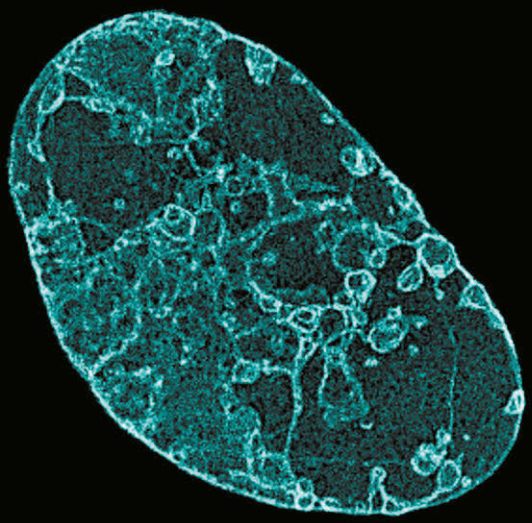
On the Evolution, Function and Pathogenesis of Intermediate Filaments

Josef Gullmets



On the back

Left: Human vimentin was transgenically expressed in *Drosophila*. This image depicts endothelial cells in a vessel called Malpighian tubule. The vessel has been dissected from a third instar *Drosophila* larvae. The cells have been stained with fluorescent antibodies against vimentin (green) and discs large (red). DNA has been visualized with DAPI (blue). The length of side of the image corresponds to 96 µm. The image was obtained using spinning disc confocal microscopy.

Right: Nestin is a cytoplasmic intermediate filament and as such forms an intricate network in the cytoplasm. This image depicts a muscle precursor cell, a myoblast. Nestin has been stained with fluorescent antibodies (red) and DNA has been visualized with DAPI (blue). The length of side of the image corresponds to 96 µm. This image was obtained with a confocal microscope. Courtesy of Elin Torvaldson.

On the cover

The lamina is a continuous protein meshwork just below the nuclear membrane. The *LMNA* mutation p.S143P results in an aggregation of lamins, which is one of the phenotypes associated with dilated cardiomyopathy (DCM). This image shows the disrupted lamina of the nucleus of an isolated fibroblast from a patient carrying the p.S143P mutation. Lamin A/C has been stained with fluorescent antibodies (cyan). The length of the nucleus is 27 µm. The image was obtained using spinning disc confocal microscopy.

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of

Intermediate Filaments

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Oss!

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List of Original Publications

I. GULLMETS J, Torvaldson E, Lindqvist J, Imanishi S, Taimen P, Meinander A & Eriksson JE. Internal epithelia in *Drosophila* display rudimentary competence to form cytoplasmic networks of transgenic human vimentin – *Submitted*

II. Lindqvist J*, Torvaldson E*, GULLMETS J, Karvonen H, Nagy A, Taimen P, Eriksson JE. Nestin contributes to skeletal muscle homeostasis and regeneration – *In revision*

III. West G*, GULLMETS J*, Virtanen L, Li SP, Keinänen A, Shimi T, Mauermann M, Heliö T, Kaartinen M, Ollila L, Kuusisto J, Eriksson JE, Goldman RD, Herrmann H, Taimen P. Deleterious assembly of the lamin A/C mutant p.S143P causes ER stress in familial dilated cardiomyopathy. *J Cell Sci* 129: 2732-43, 2016

*Equal contribution

Abbreviations

4-PBA	4-phenylbutyric acid
AA	Amino acid
ABD	Actin binding domain
ALS	Amyotrophic lateral sclerosis
ASNS	Asparagine synthetase
BAF	Barrier-to-autointegration factor
BLAST	Basic local alignment search tool
CDK5	Cyclin dependent protein kinase 5
CLN	Centrally located nuclei
CMT	Charcot-Marie-Tooth disease
CNS	Central nervous system
CytoIF	Cytoplasmic intermediate filaments
DCM	Dilated cardiomyopathy
DjIFb	Dugesia japonica intermediate filament b
EB	Epidermolysis bullosa
EBS-DM	Epidermolysis bullosa simplex, Dowling-Meara type
EDL	Extensor digitorum longus
eIF2α	Eukaryotic translation initiation factor 2A
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
EXOSC7	Exosome component 7
FISH	Fluorescence in situ hybridization
GFAP	Glial fibrillary acidic protein
GSEA	Gene Set Enrichment Analysis
H3K27me3	Trimethylation of lysine 27 on histone H3
H3K9me2	Dimethylation of lysine 9 on histone H3
IF	Intermediate filament
IFBD	Intermediate filament binding domain
INM	Inner nuclear membrane
IP	Immunoprecipitation
IPSC	Induced pluripotent stem cell
LAP2	Lamin associated polypeptide 2
LBR	Lamin B receptor
LEM	LAP2, emerin and MAN1
LINC	Linker of the nucleoskeleton and cytoskeleton
MET	Mesenchymal to epithelial transition
MSC	MAN1/Src1p/C-terminal
MTJ	Myotendinous junctions
MyBPC3	Myosin binding protein, cardiac 3
NE	Nuclear envelope
NF-H	Neurofilament heavy
NF-L	Neurofilament light
NF-M	Neurofilament medium
NIFID	Neuronal intermediate filament inclusion disease

NLS	Nuclear localization signal
NMJ	Neuromuscular junction
ONM	Outer nuclear membrane
PCD	Pre-coil domain
PNS	Peri-nuclear space
PRD	Plectin repeat domain
PTM	Post-translational modification
RT-PCR	Real-time polymerase chain reaction
SSR1	Sequence receptor subunit 1
SULT1A4	Sulfotransferase family 1A member 4
SUN	Sad1-Unc84
TA	Tibialis anterior
TD	Tudor domain
TM	Tunicamycin
ULF	Unit length filaments
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
WT	Wild type
XBP1	X-box binding protein 1
XBP1s	Spliced X-box binding protein 1
XBP1u	Unspliced X-box binding protein 1

Svensk sammanfattning

Intermediärfilament (IF) är nukleära och cytoplasmiska strukturprotein. I människans genom ingår 70 gener som kodar för 74 hittills kända intermediärfilament. Mutationer i dessa gener orsakar många allvarliga sjukdomar. IF förser cellen med en dynamisk stödstruktur som effektivt kan monteras upp och ned. IF:ens kapacitet till upp- och nedmontering möjliggör att denna stödstruktur kan ommodelleras kontinuerligt, vilket är viktigt för cellens rörlighet samt för rekonstruktionen av kärnmembranet vid celldelning. Under evolutionen har IF:en blivit mer och mer funktionellt specialiserade. Adaptionen av IF:ens funktion och deras utbredda uttryckning reflekterar hur viktig IF:en har blivit för multicellulära organismer. De nukleära IF:en är ursprunget till de cytoplasmiska intermediärfilamenten (cytoIF) och deras uttryckning är konserverad i alla multicellulära organismer. CytoIF:en är också mycket konserverade och de uttrycks i alla eumetazoa organismer, förutom i artropoderna.

Denna doktorsavhandling är indelad i tre projekt. Det första projektet behandlar varför *Arthropoda* är det enda fylum inom eumetazoerna som saknar cytoIF. I detta projekt har ett humant cytoIF, vimentin, transgent uttryckts i *Drosophila*, för att studera proteinets kapacitet att monteras och bilda IF-nätverk i artropodvävnad. Slutsatsen är att humant vimentin kan forma nätverk i epitelvävnader i *Drosophila*, vilket påvisar kompatibilitet mellan cytoIF och *Drosophila*.

Den andra studien behandlar ett mindre känt cytoIF, nestin. En nestin knock-out mus användes för att studera följderna av avsaknaden av nestin i muskelvävnad. Resultaten visar att knock-out av nestin leder till försämrad läkning av muskler. Läkning hos skelettmuskler är beroende av satellitceller, och denna studie visar att hastigheten i celldelningen är nedsatt i satellitceller som isolerats från nestin knock-out-möss.

Mutationer i nukleära IF, laminer, ger upphov till muskeldystrofi och för tidigt åldrande. I det tredje projektet som ingår i denna doktorsavhandling, undersöks en punktmutation i *LMNA*-genen. Denna mutation är associerad med dilaterad kardiomyopati (DCM). Mutationen, p.S143P i lamin A/C har identifierats i finska patienter och orsakar sent uppkommande DCM med ledningsdefekter. Patientfibroblaster, inducerade pluripotenta stamceller (iPSC) differentierade till kardiomyocyter och *Drosophila* användes för att studera fenotypen av p.S143P-mutationen. Studien visar att mutationen orsakar i patentfibroblasterna en oförmåga hos de muterade laminerna att normalt inkorporeras i laminan. De muterade

laminerna återfanns i stället i nukleoplasman och bildade ibland stora intranukleära aggregat. Dessa fenotyper ledde till endoplasmatisk retikulär (ER) stress. Dessa resultat kunde också reproduceras i kardiomyocyter deriverade från iPSC samt i *Drosophila*.

Sammanfattningsvis beskriver studierna som utförts inom ramen för denna doktorsavhandling nya strukturella och funktionella aspekter hos både nukleära och cytoplasmiska IF. Dessa resultat fördjupar vår insikt i IF:ens dynamik och kan komma att gynna utvecklingen av mediciner för att bota sjukdomar som är associerade till IF.

Abstract

Intermediate filaments (IFs) are nucleoskeletal and cytoskeletal proteins. The human IF protein family consists of 70 genes coding for 74 known proteins and their mutations cause various serious diseases. The IFs provide the cell with a dynamic scaffold that can be effectively assembled and disassembled. The assembly-property of IFs enables the IF scaffold to be continuously remolded, which is important for the motility of the cell and for the deconstruction and reconstruction of the nuclear membrane during mitosis. During evolution, the IFs have, since their emergence, become increasingly functionally differentiated. The adaptation of the IF function and spatial expression reflects how instrumental the IFs have become for multicellular organisms. The nuclear IFs, which are precursors to the cytoplasmic IFs (cytoIFs), are conserved in all multicellular organisms. The cytoIFs are also very conserved throughout the eumetazoans except for the arthropods.

This thesis is divided into three projects, the first of which deals with the question why A*rthropoda* is the only eumetazoan phyla lacking cytoIFs. We transgenically express a human cytoIF, vimentin, in *Drosophila* to study cytoIF expression and assembly in arthropod tissue. We conclude that human vimentin forms networks in epithelial tissue in *Drosophila*, suggesting some compatibility for cytoIFs in *Drosophila* specifically in epithelial tissue.

The second study deals with the less well characterized cytoIF nestin. We utilized a nestin null mouse model to investigate muscle physiology following nestin knock out. We show that nestin knock out results in impaired muscle regeneration. Skeletal muscle regeneration is dependent on satellite cells, and we could show that there was a defect in the proliferation rate of satellite cells isolated from nestin knock out mice.

Mutations in lamins give rise to muscular dystrophies and premature aging. In the third project included in this thesis, we investigate a point mutation in the *LMNA* gene associated with dilated cardiomyopathy (DCM). The mutation, p.S143P in lamin A/C, has been identified in the Finnish population and gives rise to late onset DCM with conduction defects. Patient fibroblasts, induced pluripotent stem cells (IPSCs) differentiated into cardiomyocytes and *Drosophila* were used to investigate the disease phenotype of the p.S143P mutation. We showed that in the patient fibroblasts, the mutation causes inability of mutant lamins to incorporate into the lamina. Instead, the mutant lamins are found in the nucleoplasm and frequently form intranuclear aggregates which eventually lead to endoplasmic reticulum (ER) stress.

These findings were further validated in iPSC derived cardiomyocytes as well as in *Drosophila*.

In summary, the work described in this thesis characterized new structural and functional aspects of both cytoplasmic and nuclear IFs. These findings deepen our understanding of IF dynamics and may benefit molecular targeting of IF related diseases.

Introduction

The common ancestry of species allows us to use comparative biology to make conclusions about ourselves. In the three studies that this thesis contains, different research models have been utilized to learn about the function and dysfunction of three different members of the intermediate filament (IF) protein family.

In the first study, we ask; How can there be one type of protein that is expressed in all animal groups except one? What makes that animal group special? And, is there an intrinsic property to these proteins that explains the exception to the rule? As it turns out, the cytoplasmic intermediate filaments (cytoIFs) are present it all eumetazoan organisms except the arthropods. Because cytoIFs are so conserved and present so consistently in the proteome of all other animals, the fact that they are absent exclusively from arthropods is no less than remarkable. Since organisms that are ancestors to the arthropods express cytoIFs, we know that the arthropods must have lost the cytoIFs during evolution. We provide three alternatives for how this could have happened. First, being a protein that largely governs epidermal integrity, cytoIFs could have become redundant following the emergence of the exoskeleton. Second, development of new properties in the arthropod lineage could have been incompatible with the cytoIFs and thus, there would have been a high selective pressure to replace the cytoIFs with other proteins. Third, other proteins could have evolved to be more beneficial in governing various cytoskeletal tasks, rendering the cytoIFs redundant. If cytoIFs have an intrinsic property that could explain their emergence and existence in the eumetazoan domains, and that intrinsic property could be pinpointed, it would allow for conclusions about the nature of all intermediate filaments (IFs). On this premise, we transgenically expressed human vimentin, a cytoIF, into *Drosophila*, to gain insights on what happens when arthropods regain cytoIFs.

In the second study, we have a more conventional question than in the first study; What happens when we remove a gene that encodes a particular protein. Can the consequence of a gene knock out teach us about the function of the protein? A nestin null mice strain was investigated to learn about the function of nestin, a little studied cytoIF. Nestin has been characterized as a marker for neuroepithelial stem cells. Nestin cannot form homopolymers but must co-assemble with vimentin and possibly with desmin and α-internexin. Previous studies with nestin null mice have identified that it is important for the formation of neuromuscular junctions (NMJs). However, it is known that there is nestin expression also in skeletal muscle during development. Therefore, we wanted to study nestin in the regenerating muscle. We

could show that nestin knock out causes impaired skeletal muscle regeneration. We could also show that the decrease in regeneration rate of the nestin knock out mice was due to a proliferation defect in muscle stem cells. How the muscle stem cell proliferation defect is caused by nestin knock out should be investigated further.

In the third study, our starting point is a human disease condition caused by a single heterozygous point mutation. We are asking whether we can find out how this genetic mutation is translated into an impairment in the patient. Lamins, the nuclear IFs, are when mutated involved in several human diseases. Such diseases are called laminopathies. The majority of lamin disease mutations have been reported to cause muscular dystrophies. One lamin mutation in particular, p.S143P in lamin A/C, has been linked to dilated cardiomyopathy (DCM) in the Finnish population. The symptoms typically emerge in the 40's and include conduction defects, arrhythmias and progressive heart failure. We are investigating the disease caused by the p.S143P lamin A/C mutation by studying patient fibroblasts, induced pluripotent stem cells (iPSCs) differentiated into cardiomyocytes and a transgenic *Drosophila* model. We could demonstrate that the lamina, which is the protein meshwork beneath the nuclear membrane made up by the lamins, is altered following introduction of the p.S143P mutant lamin in all of the three systems used in this study. We determined that the mutated lamins did not integrate into the lamina, but were instead found in the nucleoplasm and eventually caused endoplasmic reticulum (ER) stress response. The other phenotype that we could detect was pronounced lamin aggregation. To investigate the disease mutation *in vivo*, we expressed the p.E158P mutant *Drosophila* lamin C gene, mimicking the human p.S143P lamin A/C mutation, in a wild type (WT) *Drosophila* background. The mutation was lethal when expressed ubiquitously in the fly. However, when the mutation was expressed specifically in the salivary glands, we could detect large nuclear lamin aggregates, mimicking what was observed in the mutated human cells. Remarkably, when we performed a lethal phase analysis on the flies ubiquitously expressing the lamin mutant, we observed that the lethality occurred in the larval phase, but after a period multiple times longer than the normal larval phase. We can conclude that the mutation prevents the induction of the pupal phase. How this phenotype is connected to the human disease progression is a subject of future study.

Taken together, this thesis takes several investigative approaches to ultimately provide information about the role of the IF protein family in humans. The IF proteins are fundamental and integrated components in the eumetazoan cellular machinery, which is why basic research into their functions also increases our understanding of general cellular processes. Furthermore, the link between IFs and

disease illustrates the immediate need for applied IF research which this thesis also addresses by investigating a specific IF disease mutation.

Review of the Literature

1. The intermediate filament networks

The intermediate filament (IF) network (Figure 1) is an integrated cellular structure that provides the cell with dynamic rigidity. The IF network constitutes the end product of the IF assembly process. The network's properties vary with its subunit composition, which is decided by the individual IF's expression pattern. The IF network properties can be influenced e.g. by phosphorylation of the IF subunits, which affects their assembly.

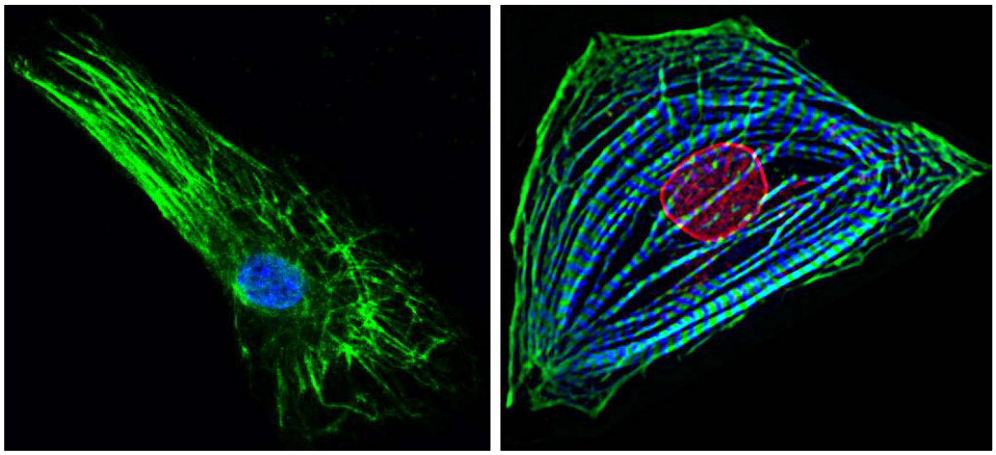

Figure 1. Cytoplasmic and nuclear IFs. *Left: Mouse myoblast where a cytoplasmic IF, vimentin, has been fluorescently labeled and is seen in green. DNA has been labeled with DAPI and is seen in blue. Courtesy of Elin Torvaldson. Right: Human cardiomyocyte where a nuclear IF, lamin A, has been fluorescently labeled and is seen in red. Myosin binding protein cardiac 3 (MyBPC3) is seen in blue and actin is seen in green.*

1.1 The general structure of IF proteins

The IF proteins have only been partially crystallized and thus, we have incomplete knowledge of their molecular structure (Chernyatina et al., 2015). What we do know from crystallization and modelling still gives us a good idea about the protein structure (Figure 2). The universal structure of the IFs is comprised of an N-terminal head domain, a rod domain and a C-terminal tail domain. The domains are separated by linker regions. The nuclear IFs, the lamins, differ from the cytoplasmic IFs (cytoIF) in having a nuclear localization signal (NLS) in the tail domain as well as a C-terminal CaaX motif, which is responsible for integration in the nuclear membrane.

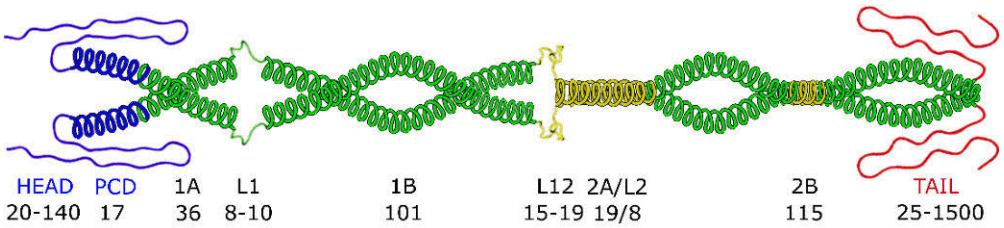

Figure 2. The IF protein structure and the coiled coil dimer*. The IF is made up by an N-terminal head domain, a central rod domain and a C-terminal tail domain. The rod domain segments are separated by short linker regions. The rod domain is alpha-helical and drives coiled coil dimer formation. Below the dimer is indicated the varying length in amino acids (AAs) of the different domains dependent on IF type. The model in this image depicts a vimentin coiled coil dimer, which, apart from the domains shared among all IFs, also has a pre-coil domain (PCD).*

The rod domain is the most conserved part of the IF structure whereas the head and tail domains are subject to most variation between different IFs (Chang and Goldman, 2004). The rod consists of three alpha-helical segments connected to each other by linker regions. The alpha-helical secondary structure is created by the heptad and hendecad repeats in the primary protein structure. The particular heptad and hendecad repeat alpha-helices allow coiled coil formation at the dimer stage. The heptad repeat is *hxxhcxc*, where *h* represents a hydrophilic amino acid (AA) residue and *c* represents a charged AA residue. The heptad repeat AA residues are named *abcdefg* (Figure 3). In the helix, one turn corresponds to 3.6 residues, where the hydrophobic position *a* and *d* will be located at the side of the helix. This promotes "zipping" of two helices at their common hydrophobic sides, creating the coiled coil. The alpha-helix structure was solved by Linus Pauling in 1951 (Pauling et al., 1951) and keratin was proposed to form coiled coils in 1952 by Pauling and Francis Crick (Crick, 1952; Pauling and Corey, 1953). The hendecad repeat has similar principle as the heptad, but with an 11-residue sequence, where one hendecad represents three turns. In the *abcdefghijk* hendecad repeat, residue *a*, *d* and *h* is involved in making up the hydrophobic core of a nearly parallel coiled coil. In the IF sequence, a stutter sequence can be found in the third segment of the rod and is a four AA sequence which introduces a discontinuity in the heptad pattern. Depending on where in the heptad repeat the stutter is found, it can induce over-winding or under-winding in the alpha-helix, affecting the structure and assembly (Brown et al., 1996).

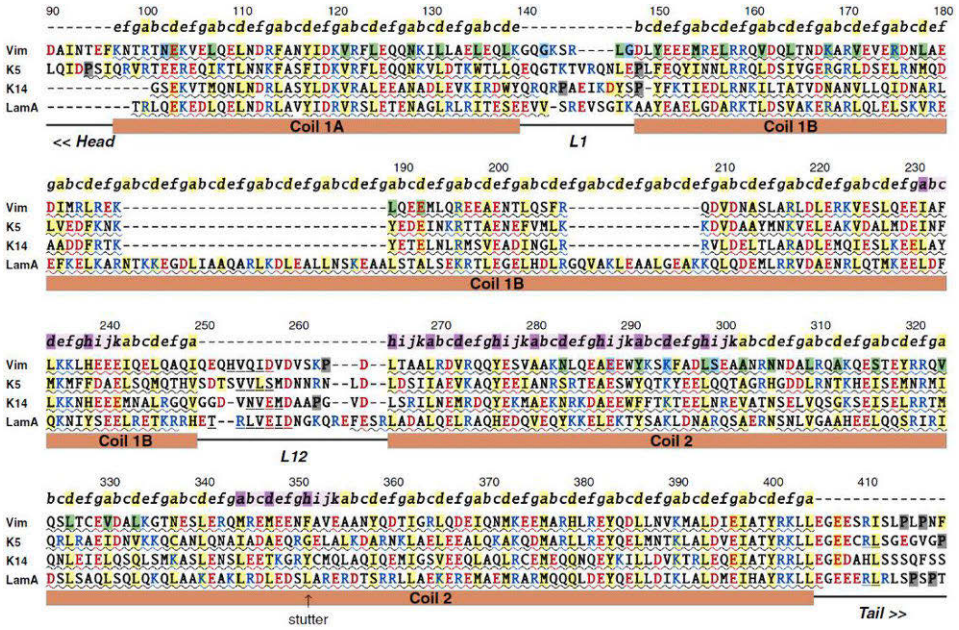

Figure 3. The alpha-helical rod domain (Chernyatina et al., 2015). *Heptad (yellow) and hendecad (purple) repeats create the alpha-helices that allow coiled coil formation. The rod domain is very conserved between different IFs. This image shows amino acid comparison of vimentin, keratins and lamin.*

1.2 The assembly of IF monomers create filaments

The IF networks are made up either by homopolymers or by heteropolymers such as vimentin and desmin (Wickert et al., 2005). The filament, which is the primary building block of the network, is assembled from the IF monomers in a specific sequence into the final product (Figure 4). In contrast to actin and microtubuli, where the polymerization requires ATP, the IF filament is assembled without the need for energy supply. Conclusions about the spontaneous assembly process of the filaments have been made during *in vitro* assembly experiments. The *in vitro* assembly experiments have shown that the filament can assemble spontaneously under physiological pH and salinity (Zackroff, 1980). Also, the tendency of the monomers to incorporate or leave the polymer is regulated by phosphorylation of key residues (Chapter 1.5). The first step of the assembly process is dimerization. The rod domain is primarily responsible for the dimerization. In the second step of the assembly process, the dimers form tetramers. Most investigators subscribe to the theory that the dimers assemble into tetramers in an antiparallel fashion (Chernyatina et al., 2012). The tetramers assemble laterally into unit length filaments (ULFs), which is the third step of filament assembly. In the fourth and last step of assembly, the ULFs anneal end to end to form the complete filament, which is thus a continuous rope-like

structure and can be thousands of ULFs long. The filament also undergoes radial compression, which leaves the IF at a diameter of approximately 10 nm. “Intermediate”, points to the diameter of the IFs, being intermediate in size to that of actin (7 nm) and microtubuli (24 nm). When assembled, the filament is not static, but it has a subunit turnover that allows for very dynamic remodeling, although it is unclear at what level of assembly the subunits are exchanged (Nöding et al., 2014).

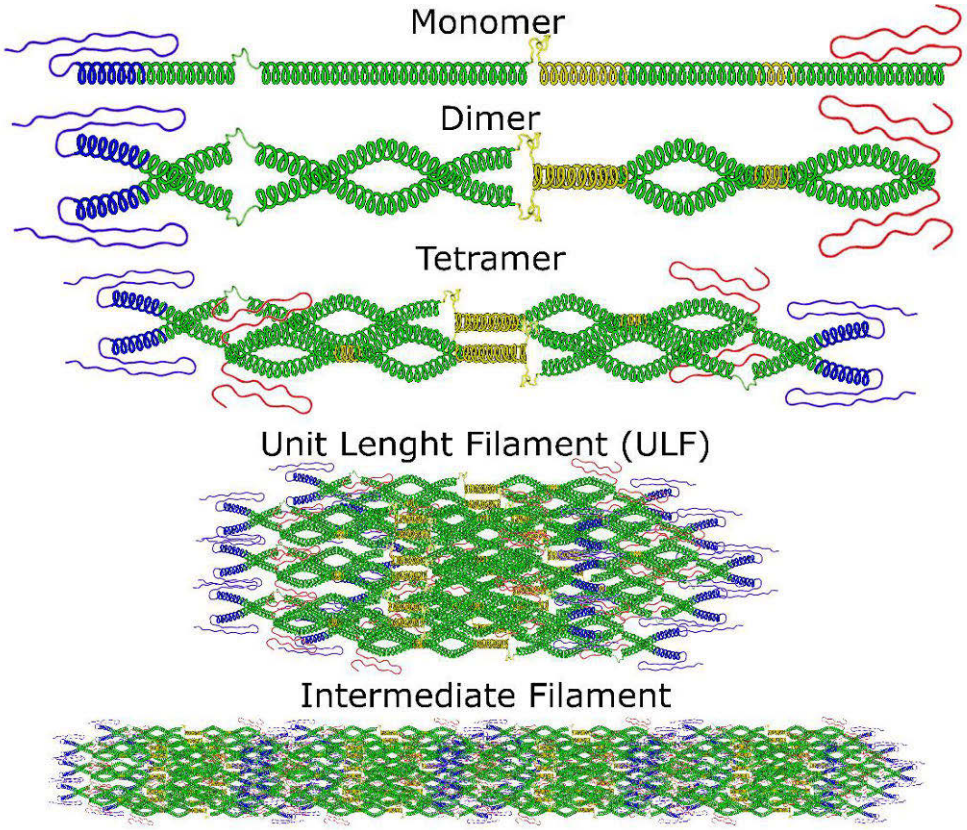

Figure 4. IF assembly*. Two IF monomers form homo- or heterodimers. The dimers then assemble into an antiparallel tetramer, which in turn form ULFs. The ULFs anneal end to end to assemble into the IF.*

Many different IFs can be made to co-assemble *in vitro*, however, whether two or more IF proteins assemble *in vivo* depends on their expression pattern and regulation, such as their phosphorylation status. In fact, because many IFs are dependent on other IFs to polymerize, then called obligate heteropolymers, the IF network composition can be regulated through the expression patterns of individual IFs (Lowery et al., 2015). The clearest example of this regulation is the keratins. The keratins are classified as type I and type II IFs, and each keratin dimer must contain compatible pairs from each group. Many keratins are only expressed in certain tissues, while

others might be expressed in several. Because the same keratin can have different partners in different tissues, this allows for increased fine tuning of the IF network properties, and a larger number of IF network compositions (Nöding et al., 2014). Since some IFs can have multiple partners, loss of a particular IF can be compensated by another. This is exemplified by the keratin 14 knock out mouse, where keratin 15 compensates for keratin 14 loss by forming filaments with keratin 5 (Lloyd et al., 1995). Knock out of keratin 5, on the other hand, is lethal (Peters et al., 2001). Astrocytes may express a combination of GFAP, vimentin and synemin (Jing et al., 2007). When GFAP and vimentin is expressed in unison, they form heteropolymers (Figure 5).

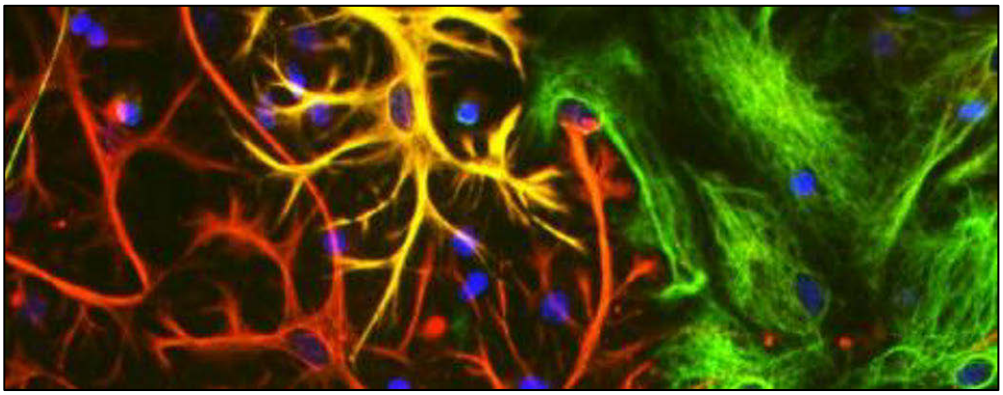

Figure 5. Co-assembly of different IFs *(EnCor Biotechnology Inc., 2013). Fibroblasts express vimentin only (green). Astrocytes express either GFAP only (red) or GFAP and vimentin. In the astrocytes that express both IFs, GFAP and vimentin form heteropolymers (yellow). 20x confocal projection.*

1.3 The interactions between IFs and their binding partners organizes the IF network

The IF is a long protein strand made up by several ULFs annealed end-to-end. The IF network, on the other hand, is an entangled, branched, highly dynamic structure that spans the whole or part of the cell. The unfolding and the complex organization of the IF network is made possible by its binding partners.

1.3.1 The IFs and their binding partners constitute a continuous structure from cell membrane to chromatin

Representatives of the IF collective is found throughout the cell in most cell types, since the cytoIFs are expressed in the cytoplasm and the lamins are found in the nuclear compartment of all cells. The cytoIFs have binding partners at the plasma

membrane as well as at the outer nuclear membrane. The lamins bind to the same protein complex on the nuclear face of the nuclear membrane as the cytoIFs bind on the cytoplasmic face of the nuclear membrane (Herrmann et al., 2007). This means that the IF networks, together with linker proteins, collectively form a structure, that is continuous throughout cell (Figure 6).

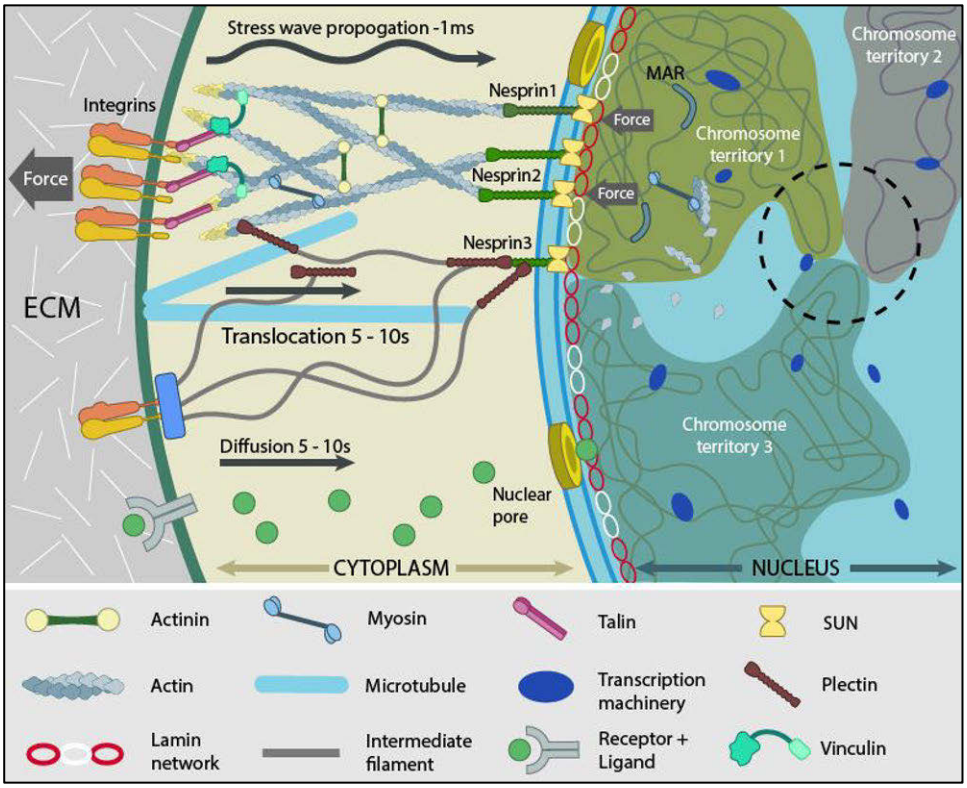

Figure 6. IFs from plasma membrane to chromatin and vice versa *(MBInfo, 2014). Through linker proteins, the cytoplasmic IF network connects to the plasma membrane and the nuclear membrane, where it is linked to the nuclear IFs, which in turn are connected to chromatin.*

1.3.2 IF binding partners in the cytoplasm

The plakins are a family of proteins with binding domains for cytoskeletal elements. The plectins are part of the plakin family and have binding sites for all three principal cytoskeletal proteins; actin, IFs and microtubuli. Plectins (and plakins) are huge proteins, containing more than 4,000 AAs. Plectins are conserved throughout the eumetazoans, however, in *Drosophila*, the plectin homolog, kakapo, has no IF binding site (Gregory and Brown, 1998). There are several plectin isoforms binding different IFs in different parts of the cytoplasm (Figure 7). The plectin structure includes an

actin binding domain (ABD), and a plakin domain which includes a SH3 domain. Plectin also has an alpha-helical rod domain promoting dimerization of the protein. The plectin C-terminal domain is made up by 6 plectin repeat domains (PRD), one of which being an IF binding domain (IFBD). Plectin 1 anchors IFs to the nuclear envelope (NE). Plectin 1a anchors IF to hemidesmomes, 1b connects IFs to mitochondria, and 1c connects IFs and microtubuli. Plectin 1d anchors IFs to z-discs. Plectin 1f connects IFs to neuromuscular junctions (NMJ), but can also associate with costameres as well as with podosomes and invadopodia, as can plectin 1K. Plectin 1f further associates with IFs and focal adhesions. The variable N-terminal domain of plectin is primarily responsible for subcellular location of the protein as well as context-dependent specific IF-binding (Figure 7) (Wiche et al., 2015). Other than plectins, there are other members of the plakin family that bind IFs. Such proteins are desmoplakins, that bind keratins to desmosomes and epiplakin which could be working as a reinforcer of the network as it preferentially binds to assembled keratins (Robert et al., 2016).

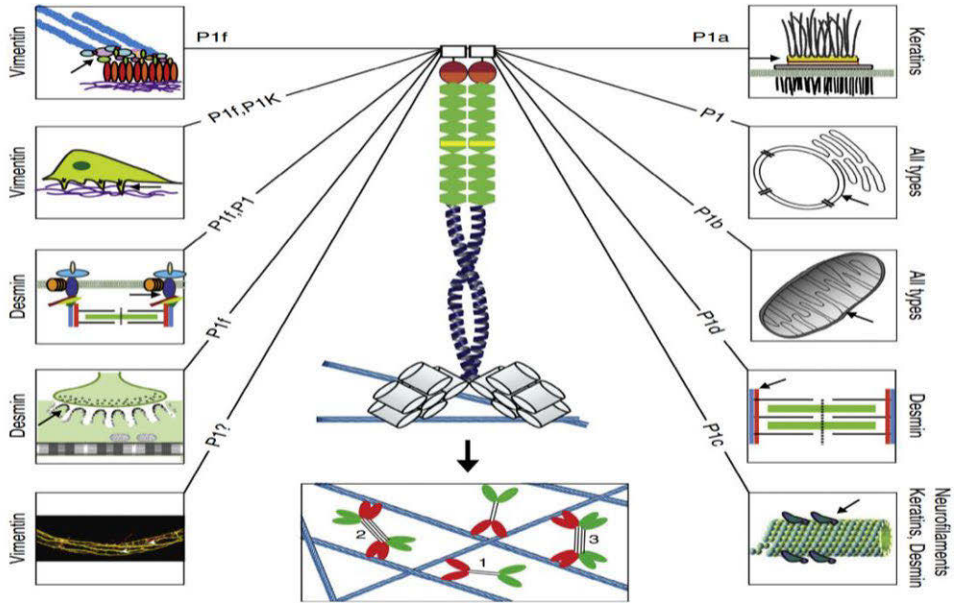

Figure 7. The plectins (Wiche et al., 2015). *Different plectin isoforms link different IFs to different components in the cytoplasm. Plectin 1a binds keratins at the desmosomes, plectin 1 anchors IFs at the nuclear membrane and plectin 1b links IF to mitochondria. Plectin 1d links desmin to z-discs, plectin 1c connects IFs to microtubuli and plectin 1f anchors IFs to focal adhesions. Plectin 1f also anchors IFs at podosomes, costameres and NMJs. Plectin 1K can also bind podosomes. The IF dimer is depicted in the center of the image. The plectin structure contains an ABD (red), plakin domain (green), SH3 domain (yellow), coiled coil (black), and at the C-terminus, 6 PRDs (white cylinders). One of the PRDs has an intermediate filament binding domain (IFBD).*

1.3.3 IF binding partners in the nucleus

The linker of the nucleoskeleton and cytoskeleton (LINC) complex, include several proteins that bind to lamins on the nuclear face of the inner nuclear membrane (INM). Through two trans-nuclear membrane protein, these linkers also bind to cytoskeletal components on the cytoskeletal face of the outer nuclear membrane (ONM) (Crisp et al., 2006). The two trans-nuclear membrane proteins of the LINC complex, the KASH domain protein nesprin and the Sad1-Unc84 (SUN) domain protein bind each other at the peri-nuclear space (PNS). While nesprins binds to cytoskeletal proteins on the cytoskeletal face of the ONM, SUN domain proteins bind lamins on the nuclear face of the INM. This is one of the anchoring points of the lamina (Figure 8).

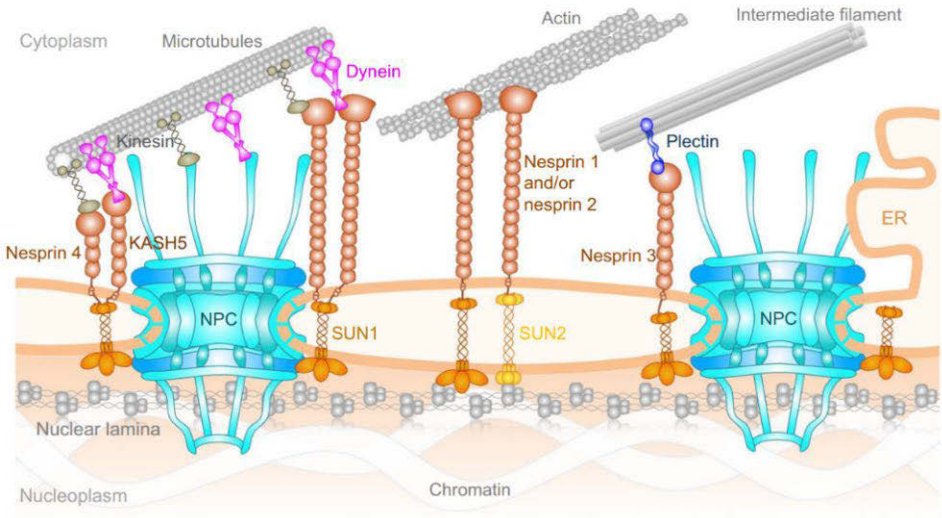

Figure 8. The LINC complex (Jahed et al., 2016)***.*** *The KASH domain proteins such as nesprins bind to the cytoskeleton on the cytoplasmic face of the ONM. SUN domain proteins bind lamin on the nuclear face of the INM. The KASH and SUN proteins bind each other in the PNS and the two proteins in turn bind cytoskeletal elements on their respective side of the nuclear membrane.*

The LEM domain proteins include several proteins that share the ability to bind Barrier-to-Autointegration Factor (BAF), which is a histone-binding and sequence-independent DNA-binding protein. The LEM domain proteins include, among others, LAP2, emerin and MAN1, from the combination of which the domain has its name. The LEM domain protein family has several members that associate with the nuclear membrane, and some of them bind lamins directly (Barton et al., 2015) (Figure 9). The lamin associated polypeptide 2, LAP2, is a protein superfamily of LEM domain proteins that bind chromatin and lamin (Foisner and Gerace, 1993; Senior and Larry,

1988). LAP2's bind BAF-DNA-complexes at a higher affinity than BAF alone (Shumaker et al., 2001). The two major LAP2 isoforms are LAP2α and LAP2β. LAP2α binds A-type lamins and is found throughout the nucleoplasm. LAP2β, on the other hand, binds lamin B1 and is tethered to the INM. LAP2α and -β most likely participates in the reorganization of the nuclear membrane and chromatin after mitosis. During mitosis, LAP2α distribution is changed from the nuclear matrix to the centrosome (Salina et al., 2002). The phosphorylation status of LAP2β varies with the assembly state of the nuclear membrane (Dreger et al., 1999).

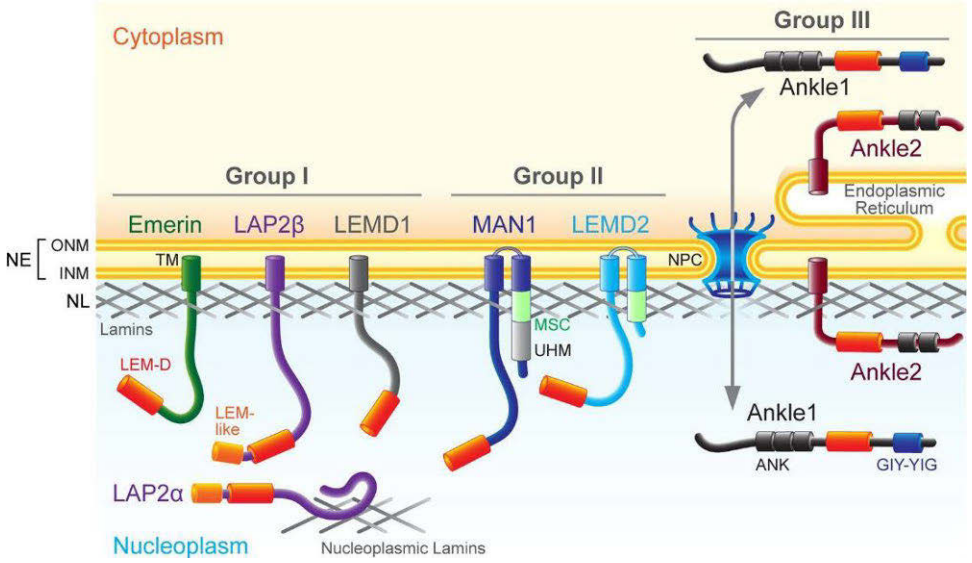

Figure 9. The LEM domain proteins (Barton et al., 2015) *The LEM domain proteins all carry the ability to bind BAF which in turn can bind histones and DNA independent of sequence. Group I of the LEM domain proteins include the LAP2 proteins, emerin and LEMD1. Group II include LEM domain proteins that also carry a MAN1/Src1p/C-terminal motif (MSC) that directly bind DNA. Group III contain LEM domain proteins, which can also be found on the cytoplasmic face of the ONM.*

The lamin B receptor (LBR), as the name suggests, binds lamin B (Schuler et al., 1994; Worman et al., 1988). LBR is embedded in the INM with its eight-time transmembrane segments. LBR has a Tudor domain, which binds to a methylated lysine residue on H4. LBR also has a basic HP1 binding domain through which chromatin can be bound (Figure 10).

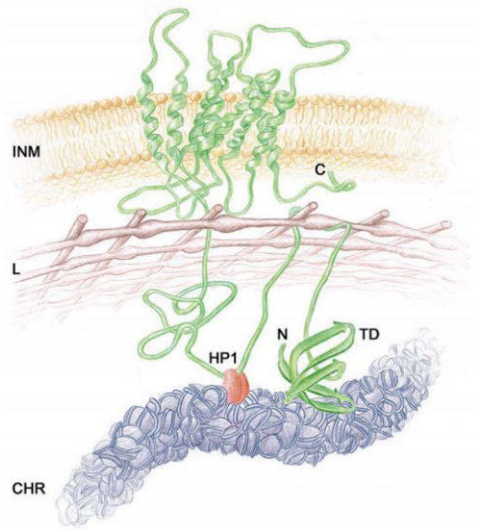

Figure 10. The lamin B receptor (LBR)(Olins et al., 2010)**.** *Graphical representation of LBR (green) interactions. The C-terminal (C) end is embedded in the INM through eight transmembrane segments. LBR binds and protrudes through the lamin B protein meshwork. LBR binds HP1 and has direct interaction with H4 via its tudor domain (TD) on its N-terminal end (N). In this way LBR can anchor both chromatin (CHR) and the lamina (L) at the INM.*

1.4 The phosphorylation of IFs regulates the assembly

IFs have been shown to be substrates for kinases and have several phosphosites (Pant et al., 1978; Sun and Green, 1978). The phosphorylation can dramatically affect the assembly properties of the subunits (Chou et al., 1989). Since the assembly of IFs happens spontaneously, phosphorylation is one of the major regulators of the IF network, as it can affect the tendency for IF monomers to incorporate into the filament. In general, phosphorylation favors disassembly and dephosphorylation conversely favors assembly (Figure 11). The phosphorylation of IFs has several functions. One of the reasons for IF phosphorylation is simply to manage remodeling of the IF network. The remodeling of the IF network favors the ability of the cell either to move itself, change its shape, relocate or rebuild organelles, or to break down cellular structures altogether (Omary et al., 2006). Since many cellular components are linked to IFs through linker proteins, the disassembly of the network releases the chain on many organelles as well as from other cytoskeletal polymers. Following IFs reassembly, on the other hand, the components can be chained to the IF network in their new position. When phosphorylation induces IF disassembly in one location, the IF subunit turnover is increased, allowing for increased motility of the cell in that location (Mendez et al., 2010). For lamins, dramatic phosphorylation of the entire lamina is coupled to a specific event in the cell, namely mitosis and the breakdown of the nuclear envelope (Ottaviano and Gerace, 1985).

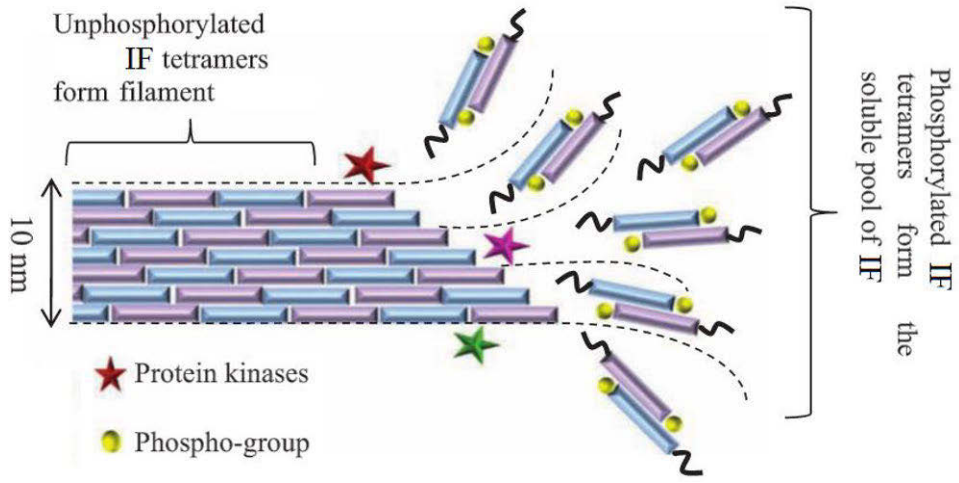

Figure 11. Phosphorylation-regulated IF assembly (Dave and Bayless, 2014)**.** *Phosphorylation mediated by kinases cause IF subunits to lose assembly preference and becomes soluble. Dephosphorylation by phosphatases cause IF subunits to gain assembly preference and integrate into the insoluble filament.*

1.5 The IFs are classified per expression pattern and structure

The IFs have been classified into six classes or types based on their structure and expression pattern (Table 1) (interfil.org)(Schweizer et al., 2006). The type I and II IFs are the keratins. Keratins assemble as heteropolymers and the classification is designed so that keratins of type I assemble with keratins of type II. The keratins can be subdivided according to their expression patterns. The three major groups are simple keratins, expressed in one-layered epithelia, barrier keratins, expressed in stratified epithelia, and hard keratins, the keratins of hair and nails. Members of the type III IFs have various expression patterns. Vimentin for example, is expressed in fibroblasts and in several other mesenchymal tissues, while desmin is expressed in muscle. Type IV are the neural IFs, however, nestin is also expressed in stem cells. A common factor for the type IV IFs is the long tail domain. Type V IFs are the nuclear IFs, the lamins. Type VI IFs include the two unconventional IFs, filensin and phakinin that are both expressed in the lens.

Table 1. Classes of human IFs *(Interfil.org). List if human IF classes and examples of IFs from each type. The IFs have been divided into six classes based on their structure and expression pattern. Examples of diseases associated with the specific IFs is also listed.*

CLASS	Genes	Proteins	Example of IF Proteins	Example of associated disease	MW (kDa)	Tissue Distribution
TYPE I	28	28	Acidic keratins	Pancreatitis, liver disease, skin fragility	40 – 57	Epithelia
TYPE II	26	26	Basic keratins		53 – 67	Epithelia
TYPE III	5	5	Vimentin	Cataract, neuropathy	57	Mesenchyme
			Desmin	Myopathy	53	Muscle
			GFAP	Neurodegenerative disease	50	Glial cells and astrocytes
			Peripherin	Neurodegenerative disease	57	Peripheral and central neurons
TYPE IV	7	7	NF-L	Neuropathy	62	Mature neurons
			Nestin	Cancer	177	Nerve and muscle stem cells
TYPE V	3	6	Lamin A	Progeria, lipodystrophy, muscular dystrophy	70	Nucleus of all cells
			Lamin C		67	Nucleus of all cells
TYPE VI	2	2	Filensin	Cataract	83	Lens fiber cells

2. The cytoplasmic intermediate filaments

The cytoIFs are expressed in the cytoplasm as an integrated part of the cytoskeleton. There are 70 cytoIF genes in humans, more than half of which encode keratins. In this thesis, two cytoIFs in particular, vimentin and nestin, are focused on.

2.1 Vimentin

Vimentin is a cytoIF that in human tissue is expressed in cells of mesenchymal origin such as fibroblasts and endothelial cells, as well as in macrophages (Korita et al., 2010). Vimentin is conserved within vertebrates, with representatives of the protein also in fish (Herrmann et al., 1996). In fish, however, fibroblasts do not express vimentin. Vimentin is able to form homopolymers (Chang and Goldman, 2004; Strelkov et al., 2003) but also heteropolymers with several other IFs, e.g. desmin, (Quinlan and Franke, 1982) GFAP and nestin (Eliasson et al., 1999) in a number of tissues. One of the clearest roles of vimentin is maintaining endothelial tissue integrity. Vimentin is expressed in endothelial cells in blood vessels and binds desmosomes, whereas vimentin removal results in leaky vessels, as seen in vimentin knock out mice (Nieminen et al., 2006). Another key role for vimentin is in cellular motility. Lymphocyte migration and fibroblast movement are mechanisms that have been shown to be disturbed by vimentin deletion and this has turned out to have consequences for processes like wound healing (Cheng et al., 2016). The association of vimentin with cancer has largely been coupled with its role in motility, as vimentin is a central protein in the EMT-MET conversion which shuttles cells between modes of higher and lower motility (Ivaska, 2011).

2.2 Nestin

Nestin was first shown to be expressed in neuroepithelial stem cells, from which the protein has its name (Lendahl et al., 1990). The protein structure of nestin differs from most other cytoIFs because of its very long tail domain, 1307 AAs. Nestin cannot form homodimers, instead, nestin forms dimers with other cytoIFs. The most frequently described assembly partners of nestin are vimentin (Eliasson et al., 1999) , and desmin (Sjöberg et al., 1994). GFAP is also described to polymerize with nestin *in vitro*, however, this observation is yet to be made *in vivo*. The co-assembly of nestin and vimentin happens at a ratio of 1:4 (Steinert et al., 1999). The expression of nestin and its incorporation into an IF network seems to introduce modulation of the properties of the host network. It is unclear what type of modulation nestin provides to the collective IF network, however, but there are some clues. In proliferating cells, nestin expression can promote vimentin disassembly into soluble subunits, suggesting

that introduction of nestin transforms the IF network in a way that it is ready for disassembly. This could be an important feature of nestin as it is expressed in many rapidly proliferating progenitor cells as well as in cancer stem cells (Neradil and Veselska, 2015). Upon differentiation, nestin expression is downregulated.

When type IV IFs assemble with other IFs, as they are obligate heteropolymers, the long tail domain has been shown to protrude radially from the filament core (Hisanaga and Hirokawa, 1988). The tail domain of nestin contains sites for post-translational modification. The type IV neurofilaments, to which nestin belongs, have been shown to have special assembly characteristics. So far, nestin has been shown to be phosphorylated, ubiquitinated and glycosylated and many of the described modifications take place on the tail (Grigelionienė et al., 1996; Sahlgren et al., 2006).

Cyclin dependent protein kinase 5 (CDK5) has been shown to phosphorylate nestin at Thr316, Thr1495 and Ser1837 (Contreras-Vallejos et al., 2014; Sahlgren et al., 2006). While these sites are phosphorylated by other CDKs during mitosis and CDK5 is not involved in cell cycle regulation, it has been suggested that CDK5 phosphorylates nestin during interphase. CDK5 is activated by p35. The cleavage product of p35, p25, has been shown to be reminiscent of cyclin A (Tarricone et al., 2001). The cleavage of p35 into p25 is mediated by calpains and has been described as a CDK5 activating event. Calpains are calcium-dependent cysteine proteases that perform their cleavage activity in cellular events such as apoptosis, motility and differentiation (Goll et al., 2003). Except the obvious cyclin-like appearance of p25, CDK5 activation is also enhanced by p35 cleavage, since p25 has a half-life 2-3 times longer than p35.

3. The nuclear intermediate filaments; the lamins

The principal structure that the lamin proteins constitute is known as the nuclear lamina. The lamina is a continuous protein meshwork layer that encompasses the chromatin and is located beneath the INM in the nuclear periphery (Figure 12). Linker proteins connect the lamina to the INM and associate it to chromatin.

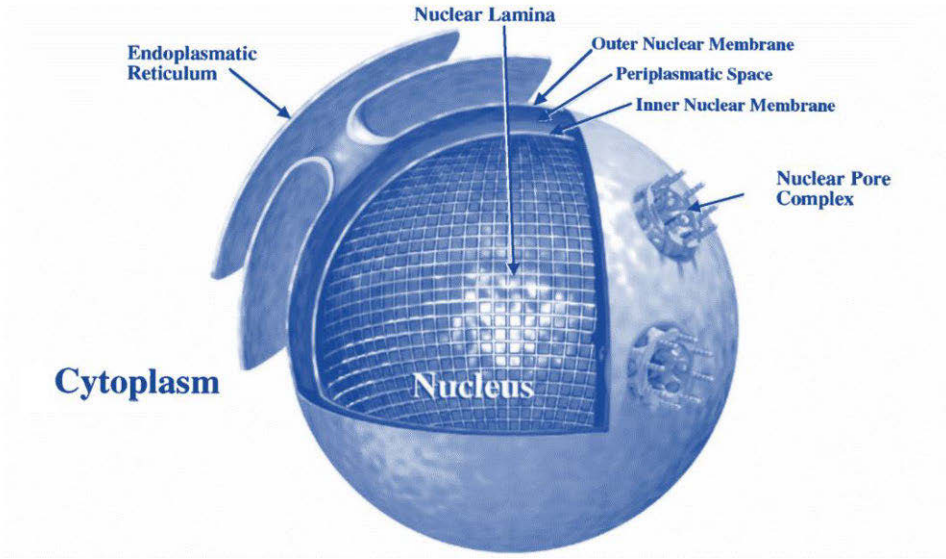

Figure 12. The lamina (Stuurman et al., 1998). *The nuclear lamina is made up by a proteinaceous meshwork of lamin filaments. Through linker proteins, the lamina is anchored to INM and cytoskeletal components. Other linkers tie the lamina to chromatin. There is also a nucleoplasmic pool of soluble lamins that interact with chromatin in the nuclear interior.*

In humans, there are three lamin genes, *LMNA*, *LMNB1* and *LMNB2*. *LMNA* encodes the A-type lamins: lamin A and lamin C. *LMNB1* and *LMNB2* encode lamin B1 and lamin B2, respectively, which are collectively known as the B-type lamins. There are additionally three minor lamin proteins encoded by the lamin genes; AΔ10, C2 and B3. Lamin A differs from the B-type lamins in that it has a much longer tail. In the tail domain, lamins A, B1 and B2 have a CaaX-box, which is post-translationally farnesylated and proteolytically cleaved in the mature lamin A. The CaaX-box, which mediated nuclear membrane anchorage, is retained in the mature B-type lamins (Dobrzynska et al., 2016). Overall, the general lamin protein structure is very similar to cytoIFs. The most obvious difference between cytoIFs and lamin, apart from cytoIFs missing the NLS and CaaX-box, is that the linker regions in coil 1 in the rod

domains, are also alpha-helical. The coil 1 is also 6 heptad repeats longer than in most cytoIFs.

Lamin assembly is slightly different from the assembly of cytoIFs. In both cases, parallel coiled coil dimers are formed, which are the principal building block of all IFs, but the tetramerization is significantly different between lamins and cytoIFs. Whereas cytoIFs form antiparallel tetramers, lamin dimers anneal head to tail forming instead a polymer of consecutive dimers. Several of these head-to-tail polymers then anneal laterally to form the lamin filament fiber (Davidson and Lammerding, 2013). Recently, the first high resolution cryo-electron tomography of the lamin meshwork has revealed, that the lamin filaments are in fact 3.5 nm in size (Turgay et al., 2017). This contrasts with the traditional 11 nm fiber size and suggests that lamins undergo a different radial compaction than cytoIFs. The A and B type lamins make up two separate meshworks (Shimi et al., 2008). These meshworks have different properties and they interact with each other, demonstrated by the fact that silencing lamin B1 leads to larger mesh size in the lamin A/C meshwork.

The lamina is recognized as a supporting structure providing the nucleus with dynamic stability. The lamina depolymerizes during mitosis along with the breakdown of the nuclear envelope and is subsequently reassembled after mitosis. The lamins have also been shown to interact with chromatin (Bickmore and van Steensel, 2013) which is why the influence of the lamin protein family on the cellular physiology is larger than that of most proteins.

4. The evolution of the intermediate filament proteins

It has been shown that all studied metazoans, multicellular eukaryotic organisms, express lamins (Fuchs and Weber, 1994). Also, sequence comparisons show, that lamins are precursors to the cytoIFs (Blumenberg, 1989). Attempts have also been made to identify IF-like proteins in unicellular organisms. The most archaic organism that has been shown to express IF-like proteins is the unicellular eukaryotic *Dictyostelium*, whose lamin-like protein is known as NE81 (Krüger et al., 2012). Furthermore, crescentin, a bacterial protein of *Caulobacter crescenti*, which can self-assemble and promotes the shape of the bacterial cell, is suggested to be an IF (Ausmees et al., 2003).

Among the eumetazoans, organisms that display true tissue organization, cytoIFs are consistently prevalent with the arthropod phyla as the only exception (Bartnik and Weber, 1989). The fact that the IFs are so conserved among multicellular eukaryotes is indicative of their crucial functionality. Since there are precursors to the arthropods that express cytoIFs, it means that the arthropods have lost their cytoIFs during evolution. Recent studies have shown that, in fact, after the disappearance of the cytoIFs from the arthropod lineage, some arthropods have regained cytoIF expression through novel lamin gene duplication and divergence (Hering et al., 2016). The lamin gene has a nuclear localization signal (NLS) that governs its localization to the nuclear compartment. The inactivation of the functionality of the NLS sequence is the first step in creating a cytoIF from a lamin following gene duplication and this has occurred several times in different lineages during evolution (Hering et al., 2016).

4.1 Epithelia and IFs emerge

Since cytoIF expression is closely associated with eumetazoans, it is conceivable that true tissue architecture has evolved in parallel with the IFs. In mammals, over 70 % of all IF genes are those of keratins, governing epithelial integrity. The tissue separating barriers, the epithelia, are instrumental in the evolution of metazoan tissue (Tyler, 2003). The epithelium allowed early metazoan organisms to define an inner space, where digestion and nutrient management could take place. The development of the internal cavity is made possible by the emergence of septate junctions, sealing the paracellular passage (Ganot et al., 2015).

4.2 The adaptation of the IF domains

Regardless on which IF's protein structure we look at, their common feature, the rod domain, is very similar. The rod domain is what defines the IFs. It allows for dimerization by the alpha-helical coiled coil, which is the first step of the network assembly. There are many other proteins that form coiled coil dimers, such as plectin and tropomyosin, and it often has the function of a spacer or "rod". The IFs are special in the sense that their rod is used as a building block, with adaptable head and tail domains, which have specific interaction with regulatory proteins (Peter and Stick, 2015). Depending on a specific IF protein's function, the head and tail domains have been adapted to best accommodate for the specific needs of the particular IF type, which is reflected in the size of the domains (Figure 13).

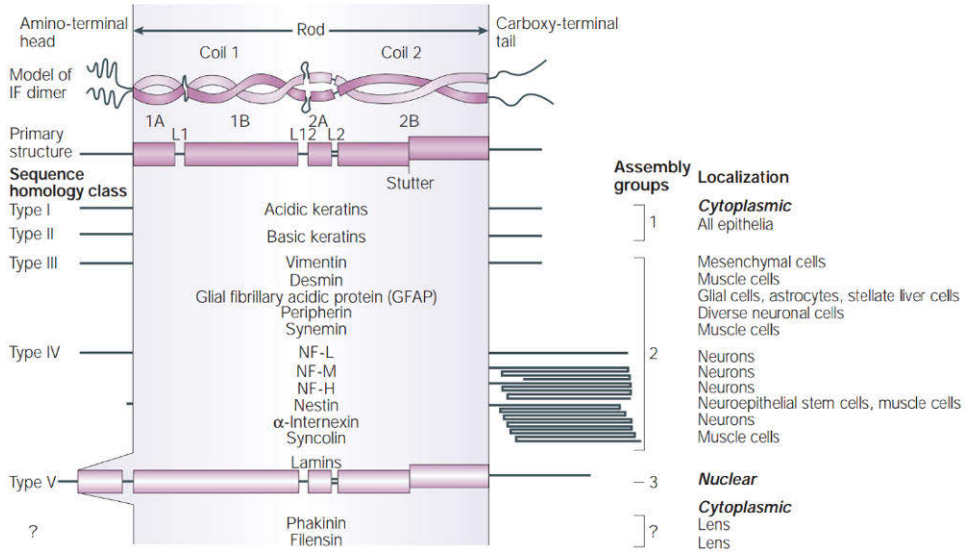

Figure 13. Conservation of the rod domain and variation of the tail domain (Chang and Goldman, 2004)**.** *The alpha-helical rod domain is the defining domain of the IFs. The tail is subject to a great deal of variation, in particular in the type IV IFs, which have a very long tail.*

5. The function of IFs

One of the aims of this thesis is to investigate the common function of the intermediate filaments. To find a universal IF function by looking at the over 70 individual IFs expressed in mammalians is difficult. During evolution, IFs have adapted to perform many distinct functions in cells and tissues. A clear view to a universal IF function is obstructed by a variety of expression patterns and functions. However, the myriad of proteins can be looked at as variation on a theme. Having a wide range of IFs in stock, allows the cell to adjust its properties through its IF expression pattern. In this section, I aim to summarize the most characteristic functions that IFs have been reported to perform.

5.1 The structural support provided by IFs

The assembly capacity together with its subunit exchange, paints the image of the IFs as a dynamic cellular scaffold. However, when it comes to the need for structural support, it can look vastly different in various tissues. One of the comparisons frequently made when it comes to differences in IF expression and function is that between fibroblasts and epithelial cells. In epithelial cells, keratins are prevalent, whereas in fibroblasts, the IFs are instead represented by vimentin. In fact, in epithelial to mesenchymal transition (EMT), which is a differentiation process that cells undergo in embryogenesis, tissue remodeling and in some cancers, keratins and vimentin are routinely used as histological markers for epithelial and mesenchymal tissue, respectively. In the two tissues, depending on the cell type, the different dynamics of the different IFs provide structural support either for a static tissue barrier or for motile soft tissue (Mendez et al., 2010). In both tissues, the IF network is highly dynamic, although it is either rigid or soft. The two examples mentioned belong to perhaps the clearest and most characteristic examples of IF structural function, but many other IF types show variations on this theme. However, finding distinct function for all known IFs in their respective tissue can be an elusive process.

5.1.1 IFs as sensors of the environment and regulation of tissue stiffness as determined by IF amount and type

The integrated IF network, including both nuclear and cytoplasmic IFs, as well as linkers and anchoring proteins, is a continuous structure from the cell membrane to the nucleoplasm. There has been evidence that mechanical forces acting on the cell membrane can be relayed by cell surface proteins such as integrin, via the cytoskeleton and lamins, eliciting a direct transcriptional response in the nucleus (Tajik et al.,

2016). It was also shown that the physical properties of the cell are very much dependent on the nuclear stiffness, which is in turn determined by which lamin type is predominately expressed. There is a correlation between lamin A type and B type protein levels and the tissue and nuclear stiffness. In soft tissues, such as brain and bone marrow, there is low lamin A and C content while there is high lamin B1 and B2 content. The inverse is true for hard tissues like bone and muscle (Swift et al., 2013) (Figure 14). Apart from determining tissue stiffness, the rigidity of the nucleus can also affect mobility. Since the nucleus is the largest cellular organelle, it is also the largest brake on migration, as the nucleus needs to be squeezed through constrictions in the extra-cellular matrix (ECM) (Harada et al., 2014). Low lamin A type levels promote soft and deformable nuclei, which favors migration through the ECM meshwork, however, the deformability comes with a price. The integrity of the nuclear envelope can be compromised at low lamin A levels if there is also mechanical stress on the nucleus, such as a tight squeeze through a constriction. If the nuclear envelope integrity is lost, however, the cell can repair the damage and continue migrating once the nucleus is intact again (Denais et al., 2016).

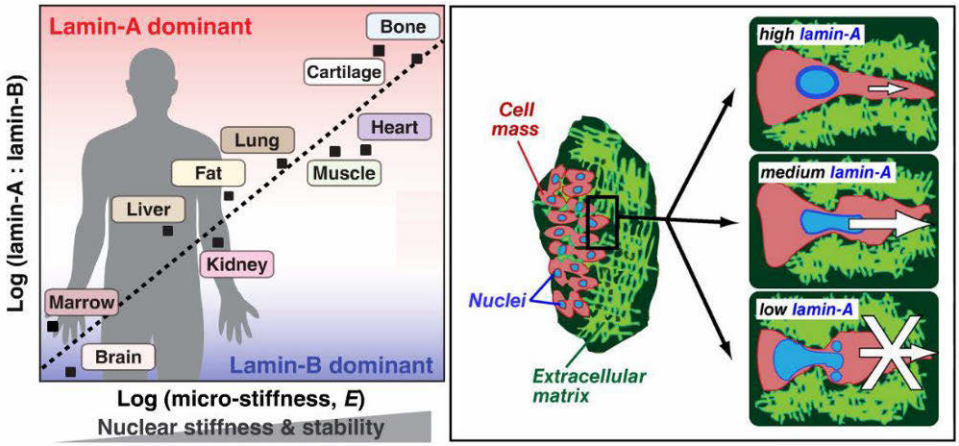

Figure 14. *Gradient of tissue stiffness correlates with lamin levels* (Harada et al., 2014; Swift et al., 2013).

5.2 The role of IFs in tissue barriers

There are two principal types of tissue barriers in metazoan organisms. One type of barrier protects the underlying tissue against the external environment or against an internal space. These are the epidermis and the internal epithelia. The other type of barriers separate solid tissues. The epithelia are one of the main sites of IF expression. The keratins are classified as the epithelial IFs and the keratin family include over 50 distinct proteins (Schweizer et al., 2006). In other words, most of the total number of IF genes and individual proteins in humans, are keratins, statistically speaking. The

epithelial barriers keratins can be divided into three principal categories (Figure 15). The first group is the intestinal simple epithelial keratins, which span the cell and connect to the desmosomes, sealing the tight junctions of the intestinal wall. This seal is important for securing that the nutrient uptake happens only through the villi, but also to prevent bacterial infection by closing access from the lumen to the underlying tissues. The second group is the stratified corneal epithelium keratins. The basal cell connects, except to desmosomes, to the hemidesmosomes at the basal membrane. There are also keratins with antimicrobial function that are released into the fluid from the corneal epithelium. The third group is the keratins of the cornified epithelium of the epidermis. The skin keratins constitute a protective shield in the outermost cornified dead cell layer which sheds continuously with mechanical wear (Geisler and Leube, 2016).

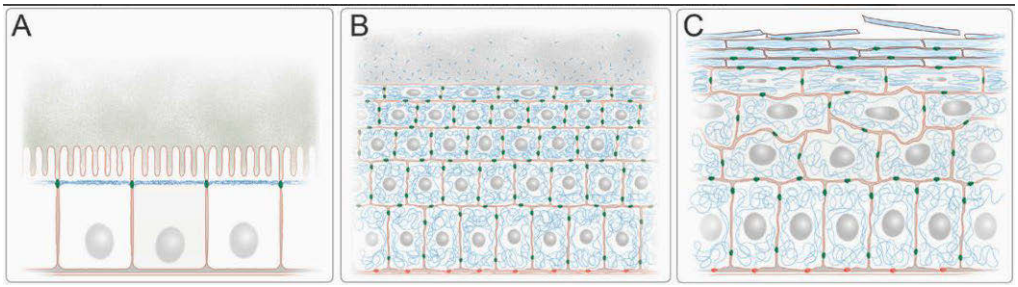

Figure 15. *Keratins in tissue barriers, epithelia* (Geisler and Leube, 2016). *(A) The first type of barrier keratins seal the intercellular passage of the intestinal epithelial cells. (B) The second type of barrier keratins those of the stratified epithelium of the cornea. (C). The third type of barrier keratin is the cornified epithelium of the epidermis. Keratins are illustrated in blue.*

5.3 IFs and motility

IFs have been shown to be involved in the motility of the cell in several ways (Chung et al., 2013). Since cells move by protruding and contracting their cytoplasm, the cell needs to be highly flexible. The migration process involves many integrated events where IFs come together in unison to enable the cellular movement. These events include phosphorylation, disassembly, IF network reorganization, subunit transport and interaction with other cytoskeletal elements. As desmosomes and hemidesmosomes are responsible for maintaining tissue integrity, IF and desmosome disintegration is coupled to promotion of migration (Kitajima, 2013) (Figure 16). Integrin adhesion is also affected by the indirect binding of IFs though plakins in the cytoplasm, and the removal of IFs from the equation can release integrins from the ECM and promote motility. The nuclear architecture is also relevant for motility and the connection of the LINC complex to cytoIFs is important for maintaining this and

enabling motility of the cell. Through these and other mechanisms, the IFs are integrated into the motility process of the cell. IFs are therefore important to processes such as wound healing (Cheng et al., 2016), angiogenesis (Dave and Bayless, 2014) and metastasis (Satelli and Li, 2011).

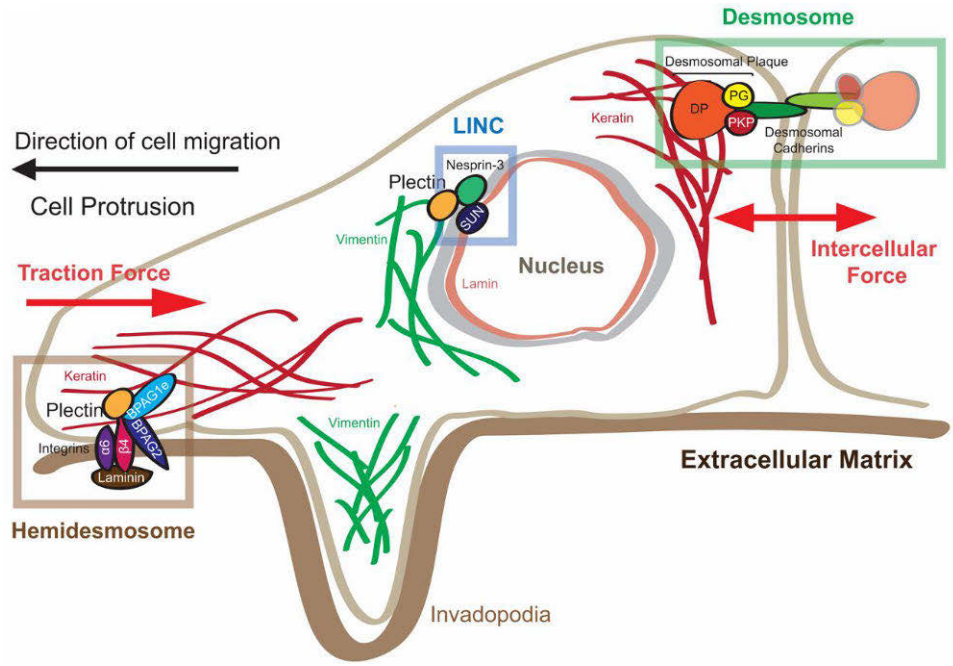

Figure 16 (Chung et al., 2013). *Desmosomes, and in epithelial cells also hemidesmosomes, are responsible for tissue integrity and anchoring of the cell and their dissociation from IFs is required for motility.*

5.4 Lamins in chromatin regulation

Since A- and B- type lamins bind to Lap2α and Lap2β, which in turn interact with chromatin, it is conceivable that lamins are instrumental in reorganizing chromatin after mitosis. Because of the division of chromatin into euchromatin and heterochromatin, there is a spatial correlation between transcriptionally active and inactive genes. Heterochromatin, which contains nongenetic or suppressed genes, tends to be localized to the periphery of the nucleus. The DNA adenine methyltransferase identification (DamID) has been successfully used to determine the association between the lamina and chromatin. This has led to the discovery of roughly 1300 lamin associated domains (LAD) throughout the chromatin. LADs contain mostly transcriptionally inactive genes and cover 35-40% of the whole genome while the other 60-65% of the chromatin is more actively transcribed (Bickmore and van Steensel, 2013). There is a correlation between LADs and the

epigenetic status of chromatin which is incompletely understood. One example of this is that the chromatin within the LADs is enriched with the repressive chromatin markers trimethylation of lysine 27 on histone H3 (H3K27me3) and dimethylation of lysine 9 on histone H3 (H3K9me2), and Lap2α binding to lamin A correlates with these sites. Another study on lamin-chromatin regulation focused on the altered gene expression in cells expressing a lamin mutant (p.E161K) causing DCM, (Mewborn et al., 2010). When profiling the gene expression of the mutant cells, genes on chromosome 13 turned out to be overrepresented among the misexpressed genes. Using fluorescence in situ hybridization (FISH), the authors show displacement of several genes involved in muscle disease. Interestingly, the entire chromosome 13 territory was displaced towards the center of the nucleus. The lamin mutation in this case could disable chromatin binding to transcription complexes at the lamina, and this could be one explanation to the altered gene expression following lamin mutation. How this intricate regulation of chromatin activity and accessibility is moderated by lamins will likely unfold in the future and mechanistically explain the reported changes in gene expression in cells expressing mutant forms of lamin (Dobrzynska et al., 2016).

5.5 IFs provide signal transduction

The mature IF is a highly insoluble structure. The soluble pool of IFs contains unassembled or disassembled subunits. Reports show that the soluble pool of IFs partake in several signal transduction pathways. IFs are phosphorylated by many kinases and while IF phosphorylation affects the assembly status, it also directly affects the size and location of the soluble pool of IFs, which can perform signal transduction. In distal axon damage, Erk1/2 is phosphorylated. Erk1/2 is then bound to soluble vimentin fragments, which is then moved by retrograde microtubuli to the cell body where Erk1/2 is activated. This process is possibly activated by calpain-mediated cleavage of vimentin, which generates the Erk1/2 binding soluble vimentin fragment (Perlson et al., 2005). Since the IFs are present throughout the cytoplasm, their shuttling between their soluble and insoluble form can act as an activation mechanism for their signaling capacity, while their association with microtubuli transport allows for their controlled localization. It is conceivable that more associations between IFs and signaling molecules will emerge in other tissues than neurons as well.

6. The pathogenesis of IF-related diseases

The IF collective is linked to more diseases than any other protein family (Omary, 2009). Since the expression pattern of IFs is so diverse, mutation of different IFs give rise to diseases in many different tissues. Diseases linked to mutation in IFs are collectively called IF-pathies, and diseases specifically caused by mutation in nuclear lamins are called laminopathies. Not only is the range of affected tissues large when it comes to IF-pathies, but the range of phenotypes and symptoms is also diverse (Figure 17).

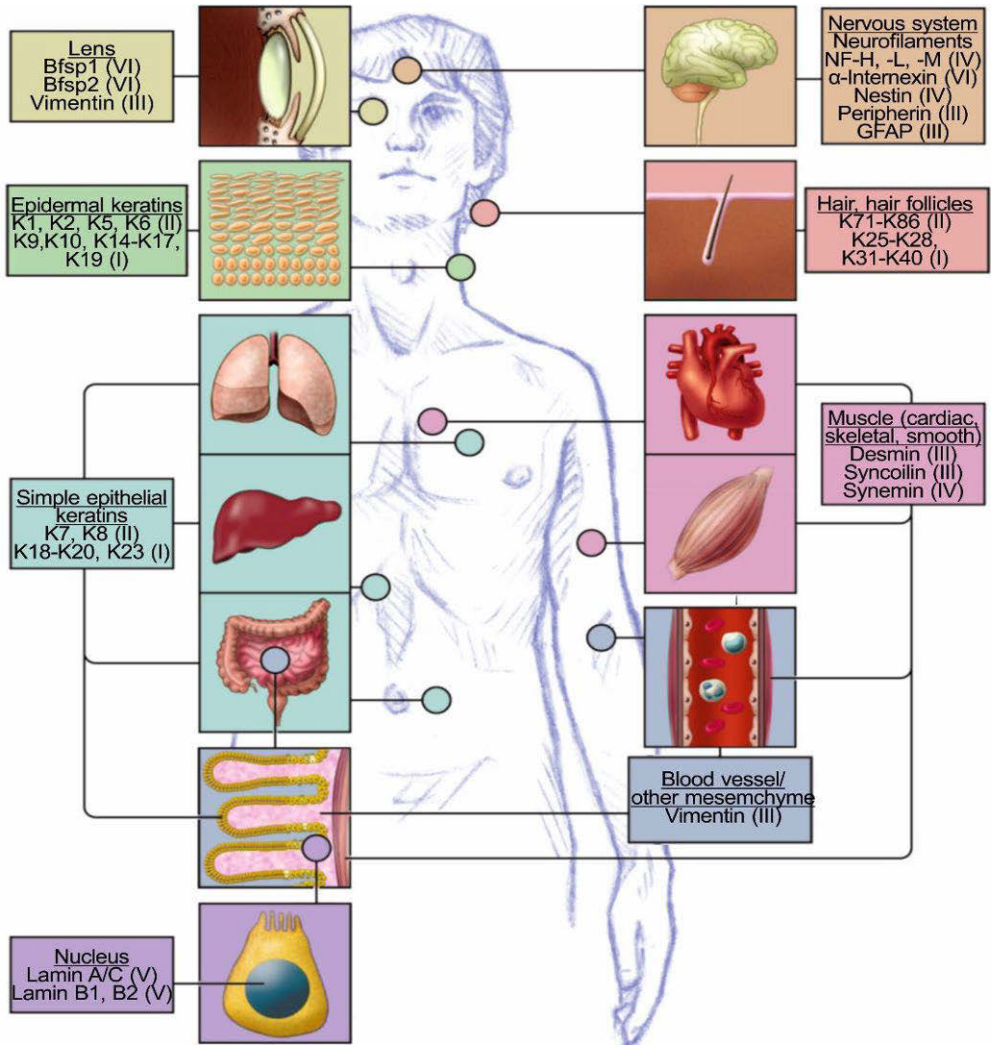

Figure 17. IFs relate to disease in many tissues (Omary, 2009). *An example of different IFs involved with diseases in tissues, illustration how specific IF proteins are expressed in certain tissues and how this relates to which tissues are affected when IFs are involved in disease.*

6.1 Skin disease

Most of the diseases discovered to be related to IFs are those of epithelia and involve mutation in certain keratins. Keratin 14 was the first IF to be implicated in disease progression as its mutation leads to epidermolysis bullosa (EB) (Coulombe et al., 1991). Other keratins have also been shown to cause disease of the skin because of their mutation, while some keratins are coupled to diseases originating in the epithelia of other organs such as cornea (K3 or K12), liver, intestine, pancreas (K8, K18 or K19), and hair (K71, K74, K75).

Most keratin related diseases are those of the skin, and they can be divided into two categories; EB, in which keratin mutation leads to severing of the connection between the epidermis and connective tissue and keratoderma, in which keratin mutation causes hyperkeratosis. There are many subtypes of these categories and EB diseases are related to mutations in K5, K14 or K9, whereas keratoderma disorders are caused by mutations in K1, K2, K5, K6c, K9, K10, K16 (interfil.org). Diseases belonging to these two categories range very much in severity from minor blistering or blushing of the skin to complete disability, because of infection or inability to move normally due to the immobility or pain of the skin (Lane and McLean, 2004). Example of severe EB is epidermolysis bullosa simplex, Dowling-Meara type (EBS-DM). EBS is caused by fragility of the keratinocyte itself because of keratin mutation, which leads to cleavage of the cell and blistering and rupture of the skin following light trauma. EBS-DM patients are constantly threatened by infection because of the disintegration of their skin and this is the most common cause of fatality in patients with this disease (Bolling et al., 2011).

Another example of severe keratoderma disorder is epidermolytic hyperkeratosis, where the suprabasal cell layer of the skin is damaged, which leads to hyperproliferation in the epidermis. The epidermal hyperproliferation in the epidermis causes ichthyotic, hardened, scaling skin. Ichthyotic skin is very inelastic and can cause skin fissures upon movement (Figure 18). Keratin-related disorders are common, with estimates of as many as 1 in 3000 of the general population (Lane and McLean, 2004).

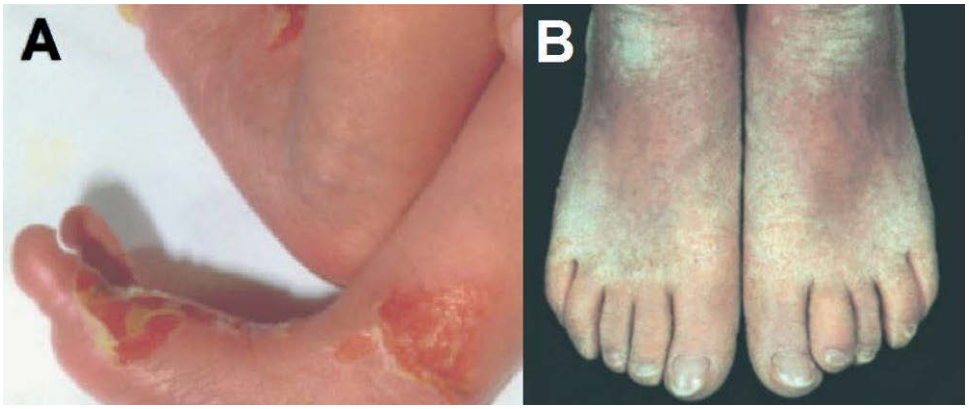

Figure 18. *EB and keratoderma disorder* (Lane and McLean, 2004). *A) Patient with a severe form of EB, epidermolysis bullosa simplex, Dowling-Meara type (EBS-DM). B) Patient with keratoderma disorder showing epidermolytic hyperkeratosis.*

6.2 IFs in neuronal disorders

There are several neuronal diseases related to IFs. In particular, mutations in type IV IFs, the neurofilaments, but some type III IFs have also been linked to neuronal disease. IFs have also been shown to play an important role in axon caliber (Friede and Samorajski, 1970). Diseases include Charcot-Marie-Tooth disease (CMT), Amyotrophic lateral sclerosis (ALS), Parkinson disease, neuronal intermediate filament inclusion disease (NIFID) and Alexander disease. Most of these diseases fall under neurodegenerative disorders (interfil.org) and the causes appear to include multiple genes and environmental factors.

CMT is a common peripheral neuropathy affecting both motor and sensory neuronal pathways. CMT has many subtypes, which differ in severity from light sensory deprivation to total paralysis of the extremities. CMT can be caused by mutation in different genes, one of them being *NEFL*, encoding neurofilament light (NF-L). CMT is caused by myelination defects and mutations in *NEFL* have been reported to show phenotypes such as swelling of the axon with either buildup or loss of myelin sheets (Liem and Messing, 2009). *NEFL* mutation can also be predisposing of CMT, without being able to singlehandedly cause the disease (Kabzińska et al., 2006). CMT affects 1 in 2500 in the overall population and 2 % of these cases are estimated to be caused by *NEFL* mutations.

ALS, also known as Lou Gehrig disease, is a rapidly progressing and fatal neurodegenerative disease. The degeneration of motor neurons is associated with IF aggregate accumulation in the axons, which in turn may be caused by IF assembly defects due to IF mutation (Liem and Messing, 2009). Mutation in all of the

neurofilaments NF-L, NF-M and NF-H but also to the type III IF peripherin have been reported in ALS.

IFs have also been linked to diseases that are caused by neuronal inclusions, but not because of IF mutation. A-internexin is the most commonly reported IF protein in such inclusions. Such diseases are called neuronal intermediate filament inclusion disease (NIFID). NIFID's are defined as diseases involving mostly IFs, but not involving synuclein nor tau, so Alzheimer's and Parkinson's disease are not part of this definition. However, IFs such as peripherin has been identified in inclusions that are found in the brain in patients with Parkinson's disease (Liem and Messing, 2009).

6.3 IFs in cataract

The optical properties of the lens are maintained by proteins including IFs. Denaturing or misfolding of proteins is very disruptive to the optical properties of the lens and the optimal opacity is maintained within slim margins. There are three IFs expressed in the lens; vimentin, filensin and phakinin. Denaturing of IFs have been shown to contribute to lens precipitates, and patients have been identified with IF mutation in *BSF2*, the phakinin gene (Jakobs et al., 2000). Also, there is one instance, where cataract has been linked to a single missense mutation, c.G596A, in the vimentin gene (Müller et al., 2009).

6.4 Laminopathies

Diseases that are caused by mutation in one of the three lamin genes *LMNA*, *LMNB1* and *LMNB2*, or in a gene encoding lamin binding partners, are collectively called laminopathies. The most common diseases caused by over 400 reported lamin mutations are muscular dystrophies and lipodystrophies. Some *LMNA* mutations lead to an extremely rare multi-organ disease that is characterized by symptoms resembling premature aging (progeria).

6.4.1 Progeria

Progeria is a severe disease that results in symptoms best described as premature aging. This devastating disease affect around 1 in twenty million people, and typically causes premature death due to heart failure or strokes in the early teens. At that time, the cardiovascular physiology of the patients resembles that of a 90-year old. Classical progeria, Hutchison Gilford progeria syndrome (HGPS), is caused by a silent *de novo* point mutation, c.1824C>T; p.G608G in *LMNA* (Eriksson et al., 2003). Although the

mutation is silent (GGC > GGT), it activates a cryptic splice site (Figure 19). Normal post-translational processing of lamin A includes farnesylation, carboxymethylation and ZMPSTE24 mediated proteolytic cleavage of the last 15 AAs of the 661 AA lamin A precursor called prelamin A. This results in a mature lamin A without the inner nuclear membrane binding CaaX motif. In the case of the p.G608G progeria mutation, the cryptic splice site causes removal of AAs 607-656, which includes the recognition site of the ZMPSTE24 protease. Therefore, mutant lamin A, called LAΔ50 or progerin, remains permanently farnesylated and is aberrantly integrated in the nuclear membrane. This causes accumulation of progerin at the lamina and progressive nuclear stiffness, deformations and herniations after each cell division. Exactly how the dysfunction of the lamina translates into a multi-organ disease that mimics the aging process is unknown, but progerin appears toxic for cells and leads to increased DNA damage and premature cellular senescence especially in tissues where lamin A is abundantly expressed, such as muscle (Zhang et al., 2011).

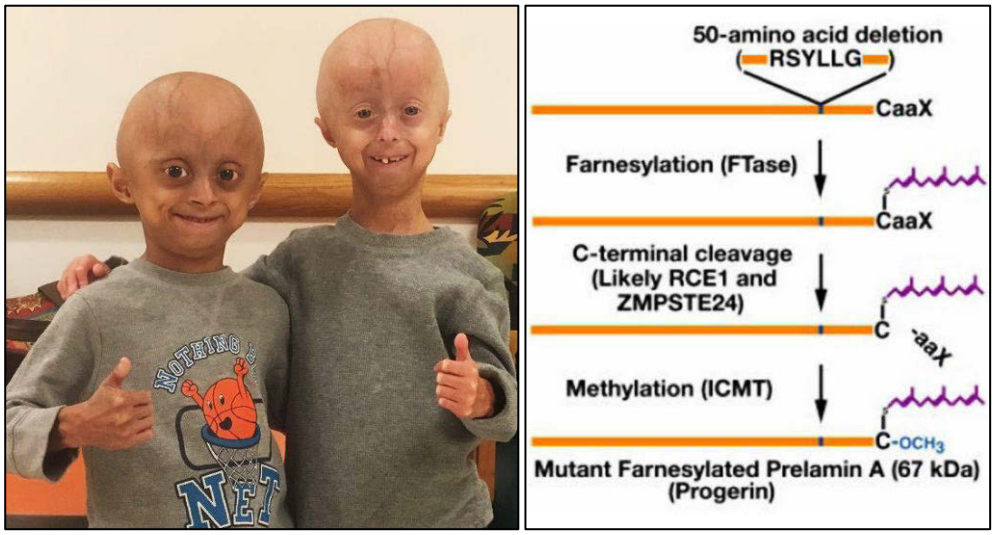

Figure 19. Hutchison Gilford progeria syndrome (HGPS). *Diego, 7, USA, Cam, 10, USA. Courtesy of The Progeria Research Foundation (progeriaresearch.org). HGPS is caused by a silent point mutation which activates a cryptic splice site in the LMNA gene. This causes a 50-amino acid deletion, including the ZMPSTE24 protease recognition site RSYLLG. Since there is no proteolytic cleavage due to the deleted recognition site, the CaaX-box is retained. The gene product of the mutated LMNA gene is called progerin, which is a permanently farnesylated lamin A that disrupts normal lamina association to the nuclear membrane. How this in turn can lead to premature aging is unclear, but lamins have interactions with chromatin and their mutation could therefore have implications on global gene expression* (Coutinho et al., 2009).

6.4.2 IFs in muscular dystrophy and lipodystrophy

There are hundreds of lamin mutations leading to muscular dystrophy. The exact pathobiology behind this connection is unclear and several potential disease mechanisms have been introduced. One plausible reason is that the tissue integrity is impaired by lamin mutation, and while the mutation affects all cells, the muscle cells are the ones affected first due to their high exposure to mechanical stress. It is also possible that the lamin mutation affects muscle specific gene expression and regulation or that there are developmental problems due to stem cell dysfunction (Davidson and Lammerding, 2013). Even less is known about the pathogenic mechanisms of lipodystrophy, leading to loss of subcutaneous adipose tissue. There is at least one example of lipodystrophy that has been shown to be caused by altered gene regulation due to lamin mutation. The p.R482W *LMNA* mutation causes increased activity of the sterol response element binding protein 1 (SREBP1), leading to increased expression of its target genes (Vadrot et al., 2015).

6.5 IFs in cancer

The IFs have, due to their tissue specific expression pattern, a long tradition as markers of tumor cell origin. Overexpression of vimentin has been linked to cancer progression (Satelli and Li, 2011). The connection between cancer and vimentin has not been completely explained due to the lack of complete knowledge of the primary function of IFs, however, in several cases the cancer-IF connection has turned out to relate to motility. Vimentin, which is a marker for mesenchymal tissue has been suggested to drive the EMT in the sense that vimentin expression could induce other genes, which drive EMT and vimentin would thus be driving cancer progression (Vuoriluoto et al., 2010). Nestin is used as a marker for cancer stem-like cells (Li et al., 2016). Lamin expression has been shown to be an indicator of patient survival in cancer (Bell and Lammerding, 2016; Foster et al., 2010). Lamin type and amount can regulate the deformability of the nucleus, and alterations in lamins can promote metastatic migration (Mendez et al., 2010). Recent studies have also shown that lamin tethering can regulate the activity of the retinoblastoma (Rb) protein, which is a cell cycle regulator. In this capacity, lamins could also be implicated in cancer progression (Kennedy and Pennypacker, 2014; Ozaki et al., 1994).

7. The model systems

In the three projects that this thesis contains, we are using several model organisms to make conclusions about IF function in humans. The models that were used were *Drosophila*, mouse, primary human cells, induced pluripotent stem cells differentiated into cardiomyocytes, and immortalized human cells lines.

7.1 *Drosophila melanogaster*

Drosophila melanogaster, the fruit fly, is an increasingly popular research model in life sciences (Figure 20). Since its first use as a research organism, *Drosophila* has been the tool in several key discoveries, the first one made by Thomas Hunt Morgan in the beginning of the 1900's, demonstrating that the chromosomes are the principal site of the material that governs inheritance (Morgan, 1910) . With this work, Morgan laid the foundation for the modern science of genetics.

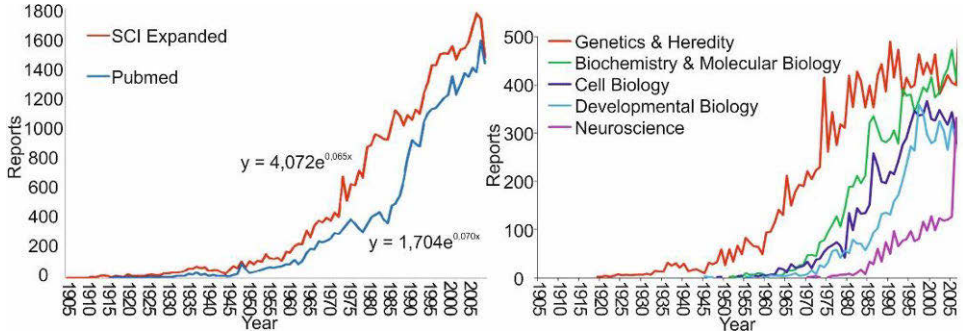

Figure 20 (Michán et al., 2010). *Number of scientific articles mentioning Drosophila in the title each year since its inception into science in Web of Science and PubMed (left). Number of scientific articles per year mentioning Drosophila in the title in different fields (right).*

Drosophila is an effective research model that has a short generation time of 9 days and is affordable to keep in stock. It is estimated that 75% of known human disease genes are conserved in *Drosophila*, making it an excellent model for biomedical research (Pandey and Nichols, 2011). Because of the history of *Drosophila* research, many genetic tools have been available for a long time for transgene expression. One of the most widely used is the UAS-GAL4 expression system (Duffy, 2002) . The transgene is placed on a plasmid under control of the UAS promoter. The fly carrying the UAS-transgene can then be crossed with a fly with tissue specific expression of the GAL4 driver. In the offspring, the GAL4 driver drives the UAS promotor which expresses the transgene. Depending of which tissue specific GAL4 driver fly is used for the cross, the fly geneticist can effectively determine where to drive the transgene.

The Chi31-integrase system can be used to integrate the transgenic sequence at a predetermined position in the fly genome (Figure 21)(Thorpe et al., 2000).

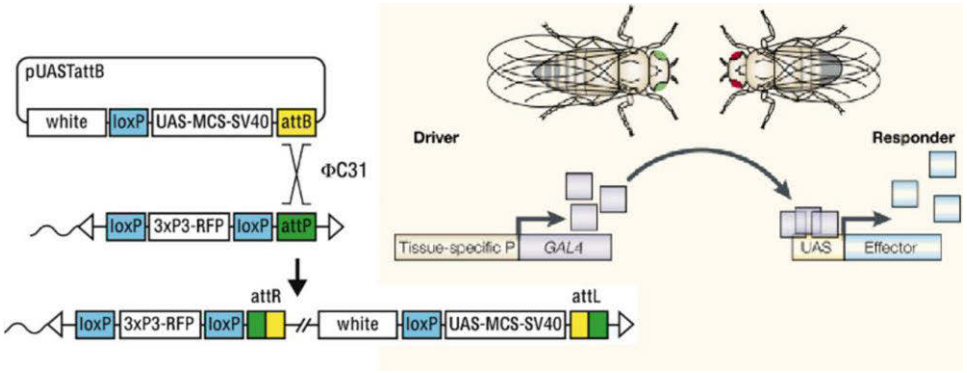

Figure 21. The Phi31-integrase mediated transgene system (Bischof et al., 2007) ***and the UAS-GAL4 expression system*** (Wimmer, 2003)***.*** *The attB-site containing plasmid is injected into fly eggs, and the Phi31-integrase activity promotes its insertion at the attP-site, enabling site-specific insertion (left). The integrated pUASattB plasmid contains an upstream activated sequence (UAS) promoter which can drive expression of the transgene. The UAS promoter is itself driven by the GAL4 driver, which can itself be driven in specific tissues. In this way, transgenes can be driven in specific tissues in Drosophila (right).*

7.2 IF knock out mice and IF disease mice models

Although *Drosophila* is an excellent research model with an extensive genetic toolbox available, researchers dealing with any model system also need to be aware of its limitations. As a mammal, the mouse physiology is very close to the human physiology. Mouse models can be very powerful in studying gene function due to the comparability to humans in most cases down to AA residue resolution. There are several IF knock out mice that have been instrumental in explaining the functions of IF as well as some less successful genetic IF knock out attempts. Vimentin null mice were initially reported not to show any phenotypes (Colucci-Guyon et al., 1994). Later studies have shown, however, that the vimentin null mice have severe wound healing defects (Cheng et al., 2016; Nieminen et al., 2006). The first lamin knock out was made by disrupting exon 8-11 of the *LMNA* gene (Sullivan et al., 1999). However, it was much later shown that this mouse was expressing a truncated form of lamin A/C. More recently, a full knock out lamin A/C mouse model was created, showing a significantly shorter life span than the mouse expressing the truncated version (Jahn et al., 2012). Both the *LMNA* mouse models show severe dilated cardiomyopathy and muscular dystrophy which highlights the connection between lamin impairment and muscle defect. Nestin knock out mice, on the other hand, have defects in

neuromuscular junction, which are principal sites for nestin expression (Mohseni et al., 2011)(Yang et al., 2011).

Several mice model have been generated expressing disease-related IF mutations. There have not been any vimentin mutant mice generated, that would express vimentin mutations identified in patients. There are, however, examples of mice strains generated that express vimentin mutants relevant for studying basic vimentin function and possible disease progression. One example is the phosphodeficient mouse, where the phosphoserines S6, S24, S38, S46, S55, S64, S65, S71, S72, S82 and S86 had all been mutated to alanine to disable phosphorylation of these residues (Matsuyama et al., 2013). The $VIM^{SA/SA}$ mice display cytokinetic failure which manifests itself as microphthalmia and cataract. Furthermore, the $VIM^{SA/SA}$ mice display defective wound healing (Tanaka et al., 2015) and these phenotypes seem to have accumulation of aneuploid nuclei in the affected tissues in common. The $VIM^{SA/SA}$ also exhibit an aging phenotype. Another vimentin mutant mouse that has been generated carries the p.R113C coil 1A vimentin mutation. This mutation also gives rise to an eye phenotype and demonstrates posterior cataract (Bornheim et al., 2008).

Several mice strains expressing disease-causing lamin mutations identified in patients have been generated. One example is the lamin A/C p.H222P mutant mouse (Arimura et al., 2005). Patients carrying this mutation suffer from Emery–Dreifuss muscular dystrophy, a striated muscle-specific laminopathy. The p.H222P homozygous mice have both skeletal and cardiac muscular dystrophy and display DCM. Mice expressing progerin by removal of the last 150 nucleotides of the *LMNA* gene to mimic the human HGPS mutation have been generated and display phenotypes similar to human progeria. These studies also showed that farnesyl transferase inhibitors can milden the progeroid phenotype resulting from progerin expression in mice (Yang et al., 2005; Yang et al., 2006). Mice expressing the HGPS lamin p.G608G mutation specifically in keratin-5 expression tissues showed many of the phenotypes associated with progeria in these tissues, including skin degeneration (Sagelius et al., 2008). Interestingly, HGPS patients seem to have unaffected cognitive development. Although expression of the p.G608G mutation specifically in brain led to neuronal nuclear distortions, the structure of the brain and the function of the adult mice appeared largely normal (Baek et al., 2015).

7.3 A model for heart cells

Primary fibroblasts can easily be obtained and cultured by biopsy. Primary fibroblasts are an almost limitless source of cells with identical genetic background as the patient

and are therefore of immense value when studying any heritable disease. However, when dealing with tissue specific diseases such as DCM, it is also crucial to look at the cells from the affected tissue, in this case cardiac muscle, to investigate tissue specific phenotypes. Unfortunately obtaining cardiac biopsy material from patients is challenging and not feasible for routine research purposes as explanted cardiomyocytes discontinue contraction under *in vitro* culture conditions. More abundant cardiac tissue material could be obtained from explanted hearts, but finding a patient with the need for heart transplant is a lengthy process and such material represents end-stage disease with various secondary alterations such as fibrosis. Also, cardiomyocytes regenerate at a very slow pace, so culturing them is slow (Kikuchi and Poss, 2012). The induced pluripotent stem cell (iPSC) technology provides an alternative source for primary cardiac cells. iPS cells are a ground-breaking technological discovery published in 2006 (Takahashi and Yamanaka, 2006), enabling scientist to dedifferentiate specialized somatic cells into pluripotent precursor stem cells by expressing the four Yamanaka factors SOX2, Klf4, cMyc and Oct4. The pluripotent precursors can then be differentiated into specific lineages such as cardiac cells by subjecting them to factors such as activin A and BMP4 (Laflamme et al., 2007). In this way, the primary patient fibroblasts that are readily available can be reprogrammed via the iPSC stage into other somatic cells with identical genetic background as the patient. Another useful cardiac cell model system, is to genetically engineer immortalized cardiomyocyte cell line to express the genetic defect of the disease to be studied. For this purpose, there is the transformed mice cardiomyocyte cell line HL1 (Claycomb et al., 1998). However, as cardiomyocytes have a low regeneration rate, the HL1 cells with their acquired high proliferation could be considered less than optimal representatives of cardiomyocytes.

Aims of the Study

The overall aim of this thesis is to improve the understanding of the IFs to benefit the common knowledge on their function and interaction. This knowledge is a platform that can be utilized for future studies to develop interventions against diseases that the IFs are involved in and to better understand the cell.

1. Transgenic expression of human vimentin in *Drosophila*

In the first study, we wanted to investigate how the transgenic cytoIFs behave in the *Drosophila* tissues when expressed in a cytoIF null background. By ubiquitous expression, we asked whether there is any cytocompatibility with vimentin in any of the *Drosophila* tissues, and if so, could such findings give clues about the universal function of IFs.

2. Muscle regeneration in nestin knock out mice

Nestin has not been extensively characterized and there have been clues that nestin could be involved in muscle stem cell behavior. In the second study, we aimed to investigate the role of nestin in muscle regeneration. We utilized the nestin knock out mouse to perform muscle regeneration studies. We also wanted to isolate primary cells from the nestin knock out mice to further study nestin involvement in muscle homeostasis.

3. Investigating the *LMNA* mutation p.S143P causing DCM

In the third study, we aimed to explain how the DCM-associated *LMNA* mutation p.S143P causes cellular disturbance in the cardiomyocyte. The primary patient fibroblasts were used in initial screenings to reveal impairments in the molecular mechanisms that the mutation gives rise to. Next, we also wanted to use iPSC-derived cardiomyocytes to confirm that what we saw in the fibroblasts was true in cardiac context. Finally, the corresponding mutant form of lamin A was expressed in *Drosophila* to test whether this would mimic the human disease phenotype, and thereby allow us to use *Drosophila* as an *in vivo* model system for *LMNA* p.S143P induced DCM.

Experimental Procedures

1. Biochemical methods

1.1 Immunoblotting

The samples were solubilized in Laemmli lysis buffer and heated at 95 °C for 5 min. The samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel samples were transferred to polyvinylidene fluoride membranes (Bio-Rad). Unspecific binding was minimized by blocking in milk. The membranes were incubated in primary antibodies overnight at +4 °C. For antibodies used, see table 2. The membranes were washed, and then submerged in horseradish peroxidase (HRP)-conjugated secondary antibodies for 1-3 h at room temperature (RT). The chemiluminescent signal was detected with enhanced chemiluminiscence reagent (ECL), and exposed on X-ray films (Fujifilm).

1.2 Immunofluorescence

Cells were grown on coverslips, and fixed with 4 % paraformaldehyde (PFA). The cells were then permeabilized with 0.1 % Triton-X 100 for 10 min and, after blocking with 1% bovine serum albumin (BSA) for 1h at RT, incubated in primary antibody overnight at +4 °C. The cells were then washed, and then incubated in fluorophore conjugated secondary antibodies for 1-3 h at RT or overnight at +4 °C. The cells were mounted in Vectashield or ProLong® GOLD (Invitrogen).

For fluorescent labeling of tissue sections, the organ was embedded in freezing medium (Tissue-Tek) and sectioned with a cryotome (Leica). The sections were then treated with the cell staining protocol above. For dissected *Drosophila* organs, a preparation microscope (Leica) was used to extract specific organs, which were fixed for 30 min in 4 % PFA +4 °C, permeabilized with 1 % Triton-X 100 for 30 min and then treated with the cell staining protocol above.

1.3 Immunohistochemistry

The tissue was collected and fixed in formalin. The tissue was then dehydrated with graded series of ethanol, and finally the ethanol was replaced with xylene. The tissue was then embedded in paraffin and sectioned with a microtome (Leica). The tissues were stained with hematoxylin and eosin or with specific antibodies. An automated staining system was used (Ventana).

1.4 Table 2: Antibodies and dyes

Primary antibodies WB=Western Blot. IHC = Immunohistochemistry. ICC = Immunocytochemistry. IF= Immunofluorescence				
Antigen	**Clone/#**	**Company**	**Application**	**Dilution**
Actin	AC-40	Sigma-Aldrich	WB	1:1000
Cdk5	DC-34	Life Technologies	WB, IP	1:1000
Cdk5	C-8	Santa Cruz	WB	1:200
Desmin	#4024	Cell Signaling	WB	1:1000
eIF2α	FL-315	Santa Cruz	WB, IF	1:300
FLAG	9A3	Cell Signaling	WB	1:1600
FLAG	SIG-25	Sigma-Aldrich	WB, IF	1:1000
GAPDH	14C10	Cell Signaling	WB	1:2000
Hsc70	SPA-810	Stressgen	WB	1:2000
Lamin A/C	4C11	Cell Signaling	WB, IF	1:1000
Lamin A	26300	Abcam	IF	1:1000
Lamin A/C	626	Novocastra	WB	1:150
Lamin B1	M20	Santa Cruz	WB	1:100
MyoD	M-318	Santa Cruz	ICC	1:100
Nestin	556309	BD Pharmingen	IHC, ICC	1:200
Nestin	611659	BD Pharmingen	WB	1:1000
p35/p25	C-19	Santa Cruz	WB	1:200
Pax7	AB_528428	DSHB	ICC	1:20
peIF2α	119A11	Cell Signaling	WB	1:1000
Synemin		Prof. Omar Skalli	WB	1:100
Troponin T	JLT-12	Sigma-Aldrich	WB	1:200
Vimentin	D21H3	Cell Signaling	WB, IF	1:200
Vimentin	V9	Sigma-Aldrich	WB, IF	1:1000
Vimentin	RV202	Abcam	WB	1:1000
Vimentin	550513	BD Pharmingen	WB	1:1000
XBP1	M-186	Santa Cruz	WB, IF	1:200
Secondary antibodies and dyes				
HRP-conjugated secondary antibodies (Vectastain)				
HRP-conjugated secondary antibodies (GE Healthcare)				
Alexa Fluor® Fluorophore conjugated antibodies (Thermo Fischer)				
BrightVision Poly-HRP secondary antibody (ImmunoLogic)				
Hoechst (1 µg/ml) or DAPI was used to visualize DNA				
Picrosirius red was used to stain collagen deposits				
Fast green FCF (Sigma-Aldrich) was used to stain injured muscle				

1.5 High salt extraction of IFs

Cells were homogenized in low detergent buffer (50 mM HEPES pH 7.4, 10 mM MgCl2, 1 mM EGTA, 0.2 % Triton X-100, 1 % PMSF and protease inhibitor). When performing high salt extraction on *Drosophila*, 100 flies were first subjected to a homogenizer. The samples were centrifuged at 14 000 g for 10 min at +4 °C. The supernatant was discarded and high detergent buffer (50 mM HEPES pH 7.4, 10 mM MgCl2, 1.5 % Triton X-100, 1 mM PMSF and protease inhibitor) was added to the pellet. After 5 min incubation, 100 U/Ml of DNase (Promega) was added, and after an additional 10 min, NaCl was added to a final concentration of 1 M. After another

5 min, the sample was centrifuged at 14 000 g for 15 min at +4 °C. The supernatant was discarded and disassembly buffer (8 M urea, 50 Mm HEPES pH 7.4, 1 mM PMSF and protease inhibitor) was added to the pellet. The samples were then centrifuged at 14 0000 g fir 15 min at +4 °C. The supernatant was then dialyzed against assembly buffer (50 Mm HEPES pH 7.4, 150 Mm NaCl, 1 mM PMSF). After, dialysis, the sample was centrifuged at 100 000 g for 15 min at +4 °C to recover filaments and the resulting supernatant was then discarded and the pellet was dissolved in disassembly buffer.

1.6 Detergent extraction and measurement

Fibroblasts were grown on coverslips. One fibroblast population was subjected to 0.5 % Triton-X for 5 min at RT. All fibroblasts were then fixed and permeabilized with 0.1 % Triton X-100. The fibroblasts were stained with antibodies against lamin A/C and DNA was stained with DAPI. The samples were fluorescently labeled with secondary antibodies and analyzed with confocal microscopy. The lamin signal from mid-plane sections of the fibroblast nuclei were analyzed by comparing a 1000-pixel area of the lamina and nucleoplasm between Triton X-100 extracted and unextracted mutant and control fibroblasts (n=20). The normalized data was analyzed with student's T-test.

1.7 Fractionation of soluble and insoluble IFs

For vimentin fractionation, S2-cells were lysed in lysis buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 0.5 % Triton X-100 and protease inhibitor (Thermo Fischer Scientific)). The samples were then centrifuged at 15 000 g for 45 min at +4 °C. The supernatant contains the soluble fraction while the pellet contains the insoluble fraction.

For lamin fractionation, the cells were subjected to 0.1 % Triton X-100, incubated for 5 min on ice and then centrifuged. The pellet was then subjected to 0.1 % Triton X-100 in lysis buffer (10mM Tris pH 7.4, 2 mM $MgCl_2$, 1x protease inhibitor (Roche)), again for 5 min on ice and then centrifuged. The two supernatants contain soluble fractions, while the pellet contains the insoluble fraction.

1.8 Kinase assay

Primary fibroblasts, isolated from 3-month-old male mice, and that had been differentiated for 24 h, were lysed in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 5 mM EDTA, 5 mM EGTA, Complete Protease Inhibitor Cocktail (Roche Diagnostics), PhosSTOP Phosphatase Inhibitor Cocktail (Roche Diagnostics)). The lysate was centrifuged, after which the supernatant was incubated with antibodies against CDK5. Protein G sepharose was added and the samples were incubated for 2.5 h. The samples were then washed with kinase reaction buffer (50 mM HEPES pH 7.2, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl2). A mixture of ATP and 3 μCi [γ-32P] ATP was added to the beads at a final concentration of 100 μM. Histone H1 was then allowed to be phosphorylated for 30 min at 30 °C. The samples were then lysed with Laemmli and run on a gel after which the samples could expose an X-ray film to detect the 32P signal.

1.9 *In vitro* protein assembly

Recombinant protein was produced as described elsewhere (Foeger et al., 2006). The recombinant protein was diluted to 0.2 g/l in storage buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 8 M urea) and dialyzed against dimer buffer 25 mM Tris-HCl buffer, pH 8.0, containing 250 mM NaCl, 1 mM DTT) for 1 h at 37 °C. A second dialysis was performed against buffers with different pH values (pH=6.5, 7.0, 7.5 or 8.0)(25 mM Mes-NaOH, 250 mM NaCl, 1 mM DTT). The samples were fixed with 0.2 % glutaraldehyde.

Recombinant protein was diluted to 0.5 g/l for assembly into paracrystals. The samples were first dialyzed against a Tris-buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA and 1 mM DTT) with 300 mM NaCl overnight at 4 °C. The samples were then diluted to a protein concentration of 0.1 g/1 and dialyzed into tris buffer repeated times with consecutively reduced NaCl concentrations. The NaCl concentrations were 250 mM, 200 mM, 150 mM, 100 mM, 50 mM, and each dialysis was done for 20 min. Samples were collected at each stage for electron microscopy.

2. Imaging

For confocal microscopy, a LSM 780, and a LSM510 confocal microscope (Zeiss) was used as well as a 3i & Zeiss spinning disk confocal microscope. For brightfield imaging of histological slides, a Pannoramic 250 Slide Scanner (3D Histech) was used.

2.1 Live cell imaging

HeLa cells were transiently transfected to express either GFP-WT-LA or GFP-p.S143P-LA. The cells were grown on coverslips and maintained in phenol red-free Leibovitz's L-15 Medium (Life Technologies, CA, USA) supplemented with 10% FCS during imaging. GFP fluorescence in half of the nucleus was bleached repeatedly for 8 secs with 488 nm every 8 sec. The fluorescence intensity was measured as a function of time.

2.2 Transmission Electron Microscopy (TEM)

Cells were grown on cover slips with grids (Mat-Tek). The samples were fixed with 5% glutaraldehyde in 0.16 mol/l s-collidine buffer, pH 7.4. The samples were then post-fixed with 2 % OsO4 containing 3% potassium ferrocyanide for 2h. The samples were dehydrated with a series of increasing ethanol concentration. The samples were then embedded with the 45359 Fluka Epoxy Embedding Medium kit. 70 nm sections were made with an ultramicrotome. The sections were then stained with 1 % uranyl acetate and 0.3 % lead citrate. The microscope used was a JEOL JEM-1400 Plus TEM with an OSIS Quemesa 11 Mpix bottom mounted digital camera operated at 80 kV acceleration voltage.

2.3 Image analysis

Software used for analysis of the images are Zeiss Zen 2012, BioimageXD, Fiji/ImageJ, CaseViewer and Pannoramic Viewer (3D Histech), Cell-IQ Analyzer (CM Technologies).

3. Cell culture, transfection and plasmids

S2 cells were cultured in Complete Schneider S2 Medium (Invitrogen) supplemented with 10% fetal calf serum, penicillin, streptomycin, and 2 mM L-glutamine. S2 cells were transfected with calcium phosphate. The pAc5.1-His_A vector was used for transfection in S2 cells.

Mouse embryonic fibroblasts lacking vimentin (MEF-vim-/-) were grown in DMEM (Sigma-Aldrich) with 10% fetal calf serum, penicillin, streptomycin, and 2 mM L-glutamine. For expressing a vector under the UAS promoter in MEF-vim-/- cells, a pSVg vector containing the Gal4 sequence was transfected with electroporation.

HeLa cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. The HeLa cells were transfected with the TransIt HeLa Monster kit (Mirus, Madison, USA).

To induce ER stress, 2 µg ml-1 tunicamycin was used (Sigma-Aldrich, MO, USA). MG132 was used to inhibit the ubiquitin-proteasome system (UPS) (10 µM, Sigma-Aldrich, MO, USA). Wortmannin was used to inhibit autophagy (10 µM, Enzo Life Sciences, Farmingdale. USA). Rapamycin was used to activate autophagy (80 ng ml-1, Sigma-Aldrich, MO, USA). To inhibit ER stress, 10 µM 4-Phenylbutyric acid (Sigma-Aldrich, MO, USA) was used.

293-T cells were used to produce lentiviral particles for transduction of fibroblasts (Alastalo et al., 2015).

The CMV2-FLAG–LA and pEGFP-myc-LA vectors were cloned as described elsewhere (Moir et al., 2000; Taimen et al., 2009). The *LMNA* p.S143P mutation was introduced with the Quick-Change II XL Site-Directed Mutagenesis kit (Agilent Technologies, CA, USA). The primer s used were 5`-GGCTCTGCTGAACCCCAAGGAGGCCGC-3´ and 5`-GCGGCCTCCTTGGGGTTCAGCAGAGCC-3`.

The pET 24a vector was used for recombinant production of lamins for *in vitro* assembly. For this purpose, the coding sequence of WT lamin A was cloned, omitting the last 18 codons.

3.1 Patients and primary cells

The Ethics Committees of the Hospital District of Helsinki and Uusimaa (HUS 387/13/03/2009) and Kuopio University Hospital approved the study. Seven patients carrying the p.S143P *LMNA* mutation were enrolled. Local anesthetics (1% lidocaine) was used to take skin biopsies from patients and healthy donors. The biopsies were washed with PBS and cut into small pieces which were attached to the bottom of a culture dish. Medium (MEM with 20 % FBS, 2 mM glutamine, vitamins, amino acids supplements and penicillin/streptomycin (all Invitrogen)) was added to the dish.

3.2 Isolation of primary mouse cells

Satellite cells were isolated by first digesting TA and EDL muscle from adult mice in Dispase II (Stem Cell Technologies) and collagenase D (Roche Diagnostics). The satellite cells were then released by titration and collected by centrifugation. The cells were cultured on gelatin-coated dishes with standard growth medium for three days. Differentiation was then induced. Standard growth medium was Dulbecco´s Modified

Eagle serum (DMEM) (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich), 20% fetal calf serum (Gibco), 10% horse serum (HyClone) and 1% chick embryo extract. Differentiation medium was 1% horse serum in DMEM with penicillin and streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate.

Myofibers were isolated by dissecting EDL muscle from 3-month-old male mice and digesting them in type I collagenase (Calbiochem). Fibers were released by careful serial titration and washing. For confocal imaging, myofibers were immediately fixed or grown in floating conditions on horse serum-coated dishes to allow satellite cell activation and then fixed. For imaging in the Cell-IQ imaging platform, myofibers were plated on Matrigel (BD Biosciences) -coated plates. Imaging could then be performed when the fibers attached to the plate.

4. Mice

Nestin KO and WT mice in a C57BL/6 background (Mohseni et al., 2011) were housed at the Central Animal Laboratory of the University of Turku. Nestin KO and WT mice, two months of age, were used for the regeneration study. An incision was performed with a razor blade through the skin and half of the tibialis anterior (TA) muscle. The mice were anesthetized for the procedure and received buprenorphine (Temgesic, Reckitt Benckiser Healthcare) 0.1 mg/kg i.p. 0 and 8 h post-operative as well as carprofen (Rimadyl, Pfizer Animal Health) 5 mg/kg s.c. 0, 24 and 48 h post-operative as pain relief. Carbon dioxide inhalation followed by cervical dislocation was the euthanization procedure and the experiments were performed according to the Finnish Act on Animal Experimentation (62/2006) and under the permission of the Finnish National Animal Experiment Board.

4.1 Body composition measurement

A nuclear magnetic resonance Echo-MRI body composition analyzer (EchoMRI LLC) was used to determine the amount of fat and lean mass of the mice.

5. Fly husbandry

To generate transgenic flies, cDNA was inserted into the pUAST-attB vector. The plasmids were injected into fly eggs (Bestgene). The UAS-transgenes were expressed ubiquitously under control of the DaGal4 driver. Yw and TM6B fly strains were used as balancers.

6. Microarray

Isolated patient and control fibroblasts were harvested and RNA was extracted with RNeasy kit (Qiagen). The RNA quality was tested with a NanoDrop spectrophotometer ND-1000. The Finnish microarray and Sequencing Centre (http://www.btk.fi/microarray-and-sequencing/) performed a whole genome expression analysis with an Illumina Human HT-12 v.4, as well as expression bead chip and data analysis. The Gene Set Enrichment Analysis (GSEA) software and MSigDB (http://www.broadinstitute.org/gsea/index.jsp) was used to further process the data. The data analysis was focused on gene set from the Reactome pathway and a heatmap was made with two-sided t-test the GENE-E. The Pearson correlation analysis was made with the MeV software.

7. Statistics

For statistical analysis, GraphPad Prism 6 was used. Statistical significance was determined using Student´s t-test, unpaired Student´s t-test with Welch´s correction or Mann-Whitney test when samples were not normally distributed (D'Agostino-Pearson omnibus normality test or Shapiro-Wilk normality test) depending on the experimental setup. Mean values are presented as ± standard error of the mean (SEM). $P<0.05$ is considered significant and marked with asterisk ($p<0.05$= *, $p<0.005$= **, $p<0.001$= ***, $p<0.0001$= ****), n.s. stands for not statistically significant ($p>0.05$).

8. Sequence comparison and domain search

The protein sequence comparison and the cladogram were made using ClustalW2 (http://www.ebi.ac.uk/). The MOTIF Search tool, (http://www.genome.jp/tools/motif/), was used to identify filament-like domains.

Results and Discussion

1. The evolution of cytoplasmic intermediate filaments from the arthropod perspective

Nuclear lamins are the ancestors of all IFs and present in early metazoans (Blumenberg, 1989). It has been shown that cytoIFs have emerged several times during evolution through duplication of the lamin gene and inactivation of its NLS (Hering et al., 2016). It is known that cytoIFs are very widespread in all eumetazoan with a single exception in the very characteristic phyla that is the arthropods. It is also known that other earlier protostomes that are ancestors of the arthropods had cytoIFs (Bartnik and Weber, 1989). Three important questions can be formulated to approach the fundamental functions of IFs by evolutionary reasoning:

(A) What characteristics had lamins acquired in early eumetazoan evolution that would be beneficial in the cytoplasmic context?

(B) What functional need did the cytoplasmic IFs satisfy when they were selected for to be expressed in the cytoplasmic compartment?

(C) What was the reason for the disappearance of the cytoIFs from the arthropod lineage?

The answers to these questions contain information about the fundamental function of the IFs. This information would be of immense value, because it would enable the understanding of a protein family that is at the center of many human diseases. To tackle these questions, we decided, as one sometimes does when faced with a conundrum, to focus on the exception to the rule. The rule and the exception are very clear; all multicellular organisms with true tissue organization express nuclear and cytoplasmic IFs. The arthropods are the exception and express only nuclear IFs. We approached this inconsistency by reintroducing a generic cytoIF into an arthropod, the fruit fly, *Drosophila melanogaster*. We chose *Drosophila* because it is a common research model with genetic tools readily available. We chose human vimentin because it is a widely researched IF and because it can assemble into homopolymers, unlike keratins for example.

1.1 CytoIFs can be expressed in arthropods without gross phenotype

In transfected S2 cells, which are macrophage-like in origin, human vimentin formed large aggregates (I, Figure 1A). We expressed vimentin ubiquitously using the Daughterless Gal4 driver to drive expression of the UAS-Vimentin construct that we had cloned and incorporated into a *Drosophila* line. We detected the expression with

vimentin antibodies and could see that the expression was successful (I, Figure 1B-D). The transgenic flies were fertile and had normal body morphology. We did longevity and weight measurements to rule out gross physiological phenotypes due to vimentin expression (I, Figure 1E-F). Although more substantial analyses are needed to rule out any physiological defects due to vimentin expression, these results prove that there is nothing intrinsically toxic or harmful in cytoIF expression in arthropods that would have been driving negative selection of cytoIF during evolution. This has also been proven by others by expressing keratin 14 and 5 in *Drosophila* (Bohnekamp et al., 2015), which gave rise to IF assembly without apparent harmful phenotypes.

1.2 Biochemical analysis confirms cytoIF absence in *Drosophila*

Since *Drosophila* is a common research model, it is among the organisms, which have had their genomes completely sequenced (Adams, 2000). Thus, we could easily search for IF homologues in the *Drosophila* genome database by Basic Local Alignment Search Tool (BLAST). We did not find any matches with IFs in the *Drosophila* genome by this method (data not shown). However, IF conservation tends to be more structural than sequential, meaning for example that maintaining the coiled coil structure is not down to a specific AA, but rather to a pattern of AA classes. Therefore, we decided to use a biochemical approach to test the claims that arthropods do not have cytoIFs. The so called IF-preparation that we used, takes advantage of the insolubility of the assembled IFs to isolate IFs. We then identified the proteins that we isolated by mass spectrometry. By this method, we first showed that we could isolate lamin from S2 cell lysates (I, Figure 2A). Thereafter, we decided to repeat the experiment on whole adult flies to rule out eventual tissue specific expression of any cytoIFs. However, there were so many protein bands (>100) in our isolation that we decided to run a mass spectrometry on the whole separated gel lane. Mass spectrometry showed that we isolated human vimentin from our transgenic flies as well as the two *Drosophila* lamins, lamin C and lamin Dm0. While none of the other proteins identified were candidates for IFs and we decided to perform an IF-like domain search on them. Apart from the known IFs in our isolate, the only identified protein having an IF-domain was tropomyosin 2 (I, Figure 2B, Table S1). This is of particular interest as an atypical tropomyosin with IF-like properties was recently reported in *Drosophila* (Cho et al., 2016).

1.3 Mesenchymal tissues show low vimentin network regularity in the transgenic *Drosophila*

Several tissues in the transgenic flies expressing human vimentin were stained and analyzed with confocal microscopy. In adipose tissue (I, Figure 2C-D), and in muscle (I, Figure 2E-F), vimentin formed small cytoplasmic aggregates with no detectable regularity. Since vimentin was recovered in the insoluble fraction in the transfected S2 cells (I, Figure S4), it most likely assembled to some degree also in the mesenchymal tissues. These observations suggest that there was tissue specific interaction between vimentin and endogenous proteins.

1.4 Transgenic human vimentin assembles into a network in internal epithelial in *Drosophila*

One of the tissues that stood out above others was internal epithelium. In Malpighian tubules, as the clearest example of this, vimentin assembled into nuclear cage-like structures that transverse the epithelia across cell margins (I, Figure 3D-F). Epithelium is one of the principal tissues of cytoIF expression in mammals, but having lost cytoIFs during evolution, it could be expected that the arthropods could not differentiate between cytoIF types when it comes to tissue specific interaction. Nonetheless, the internal epithelium displayed very regular vimentin structures in the transgenic flies. Other internal epithelia such as intestine (I, Figure 3A-C) and salivary gland (I, Figure 3G-H) also displayed nuclear cage-like structures. It would obviously be of great interest to know what proteins vimentin is interacting with in these tissues. Could there be intrinsic properties that allow vimentin to bind to more generic proteins in the epithelium, even in the absence of its traditional binding partners such as the plectins, which in *Drosophila* has no IF-binding domain (Gregory and Brown, 1998)?

1.5 Internal and external barriers are principal tissues for archaic cytoIF expression

Compiling data from the literature on cytoIF expression among eumetazoan organisms, paints a clear picture of tissues where IFs are preferentially expressed (I, Figure 4A). There are several phyla which have epidermal cytoIFs as their only cytoIFs. The most characteristic phylum with only epidermal cytoIFs is perhaps *Platyhelmintha*, the flatworm. The flatworm is itself a remarkable organism with seemingly limitless regeneration capacity. The flatworm *Dugesia japonica* expresses a cytoIF named DjIFb in its external epithelium. Interestingly, DjIFb is only expressed

when the epithelium is intact. DjIFb expression is inactive along the body margin of the regenerating tissue, but as soon as the regeneration is complete, DjIFb expression resumes (Tazaki et al., 2002). In a large part of the eumetazoan phyla, there is both epidermal and internal epithelial expression. This cytoIF expression group is represented for example by *Caenorhabditis elegans*, another common research model. *C. elegans* has eleven genes coding for 14 epidermal and internal epithelial cytoIFs and one gene coding for its single lamin (Carberry et al., 2009). To make conclusions about which of its cytoIFs evolved first, we performed a sequence comparison between all 11 cytoIFs and lamins. The results show that the nucleotide sequence of the epidermal cytoIFs is more homologous with lamin than that of internal epithelial cytoIFs, (I, Figure 4B) suggesting that epidermal cytoIFs were most likely the first ones to evolve. In fact, it is reported that this has indeed happened more than once during evolution. A recent report shows that tardigrades, among others, have regained cytoIF expression through lamin gene duplication after their cytoIF disappearance along with the arthropods (Hering et al., 2016) and in each of these cases, the cytoIF was expressed in the epidermis.

We speculate, since *Drosophila* is viable with cytoIF expression, that the arthropod lineage lost their cytoIFs because of the emergence of the exoskeleton or by the emergence of the IF-like tropomyosin Tm1-I/O. It would be interesting to know if Tm1-I/O is conserved throughout the arthropods and, whether it was evolved before or after disappearance of cytoIFs from the arthropod genome. By shedding the NLS, lamins introduced IF assembly and dynamics into the cytoplasm. The bacterial IF-like protein crescentin forms a sturdy scaffold to support cell shape (Ausmees et al., 2003) that needs to reassemble for each division. This reassembly is comparable to what the nucleus undergoes during mitosis in eukaryotic organisms. Because lamins have such a long evolutionary history, with lamin-like proteins in yeast (Krüger et al., 2012), and since crescentin is present in prokaryotic organisms, it is conceivable that lamins could have been involved when the eukaryotic nucleus was evolved. The integration in chromatin regulation through LADs is indicative of the long history of parallel evolution of chromatin and lamins.

Barrier integrity maintenance is one of the core functions of IFs in the cytoplasm, and in the several reported instances of pan-arthropods regaining cytoIF expression, such cytoIFs have been expressed in the barriers. The epidermal tardigrade cytoIF cytotardin has similar appearance in epidermis as vimentin in *Drosophila* internal epithelium (Hering et al., 2016). Isomin is expressed in the intestine of *Isotomurus*, the only animal known to express cytoIFs in the intestine alone (Mencarelli et al., 2011). The fact that some arthropods have shed the cytoIFs illuminates that in

eumetazoans cytoIFs are indispensable, or replaced by something equally indispensable.

One of the major findings in the transgenic *Drosophila* was that the transgenically expressed vimentin formed networks in the cytoplasm of many internal epithelia. This result promptly suggests that there are proteins linking the vimentin network to the cell membrane and possibly to the nuclear membrane. The proteins that mediate the observed compatibility with human vimentin in the internal epithelial membranes in *Drosophila* should be identified. We must, however, consider the possibility that there are no protein linkers binding to the transgenic vimentin. When considering the vimentin expression in internal epithelia, we must remember that these epithelial cells are very thin. Perhaps, since IFs have spontaneous polymerization capacity, could vimentin assemble into a semi-regular entity that would more or less fill the limited space around the nucleus in the epithelial cells, and provide us with the "nuclear cage" that we saw in the stainings (I, Figure 3). This possibility needs more investigation to be ruled out.

The most obvious next step for vimentin expression in *Drosophila* would be to express a mutant form of human vimentin. Although there are many artificial mutations that have been reported to change the properties of vimentin, the only reported human disease mutation is, to my knowledge, c.G596A. This causes the missense mutation p.E151K which induces vimentin assembly defects and has been identified in a cataract patient (Müller et al., 2009). Expressing this mutation could provide another dimension for studying the vimentin network by comparing the assembly-competent and incompetent IF networks. More importantly, it would potentially allow for the use of the transgenic vimentin *Drosophila* as a human disease research model for cataract.

2. Fine-tuning of the IF network through the expression of specific IF proteins; learning from the nestin knock out mouse

Immunostaining of WT rat skeletal muscle shows nestin expression in neuromuscular junctions (NMJ) and in myotendinous junctions (MTJ)(Vaittinen et al., 1999). Nestin cell studies show nestin being regulated by CDK5 phosphorylation (Sahlgren et al., 2003). Depletion of nestin has been shown to affect Cdk5 regulation and Cdk5 activity affects the proliferation rate of myogenic precursor cells (Pallari et al., 2011). Like other IFs, nestin has a specific expression pattern. It is expected that the different IFs would perform tasks for which they have become specialized in their respective tissues. Since nestin is expressed in myoblasts during development (Sejersen and Lendahl, 1993), it is conceivable that nestin would be detectable in regenerating skeletal muscle, where myoblasts have an important role. We wanted to find out if the modulation of the IF network by the introduction of nestin could be important for normal muscle function and regeneration. For this study, we obtained nestin knock out mice previously generated (Mohseni et al., 2011), allowing *in vivo* investigation of muscle function in a nestin compromised animal.

2.1 Nestin null mice have lower body weight due to lower lean mass

Extensive phenotyping of the general physiology and morphology of the nestin -/- mice showed that the male nestin null mice had lower total body weight than WT male mice at three months of age (II, Figure 1A). The decrease in total body weight was not due to consumption or significant change in activity (II, Figure S1) and the difference in total weight became more pronounced in older male mice (II, Figure 1B). An MRI body composition analysis revealed that the male null mice have lower lean mass, a difference that increased with age (II, Figure 1C-D). However, the phenotype did not seem to be gender specific, since we could also show a lower muscle mass in female null mice when compared to WT females at the age of three months (II, Figure S2A-B).

2.2 Nestin null mice show increased muscle regeneration in uninjured conditions

We next dissected three individual hind leg muscles from male nestin null and WT mice and performed weight measurements to determine if the lean mass weight phenotype could be detected on a single muscle level. There were significant differences between WT and null tibialis anterior (TA) and extensor digitorum longus (EDL) muscles, but not in soleus (II, Figure 2A). We also measured the sectional area

of soleus and EDL muscle, but there was no significant difference between nestin null and WT mice. However, when quantifying the number of centrally located nuclei (CLN) in muscle cross sections, there was a higher number of baseline regenerating muscle fibers in nestin null muscle compared to WT (II, Figure 2D-E). Such increased number of CLN in muscle fibers is a marker for regeneration (Yin et al., 2013).

2.3 p35 is upregulated in differentiating nestin null myoblasts

Since nestin depletion has been shown to cause Cdk5 related muscle defects both *in vitro* (Pallari et al., 2011) and *in vivo* (Mohseni et al., 2011), and since Cdk5 is important for myogenic differentiation, we isolated primary myoblasts to investigate if there were any phenotypes relating to muscle differentiation in the nestin null cells. Myoblasts were stained for imaging and the presence and absence of nestin in the WT and null cells was confirmed by immunoblotting at the same time points (I, Figure 3A-B). Upon differentiation up to 72 h, the Cdk5 activator p35 was upregulated in differentiating nestin null myoblasts when compared to WT controls (II, Figure 3C). There was, however, no difference in pace of regulation of the muscle differentiation markers troponin-T and desmin nor in p25, which is the cleaved and active form of p35. To investigate whether the detected increase in p35 in differentiating nestin null primary myoblasts could increase the Cdk5 activity relative to WT cells, we performed a kinase assay. Immunoprecipitated (IP) Cdk5 from nestin null or WT primary myoblasts were allowed to phosphorylate histone H1 in the presence of γ-32P-ATP. We could show that Cdk5 IP:d from nestin null primary myoblasts incorporated more γ-32P-ATP into histone H1 than Cdk5 from WT myoblasts (II, Figure 3D). It is plausible that the increased Cdk5 activity is caused by nestin depletion leading to p35 increase.

2.4 Nestin null mice have a satellite cell proliferation defect

Because there are reports that nestin is expressed in satellite cells and marks the quiescent satellite cells (Day et al., 2007), we isolated satellite cells by culturing skeletal muscle fibers from the nestin null and WT mice. After 72 h of isolation and culturing myofibers on growth medium, we stained the cells with antibodies against MyoD, a marker for quiescent and activated satellite cells and Pax7, a marker of committed satellite cells. After isolation of the myofibers, there was no difference in the number of Pax7 positive cells between nestin null and WT satellite cells (II, Figure 4A). Similarly, the numbers of MyoD, Pax7 and MyoD/Pax7 positive cells in the activated myofibers were equal in nestin null and WT genotypes after 72 h of culture/differentiation (II, Figure 4B). Since we could see an induction of baseline

regeneration in the nestin null mice muscle and since myogenic precursors express nestin under normal conditions, we analyzed if there were any alterations in the proliferative capacity between isolated nestin null and WT satellite cells. We recorded satellite cells for 96 h after myofibers had been plated on Matrigel, and we tracked the satellite cell movement and proliferation during this time. We could show that the interval between divisions was significantly delayed in the nestin null cells compared to WT, by 1 h on average (II, Figure 4C). We also counted cell number over time by live CellIQ imaging and found that nestin null satellite cell numbers were increasing at a slower rate than the WT satellite cells (II, Figure 4D), corresponding to the delay in replication time.

2.5 Nestin null mice have impaired muscle regeneration

We next investigated the muscle regeneration in the nestin null and WT mice *in vivo* by performing an incision wound assay. TA muscle of 2-month old nestin null and WT control mice were subjected to incision wound and a subsequent regeneration was followed for 28 days. In WT mice, nestin expression was induced following incision wound in the TA muscle and readily detectable in all selected timepoints; 3, 7, 14 and 28 days post injury (II, Figure 5A). The regenerating area, identified by muscle fibers with CLN, was significantly larger in nestin null mice TA muscle 28 days after incision when compared to WT (II, Figure 5B). There were no evident changes between the nestin null and WT in scar formation as analyzed by H&E and Sirius red (collagen) staining at 28 days post injury. Therefore, we conclude that the genetic depletion of nestin leads to impaired muscle regeneration. While nestin has been shown to form heteropolymers with desmin in muscle, induction of nestin following injury may indicate a supporting role for nestin in myofiber regeneration and especially in satellite cell proliferation.

As different IFs are expressed in different spatial and temporal manner, comparing the knock out phenotypes between these IFs is important to gain insights on their function. The nestin knock out has, as this project has demonstrated, a muscle regeneration defect, proliferative defects, at least in some myogenic stem cells, and deregulation in CDK5 signaling. Synemin is a type III cytoIF which is expressed at costameres and z-discs. Synemin knock out mice display biomechanical defects in muscle fibers (García-Pelagio et al., 2014). Desmin, also a type III cytoIF, is one of the major cytoIFs in muscle. In desmin null muscle fibers, altered fiber connectivity and nuclear linkage was observed (Shah et al., 2004). In keratin 19 null mice, there is a loss of costameres, and an accumulation of mitochondria under the sarcolemma (Lovering et al., 2011). Lastly, lamin A/C null myoblasts display impaired

differentiation (Frock et al., 2006). The reason for the similarity of phenotypes between different IF knock out mice could be that the different IFs cooperate to optimize the mechanical properties of the tissue and that this modulation is regulated by their expression pattern. Another explanation could be that IFs are involved in signaling pathways that are critical to the tissues from which they were removed. Nestin is phosphorylated by CDK5 and, thus, we assume that nestin is downstream of CDK5 from a signaling perspective. Since CDK5 regulation is affected in nestin KO-mice, it would also suggest that normally, there are downstream effects of nestin induced by CDK5. However, we cannot at this point rule out the possibility that nestin dynamics and mechanical support are required for normal myogenic proliferation. To investigate whether signaling events take place downstream of nestin, this subject needs further investigation.

3. Studying the mechanisms of DCM caused by the p.S143P mutation in lamin A/C

Dilated cardiomyopathy (DCM) is a severe heart disease that leads to dilation of one or both ventricles, and causing decreased contractile force. DCM is frequently associated with arrhythmias that may cause sudden death. After mutations in titin, mutations in *LMNA* encoding for lamin A and C, are the most common cause for familial DCM (Hershberger et al., 2013). In Finland, the *LMNA* founder mutation p.S143P is estimated to be responsible for up to 10% of hereditary DCM cases (Kärkkäinen et al., 2004).

3.1 The *LMNA* p.S143P mutation causes mislocalization of lamin A/C and intranuclear aggregation

To investigate the cellular mechanisms of the DCM causing *LMNA* mutation p.S143P, we first studied the morphology of the lamina in different cellular systems. In patient fibroblasts, we frequently observed aggregation of lamin A/C and a less pronounced overall lamina structure. This is because the mutant lamin monomers were unable to integrate normally into lamin polymers, but instead favored the formation of dense aggregates *in vitro* (III, Fig 4). In analogue, lentiviral transduction of WT fibroblasts with a plasmid encoding FLAG-tagged p.S143P lamin A led to formation of distinct round aggregates detected by FLAG antibody (Figure 22, unpublished data, III, Figure 1A-B). This confirms further that the mutant lamin monomers cannot integrate properly into the lamina.

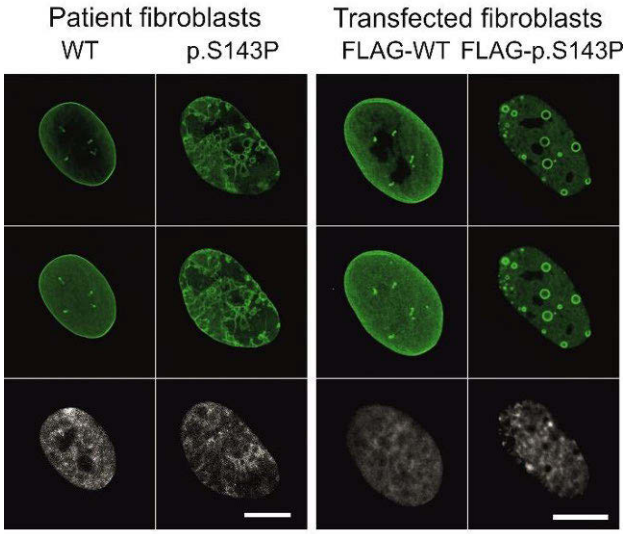

Figure 22. *Left: Primary WT and patient fibroblasts. Lamin A/C has been fluorescently labeled with antibodies and is seen in green. Right: WT control fibroblasts transfected with FLAG-tagged WT or p.S143P-lamin A. FLAG antibody staining is seen in green. The top row shows a confocal slice and the middle row shows a maximum projection. In the bottom row, DNA has been labeled with DAPI. Scale bar: 10 um.*

We next stained left ventricular cardiac tissue from a patient carrying the p.S143P mutation to examine if the lamin aggregates that we had observed *in vitro* were visible also in patients. Few intranuclear lamin aggregates were detected in cardiac tissue obtained from a p.S143P mutation carrier while similar structures were not observed in controls without *LMNA* mutation (III, Figure 1C). These aggregates, however, were only infrequently seen suggesting that they may represent end stage of the cellular phenotype and sensitize for cell death. By exposing the patient fibroblasts to Triton-X extraction and examining the lamina, it became clear that the mutant lamin is more soluble and therefore more prone to mislocalize into the nucleoplasm (III, Figure 1D-E). This would suggest that a lower amount of lamin monomers is incorporated into the lamina in the patient fibroblasts. These results were further confirmed by a solubility assay. The lysed patient fibroblasts and WT controls were dissolved first in low detergent, centrifuged and collected and then dissolved in detergent along with high salt concentration. In the control cells, lamin A/C was recovered in the insoluble fraction, but in the patient cells, some of the lamin A/C was recovered in the detergent with high salt fraction. These results indicate that the mutant p.S143P lamin A/C is more soluble. The increased solubility could be caused by the mutation in several ways. One plausible explanation is, that the amino acid substitution S>P, that the mutation induces, causes a conformational change that promotes the display of more soluble amino acids on the surface of the lamin monomer. Another possible explanation is, that the mutated serine is a phosphoserine that is connected to an inducible change in solubility. If the proline mimics a permanently phosphorylated serine at the position which is occupied by a serine in the WT lamin A/C, this could induce solubility and decrease the capacity of the mutant lamin A/C to incorporate into the lamina. Alternatively, if the serine is indeed a phosphosite which is needed to regulate solubility, its removal and replacement by a non-phosphorylateable proline could shift the solubility of the protein under certain conditions.

To visualize the aggregates in more detail, HeLa cells expressing either FLAG- or GFP-tagged p.S143P mutant lamin A were prepared for transmission electron microscopy (TEM) analysis. In both cases the aggregates appeared highly solid and electron dense, presumably containing compacted lamins (III, Figure 2C-D). To investigate the mobility of the mutant lamin in living cells, a fluorescence loss in photobleaching (FLIP) analysis was used. After repeated photobleaching of one half of the nucleus, a more significant loss of fluorescence signal in both the nucleoplasmic and the lamina regions was observed in GFP-p.S143P-lamin A expressing cells when compared to GFP-WT-lamin A. This shows that the overall mobility of mutant lamin A is increased (III, Figure 3).

3.2 p.S143P *LMNA* mutation causes an assembly defect

We also performed *in vitro* assembly experiments with recombinant protein to investigate polymerization capacity of the p.S143P lamin A. While WT lamin A assembled into regular filamentous structures (III, Figure 4A-C), the p.S143P mutant lamin A reached an irregular and aggregated state (III, Figure 4E-F). When mutant and WT lamin A was assembled in a 1:1 ratio, the polymers reached partial organization but no structures comparable to those assembled from WT lamin A only (III, Figure 4G-I). These results prove that even if mutant lamin A can partially integrate into lamin polymers, its partial integration interferes with the normal assembly steps of lamin A. These results highlight how disruptive this particular lamin mutation is. If the mutation would have had a mild effect on assembly, the 1:1 ratio mixture of WT and mutant lamins could still have generated some normal filaments, since there are plenty of WT lamins that could have assembled on their own even with the mutant lamins present. Conversely, if the mutant lamins would not have had any potential to assemble, they would have been excluded from the polymer completely and again, the WT lamins would have assembled to create some normal filaments. In the experiment, however, no normal filaments formed, and this points to that the mutant not only integrates with the WT lamins into the polymer, but restricts its continued formation.

3.3 The *LMNA* mutation p.S143P causes endoplasmic reticulum (ER) stress

After our thorough biochemical analyses, we turned our focus to the transcriptome of the p.S143P lamin A/C patient cells. A whole genome expression analysis showed that the patient cells carrying the mutant and the control cells formed two distinct groups by Pearson metrics (III, Figure 5A). A heatmap of all gene expression changes also showed two clear groups representing patients and controls (III, Figure 5B). A Gene Set Enrichment Analysis (GSEA) was performed on the transcription data to analyze the expression changes in subgroups of genes associated with specific processes. The analysis showed that changes in expression of unfolded protein response (UPR) genes were overrepresented in the patient cells (III, Figure 5 D). Several genes involved in the UPR were noted on the top of the list, such as X-box binding protein 1 (XBP1) (III, Figure 5C). The UPR is typically caused by accumulation of unfolded or misfolded proteins at ER and thus, causing ER stress.To investigate if the ER stress pathway is indeed affected in the cells with the p.S143P lamin A/C mutation, we performed a real-time polymerase chain reaction (RT-PCR) on key UPR transcripts. One of these is a transcription factor XBP1 which under

normal conditions remains unspliced (XBP1u) and resides in the cytoplasm. Upon ER stress, the inositol requiring enzyme 1 (IRE1α) induces splicing of a 26-nucleotide sequence from XBP1u mRNA to yield the activated form, spliced XBP1 (XBP1s). When translated, the splicing results, through a frame shift, in the shorter XBP1u, and the longer XBP1s proteins (III, Figure 5E). Consistently, we detected a significant induction of the activated XBP1s in lamin mutant cells, along with UPR related genes *exosome component 7 (EXOSC7)*, *sulfotransferase family 1A member 4 (SULT1A4)*, *asparagine synthetase (ASNS)* and *sequence receptor subunit 1 (SSR1)* (III, Figure 5F). There was significantly more activated XBP1s in primary patient fibroblasts when compared to controls (III, Figure 5G). Also, eukaryotic translation initiation factor 2A (eIF2α) was induced in its phosphorylated form in the primary patient fibroblasts. In its phosphorylated state, eIF2α suppresses protein translation during ER stress.

To confirm that the ER-stress was specifically induced by the mutant lamin, control primary fibroblasts were transfected with FLAG-tagged WT and mutant lamin A constructs. Both phospho-eIF2α and XBP1s were enriched in mutant lamin expressing fibroblasts relative to controls (III, Figure 6A-B). To evaluate if we could increase lamin aggregate formation by inducing ER stress with tunicamycin (TM), the numbers of cells with lamin aggregates after 0, 4, 6 and 24 hours of TM treatment were determined. There was an increased number of cells with aggregates in the lamin mutant expressing cultures after 4 hours of TM treatment, but a decreased number after 24 hours of treatment (III, Figure 6C). To investigate if the proportional decrease in cells was due to cell death, we also analyzed cell count before and after TM treatment. The results showed that there were significantly less viable cells in mutant expressing cultures after TM treatment suggesting that mutant lamin may sensitize cell for cell death (Figure 6D). Since ER-associated degradation is mediated through the ubiquitin-proteasome pathway, we also inhibited the proteasomal degradation with MG132, which significantly increased the number of aggregates in lamin mutant transfected cells (III, Figure 6E). Finally, to test whether interference with the UPR can affect aggregate formation, we inhibited the UPR by 4-phenylbutyric acid (4-PBA) treatment. In both untreated and TM treated cells, 4-PBA treatment decreased the number of cells with aggregates (III, Figure 6F). In summary, these results suggest that p.S143P lamin A/C causes UPR either directly or indirectly, and alleviating ongoing UPR may have beneficial effects on processing of mutant lamin and cell viability.

3.4 IPSC-derived cardiomyocytes expressing p.S143P *LMNA* mutation

To test the effect of p.S143P lamin A/C on human cardiomyocytes, the primary patient and control fibroblasts were reprogrammed into iPSCs, further differentiated into cardiomyocytes (CMs) and stained for lamin A and cardiac specific markers. The stainings confirmed that there were less lamin A incorporated into the lamina structure and more nucleoplasmic lamins in the mutant CMs (Figure 23, unpublished data). However, while lamin aggregates were occasionally observed in undifferentiated and immature cells (data not shown), they were not found in the nuclei of mature cardiomyocytes. This may reflect the toxic nature of aggregates for CM maturation under *in vitro* conditions.

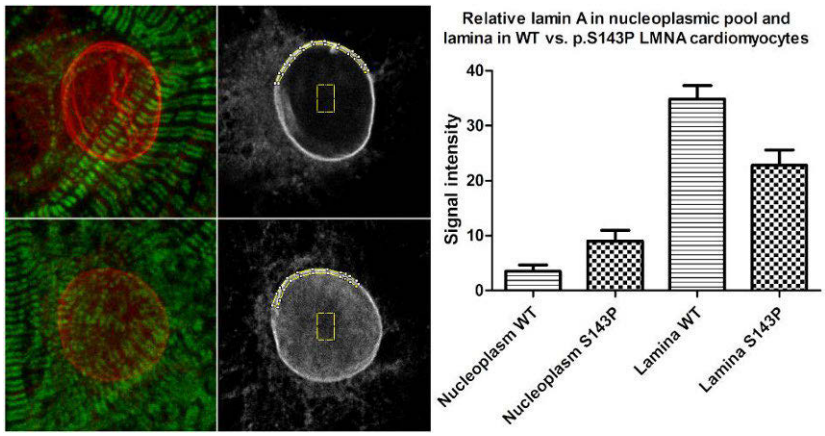

Figure 23. *The amount of nucleoplasmic lamin was compared between iPSC derived cardiomyocytes carrying the WT and the p.S143P LMNA gene. Lamin A is seen in red and cardiomyocyte-specific myosin binding protein cardiac 3 (MyBPC3) in green. The images on the left show maximum projections covering the entire lamina region and sarcomeric structures. The images on the right show single midplane confocal sections from the center of the nucleus. The top row shows WT and the bottom row shows mutant cardiomyocytes (n=4).*

These results confirm that the nucleoplasmic phenotype can be seen also in cardiomyocytes. The fact that there are no intranuclear aggregates in the mutant cardiomyocytes however, is puzzling. In being dedifferentiated to a pluripotent stem cell stage, it is possible that there was minimal lamin A-type expression, since B-type lamins are predominately expressed in stem cells. It is plausible that the aggregate formation is induced by many iterations of nuclear mitotic disassembly and reassembly over a sustained period. Another possibility is simply that the cells that develop aggregates die. If this is the case specifically in the cardiomyocytes, it could

be connected to the cardiomyopathy that the patients suffer from and should be investigated further.

3.5 Expression of the *Drosophila* lamin C mutant p.E158P mimics the human p.S143P lamin A/C *in vivo*

To investigate the effects of studied lamin mutation in an *in vivo* model, we expressed p.E158P mutant *Drosophila* lamin C (mimicking the human p.S143P lamin A/C) in fly in a WT background (unpublished data). The mutation was lethal when expressed ubiquitously in the fly. However, when expressed specifically in the salivary glands, we could detect large nuclear lamin aggregates that were similar to those seen in the human patient cells (Figure 24, unpublished data). Remarkably, when we performed a lethal phase analysis on the flies ubiquitously expressing the lamin mutant, the fatality took place at the larval phase, but after a period multiple times longer than the larval phase normally takes. We can conclude that the mutation inhibits the induction of the pupal phase. If this defect in lamin function is comparable to part of the mechanisms behind the human disease pathogenesis is a subject of future study. Nevertheless, these results show that *Drosophila* can be used to model the consequences of this lamin mutant *in vivo*. This model system might be used as a platform to screen potential small molecular drugs.

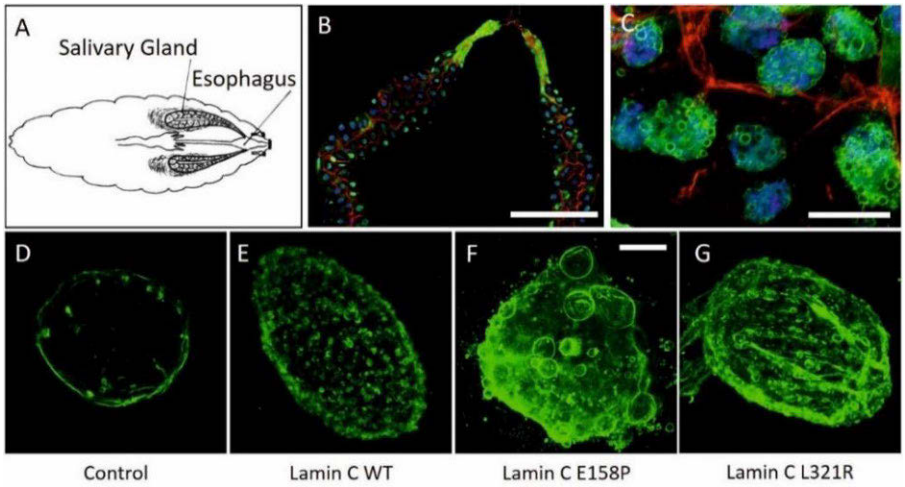

Figure 24. *To investigate the nuclear morphology in the Drosophila expressing the lamin mutants, we dissected and stained salivary glands for confocal imaging (A-B). The nucleus of the Drosophila expressing the human DCM mutation shows similar aggregates as the human patients (C). Normal WT Drosophila salivary gland nucleus (D), lamin WT overexpression control (E), DCM mutant expression (F), Control mutant expression (G). The specimen was stained against lamin C (Green), DNA (Blue), Actin (Red). Scale bars: B – 100 µm, C – 50 µm, D-G– 10 µm.*

These studies illustrate the detrimental effects of p.S143P lamin A/C in cell nucleus and in living organism. This new knowledge may turn out to be critical in the future when novel interventions against DCM are designed and tested. To our knowledge, this is the first study to link laminopathies to cellular ER stress. The remaining question in this respect is whether the ER stress is a cause or result of the disease but previously alterations in ER stress have also been reported in DCM caused by mutations in other genes such as KDEL (Hamada et al., 2004). The p.S143P mutation causes production of aberrant lamin protein and based on our findings it impairs lamin assembly as well as lamin incorporation into the lamina. Since lamin A/C is such a redundant protein, it is perhaps not surprising that massive misfolding would burden the UPR. Alternatively, defects in lamina structure could interfere with the architecture of INM and ONM, the latter being continuous with the ER.

Tissues that are under heavy mechanical stress also rely on other cytoskeletal components than IFs. In the case of muscle, cytoskeletal and nuclear actin are crucial proteins in mechanical function. Studies have not only shown that lamin mutation can affect actin dynamics, but also that this dynamic can affect gene regulation. This is the case with megakaryoblastic leukaemia 1 (MKL1), which is a transcription factor that exposes its NLS following dissociation from actin, promoting its translocation and transcriptional activity. The *LMNA* p.N195K mutation displays altered binding to emerin which in turn is responsible for change in actin dynamics causing deregulation of MKL1 (Ho et al., 2013). Serum response factor (SRF) expression can be induced by MKL1, and SRF deregulation has been shown to cause DCM (Parlakian et al., 2005). This is a mechanism of progression of DCM that should be investigated carefully also in the case of the p.S143P lamin A/C mutation.

A recent study showed that in transgenic mice expressing the human DCM related LMNA mutation p.H222P, WNT signaling was decreased in the heart. The decreased WNT signaling correlated to decrease in the gap junction protein connexin 43. Furthermore, the activation of WNT by 6-bromoindirubin-3'-oxime, was coupled with increased connexin 43 and improved heart function (Le Dour et al., 2017). Therefore the WNT signaling pathway should also be investigated in the p.S143P lamin A/C mutation.

To understand the mechanisms behind cardiomyopathy, it is important to test and validate the mutant lamin phenotype and our findings specifically in cardiac cells. Since many lamin mutations have cardiac specific defects, finding tissue specific phenotypes could prove crucial in explaining the disease progression due to the p.S143P mutation. Such cardiac tissue specific phenotypes could be completely overlooked if we fail to find a reliable cardiac model to study the mutation in. One possible explanation to why cardiac muscle is primarily affected by this lamin

mutation, could be that cardiac muscle is simply subjected to higher physical strain than other tissues *in vivo*. In such case, many cell types would not be expected to show severe defects due to the mutation under normal cell culture conditions. The mutation phenotype would then not reveal itself *in vitro* unless the cells are stressed to a significant degree.

Concluding Remarks

The lamins are precursors of cytoIFs. Simple IF-like proteins such as NE81 of yeast and crescentin of prokaryotes, on the other hand, are most likely precursors of lamins. Instead of asking what functional need the cytoIFs satisfied by being expressed in the cytoplasm, one could ask the equally valid question of what functional need the lamins satisfied in becoming nuclear proteins in the first place. My notion is that the answer to both questions is the need for dynamic and integrated assembly. The constant renewal of the cell is itself a process of assembly and reassembly. All larger organellar structures in the cell depend on the assembly of constituents that can be manufactured by the cellular machinery. The cytoskeletal proteins create large entireties from identical subunits. Subtle changes in a monomer subunit become enormous changes in the polymer structure. This is the reason that understanding how IF mutation causes disease is challenging. The translation of genotype of the IF protein into phenotype of the IF network is not intuitive and needs precise examination.

We are on the brink of a revolution in cellular biology, where new powerful techniques will reshape the way that we can study the cell. Single cell transcriptomics will enable us to more carefully than ever study the effect of cellular events. Reprogramming of cells lets us initiate differentiation events in cells. Combining cellular reprogramming and transcriptomics enables us, with great precision, to induce a specific transition from one cell type into another and to measure precisely what steps the cell needs to take to facilitate that transition. Such studies enable us to reveal exactly which protein expression onset is activated at a differentiation threshold. Furthermore, high resolution transcriptomics makes possible precise readouts after induction of cellular responses to chemical and mechanical intervention. For intermediate filament research, such studies will be of immense importance, as the cell carefully selects between its repertoire of 70 intermediate filament genes to fit the needs of the cell's differentiation state as well as physical status at any given moment. Combined with new live cell imaging techniques, such technologies will reveal to us what needs the cell fulfills and what action it is taking, when it decides to call a specific IF into action. That is information that we need to fully understand the function of the intermediate filaments and their related diseases.

Josef Gullmets Turku 2.5.2017

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16	Current Opinion in Cell Biology	4063620114446
17	Journal of Clinical Investigation	4063640672600
19a	The Progeria Research Foundation	With permission
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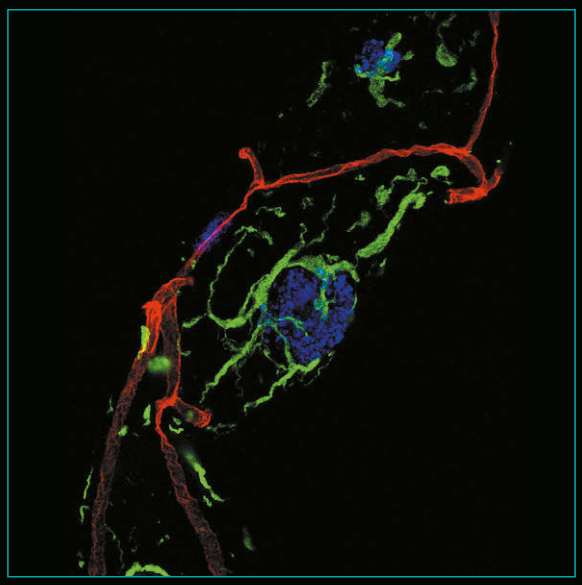
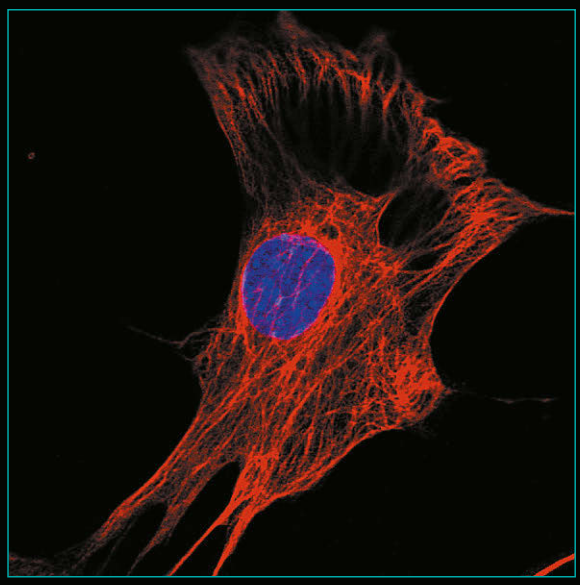
21a	Proceedings of the National Academy of Sc.	Copyright 2008 PNAS
21b	Nature Review Genetics	4076940006020

The common ancestry of species allows us to use comparative biology to draw conclusions about ourselves. In the three studies included in this thesis, different model organisms have been utilized to learn about the functions of three different members of the intermediate filament (IF) protein family.



▲ In the first study, human vimentin is transgenically expressed in *Drosophila* to study cytoplasmic IF expression and function.



▲ In the second study, a nestin null mouse model is utilized to investigate the role of nestin in muscle physiology and regeneration.

▶ The third project included in this thesis investigates a point mutation in the *LMNA* gene associated with dilated cardiomyopathy (DCM). The mutation, p.S143P in lamin A/C, has been identified in the Finnish population and gives rise to late onset DCM with conduction defects.

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