OS cells. All tested cell types contain more lamin C than lamin A present. I also compared my results with the amounts of LAP2 α , lamin A and lamin C within a cell obtained in a study by Beck et al., 2011. Martin Beck and Alexander Schmidt investigated the complete proteome of U2OS cells by quantitative tandem mass spectrometry [for more information see (Beck et al., 2011)] and generated a compilation of protein abundance scores for 73% of the U2OS proteome. The copy number per cell was 1.5×10^6 for lamin A/C and 3.1×10^4 for LAP2 α (Figure 4.3.A yellow bars). These are clear lower amounts (20 times lower for lamin A/C and a 300 times lower for LAP2 α) than the quantities determined in my study. However, the differences in the preparation and calculation methods used in the two studies could explain the variation in the respective data. Additionally, the use of U2OS cell lines which might be slightly different in the two studies and may have varied in their proliferation capacities could also contribute to differing total values.

The graph in figure 4.3B displays the number of soluble molecules per cell and reveals that in all three cell lines the amounts of LAP2 α in the soluble fractions are similar to those in the total fractions. This is an expected result as LAP2 α is a very dynamic protein localized throughout the nucleoplasm (Dechat et al., 2004). Lamin A and lamin C on the other hand show lower amounts in the soluble fraction compared to the total fraction which can be attributed to their distribution between the insoluble lamina-associated and the soluble nucleoplasmic pools. Figure 4.3C represents the soluble pools as percentage of the total protein quantities under the lysis conditions used (see Material and Methods). In BJ1 and HeLa cells approximately 30% to 40%, respectively, of lamin A and C are soluble. In U2OS cells, 20% of lamin A and 30% of lamin C are soluble. As indicated above, in all three cell lines LAP2 α is almost completely soluble.

To get an idea about the stoichiometric relation between lamin A/C and LAP2 α molecules within a cell the relative lamin A/C to LAP2 α ratios in the soluble and total fractions of HeLa, BJ1 and U2OS cells were calculated (Figure 4.3D). Regarding the total protein amounts about 3 to 4 times more lamin A/C than LAP2 α is present in HeLa and U2OS cells. In the soluble protein pool the lamin to LAP2 α molecule ratio decreases to nearly 2:1 in HeLa and 1:1 in U2OS, indicating that for every lamin dimer one LAP2 α molecule is available in HeLa. BJ1 cells show a different pattern. As mentioned above BJ1 cells contain more lamin A/C and less LAP2 α compared to the other two cell lines. In the total fraction the lamin A/C to LAP2 α ratio is 47:1, which decreases to 14:1 in the more informative soluble fraction, which is still a lot higher than in HeLa and U2OS cells. Interestingly, the BJ1 data correspond well with the calculated U2OS cell ratios in the Beck et al. paper, in which 48 times more total lamin A/C

than LAP2 α was reported. This is a much higher ratio than the 3:1 ratio in U2OS cells found in my study.

The amounts of lamin A/C and LAP2 α differ significantly between the cell lines. It appears that fast proliferating cell lines, such as HeLa cells, contain more LAP2 α than slower proliferating cells (e.g. BJ1 cells). These differences are also reflected in the stoichiometric relation between lamin A/C and LAP2 α . Concerning the soluble pools, which are more informative regarding the properties, structure and regulation of the nucleoplasmic A-type lamins, the molecular ratio of lamin A/C to LAP2 α within a single cell can be expected between 1:1 to 14:1 depending on the cell line.

Protein	HeLa		BJ1		U2OS	
	total	soluble	total	soluble	total	soluble
LAP2α	9.3 x10 ⁶ ±3.3 x10 ⁶	8.4 x10 ⁶ ±3.9 x10 ⁶	4.3 x10 ⁶ ±5.1 x10 ⁶	5.9 x10 ⁶ ±4.8 x10 ⁶	9.5 x10 ⁶	8.9 x10 ⁶
Lamin A	1.6 x10 ⁷ ±6.9 x10 ⁶	6.4 x10 ⁶ ±3.2 x10 ⁶	3.1 x10 ⁷ ±1.1 x10 ⁷	1.0 x10 ⁷ ±5.4 x10 ⁷	9.6 x10 ⁶	2.1 x10 ⁶
Lamin C	2.1 x10 ⁷ ±9.9 x10 ⁶	8.2 x10 ⁶ ±2.8 x10 ⁶	6.5 x10 ⁷ ±8.8 x10 ⁶	1.9 x10 ⁷ ±9.5 x10 ⁶	1.9 x10 ⁷	6.4 x10 ⁶
Lamin A/C	3.7 x10 ⁷	1.5 x10 ⁷	9.7 x10 ⁷	2.9 x10 ⁷	2.9 x10 ⁷	8.4 x10 ⁶
Protein	HeLa w/o		BJ1 w/o		U2OS w/o	
	total	soluble	total	soluble	total	soluble
LAP2α	1.2 x10 ⁷	1.0 x10 ⁷	9.9 x10 ⁶	9.5 x10 ⁶	6.8 x10 ⁶	6.7 x10 ⁶
Lamin A	1.7 x10 ⁷	3.2 x10 ⁶	4.6 x10 ⁷	2.4 x10 ⁶	6.0 x10 ⁶	1.6 x10 ⁶
Lamin C	2.5 x10 ⁷	7.1 x10 ⁶	1.4 x10 ⁸	9.8 x10 ⁶	1.3 x10 ⁷	5.4 x10 ⁶
Lamin A/C	4.2 x10 ⁷	1.0 x10 ⁷	1.9 x10 ⁸	1.2 x10 ⁷	1.9 x10 ⁷	7.0 x10 ⁶

Table 4.1: Calculated amount of molecules per cell in HeLa, BJ1 and U2OS. Presented is the number of molecules per cell in the total cell lysate and soluble fraction. Mean ± Standard Deviation is shown from three independent experiments. w/o indicates that analysis was done after removal of mitotic cells by mechanical shake off.

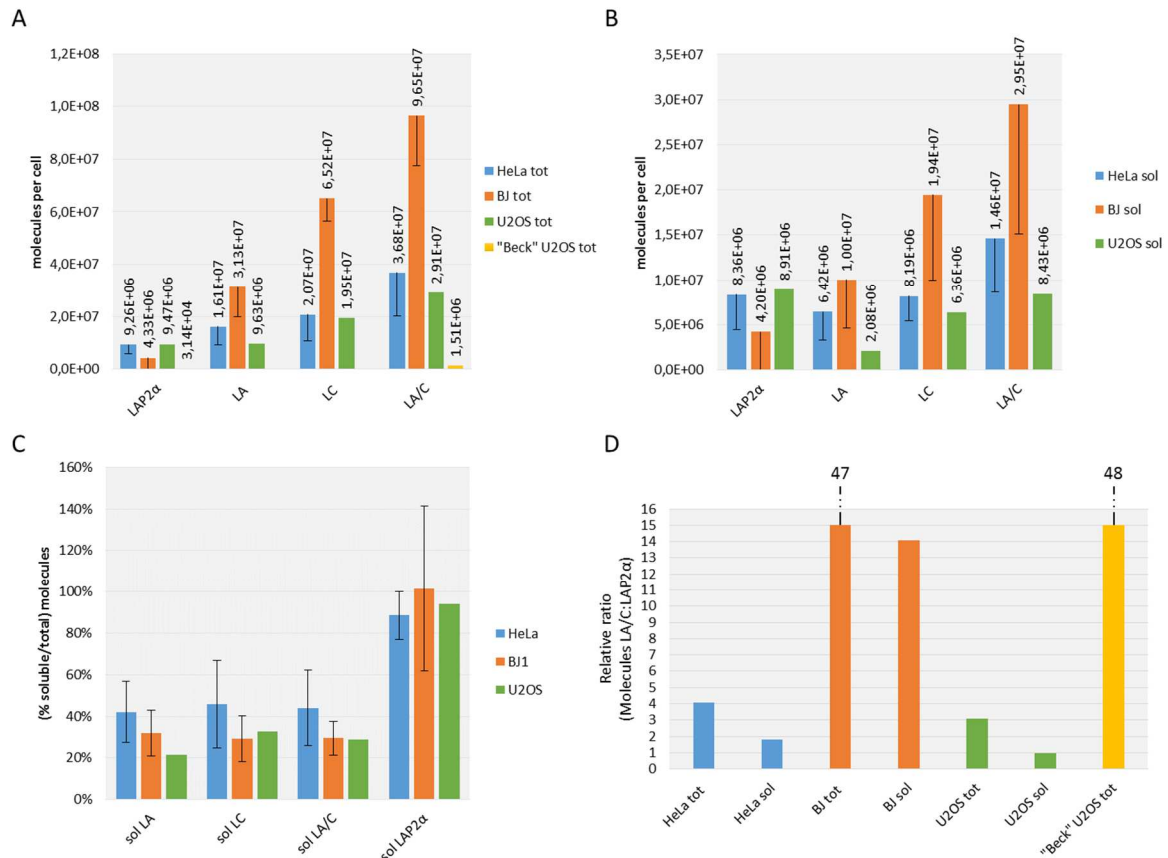


Figure 4.3: Comparison of LAP2 α , Lamin A and Lamin C in HeLa, BJ1 and U2OS cells. A) Calculated number of protein molecules per cell in total cell lysates including adopted data from (Beck et al., 2011). B) Number of molecules per cell in soluble fractions. C) Percentage of soluble over total molecules. D) Relative ratios of Lamin A/C to LAP2 α molecules per cell in total amount (tot) and soluble pool (sol). Yellow bar displays calculated ratio from adopted lamin A/C and LAP2 α abundances from (Beck et al., 2011).

4.2.3 The lamin A/C to LAP2 α ratio does not change significantly after removal of mitotic cells.

The experiments described in chapter 4.2.2 were repeated with HeLa, BJ1 and U2OS cells depleted of mitotic cells by mechanical shake-off. Since A-type lamins become soluble during mitosis (see Introduction), the mitotic lamin pool may affect the calculated amount of soluble lamins A/C in interphase cells, depending on the number of mitotic cells in the culture. While HeLa and U2OS cells show no differences in the total number of LAP2 α , lamin A and lamin C molecules independent of whether they contain or lack mitotic cells, total cell lysates prepared from purely interphase BJ1 cells contain more of these proteins compared to mixed cultures (compare Figure 4.4A with Figure 4.3A). When comparing only molecule numbers in the soluble fractions of interphase and mixed cell cultures, the amount of soluble lamin A/C molecules decreases from ~40% to ~25% in HeLa cells and from ~30% to ~7% in BJ1 cells when mitotic cells were removed prior to the fractionation (Figure 4.4B). This is in agreement

with the assumption that in mitotic cells the amount of soluble lamin A/C increases due to the breakdown of the NE and the disassembly of the lamina. The result regarding the solubility of LAP2 α did not change when mitotic cells were removed prior to the fractionation and remained close to 100%. The altered experimental conditions lead to a change in the lamin A/C to LAP2 α ratio (Figure 4.4C), which is most pronounced in BJ1 cells. There the ratio changes from 48:1 to 19:1 for the total fraction and from 47:1 to 1:1 for the soluble fraction.

However, as the data for the lysates containing solely interphase cells derived only from one experiment they have to be taken with care and would need further verification.

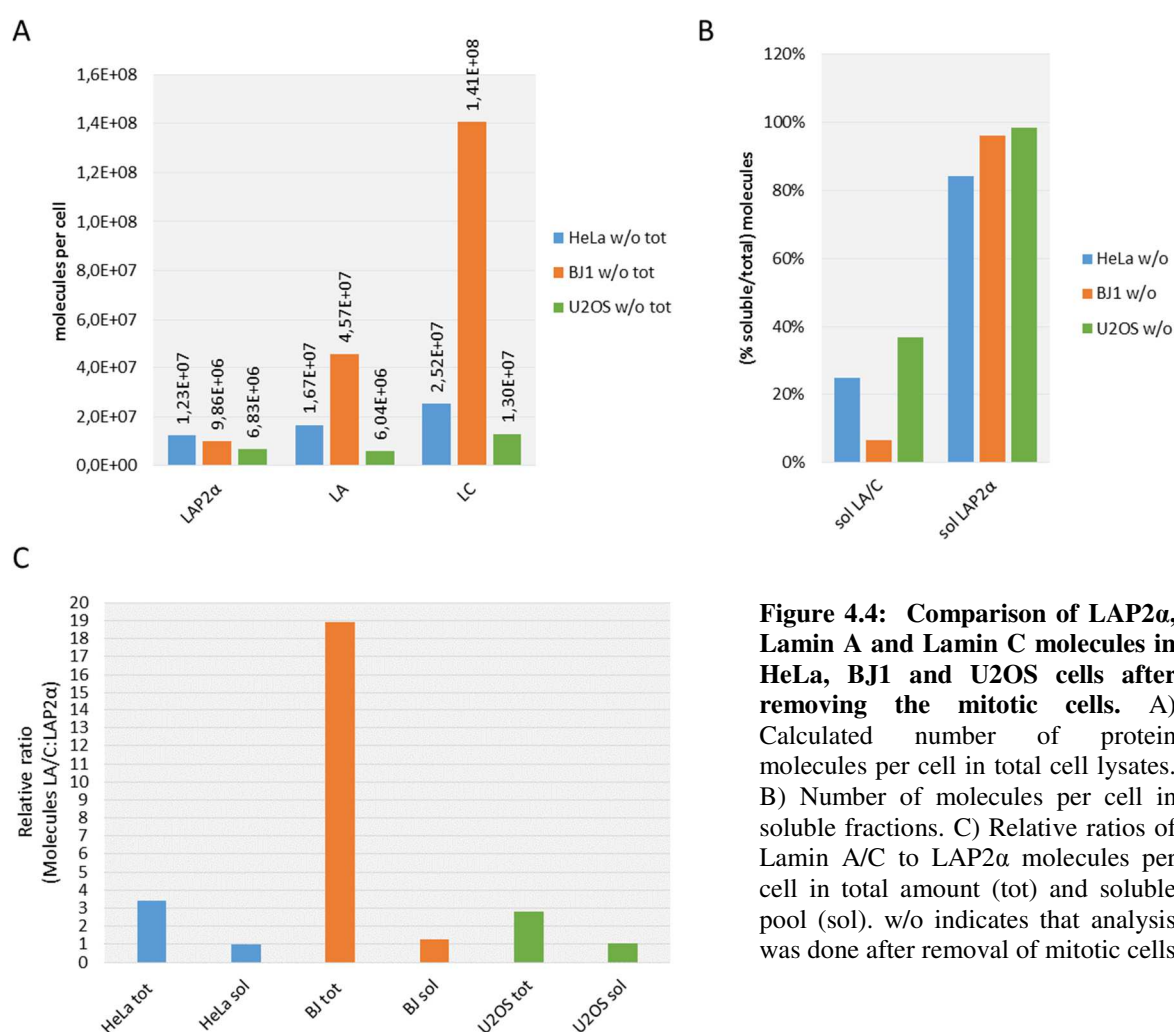


Figure 4.4: Comparison of LAP2 α , Lamin A and Lamin C molecules in HeLa, BJ1 and U2OS cells after removing the mitotic cells. A) Calculated number of protein molecules per cell in total cell lysates. B) Number of molecules per cell in soluble fractions. C) Relative ratios of Lamin A/C to LAP2 α molecules per cell in total amount (tot) and soluble pool (sol). w/o indicates that analysis was done after removal of mitotic cells

4.3 Influence of LAP2 α on the formation of lamin structures *in vitro*

The main part of this work was to investigate a potential interfering effect of LAP2 α on the assembly properties of A-type lamins. Is LAP2 α able to impair lamin polymerization and thus incorporation into the lamina by binding to lamin molecules and keeping them soluble in the nucleoplasm? Here, *in vitro* experiments were performed in which assembly of lamin A into

paracrystals and filaments, was induced either in the presence or absence of LAP2 α . Possible alterations in lamin polymerization and structure formation were investigated by negative staining electron microscopy. In addition, lamin assembly was monitored by different sedimentation assays to gain information about assembly state and intermediates, sample heterogeneity and molecular stoichiometry of the complex.

4.3.1 Solubility of lamin A and LAP2 α at *in vitro* lamin assembly conditions

An initial aim of this study was to establish a working protocol for lamin A assembly that was also suitable for LAP2 α and could be used for the subsequent analyses, such as eppendorf centrifugation, analytical ultracentrifugation and sucrose density gradient centrifugation. One problem that occurred throughout this work was the high insolubility of LAP2 α at low NaCl concentrations (Figure 4.5A). Comparison of LAP2 α solubility between denaturing conditions (8 M Urea) and assembly conditions with decreasing salt concentrations revealed that in 300 mM NaCl about 50% of LAP2 α were soluble which dropped to nearly 0% in 100 mM and 50 mM NaCl. Lamin A precipitated as expected in 200 mM and lower NaCl concentrations due to the formation of higher order structures (Figure 4.5B). LAP2 α and lamin A were never completely soluble, as even in 8 M Urea both proteins were found in the pellet fraction to various extent, most likely due to unspecific aggregation into large insoluble complexes (Figure 4.5). Especially the insolubility of LAP2 α at low salt concentrations at which lamin assembly can be studied, was problematic as the aggregation of LAP2 α led to loss of soluble LAP2 α and may influence lamin polymerization in the *in vitro* assay. In order to find out more about formed LAP2 α complexes analytical ultracentrifugation was performed. Recombinant LAP2 α was analyzed by sedimentation velocity ultracentrifugation after it had been dialyzed into buffers containing either 300 mM NaCl or 100 mM NaCl.

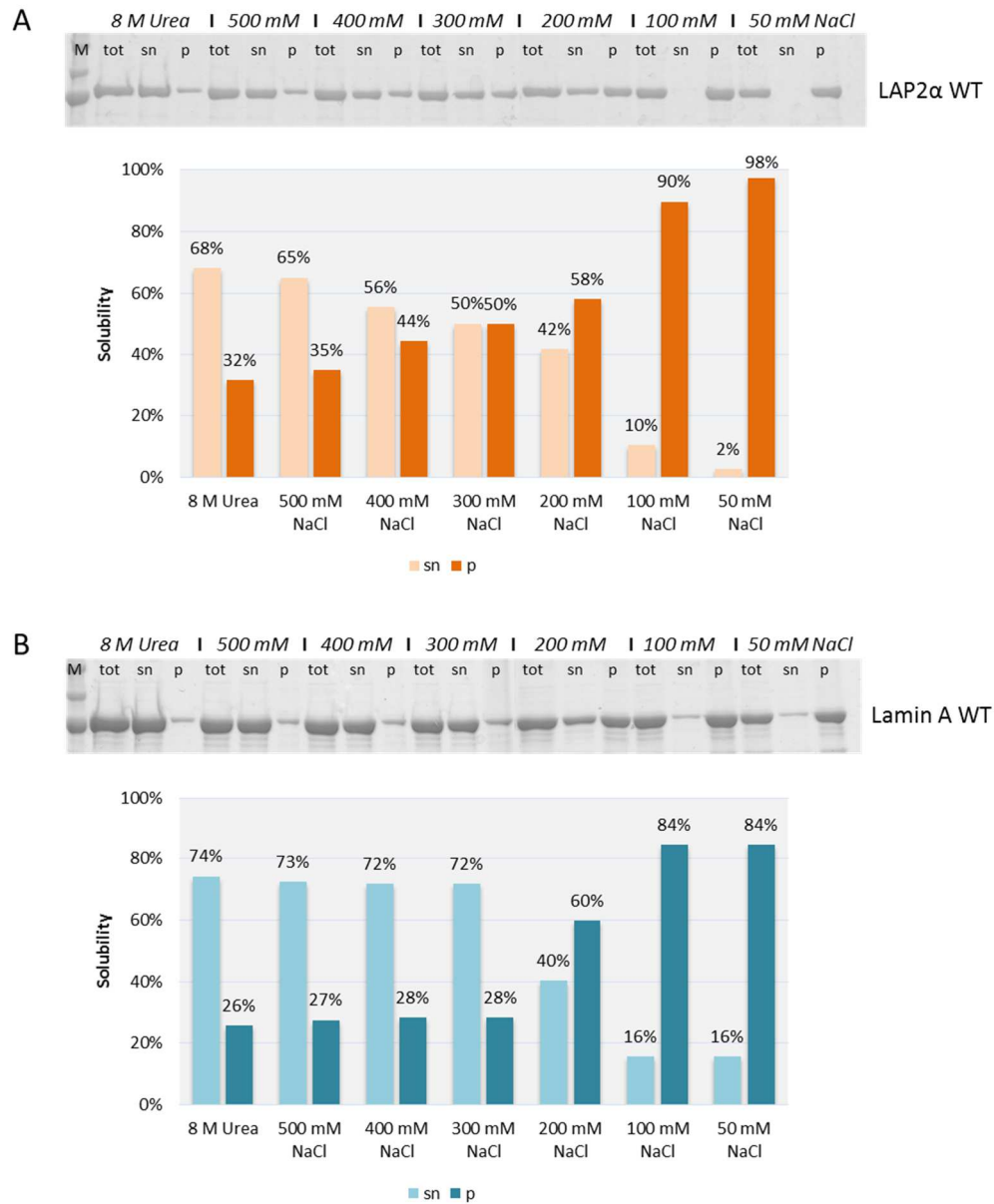


Figure 4.5: Solubility of lamin A WT and LAP2 α at different buffer conditions. Samples were analyzed at denaturing conditions in 8 M Urea and renatured in buffers with decreasing NaCl concentrations. A) LAP2 α WT and B) lamin A WT supernatant (sn) and pellet (p) fractions were analyzed by SDS-PAGE after dialysis into the proper buffer and a 10 min centrifugation step in an Eppendorf table centrifuge at 13.000 rpm.

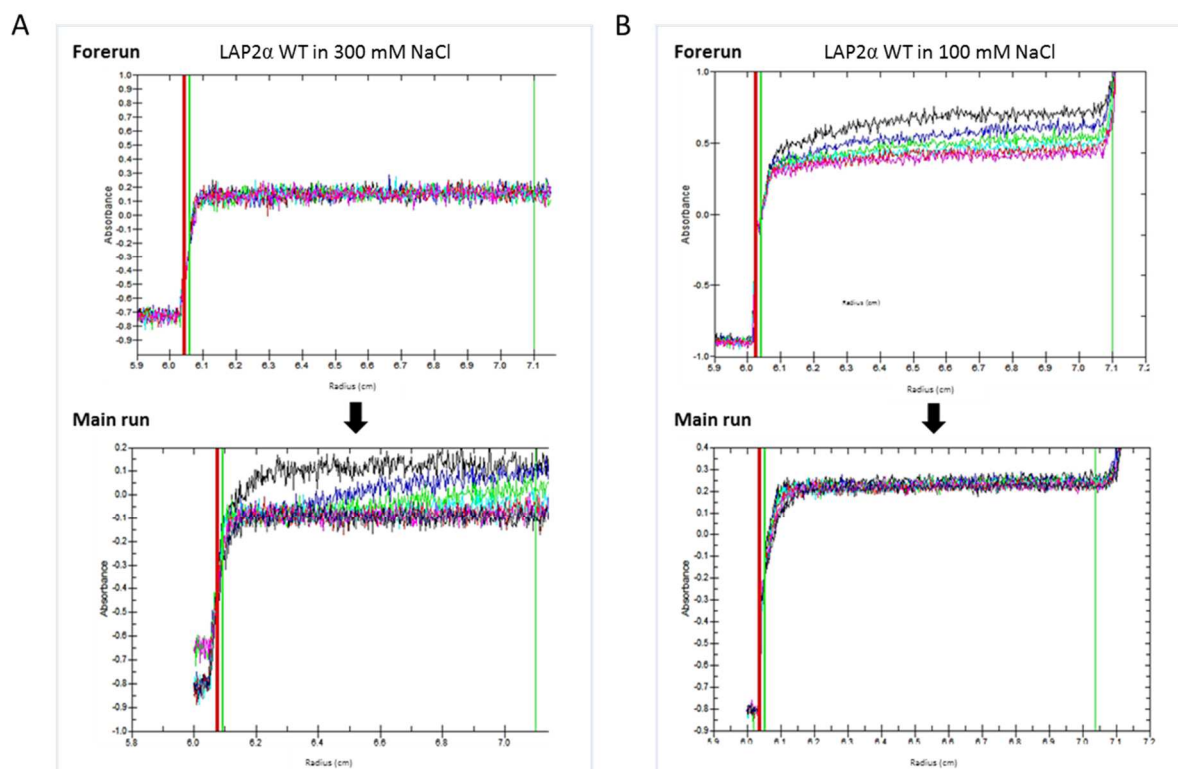


Figure 4.6: Sedimentation velocity analytical ultracentrifugation of LAP2 α WT. LAP2 α WT was dialyzed into paracrystal assembly buffers with either A) 300 mM or B) 100 mM NaCl. The SV run was carried out in an Optima XL-A analytical ultracentrifuge equipped with an An-60 Ti Rotor. Forerun: 700 x g; Main run: 130 000 x g. Depicted are scans across the centrifugation cell taken at different time points during the SV run. Coloured lines represent the movement of the sedimentation boundaries for LAP2 α WT in A) 300 mM NaCl, 10 mM Tris.HCl, 0.2 mM DTT, pH 7.5 and B) 100 mM NaCl, 10 mM Tris.HCl, 0.2 mM DTT, pH 7.5.

The graphs in Figure 4.6 show scans of the moving protein boundary in the sedimentation tube depicted as absorbance at 280 nm at different time points versus the radial position in the tube cell. In the forerun (700 x g) in 300 mM NaCl the boundaries (colored lines) are very close indicating that most of the molecules settled together. Only at 130 000 x g (Main run) some particles settled faster than the others molecules suggesting that the sample was heterogeneous and contained aggregates (Figure 4.6A). These findings are consistent with the data presented in figure 4.5A showing that 50% of LAP2 α precipitated at 13.000 rpm in an eppendorf table centrifuge in 300 mM NaCl conditions due to aggregation. In 100 mM NaCl the situation was worse (Figure 4.6B). Already in the forerun the LAP2 α sample appeared much more heterogeneous containing large aggregates (black line), big enough to sediment at low speed. These aggregates, probably formed by self-interaction of LAP2 α at low salt conditions, also settled by centrifugation in the eppendorf table centrifuge. There 90% of the protein precipitated under these conditions (Figure 4.5A). Since all large particles precipitated already in the forerun at 700 x g the subsequent main run at 130 000 x g revealed a small homogenous group of molecules with approximately the same molecular weight and shape. This was indicated by

their collective migration points revealing a S^* value of 5-6 for LAP2 α (in detail see chapter 4.3.3.5).

Further tests revealed that the precipitation of LAP2 α under low salt conditions seemed to be triggered by its C-terminal domain. The relevant carboxyl-terminal region contains six of a total of ten cysteine residues present in full length LAP2 α (Figure 4.8C). Therefore, it is likely that in weak reducing conditions, these cysteine residues cause aggregation by forming intermolecular disulphide bridges. As recommended in literature all lamin assembly buffers used in this work initially contained 1 mM DTT (Heitlinger et al., 1992; Karabinos et al., 2003). Increasing the concentration of the reducing agent DTT from 1 mM to 50 mM led to an increase in LAP2 α solubility under 100 mM NaCl conditions from nearly 0% to about 50% (Figure 4.7A). A truncated LAP2 α mutant, LAP2 α^{1-414} , lacking the last 279 amino acids of the C-terminus including the six cysteine residues, is much less prone to form oligomers caused by oxidation. Accordingly about 60% of the mutant protein was soluble in 100 mM NaCl and 1 mM DTT conditions, and solubility did not significantly increase upon higher DTT concentration (Figure 4.7B). These results indicate that preventing the oxidation of the cysteine residues is crucial for keeping LAP2 α soluble.

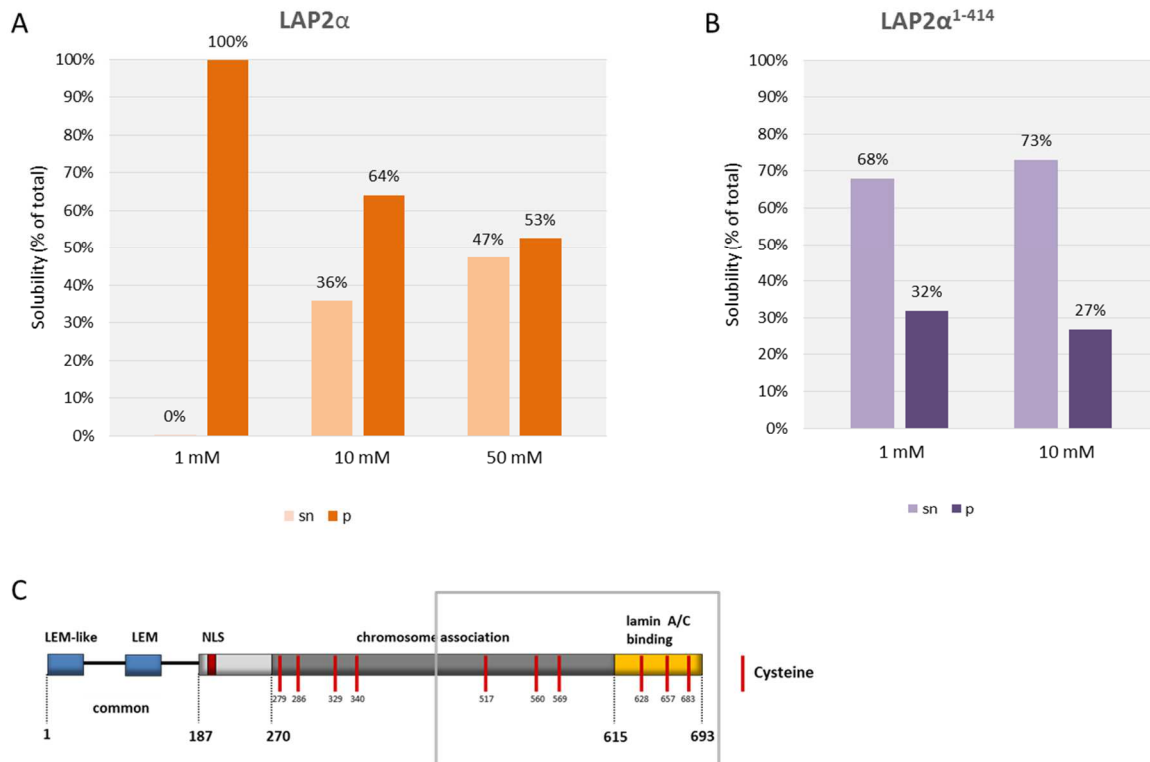


Figure 4.7: Influence of different DTT concentrations on LAP2 α solubility. Recombinant A) full length LAP2 α and B) LAP2 α ¹⁻⁴¹⁴ were dialyzed into 100 mM NaCl, 10 mM Tris.HCl, pH 7.5 supplemented with either 1 mM, 10 mM or 50 mM DTT. Samples were analyzed by SDS-PAGE after a 10 min centrifugation step in an Eppendorf table centrifuge at 13.000 rpm to obtain supernatant (sn) and pellet (p) fractions. C) Schematic representation of full length LAP2 α . Depicted in red are cysteine residues. The grey box represents the last 279 amino acids lacking in the truncated LAP2 α ¹⁻⁴¹⁴.

4.3.2 Strong reducing conditions prevent lamin assembly

Next the 50 mM DTT conditions were tested in the lamin *in vitro* assembly assays. Therefore lamin A, either in the presence or absence of LAP2 α , was dialyzed against paracrystal assembly buffer or filament assembly buffer, each supplemented with 50 mM DTT. Subsequently, all samples were analyzed by sucrose gradient centrifugation. The collected fractions were further analyzed by SDS-PAGE and the coomassie stained bands by density measurements. Sucrose density gradient centrifugation allows detecting alterations in the sedimentation pattern of lamin A and LAP2 α upon protein-protein interactions. Figure 4.8 shows one example of a stained gel and the respective blots of lamin A WT and LAP2 α WT at low salt conditions (100 mM NaCl). In this experiment LAP2 α showed no sign of aggregation as it was only present in fraction 2 to 3 indicating that it was almost completely soluble. Interestingly, also lamin A WT was only found in the low sucrose density region (fractions 1 to 2). This suggested that lamin A remained soluble in conditions under which it usually formed paracrystals, when 50 mM instead of 1 mM DTT was used. Hence, the high DTT concentrations

apparently impairs the formation of paracrystals. Accordingly I did not see any changes in the sedimentation profiles in LAP2 α and lamin A alone versus the mixed sample (Figure 4.8C and D).

Similar results were obtained at filament assembly conditions (Figure 4.9). Independently of lamin A LAP2 α was mainly present in fraction 2 and therefore mostly soluble (Figure 4.9D). Also lamin A remained soluble and showed no indication for polymerization (Figure 4.9A and B). In summary strong reducing conditions seemed to interfere with the formation of lamin filaments.

As the use of high DTT concentrations precluded lamin A assembly all further experiments were performed with assembly buffers containing 1 mM DTT to induce lamin polymerization, but in most cases LAP2 α was centrifuged before analysis to remove aggregates.

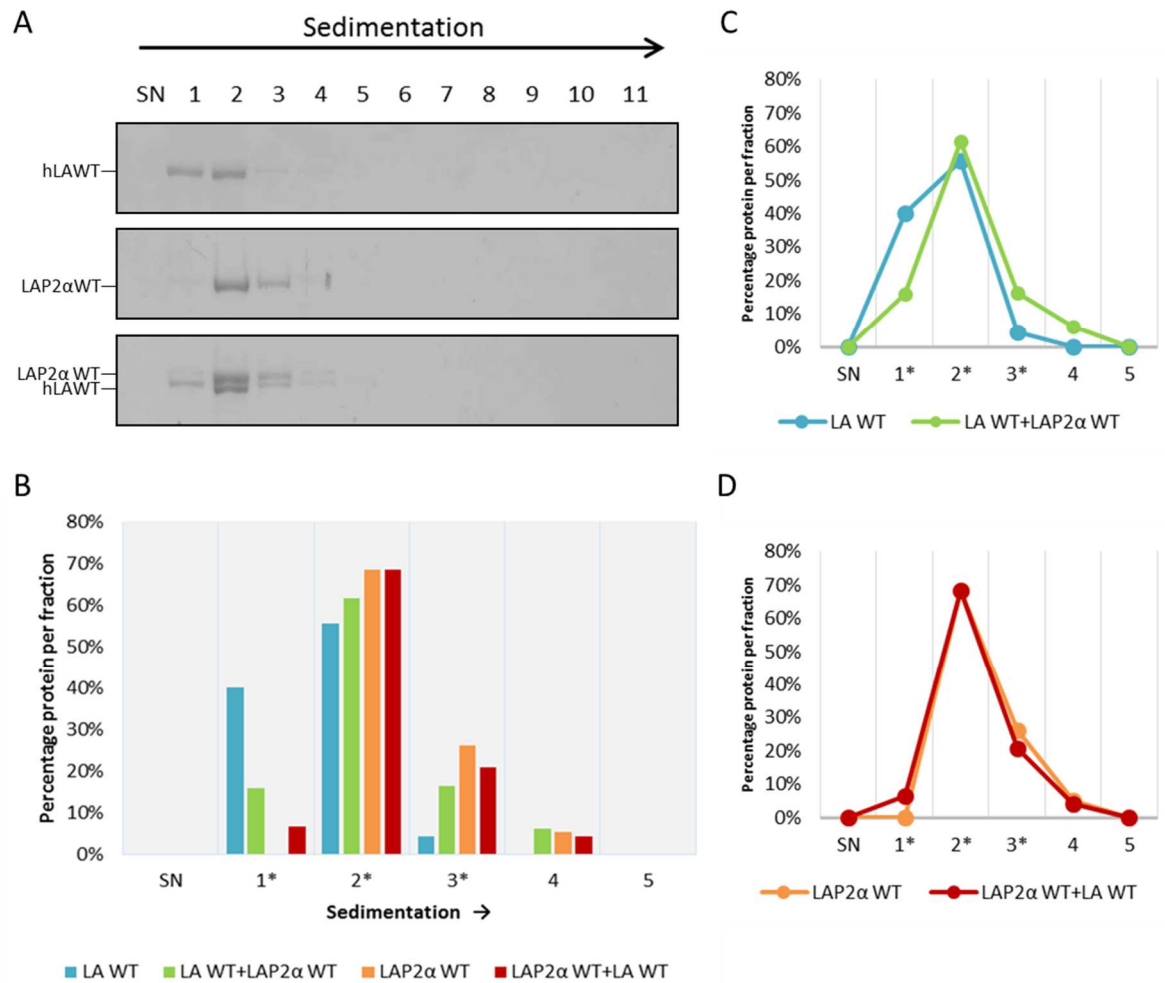


Figure 4.8: Sucrose density gradient centrifugation after dialysis into 100 mM NaCl, 10 mM Tris.HCl, 50 mM DTT, 10% sucrose, pH 7.5. Lamin A WT and LAP2α WT were dialyzed either alone or together into paracrystal assembly buffer containing 50 mM DTT. Afterwards samples were separated in a 10% to 30% sucrose gradient in a Beckman Optima L-70 ultracentrifuge equipped with an SW-40 Rotor at 190.000 x g for 20 hours. Fractions were collected from top to the bottom, starting with the applied protein sample (SN), and analyzed by SDS-PAGE. The strong density of the bands were measured using ImageJ software and the amount of protein per fraction was calculated as percentage rate of the applied total protein quantity. Fractions containing the marker protein BSA are indicated by an asterisk. A) Exemplary coomassie stained gel of fraction SN to 11 is shown. The calculated protein percentage was blotted as bar chart (B) or as curve chart (C-D).

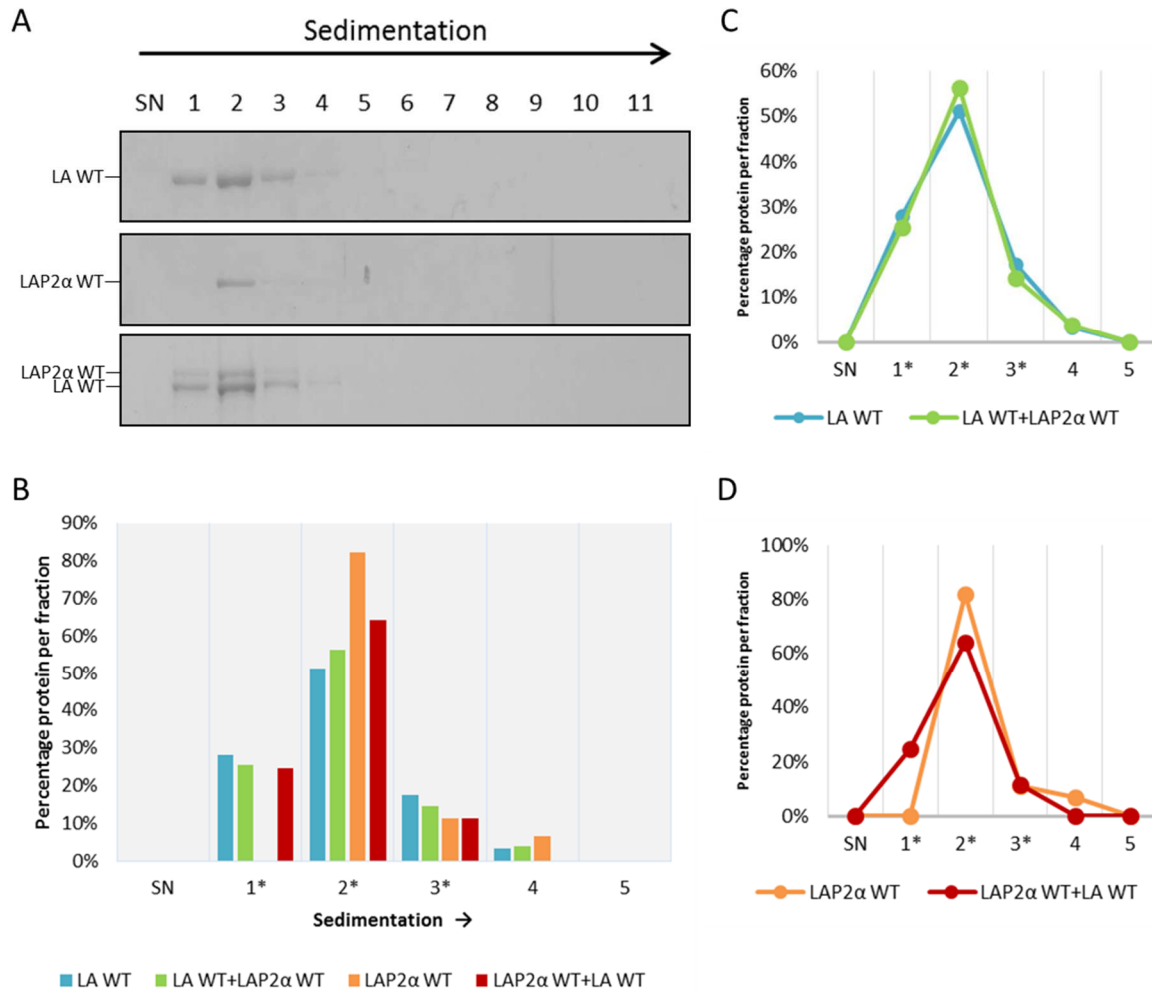


Figure 4.9: Sucrose density gradient centrifugation after dialysis into 250 mM NaCl, 25 mM MES, 50 mM DTT, 10% sucrose, pH 6.5. Lamin A WT and LAP2α WT were dialyzed either alone or together into filament assembly buffer containing 50 mM DTT. Samples were separated in a 10% to 30% sucrose gradient at 190.000 x g for 20 hours. Collection of the fractions and density measurements of the protein bands were done as described in figure 4.8. A) Exemplary coomassie stained gel of fraction SN to 11 is shown. The calculated protein percentage was blotted as bar chart (B) or as curve chart (C-D).

* BSA fractions

4.3.3 LAP2α interferes with lamin A paracrystal assembly *in vitro*

The formation of lamin structures *in vitro* depends on the buffer conditions as described in chapter 3.2.5. To examine the influence of LAP2α on the assembly of A-type lamins into paracrystals recombinant lamin A was stepwise dialyzed against buffers with decreasing salt concentrations either in the presence or absence of either LAP2α WT or mutant.

4.3.3.1 *LAP2 α interacts with lamin A at high salt conditions and prevents lamin A assembly*

Normally, lamin A assembly into paracrystals does not occur at NaCl concentrations of 300 mM or higher. Under these conditions sucrose gradient analyses revealed a possible interaction between lamin A WT and LAP2 α WT (Figure 4.10). Lamin A WT alone sedimented to fractions 1 to 4 with the highest concentration found in fraction 2 (Figure 4.10B). Since lamin A sedimented in a broad peak, it is likely that it formed various intermediate structures of different size and shape. LAP2 α WT alone exhibited a more uniform distribution and was mainly detected in fractions 2 and 3. Strikingly, lamin A WT and LAP2 α WT together showed an altered sedimentation pattern, as both proteins were slightly shifted towards higher sucrose densities, indicating the formation of larger hetero-complexes (Figure 4.10). Lamin A WT sedimented in fractions 3 and 4 in the presence of LAP2 α WT, compared to fraction 2 when assembled alone (Figure 4.10C). Similarly, LAP2 α WT sedimentation peaked around fractions 3 and 4 in the presence and around fractions 2 and 3 in the absence of lamin A WT (Figure 4.10D). The fact that the LAP2 α /lamin A ratio was always similar, independently of the total amount of protein present in the individual fractions and the sucrose density, suggests a high affinity between the two proteins even at high salt concentrations.

The finding that lamin A and LAP2 α bind to each other and form a complex raises the question on which molar ratio the proteins exist in such a complex. By quantifying the protein bands for each fraction it was possible to calculate the binding stoichiometry. The molecular lamin A to LAP2 α ratio was found to be in the range of 2.5:1 in the fractions 3 to 5 (Figure 4.10A).

Control experiments using binding deficient mutant proteins support the hypothesis that lamin A and LAP2 α form complexes in vitro. First lamin A¹⁻⁵⁴² was used as another positive control as it still contained the Ig-fold, likely involved in LAP2 α binding but lacking the last 104 residues of the C-terminal domain. In the presence of LAP2 α this lamin A mutant showed a similar, although slightly weaker, shift towards higher sucrose densities compared to lamin A WT. In the absence of LAP2 α WT lamin A¹⁻⁵⁴² was present in fractions SN to 4 (Figure 4.11A and B), peaking at fraction 1, whereas in the presence of LAP2 α WT the peak was shifted to fraction 2 (Figure 4.11C). LAP2 α WT on the other hand displayed no such defined shift in the presence of lamin A¹⁻⁵⁴², but the peak seemed to broaden (Figure 4.11D). These data indicate that the lamin A C-terminus may also contribute or regulate binding to LAP2 α . The calculated molecular ratio of the proteins was 1:1 in fraction 2.

As a negative control lamin A¹⁻⁴³⁷ was used, lacking the last 209 amino acids including the Ig-fold and thus the LAP2 α binding site. Other than lamin A WT and lamin A¹⁻⁵⁴², lamin A¹⁻⁴³⁷ sedimented independently of LAP2 α WT and was always enriched in fraction 1 (Figure 4.12). Consistently LAP2 α WT also showed no change in its sedimentation profile in the presence versus absence of lamin A¹⁻⁴³⁷ (Figure 4.12D). As another negative control LAP2 α ¹⁻⁴¹⁴ was used, lacking the last 277 amino acids of LAP2 α WT including the lamin A/C binding domain. The presence of LAP2 α ¹⁻⁴¹⁴ caused no change in the sedimentation pattern of lamin A WT (Figure 4.13A and B). Lamin A WT, with or without LAP2 α ¹⁻⁴¹⁴, was mainly present in fraction 2, whereas LAP2 α ¹⁻⁴¹⁴ was found in fractions 1 and 2 (Figure 4.13C and D). Since both proteins did not show any shift towards higher sucrose densities when mixed it can be concluded that they did not interact.

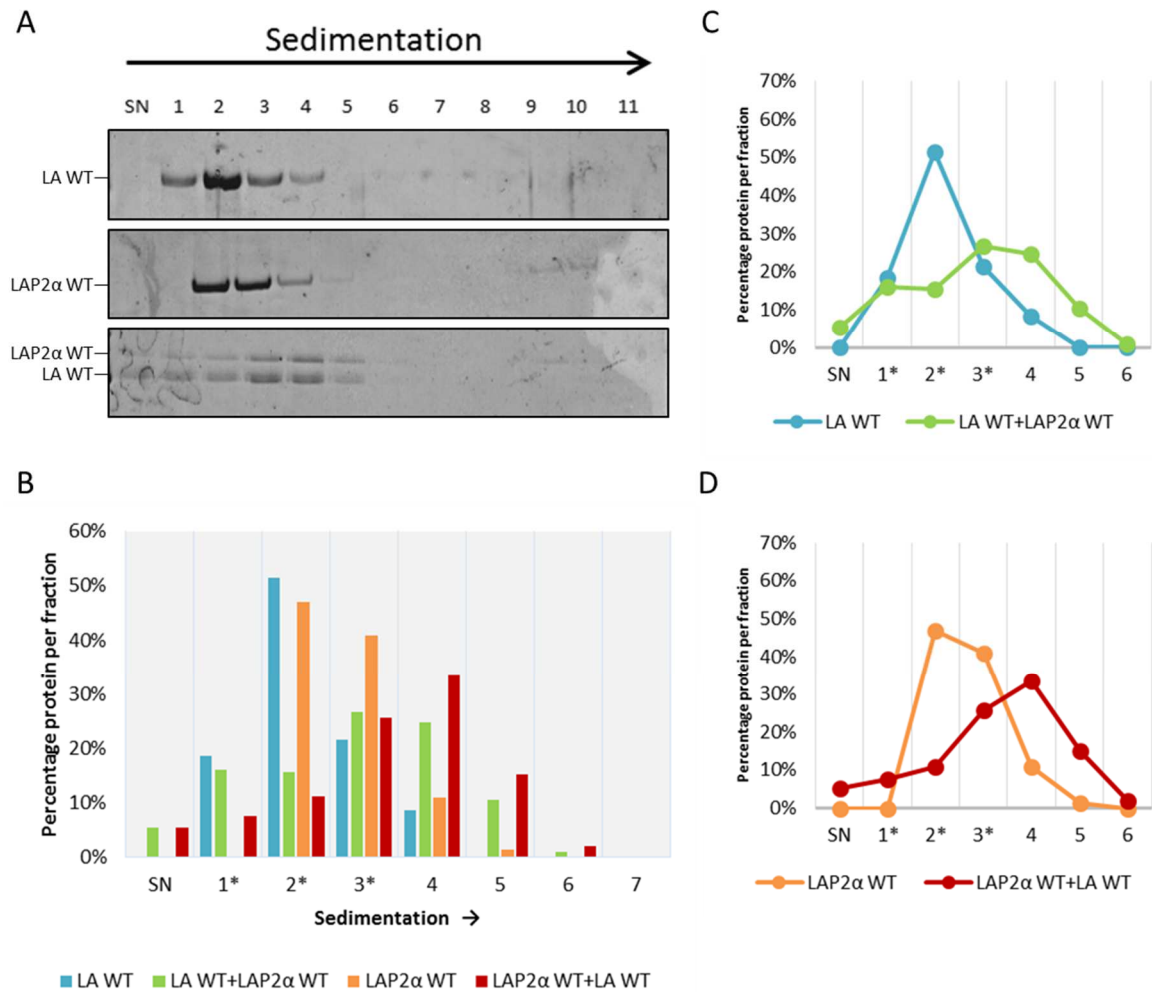
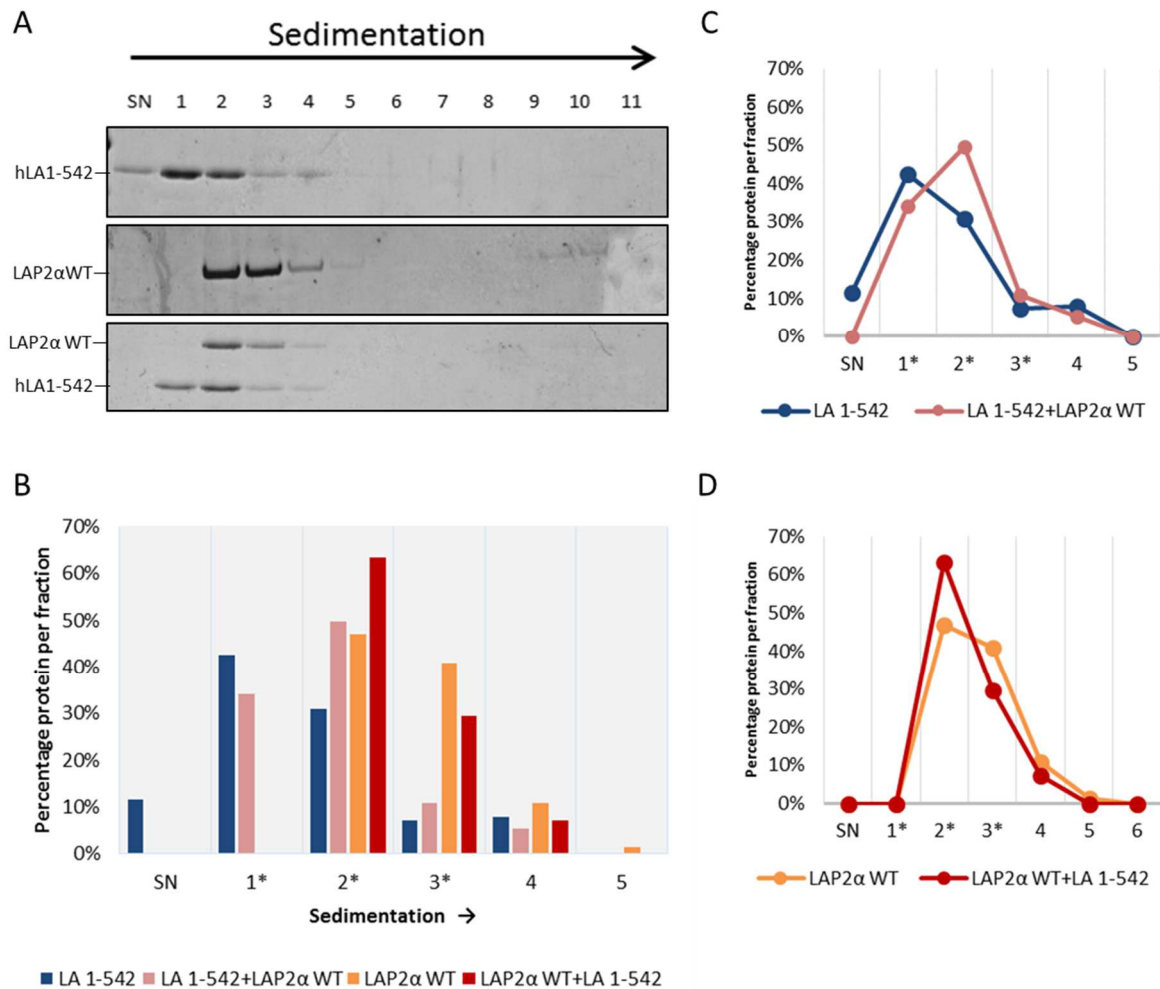


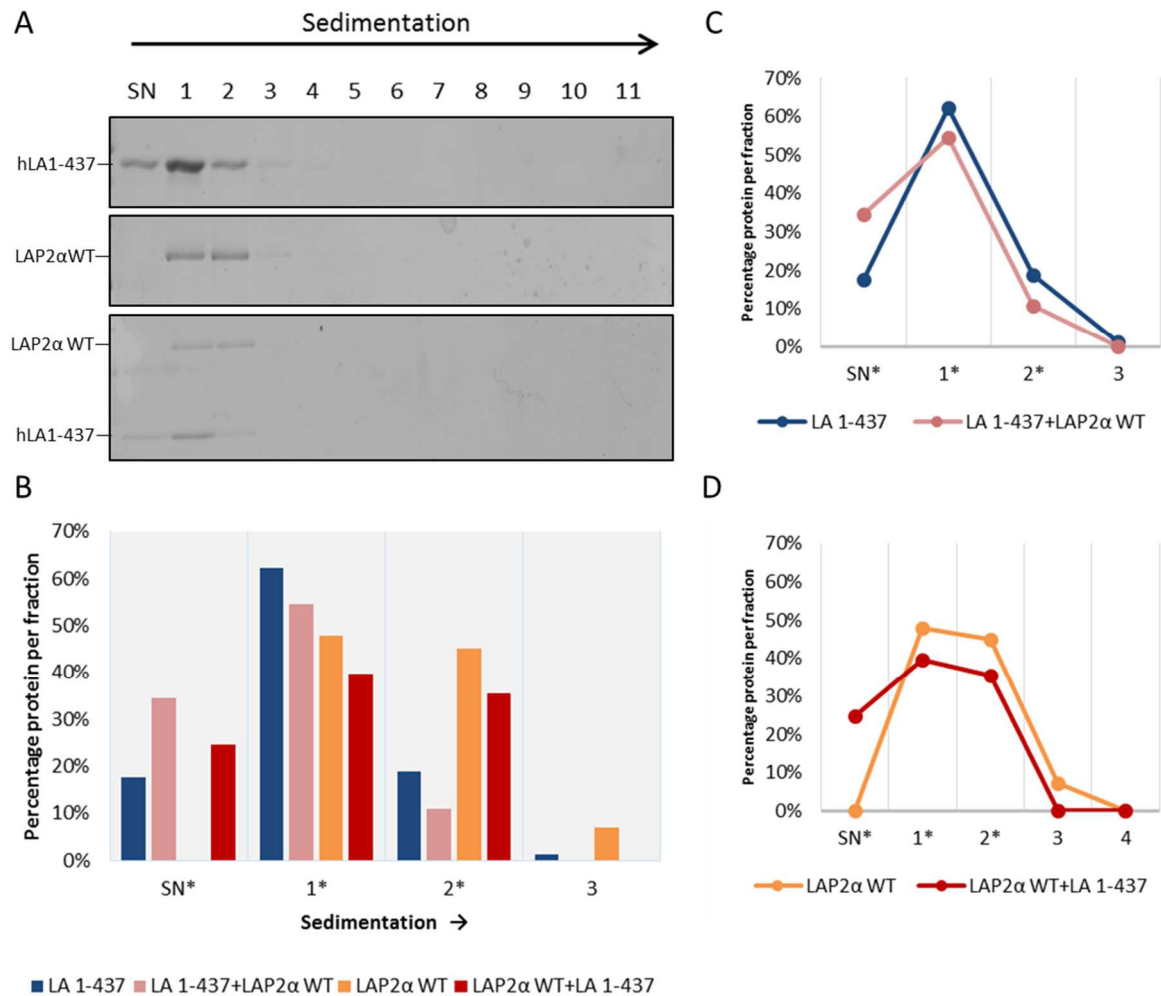
Figure 4.10: Sucrose density gradient centrifugation after dialysis into 300 mM NaCl, 10 mM Tris.HCl, 1 mM DTT, 10% sucrose, pH 7.5. Lamin A WT and LAP2α WT were dialyzed either alone or together into paracrystal assembly buffer. Samples were separated in a 10% to 30% sucrose gradient at 190.000 x g for 20 hours. Collection of the fractions and density measurements of the protein bands were done as described in figure 4.8. A) Exemplary coomassie stained gel of fraction SN to 11 is shown. The calculated protein percentage was blotted as bar chart (B) or as curve chart (C-D).

* BSA fractions



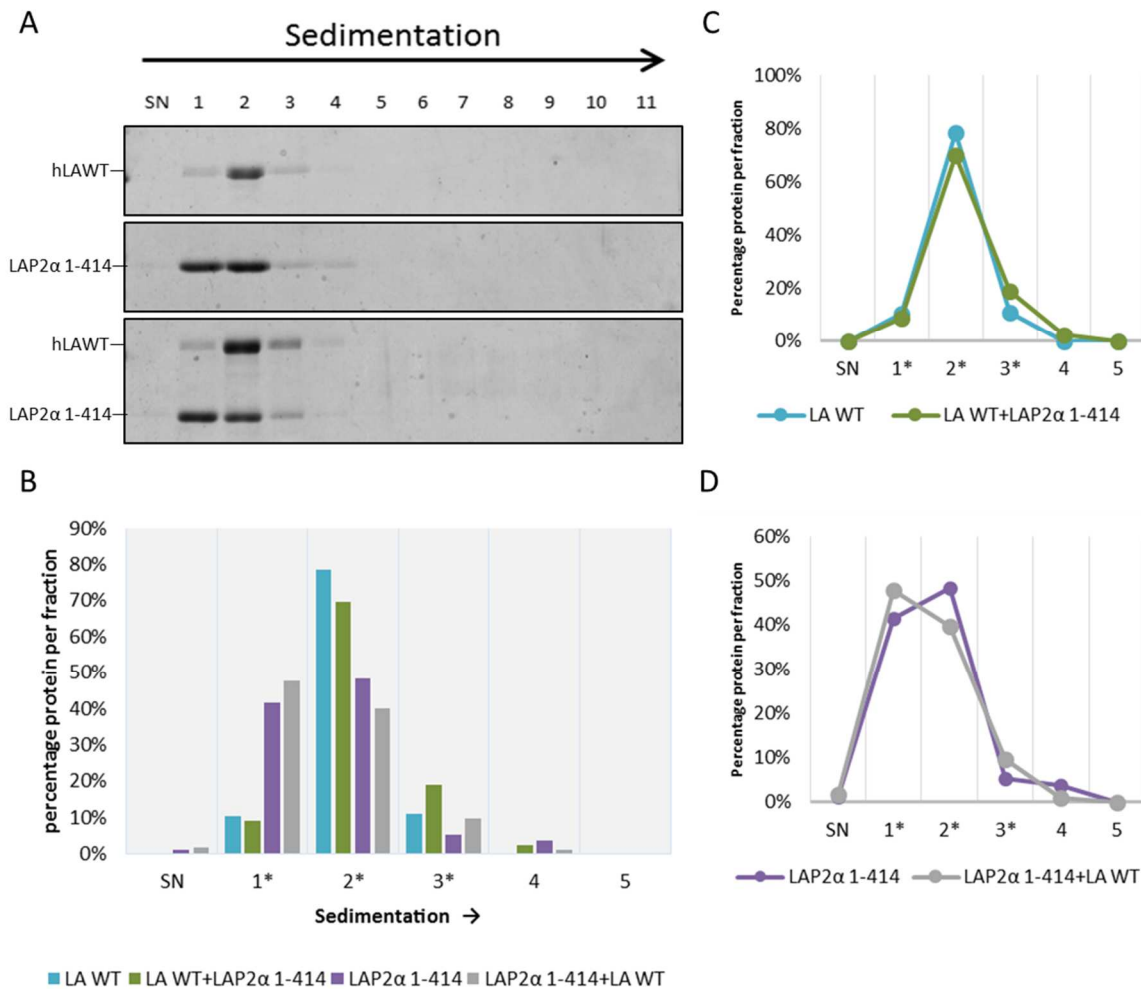
4.11: Sucrose density gradient centrifugation after dialysis into 300 mM NaCl, 10 mM Tris.HCl, 1 mM DTT, 10% sucrose, pH 7.5. Lamin A¹⁻⁵⁴² and LAP2α WT were dialyzed either alone or together into paracrystal assembly buffer. Samples were separated in a 10% to 30% sucrose gradient at 190.000 x g for 20 hours. Collection of the fractions and density measurements of the protein bands were done as described in figure 4.8. A) Exemplary coomassie stained gel of fraction SN to 11 is shown. The calculated protein percentage was blotted as bar chart (B) or as curve chart (C-D).

* BSA fractions



4.12: Sucrose density gradient centrifugation after dialysis into 300 mM NaCl, 10 mM Tris.HCl, 1 mM DTT, 10% sucrose, pH 7.5. Lamin A¹⁻⁴³⁷ and LAP2α WT were dialyzed either alone or together into paracrystal assembly buffer. Samples were separated in a 10% to 30% sucrose gradient at 110.000 x g for 20 hours. Collection of the fractions and density measurements of the protein bands were done as described in figure 4.8. A) Exemplary coomassie stained gel of fraction SN to 11 is shown. The calculated protein percentage was blotted as bar chart (B) or as curve chart (C-D). Note that, due to lower speed, all samples and BSA run slightly shifted compared to figure 4.8-4.11, 4.13 and 4.14.

* BSA fractions



4.13: Sucrose density gradient centrifugation after dialysis into 300 mM NaCl, 10 mM Tris.HCl, 1 mM DTT, 10% sucrose, pH 7.5. Lamin A WT and LAP2α¹⁻⁴¹⁴ were dialyzed either alone or together into paracrystal assembly buffer. Samples were separated in a 10% to 30% sucrose gradient at 190.000 x g for 20 hours. Collection of the fractions and density measurements of the protein bands were done as described in figure 4.8. A) Exemplary coomassie stained gel of fraction SN to 11 is shown. The calculated protein percentage was blotted as bar chart (B) or as curve chart (C-D).

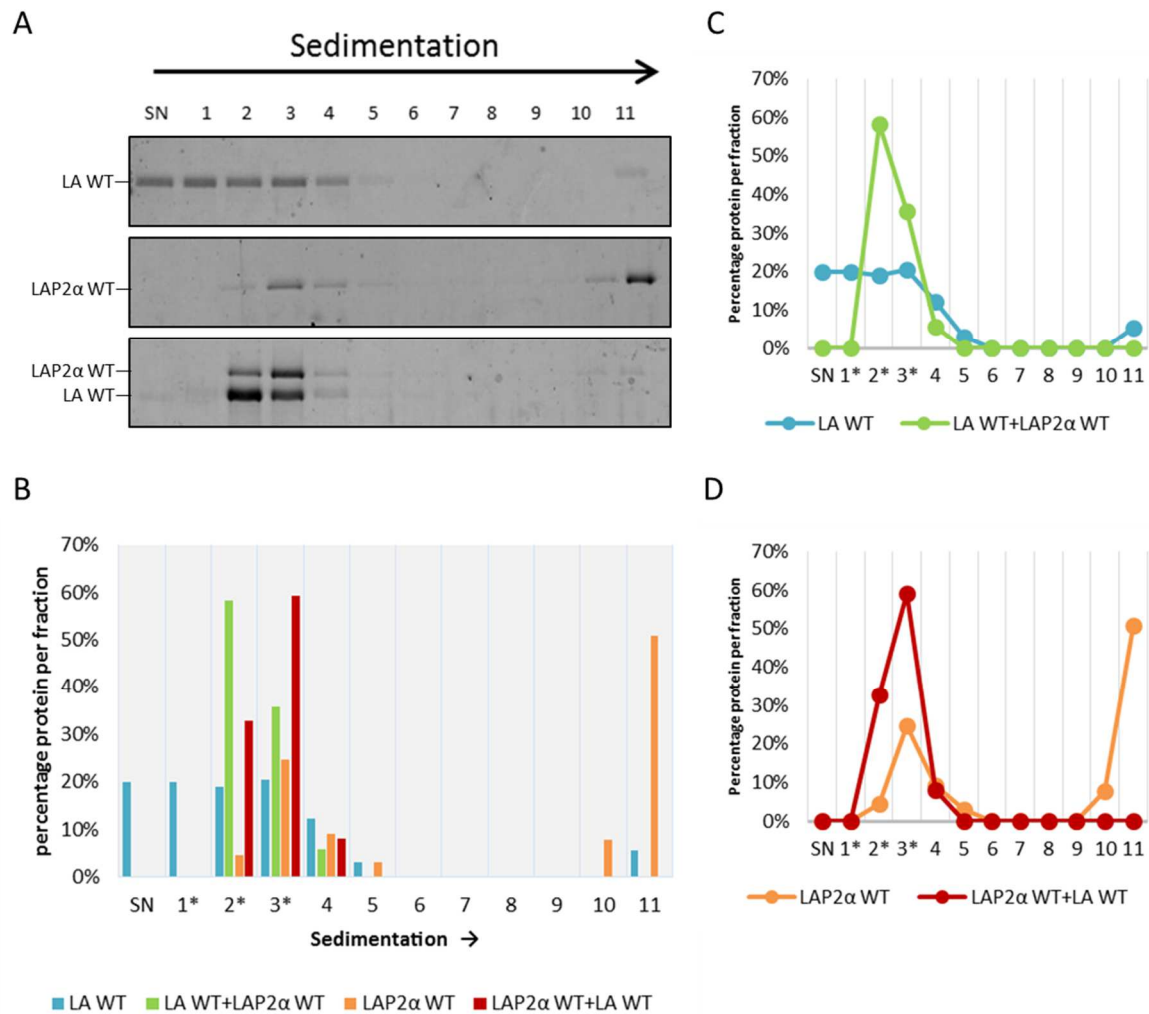
* BSA fractions

4.3.3.2 *Mutual influence of lamin A and LAP2α at paracrystal assembly conditions*

Next the influence of LAP2α on lamin A paracrystal assembly was analyzed by sucrose density gradient centrifugation. As mentioned before, lamin A assembles into paracrystalline structures in paracrystal assembly buffer at salt concentrations below 200 mM. The following experiments were performed in 100 mM NaCl buffers in which paracrystals fully develop [see chapter 4.3.3.4 and (Foeger et al., 2006)]. Similar to the 300 mM situation (see chapter 4.3.3.1) lamin A alone showed a broad distribution between fractions SN to 4 suggesting relatively small lamin structures with various sizes and shapes, likely intermediates of the assembly

process (Figure 4.14A and B). A small amount of lamin was present in fraction 11, likely representing assembled paracrystals. Taken into account that under low salt conditions lamin A should efficiently form paracrystals, I expected a larger amount of lamin A in this pellet. I assume that large structures in the pellet got lost as they stick tightly to the bottom of the tube. Nevertheless, focusing on the assembly intermediates lamin A showed a clearly different sedimentation profile in the presence versus absence of LAP2 α and was mainly accumulated in fractions 2 and 3 (Figure 4.14C). When LAP2 α was analyzed alone, it sedimented to some extent in fractions 3 and 4, but as expected under this low salt concentration the main amount was found in the pellet fraction 11, most likely representing large aggregates (Figure 4.14D). In the presence of lamin A however, nearly no large LAP2 α structures were detectable and the main fraction of the protein co-sedimented with lamin A in fraction 2 and 3. Since both proteins showed similar sedimentation patterns when mixed together and the presence of lamin A prevented aggregation of LAP2 α , it can be concluded that they interacted and formed complexes.

Assuming a complex formation, the calculated molecular lamin A to LAP2 α ratio was 5:1 in fraction 2 and 1:1 in fraction 3 (Figure 4.14A). The latter corresponds with the estimated ratio of 1:1 in the soluble pool of HeLa, BJ1 and U2OS cells as described in 4.2.3. This would support the idea that at specific conditions lamin A and LAP2 α interact in a certain stoichiometry.



4.14: Sucrose density gradient centrifugation after dialysis into 100 mM NaCl, 10 mM Tris.HCl, 1 mM DTT, 10% sucrose, pH 7.5. Lamin A WT and LAP2α WT were dialyzed either alone or together into paracrystal assembly buffer. Samples were separated in a 10% to 30% sucrose gradient at 190.000 x g for 20 hours. Collection of the fractions and density measurements of the protein bands were done as described in figure 4.8. A) Exemplary coomassie stained gel of fraction SN to 11 is shown. The calculated protein percentage was blotted as bar chart (B) or as curve chart (C-D).

* BSA fractions

4.3.3.3 Presence of LAP2α WT changes lamin A paracrystal structures

The aim of this part of my work was to monitor the influence of LAP2α WT and LAP2α¹⁻⁴¹⁴ on the formation of higher order lamin A structures by electron microscopy. As in the previous experiments lamin A paracrystal assembly was induced by stepwise dialysis using buffers with decreasing NaCl concentrations either in the presence or in the absence of LAP2α WT or mutant, respectively. The so prepared samples were then negatively stained and examined in a Zeiss EM910 electron microscope. Assembled alone, lamin A formed quite large paracrystals with a defined pattern (Figure 4.15A). The background of the grid was clean and no crystal fragments or unspecific lamin aggregates could be observed. In contrast, in the

sample containing lamin A and LAP2 α WT fewer large paracrystalline structures were found, and additionally filamentous structures, aggregates and possibly paracrystal fragments were detected. These data suggest an influence of LAP2 α on the formation of lamin A paracrystals.

In the presence of LAP2 α^{1-414} the formed paracrystals were present at similar numbers as in the lamin WT sample alone and regularly shaped (Figure 4.15C). However these structures differ from lamin A structures alone in the axial repeat patterns formed by lamin Ig-folds lying upon each other and thereby intensifying the negative stain. (Figure 4.15: white arrows). While lamin A WT alone exhibited clear and readily recognizable repeat patterns, lamin A + LAP2 α^{1-414} showed less distinct patterns which could only be observed at the edge of the paracrystals. Intriguingly, the axial repeats were never observed in the lamin A WT sample containing LAP2 α WT, probably due to binding of LAP2 α WT to the carboxy-terminal domain of the lamin and thus covering the banded pattern.

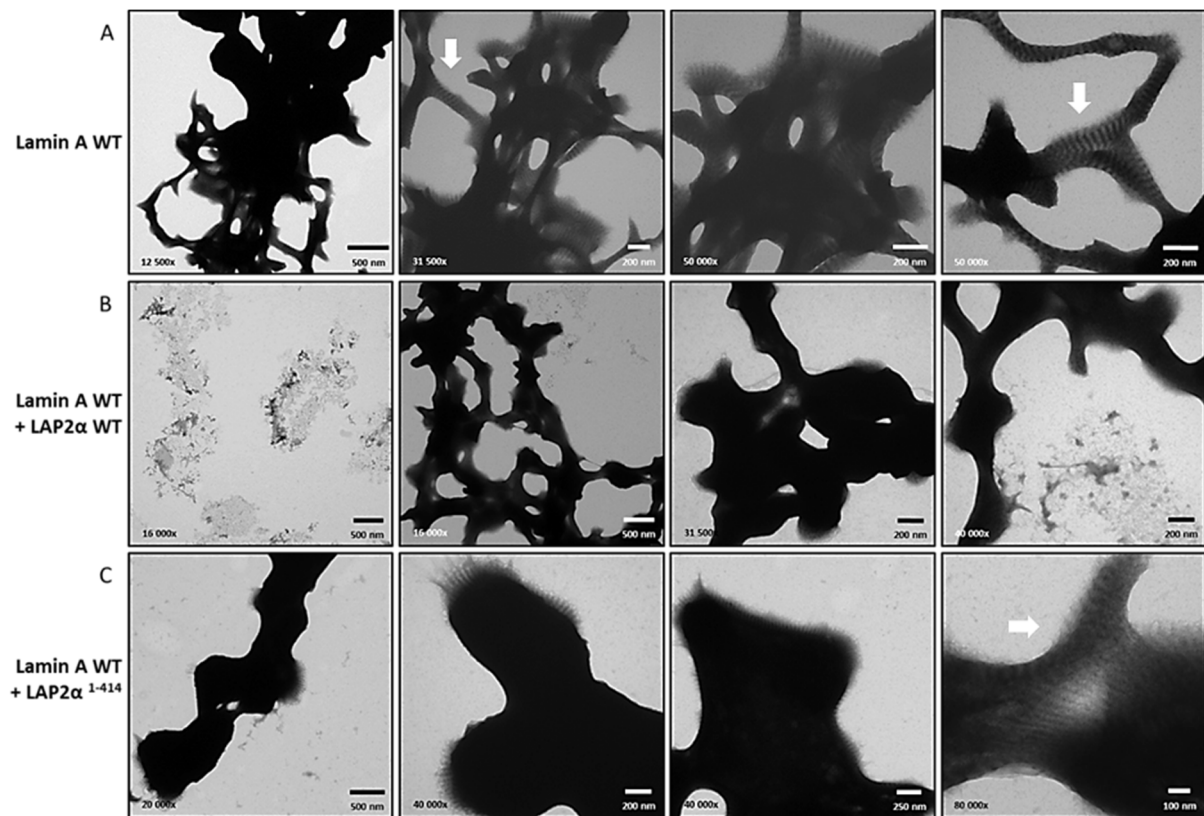


Figure 4.15: Electron microscopy images of lamin A WT structures formed in paracrystal assembly buffer. Recombinant lamin A was dialyzed, either A) alone, B) with LAP2 α WT or C) with LAP2 α^{1-414} , into 100 mM NaCl, 10 mM Tris.HCl, 1 mM DTT, pH 7.5 to induce paracrystal formation. Samples were prepared by negative staining using 2% uranyl acetate. Distinct axial repeats (arrows) were visible only in A) and C).

4.3.3.4 Increased lamin solubility in the presence of LAP2 α and vice versa

As an alternative approach the influence of LAP2 α WT on lamin A assembly was studied by sedimentation analysis in an Eppendorf table centrifuge at 13.000 rpm for 10 min. After paracrystal assembly (100 mM NaCl) and subsequent centrifugation total, supernatant and pellet fractions were quantitatively analyzed as described in Materials and Methods. In Figure 4.16A an exemplary coomassie stained gel is shown depicting lamin A and LAP2 α WT samples. When assembled alone most of lamin A was found in the pellet fraction. Strikingly, in the presence of LAP2 α WT it remained mainly soluble. In line, also LAP2 α WT became more soluble in the presence of lamin A. Since the results showed some variation between individual experiments, the increase in solubility was further quantitatively analyzed. These analyses from five independent experiments revealed a statistically significant increase in the solubility of lamin A in the presence of LAP2 α at low salt paracrystal assembly conditions (Figure 4.16B; n=5, p=0.019). Nearly twice as much lamin A was soluble in the presence versus absence of LAP2 α (42% w/o LAP2 α vs. 77% with LAP2 α), indicating that LAP2 α WT impaired lamin A assembly and solubility. Apparently LAP2 α WT also became more soluble in the presence of lamin A (Figure 4.16C; n=4, p=0.228). Interestingly, also LAP2 α ¹⁻⁴¹⁴ appeared to have an effect on the solubility of lamin A. Although the truncated LAP2 α mutant lacked the lamin A/C binding its presence during lamin A assembly increased the solubility of lamin A up to 20% (Figure 4.16D).

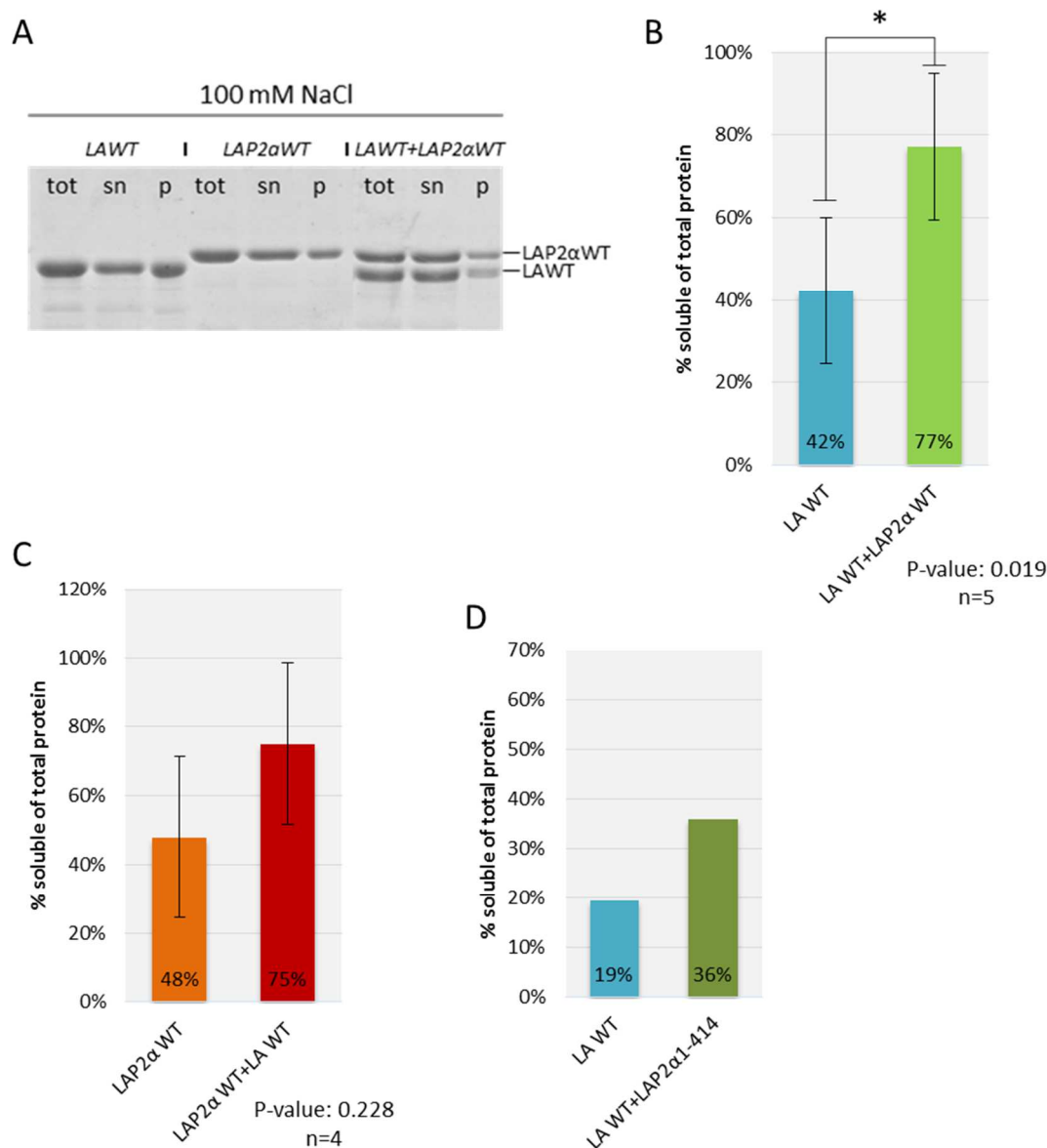


Figure 4.16: Effect of LAP2 α on the solubility of lamin A and vice versa. Lamin A WT was dialyzed into 100 mM NaCl, 10 mM Tris.HCl, 1 mM DTT, pH 7.5 either alone, with LAP2 α WT or with LAP2 α ¹⁻⁴¹⁴. Removed aliquots were spun at 13,000 rpm for 10 min to get total, supernatant and pellet fraction. Samples were separated by SDS-PAGE, stained with coomassie blue and analyzed by density measurements using *ImageJ* software. A) Exemplary coomassie stained gel of total (tot), supernatant (sn) and pellet (p) fraction is shown. B) Solubility of lamin A increased statistically significant in the presence of LAP2 α WT. C) Likewise LAP2 α solubility is increased in the presence of lamin A. D) Increased solubility of lamin A upon adding the truncated LAP2 α ¹⁻⁴¹⁴ is shown.

4.3.3.5 Analysis of assembly by analytical ultracentrifugation

Parallel to the sedimentation assays done in the Eppendorf centrifuge, sedimentation velocity (SV) analyses were performed. Sedimentation velocity analysis is an analytical ultracentrifugation (AUC) method to study the size and shape of complexes. It monitors the

movement of molecules in solution in response to a centrifugal force by measuring the light absorption at 280 nm. The sedimentation coefficient (S^*) is defined as the ratio between a particle's sedimentation velocity and the applied acceleration, and depends on molecular shape and molecular weight of proteins. Since hydrodynamic friction affects elongated and large proteins more than globular and small ones, the latter result in smaller sedimentation coefficients. In this work SV was used as a tool to study protein shape and conformation, homogeneity of a sample, possible aggregation and at best the formation of protein complexes and their binding stoichiometry.

The initial analysis of LAP2 α was described in chapter 4.3.1 and revealed the formation of LAP2 α aggregates of various sizes at lamin assembly conditions. In order to investigate the influence of LAP2 α on lamin A polymerization, samples were first dialyzed into 300 mM, 200 mM, 100 mM or 50 mM paracrystal assembly buffers containing reduced DTT concentration to prevent interference with absorption. A short centrifugation step followed to get rid of very large particles that would disturb the measurements. Afterwards, samples were diluted to a final concentration of 0.1 g/l for samples containing only lamin A or LAP2 α and 0.2 g/l for samples containing lamin A and LAP2 α together. Table 4.2 summarizes the data of each SV run. The final calculation revealed the sedimentation coefficient distribution that displays the sedimentation coefficient as the peak maximum and the concentration of the respective samples as the area under the peak.

In 300 mM NaCl paracrystal assembly buffer most of LAP2 α WT formed aggregates as shown before (Chapter 4.3.1), while lamin A was still mainly soluble (Figure 4.17A). The absence of a peak in the region from 15-30 S^* indicated that larger aggregates were successfully removed in the preceding centrifugation step. A closer look at the region between 0 and 12 S^* revealed a peak for lamin A WT (light blue) around a S^* value of 4, which correlates with previous data showing a S^* value of 3.6 for a dimeric protein (Aebi et al., 1986). In contrast, LAP2 α WT (dark blue) depicted a broader, flattened peak that points to a more heterogeneous sample, centered at a S^* value of ~6. To study the interaction of lamin A WT with LAP2 α WT the proteins were either mixed in urea before dialysis (green line) or after dialysis in 300 mM NaCl assembly buffer (black line). Since sedimentation assay in the Eppendorf centrifuge (data not shown) and AUC revealed no differences between the two approaches, mixing already in urea was subsequently used due to easier handling. Surprisingly, lamin A WT+LAP2 α WT had a similar S^* value of ~4 as lamin A WT alone. If an interaction would occur under these conditions a peak shift towards higher S^* values is expected. Thus, either the proteins did not interact or the binding caused conformational changes that slowed down the complex in the

AUC. Since sucrose gradient centrifugation analyses indicated that LAP2 α and lamin A interacted to some extent even in 300 mM conditions I favor the second possibility.

A slightly different behavior was observed in 100 mM NaCl compared to the 300 mM situation (Figure 4.17B). While the S^* values for lamin A WT (green line) and LAP2 α WT (dark blue line) were the same in both conditions (4 and 6, respectively), the area under the peak decreased slightly in the case of lamin A and significantly increased for LAP2 α . Furthermore, the large amount of particles with S^* values <2 indicates that smaller particles were present, most likely degradation products. In contrast to 300 mM NaCl lamin A WT+LAP2 α WT (black line) showed a maximal S^* value around 6, which correlates with the S^* value for LAP2 α , but is higher than that for lamin A. Again, if the two proteins would interact and form complexes S^* values in the range between 9 and 10 or even higher are expected.

The calculated graph in figure 4.17C depicts the sedimentation coefficient distribution from AUC runs of lamin A WT+LAP2 α WT assembled in 300 mM (green line), 200 mM (black line), 100 mM (light blue line) and 50 mM NaCl (dark blue line). Obvious were the continuously decreasing protein concentrations, probably due to increased polymerization and/or formation of complexes/aggregates in lower salt concentrations. These large particles were most likely removed in the last centrifugation step before the AUC, and therefore the analyzed samples contained mainly smaller and soluble molecules. Nevertheless, a slight shift in S^* values from ~ 4 to ~ 6 could be observed with decreasing salt concentrations.

These data indicate that sedimentation velocity analytical ultracentrifugation is not the preferred method for studying lamin A assembly as most of the assembled structures are removed in the pre-clearing step, but it can depict formed assembly intermediates.

Run	Protein	Concentration [mg/ml]	Buffer	Sedimentation coefficient S* [Svedberg]
A	LA WT	0.1	300 mM NaCl	4
	LAP2 α WT	0.1	10 mM Tris.HCl	6
	LA WT+LAP2 α WT	0.2	0.2 mM DTT	4
			pH 7.5	
B	LA WT	0.1	100 mM NaCl	4
	LAP2 α WT	0.1	10 mM Tris.HCl	6
	LA WT+LAP2 α WT	0.2	0.2 mM DTT	6
			pH 7.5	
C	LA WT+LAP2 α WT	0.1*	300 mM NaCl/	4
	LA WT+LAP2 α WT	0.1*	200 mM NaCl/	4-5
	LA WT+LAP2 α WT	0.1*	100 mM NaCl/	4-5
	LA WT+LAP2 α WT	0.05*	50 mM NaCl	5-6
			10 mM Tris.HCl 0.2 mM DTT pH 7.5	

Table 4.2: Sedimentation velocity experiments. Conditions for each run as depicted in Figure 4.17.

Speed: Forerun 700 x g, Main run 130 000 x g.

* Lower concentration due to lower protein amounts at the beginning and loss during dialysis.

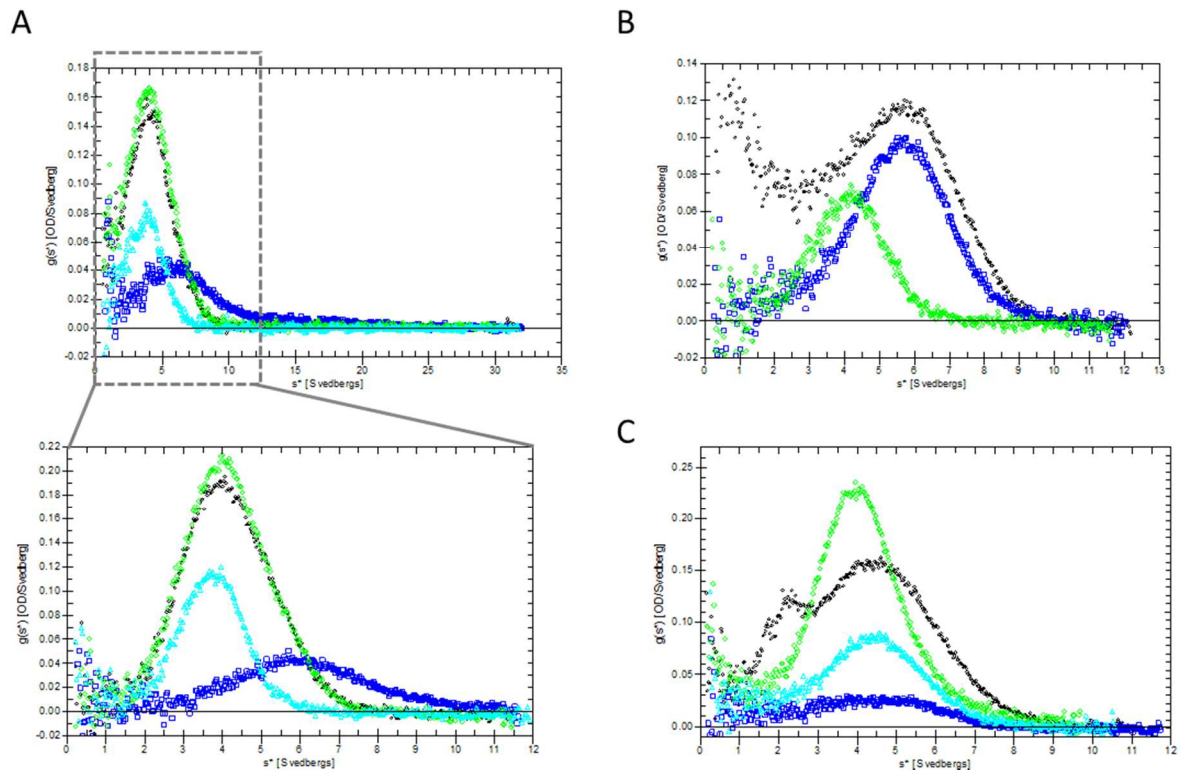


Figure 4.17: Sedimentation velocity analytical ultracentrifugation of LAP2 α WT and lamin A WT. LAP2 α WT and lamin A WT were dialyzed either alone or together into paracrystal assembly buffers with various NaCl concentrations. The SV run was carried out in an Optima XL-A analytical ultracentrifuge equipped with an An-60 Ti Rotor. Forerun: 700 x g; Main run: 130 000 x g. A) – C) Calculated graphs depicting the sedimentation coefficient distribution. The area under the peak displays the concentration of the sample. A) Samples: LA WT (light blue), LAP2 α WT (dark blue), LA WT+LAP2 α WT (black and green) in 300 mM NaCl. B) Samples: LA WT (green), LAP2 α WT (dark blue), LA WT+LAP2 α WT (black) in 100 mM NaCl. C) Sample: LA WT+LAP2 α WT in 300 mM NaCl (green), 200 mM NaCl (black)^o, 100 mM NaCl (light blue) or 50 mM NaCl (dark blue).

^oPeak at S*2 treated as outlier.

4.3.4 Influence of LAP2 α on lamin filament assembly

Next the influence of LAP2 α on the *in vitro* formation of lamin A filaments was investigated. Lamin A filament assembly was done as described in Materials and Methods in the presence or absence of LAP2 α . The assembled lamin structures were subsequently analyzed by negative staining electron microscopy and by sedimentation analyses regarding their size, shape and solubility.

4.3.4.1 Electron Microscopy reveals subtle effects of LAP2 α on lamin filament assembly

Samples containing lamin A were assembled into filaments, either alone or in the presence of LAP2 α WT or LAP2 α ¹⁻⁴¹⁴, negatively stained and examined in a Zeiss EM910 electron microscope (Figure 4.18). While lamin A filaments assembled alone were well

structured and defined with even edges (Figure 4.18A), filaments assembled in the presence of LAP2 α WT contained regions where their surface was slightly rougher, uneven and disrupted (Figure 4.18B). Interestingly, the lamin A+LAP2 α^{1-414} filaments looked similar to those where full length LAP2 α was added (Figure 4.18C). Additionally, both mixed samples exhibited a darker shading of the filamentous structures, probably an effect of more intensive negative staining due to association of full length LAP2 α or the truncated mutant (Figure 4.18B and 4C). Therefore, based on the electron microscopy images no differentiation between LAP2 α^{1-414} and LAP2 α WT with respect to their influence on lamin A assembly could be made. Although LAP2 α^{1-414} is lacking the C-terminal lamin binding domain it seemed to associate with the formed filaments unspecifically and thereby affecting lamin A assembly. This is in agreement with the data obtained from the paracrystal assembly studies in which the mutant LAP2 α protein likely also bound to lamin A and affect the formation of lamin A paracrystals (see chapter 4.3.3.3). In summary the data indicate that LAP2 α affects lamin polymerization into filaments, however, these effects were less clear than in the paracrystal assembly assays.

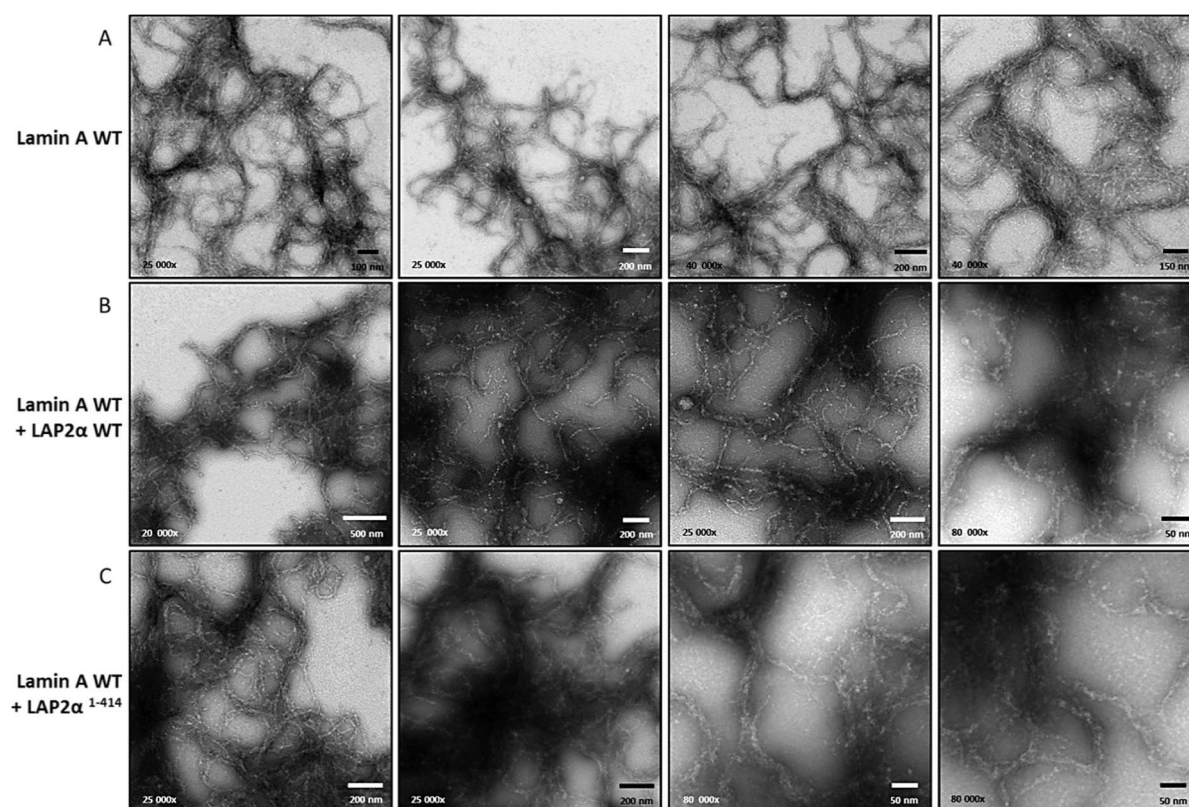


Figure 4.18: Electron microscopy images of lamin A WT structures formed in filament assembly conditions. Recombinant lamin A was dialyzed, either A) alone, B) with LAP2 α WT or C) with LAP2 α^{1-414} , into 250 mM NaCl, 25 mM MES, 1 mM DTT, pH 6.5 to induce filament formation. Samples were prepared by negative staining using 2% uranyl acetate.

4.3.4.2 *Lamin A shows slightly increased solubility upon addition of LAP2 α*

In addition to electron microscopy, lamin A filament assembly was analyzed with respect to lamin A solubility by sedimentation assays in an Eppendorf table centrifuge. After assembly the various samples (lamin A WT, lamin A WT+LAP2 α WT, lamin A WT+ LAP2 α ¹⁻⁴¹⁴, lamin A¹⁻⁴³⁷ and lamin A¹⁻⁴³⁷+LAP2 α WT) were spun at 13.000 rpm for 10 min. Aliquots were taken before and after the centrifugation to get a total, supernatant and pellet fraction and all samples were separated by SDS-PAGE. Quantitative analysis of the Coomassie stained gels was done using *ImageJ* software. Lamin A alone was to a great extent (up to 70%) soluble under filament assembly conditions (Figure 4.19A). The lamin A solubility increased slightly (15% - 18%) in the presence of LAP2 α WT and LAP2 α ¹⁻⁴¹⁴, respectively. Interestingly and in agreement with the data obtained by electron microscopy, also LAP2 α ¹⁻⁴¹⁴ appeared to have some influence on lamin A filament assembly, as it increased the solubility of lamin A despite the lack of the lamin A/C binding domain (Figure 4.19A). Lamin A¹⁻⁴³⁷, which was used as negative control, lacking the last 209 amino acids of the C-terminus including the LAP2 α interaction region, showed no increase in solubility in the presence of LAP2 α WT, as expected. This is in agreement with the data obtained from sucrose gradient centrifugation experiments (see chapter 4.3.3.1), confirming that the carboxy-terminal domain of lamins A/C is responsible for LAP2 α interaction. However, it must be noted that already more than 80% of lamin A¹⁻⁴³⁷ were soluble in the absence of LAP2 α .

In contrast to the situation in the paracrystal assembly studies, lamin A remained highly soluble when assembled into filaments. Even though electron microscopy revealed defined filamentous structures (Figure 4.18), the high NaCl concentration (250 mM) in the filament assembly buffer may interfere with lamin polymerization and keep the filaments small. This hypothesis is supported by observations that lamin polymerization started at around 200-250 mM NaCl (Figure 4.5). To investigate whether formed lamin filaments were too small to precipitate at the used speed lamin A WT, LAP2 α WT and lamin A WT+LAP2 α WT samples were spun at increasing centrifugation speed (4.000, 7.000, 9.000, 11.000 and 13.000 rpm, respectively) following filament assembly. Supernatant and pellet fractions were subsequently analyzed by quantitative SDS-PAGE and the percentage of the respective proteins present in each fraction was calculated. The percentage of lamin A WT and LAP2 α WT present in the pellet fractions increased slightly with increasing centrifugation speed (from 22% to 38% for LAP2 α WT and from 22% to 30% for lamin A WT) (Figure 4.19C and E). The LAP2 α WT sample appeared structurally slightly more heterogeneous showing a stronger increase of protein present in the pellet with increasing speed.

Strikingly, the majority of lamin A WT (82-89%) as well as of LAP2 α WT (85-87%) remained soluble independent of the centrifugation speed when present in the mixed sample (Figure 4.19D and F). Therefore it can be suggested that lamin A and LAP2 α have a mutual impact on the aggregation state of the respective binding partner and that the formed complexes have a uniform particle size, are small and have reduced tendency to aggregate.

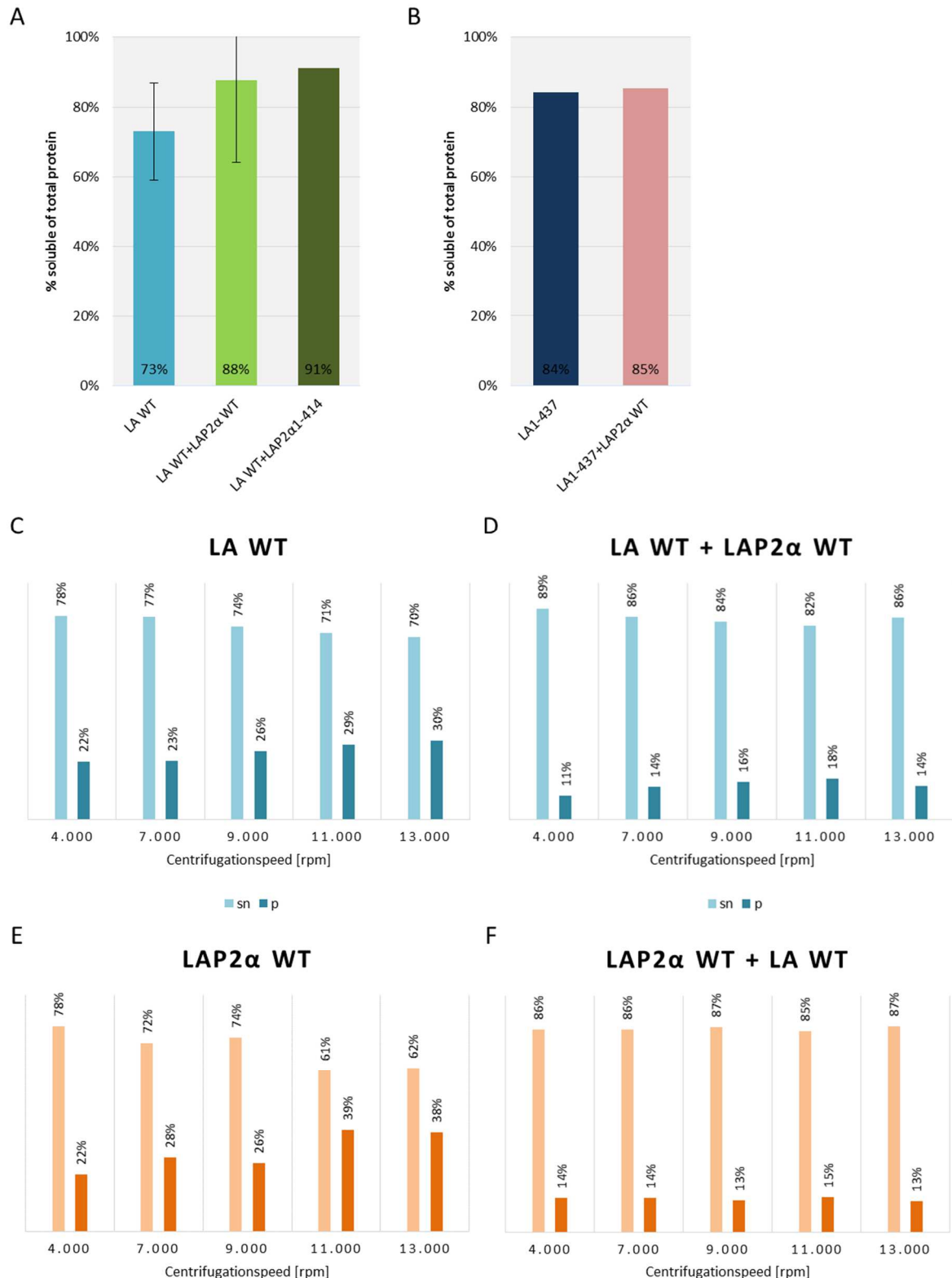


Figure 4.19: Influence of LAP2α on lamin A at filament assembly. Samples were dialyzed against 250 mM NaCl, 25 mM MES, 1 mM DTT, pH 6.0. A) and B) Aliquots were centrifuges at 13.000 rpm for 10 min to get soluble and pellet fraction. Samples were separated by SDS-PAGE, coomassie stained and analyzed by density measurements with *ImageJ* software. A) Slightly increased solubility of lamin A is detected in the presence of LAP2α WT and LAP2α¹⁻⁴¹⁴. B) No change in solubility of lamin A¹⁻⁴³⁷ is detectable in the presence of LAP2α WT. C) – F) Aliquots were spun at 4.000 to 13.000 rpm. Soluble and pellet fraction were quantified as described above. C) and E) show decreased solubility with increasing centrifugation speed. D) and F) Continued high solubility of lamin A WT and LAP2α WT in the mixture was observed at all centrifugation

5 Discussion

5.1 Abundance of lamin A/C and LAP2 α in cells

5.1.1 Quantitative measurements *in vivo*

During interphase lamin A/C and LAP2 α form a complex that is involved in regulating cell cycle progression, transcription and chromatin organization (Dechat et al., 2010b; Naetar and Foisner, 2009). Determining the abundance of lamins and LAP2 α within a cell is necessary to obtain insight into the possible stoichiometric composition of these intranuclear complexes *in vivo*. As the quantities of lamin A, lamin C and LAP2 α in human cells were still unknown, quantification of these proteins in HeLa, U2OS and hTERT BJ1 cell lines was a main aim of this work.

Concerning the abundance in total cell lysates it seems that fast proliferating cell lines, like HeLa and U2OS, contain more LAP2 α than slow proliferating cells, like BJ1. On the contrary lamin A and C are present in higher amounts in slow proliferating cells than in HeLa and U2OS. Lamin C was most abundant in all three cell lines. The percentage of soluble molecules amounts to an average of one third of the total quantity of lamin A and C. LAP2 α displays in all cell lines almost complete solubility as expected as it is a nucleoplasmic protein lacking any transmembrane domain. The presence of 30% to 40% of soluble A-type lamins differs from the results from Kolb *et al.* (Kolb et al., 2011), which revealed a solubility of up to 10% for A-type lamins in HeLa, U2OS and human fibroblasts. The different protein extraction procedures are most likely the reason for the different lamin solubilities.

5.1.2 Molecular stoichiometry of lamin A/C-LAP2 α complex *in vivo*

The stoichiometric ratio of lamin A/C to LAP2 α *in vivo* displays that in fast proliferating cells 3 to 4 times more lamin A/C than LAP2 α is present, whereas the ratio in BJ1 cells is up to 47:1. More interestingly, the soluble pool, where lamin A/C-LAP2 α complex formation likely takes place, suggests interaction of the molecules at 1:1 ratio in HeLa and U2OS and 14:1 in BJ1 cells.

Probably more informative are data obtained from purely interphase cell lysates after the mechanical removal of mitotic cells as the formation of nucleoplasmic lamin A/C-LAP2 α complexes occurs during G1 phase and lamins A/C are completely soluble in mitotic cells. In the soluble pool of all three cell lines this analysis displays an abundance ratio of lamin A/C to LAP2 α of 1:1, suggesting that the complexes consist of equal amounts of lamin A/C and LAP2 α molecules.

Based on the assumption that lamin A/C and LAP2 α interact in a 1:1 ratio, one can assume that the more LAP2 α is present within the nucleoplasm the more lamins can be bound if no other mechanisms regulate the interaction. Hence, HeLa and U2OS cells should exhibit more nucleoplasmic lamins due to higher LAP2 α levels than BJ1 cells, which is exactly what I got in pure interphase cells. Overall this study provides the first insight into the abundance and stoichiometry of lamins and LAP2 α within a cell.

5.2 Influence of LAP2 α on lamin A assembly

5.2.1 Limitations of the lamin A assembly in the presence of LAP2 α

In order to investigate the influence of LAP2 α on lamin polymerization, *in vitro* lamin A assembly assays were performed in the absence and presence of LAP2 α . Soluble LAP2 α molecules were expected to bind to lamin A molecules, thereby preventing their assembly into higher order structures as filaments or paracrystals. However, conditions in which lamin polymerization has been expected were suboptimal for LAP2 α . Precipitation of LAP2 α was a recurring problem throughout all *in vitro* lamin assembly experiments. Even in 8 M urea both lamin A and LAP2 α show up to 30% insolubility. This is not that surprising as urea is not the ultimate denaturing agent and for example freezing of protein samples in urea can lead to aggregation. However, contrary to lamin A, which shows no increase in precipitation upon dialyzing into non-urea conditions, LAP2 α 's solubility rapidly decreased when kept in buffers lacking urea.

The insolubility of LAP2 α was most likely triggered by aggregation due to oxidation of the cysteine residues in the carboxy-terminal domain. This hypothesis is supported by the fact that the solubility of LAP2 α was increased by using higher concentrations of the reducing agent, here DTT. Therefore an attempt to overcome the precipitation of LAP2 α during *in vitro* assembly experiments was to increase the reducing environment of the buffer. This indeed solved the problem of insoluble LAP2 α but, unfortunately, it also impaired the polymerization of lamin A and was therefore not useable in the assays. This insolubility of LAP2 α in assembly buffers, however, revealed an influence of lamin A on the solubility of LAP2 α . Thus there is a mutual influence of LAP2 α and lamin A preventing aggregation of the proteins.

Despite these problems this work revealed some important properties of LAP2 α . Analytical ultracentrifugation sedimentation velocity showed that LAP2 α has a molecular mass of 75.5 kDa and a sedimentation coefficient of $S^* 6$ in the non-aggregated state. Assuming from Erickson *et al.* that a molecular mass difference of 10 kDa corresponds with a S^* value difference of approximately 1 for globular proteins (Erickson, 2009) and taking into account

that Bovine serum albumin, which is about 10 kDa smaller than LAP2 α exhibits a S^* value of 4.6, LAP2 α may behave like a globular protein in AUC. As the samples for the analytical ultracentrifugation had to be centrifuged before the run to remove larger aggregates, the obtained values are representative only for the soluble protein fraction. LAP2 α reveals a S^* of 6 in both, high salt and low salt, conditions. Assuming that serum albumin was tested as a monomer in Erickson *et al.* this would indicate that LAP2 α is present as a monomer at these conditions, which is not in agreement with atomic structures of LAP2 α C-terminus predicting LAP2 α dimers. Thus LAP2 α may form large but filamentous extended structures. Soluble non-assembled lamin A reveals a S^* value of 4 in analytical ultracentrifugation. Compared to Aebersold *et al.* where a S^* 3.6 for dimeric lamin A is proposed, this would confirm that lamin A dimer is the basic unit of the soluble pool (Aebersold *et al.*, 1986).

In summary, the analytical ultracentrifugation analysis could not be used for following lamin assembly, as the formed aggregates/complexes precipitated already in pre-sedimentation steps but it provided some useful informations on soluble proteins.

Sucrose density gradient centrifugation analysis was more useful for studying lamin A/C-LAP2 α interactions. LAP2 α alone in high salt buffers and in the presence of 50 mM DTT sedimented together with Serum albumin. Assuming that Serum albumin, used as a marker, sediments as a monomer with a molecular weight of 66 kDa the data were consistent with LAP2 α being a globular monomer or an elongated oligomer in non-assembly conditions. Less reducing conditions and lower salt leads to self-interaction of LAP2 α into large aggregates. Lamin A shows similar properties than LAP2 α on gradient gels in non-assembly conditions. As lamin A is known to exist as a dimer it is plausible that LAP2 α also exists as dimer. In 100 mM NaCl buffer lamin A structures exhibit a heterogeneous size distribution reaching from dimers to slightly larger oligomers.

5.2.2 Mutual influence of lamin A and LAP2 α

5.2.2.1 ... at paracrystal assembly conditions

According to my results LAP2 α and lamin A influence each other's solubility in paracrystal assembly conditions. Interaction of these proteins A in non-assembly conditions (300 mM NaCl) is indicated by changes of sedimentation profiles of both proteins in the sucrose gradient centrifugation upon mixing LAP2 α and lamin A compared to samples of these proteins alone. The lamin A+LAP2 α mixture exhibited a broad distribution over the gradient pointing to a heterogeneous size distribution of particles. The main peak, however, displayed a shift towards larger sizes in the mixed samples compared to lamin A and LAP2 α alone. Support for

an interaction of the proteins comes from the fact that all negative controls do not show these alterations. A possible stoichiometric ratio between lamin and LAP2 α molecules at this high salt conditions was estimated as 3:1 or 2:1.

This number is slightly higher than the 1:1 ratio in the soluble pool of fractionated HeLa, BJ1 and U2OS cells as described in 4.2.3. The high salt non-assembly conditions used in the sucrose gradient experiment might have favored the formation of different, non-physiological complexes, compared to the situation *in vivo*. However, in the cell fractionation experiments not all lamin A and LAP2 α present in the soluble fraction must interact with each other and form a complex.

Lower salt concentrations (100 mM NaCl) increased the molecular ratio of lamin to LAP2 α in the fractions to 1:1. This corresponds well with the *in vivo* calculated ratio in the soluble pool of purely interphase cells. Another evidence for a strong interaction of the proteins is the fact that lamin A is able to prevent self-aggregation and thereby precipitation of LAP2 α . Similar to the sucrose gradient centrifugation results also the Eppendorf centrifugation assay showed, at low salt lamin assembly conditions, significant increase in solubility of lamin A in the presence of LAP2 α and vice versa. Electron microscopy also depicted some kind of interference of LAP2 α with the formation of paracrystalline structures. Furthermore, closer inspection of the images indicated that LAP2 α binds lamins close to the Ig-fold motif, as the axial repeat pattern of lamin A paracrystals, caused by overlapping Ig-folds, was not visible in the presence of LAP2 α . This was probably due to the binding and thereby covering by LAP2 α .

Interestingly, also the mutant lamin A-binding deficient LAP2 α^{1-414} , used as a negative control, showed similar influence on lamin A paracrystal patterns as the LAP2 α wild type. The Eppendorf centrifugation assay also showed a slight increase in solubility of lamin A in the presence of LAP2 α^{1-414} which was less pronounced than the effect of LAP2 α wild type. Although Dechat *et al.* provided evidence by blot overlay assays that the N-terminal domain of LAP2 α does not bind lamins (Dechat et al., 2000a), my work found some effect of LAP2 α^{1-414} on lamin A. It cannot be excluded that additional binding regions of LAP2 α or further modifications are responsible for the binding of lamins. Alternatively, these proteins could bind unspecifically due to the high concentration in the mixture.

5.2.2.2 ... at filament assembly conditions

The lamin filament assembly assays showed two problems. First, the high salt concentrations of 250 mM in the filament assembly buffer may decrease binding of LAP2 α to lamin A. Second, even though the electron microscopy images indicated the formation of well-

structured filaments, these filaments did not efficiently precipitate in the centrifuge. In the Eppendorf centrifugation assay, lamin A was soluble up to 70% in these conditions and this increased only slightly upon adding LAP2 α as well as LAP2 α ¹⁻⁴¹⁴.

Nevertheless, sedimentation experiments with increasing speed under filament assembly conditions indicated some mutual influence of lamin A and LAP2 α . LAP2 α was able to inhibit the formation of larger lamin structures and likewise LAP2 α solubility increased in the presence of lamin A. These observations are consistent with interaction of the proteins.

5.3 Conclusion

This work presents results that points towards interaction of LAP2 α and lamin A/C and possible regulation of solubility and lamin A assembly. Based on the Quantification experiments *in vivo* sufficient amounts of lamin A/C and LAP2 α molecules are present in the nucleoplasmic pool of interphase cells to enable complex formation in an equal molecular stoichiometry (1:1). Thus each LAP2 α molecule would bind one lamin A/C and preventing its assembly into the lamina.

In vitro experiments confirmed this stoichiometric ratio by revealing also a 1:1 lamin A to LAP2 α ratio at lamin assembly conditions. Furthermore several observations point towards a role of LAP2 α in impairing lamin A assembly.

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7 Curriculum vitae

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Final Bachelor’s Thesis:
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