Development of new tools for studying nuclear lamin function

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Abbreviations

AP-1  activation protein 1
CaaX  (cysteine-aliphatic-aliphatic-any amino acid) motif
CHO-K1  Chinese Hamster Ovary K1 cells
EDMD  Emery-Dreifuss muscular dystrophy
EGFP  enhanced green fluorescent protein
FITC  fluorescein isothiocyanate
FPLD  familial partial lipodystrophy
INM  inner nuclear membrane
KD  knockdown
LMNA  A-type lamin gene
LMNB1  lamin B1 gene
LMNB2  lamins B2 and B3 gene
MEF  mouse embryonic fibroblast
NE  nuclear envelope
NLS  nuclear localization sequence
NPC  nuclear pore complex
ONM  outer nuclear membrane
PCR  polymerase chain reaction
Rb  retinoblastoma protein
RNAi  RNA interference
shRNA  short hairpin RNA
siRNA  small interfering RNA
tet  tetracycline
TR  tetracycline repressor
TxR  texas red
WT  wild type
ZMPSTE24  zinc metalloproteinase ste24 (prelamin A processing)
Preface

During this traineeship I have studied lamins, nuclear intermediate filaments with which I was unfamiliar until I attended a lecture during my third year of the Molecular Life Sciences bachelor. Just before I had to choose a trainee post, a so called ‘caput college’ on the presumed role of lamins in disease was thought by Frans Ramaekers. This topic immediately got my attention and made me decide to do a work placement at the Department of Molecular Cell Biology under supervision of Jos Broers on lamins. Besides learning much about these interesting proteins, I learned many new practical skills and had the opportunity to make some great microscopic images.

Acknowledgements

I wish to express my acknowledgements to Jos Broers for supervising me during this project. I would like to thank Jorike Endert, Mieke Henfling, and Helma Kuijpers for helping me at the lab. I also wish to thank Bert Schutte, Kevin Hochstenbach, and Frederik Houben for carefully reading this thesis and making good suggestions. I thank Rob for his patience and understanding during the time of writing this thesis.
Abstract

Lamins are nuclear intermediate filament proteins encoded by the *LMNA*, *LMNB1*, and *LMNB2* genes. They form a filamentous meshwork underneath the inner nuclear membrane (INM) and intranuclear structures. Lamins bind to many nuclear proteins and participate in various intranuclear activities. They can be divided in two subgroups; A- and B-type lamins. While A-type lamins are differentially expressed, B-type lamins are expressed in all cells and are essential for cell survival. Mutations in *LMNA* were found to cause an array of seemingly unrelated diseases. However, no such mutations have been found in *LMNB1* or *LMNB2*. To study lamin A/C and lamin B2 function, two different model systems were developed. To study the functions of A-type lamins an *in vitro* model that allows controllable down- and upregulation of A-type lamin expression according to the ‘Tet-On’ system was developed. This system permits the study of these proteins in genetically identical cells and probably reduces the effects of inter-cell line variability. Controllable lamin A/C expression was achieved in HCT116 colon cancer cells. Lamin A/C knockdown had several effects on these cells; increase in proliferation, altered morphology, and reappearance of the gap-junction protein connexin 43. These findings support the previously suggested role of lamins in regulating cell proliferation. Altered cell-cell adhesion may be caused by an effect of A-type lamins on gene regulation. However, these differences were not confirmed in cells with tetracycline controllable lamin expression. Since cell biological experiments were only started recently these results need further research.

The two main B-type lamins are lamin B1 and B2. Relatively little is known about lamin B2. To investigate lamin B2 function, a lamin B2-enhanced green fluorescent protein (EGFP) expression vector was constructed and transfected into mammalian cells. High lamin B2-EGFP expression resulted in nuclear growth and numerous nuclear abnormalities. Lamin B2 dynamics during mitosis were studied by time-lapse imaging with a fluorescence confocal laser scanning microscope. Interestingly, lamin B2 only reassembled after cytokinesis has completed. This emphasizes differences in lamin B1 and lamin B2 function during mitosis, since lamin B1 reassembles at late anaphase to early telophase. Lamin B2 even seems to relocate into the nucleus after A-type lamins have reassembled.
1 Introduction

Since the discovery of harmful mutations in the lamin A/C gene (LMNA) seven years ago, A-type lamin research has regained attention. Lamins belong to the intermediate filament protein superfamily and form a major part of the nucleoskeleton. The mechanism by which mutations in these karyoskeletal proteins cause disease and alter nuclear function remains to a great extent a mystery. To explain the normal functioning of A-type lamins and the phenotypic diversity observed in laminopathies, several not mutually exclusive theories have been proposed. Firstly, based on lamin structure and localization they provide a structural framework that influences cellular stability and mechanics similar to other cytoskeletal proteins. Secondly, by interacting with nuclear proteins, A-type lamins could influence the functioning of these proteins or act as regulators of gene expression. At this moment, supportive data for both theories have been published, which underlines the complexity and variety of A-type lamin functions.\(^1\) It is obvious that many aspects about this topic have to be clarified and that the theories describing lamin A/C functioning need fine-tuning. A great number of open questions make A-type lamins a challenging research topic.

In contrast to the number of papers attributed to A-type lamins, much less has been published on B-type lamins. In particular lamin B2, one of the three B-type lamin subtypes, is getting little attention. The importance of B-type lamins for basal cell functioning is indicated by their ubiquitous expression and their necessity for cell survival.\(^2\) Which essential cellular needs B-type lamins fulfill are largely unknown. Even less is clear about the distinct functions of the lamin B subtypes. Certainly, B-type lamin research has to catch up with that regarding A-type lamins.

1.1 Lamin essentials

The eukaryotic nucleus is enclosed by the nuclear envelope (NE), consisting of two distinct but interconnected lipid bilayer membranes that separate the nucleoplasmic and cytoplasmic activities. The two NE membranes are termed the inner and outer nuclear membranes (INM and ONM, respectively), of which the latter is in continuity with the endoplasmic reticulum. Both membranes have their own unique protein composition.\(^3,5\) The INM and ONM are separated by an approximately 50 nm wide lumen (the perinuclear space) and contact each other at sites were macromolecular assemblies called nuclear pore complexes (NPCs) are
These NPCs form selective aqueous channels that provide a nucleocytoplasmic transport pathway for macromolecules up to 35-40 nm in diameter. At the nucleoplasmic face of the INM the nuclear lamina is situated; a meshwork of protein polymers. This lamina is composed of nucleus-specific members of the intermediate filament protein family (the lamins), and lamin associated proteins. Lamins can be divided in the closely related A-type and B-type lamins, which are encoded by *LMNA*, *LMNB1*, and *LMNB2* genes. Alternative splicing of the *LMNA* transcript generates 4 A-type lamin variants; lamins A, AΔ10, C1 and C2. Lamins A and C are the main *LMNA* products. They share the first 566 amino acids and have 98 and 8 unique amino acids respectively. The developmentally regulated A-type lamins are virtually absent or less abundant in non-differentiated cells, cells of neuroendocrine origin, and some hematopoietic cell types. In contrast, the B-type lamins, encoded by *LMNB1* (lamin B1) and *LMNB2* (lamins B2 and B3), are constitutively expressed and are indispensable for cell and organism survival. Each cell has at least one lamin B subtype, and both lamin B1 and B2 are expressed in almost every cell type. Lamin B3 has been identified as an alternative splicing product of the *LMNB2* gene and turned out to be restricted to mouse spermatocytes.

1.2 Lamin structure....

The structure of lamins and their organization in higher order assemblies has been studied extensively during the past three decades (reviewed by Stuurman et al. 1998). Lamins have the typical tripartite molecular organization of all intermediate filaments: a central α-helical ‘rod’ domain situated next to short N-terminal ‘head’ and long C-terminal ‘tail’ domains. The rod domain drives the parallel binding to a similar α-helix to form a lamin dimer. Lamin dimers are probably the basic structural units of lamin polymers. At the C- and N-terminal ends of the rod domain are highly conserved segments that are necessary for the assembly of lamin dimers at a higher order level, such as head-to-tail polymerization. The exact higher order organization of lamins is still unknown. While in Xenopus a meshwork of filaments can be observed, filament formation can not be demonstrated in other organisms. Another evolutionary conserved lamin motif is the nuclear localization sequence (NLS) which is required to target lamins to the nucleus. Besides this, several phosphoacceptor sites control the assembly and disassembly of the lamin meshwork by hyperphosphorylation (disassembly) and dephosphorylation (reassembly) during mitosis. In addition, lamins (except lamin C) have a C-terminal CaaX (cysteine-aliphatic-aliphatic-any amino acid) box which is substrate for a sequence of posttranslational modifications, and which targets lamins to the INM.
Interestingly, this box is proteolytically cleaved from the lamin A precursor (prelamin A) to generate mature lamin A, while B-type lamins retain the CaaX motif.\(^5\)

After discussing the molecular structure of lamin subunits, now will follow a short discussion about the \textit{in vivo} organization of lamins. As already mentioned, lamins are mainly located underneath the INM. Besides their presence in the nuclear lamina, there is substantial evidence that lamins are also present in the nuclear interior. These intranuclear lamins are described as intranuclear foci or fibers,\(^12-15\) and nuclear channels where lamins line invaginations of the NE.\(^16\) Lamins are also present as a diffuse nucleoskeleton, termed the ‘veil’.\(^3,15,17\) Although there are many clues about lamin location, dynamics, and interactions, identifying the \textit{in vivo} lamin molecular organization has been difficult and the exact higher level of molecular organization has not yet been established.\(^3\) In several publications it is suggested that lamins exist as two distinct assembly states. A relatively stable and immobile lamin organization is present at the lamina, whereas the nucleoplasmic lamin pool exchanges its lamins more rapidly.\(^3\) To this day it is not known whether there is a difference in quaternary structures of these differentially located lamins.

\subsection{1.3 \textit{and function}}

Various functions are attributed to lamins (see reviews in references \(^3\) and \(^18\)). They have a major structural role in the cell by determining nuclear shape and size and by organizing the nuclear pore complexes in the NE. The reassembly of the NE during mitosis also requires the presence of lamins. Furthermore, numerous nuclear proteins interact with lamins and a vast array of nuclear proteins was shown to depend on lamins for their proper localization at the INM, e.g. emerin. Finally, lamins are thought to play a role in many nuclear activities, including the elongation phase of DNA replication, mRNA transcription and processing, and gene regulation.\(^3,18\)

The differences in tissue distribution of A- and B-type lamins\(^9\) suggest distinct roles for these two groups of lamins. Supportive data came from RNAi experiments. Cells stopped growing and eventually went into apoptosis when lamin B1 or B2 was knocked down, illustrating that B-type lamins are essential for cell viability. A-type lamin targeting by siRNA, in contrast, resulted in phenotypically distinct but still viable cells.\(^2\) The functional differences between A- and B-type lamins are further exemplified by the presence of harmful mutations in \textit{LMNA}.\(^19\) To this day, an attempt to find such mutations in B-type lamins has failed.\(^10\) The general opinion is that A-type lamins have a role in the proper functioning of differentiated,
adult tissues. Obviously, B-type lamins are involved in a number of critical processes, for example mitosis.\textsuperscript{20}

Apparently, lamin functions are numerous and diverse. In the next sections, several aspects of lamin functioning will be set out in detail. First, the role of lamins, in particular B-type lamins, in mitosis is discussed. Next, current knowledge of A-type lamins is addressed by summarizing the understanding of laminopathies and describing two main hypotheses regarding lamin A/C functioning.

1.4 Lamin behavior in mitosis

Mitosis is the phase of the cell cycle at which nuclear division takes place. In most eukaryotic cells the NE is disassembled at the onset of cell division (‘open’ mitosis), which is dissimilar from a ‘closed’ mitosis, in which the integrity of the NE is retained. During this process the nuclear lamina has to be depolymerized and the NE is fragmented.\textsuperscript{21} Lamin phosphorylation by cdc2 (Cdk1) is needed to disassemble the lamin polymer network at the onset of mitosis.\textsuperscript{22,23} NE breakdown and lamina disassembly seem to be highly orchestrated. However, the temporal organization of this process is still elusive. It has been postulated that at late prophase, B-type lamin polymers become fragmented before they completely dissolve; in contrast, A-type lamin polymers are depolymerized fast at early prophase. B-type lamins are associated with the NE in early mitotic cells,\textsuperscript{24} and the main fraction of B-type lamins remains associated with membranes during the remainder of mitosis.\textsuperscript{25} The A-type lamins behave otherwise; they first diffuse into the nucleoplasm and later into the cytoplasm and are not bound to membranes.\textsuperscript{24}

In order for mitosis to proceed, the NE has to be broken down. Spindle microtubuli may mediate NE disruption by stretching and ultimately tearing the NE.\textsuperscript{26} Moreover, it has been proposed that NE breakdown is dependent on the microtubule associated motor protein dynein which binds the NE. By its minus-end-directed motility, dynein moves along microtubules towards the centrosome. The NE is stretched by this movement and will rupture at the point of maximal tension.\textsuperscript{27} Current understanding of mitosis suggests that B-type lamin fragmentation is needed to weaken the lamina such that tearing of the NE can occur.\textsuperscript{21} B-type lamins also appeared to have a direct role in mitosis by assisting mitotic spindle formation and creating a spindle matrix that tethers the so-called ‘spindle assembly factors’. It was suggested that lamin B2 is the structural component of the spindle matrix that regulates microtubule dynamics during mitosis.\textsuperscript{20} Another event that presumably helps NE breakdown is
phosphorylation of NPCs. The phosphorylated nucleoporins leave the NPCs and thereby permeabilize the NE. During telophase the NE reassembles around the condensed chromosomes. There is some controversy in literature about the temporal organization of lamin reassembly. Lamin reassembly is probably assisted by dephosphorylation and depends on recruitment of protein phosphatase 1 (PP1) to the INM. It is still matter of debate whether lamin B1 assembles before or after the NE and the NPCs are formed. Lamin A accumulates in the nucleoplasm after NE and NPC formation and is later gradually incorporated into the lamina. Some data support an active participation of lamins in NE assembly, by assisting chromosome decondensation, NE formation, and NPC assembly. Certainly, the last word in regard to lamina and NE reassembly has not yet been spoken.

1.5 The laminopathies
Although A-type lamins are dispensable for cell functioning, they have an important role in differentiated tissues, inasmuch as alterations in these proteins cause an overwhelming number of inherited diseases (reviewed in Jacob and Garg, reference 19). Mutations causing disease in A-type lamins were only recently identified and boosted interest in these proteins. In 1999, it was shown that four LMNA mutations cosegregated with the autosomal dominant Emery-Dreifuss muscular dystrophy (EDMD) phenotype. During the same year, Lmna was knocked out in mice to further examine the roles of lamins A and C. The Lmna knockout mouse model phenotypically resembled EDMD pathology, including muscular dystrophy and mislocalization of the INM protein emerin. To date, approximately 7 years after the initial discovery of harmful LMNA mutations, at least 11 different diseases are described to be caused by A-type lamin mutations and probably more will be identified in the future. The LMNA disorders are collectively referred to as laminopathies. Among these diseases are striated muscle disorders, partial lipodystrophies, peripheral nerve diseases, and rare premature aging syndromes such as progeria. Surprisingly, these diseases are clinically distinct and affect various cell types. Involved tissues are striated muscles (e.g. EDMD), adipose tissue (familial partial lipodystrophy type 2, FPLD2), and peripheral nerve cells (Charcot Mary Tooth disorder type 2, CMT2). The premature aging syndromes affect many tissues and are therefore also called ‘systemic laminopathies’. Only recently, both mutations in LMNA or in the prelamin A protease gene ZMPSTE24 were discovered in patients having the rare disorder ‘restrictive dermopathy’. These mutations
cause either accumulation of truncated (in the case of \textit{LMNA} mutations) or normal prelamin A (in the case of \textit{ZMPSTE24} mutations), due to the defective prelamin A processing.\textsuperscript{32}

It has been suggested that the heterogeneity of laminopathies can be partly explained by the location of the mutation in the lamin molecule. Clustering of disease phenotypes and statistical analysis has led to a division of laminopathies in two groups, one containing muscle diseases, atrial fibrillation, and CMT, the other encompasses progerias, mandibuloacral dysplasia, and lipodystrophy. This division suggests a difference in pathologic mechanisms for these two groups.\textsuperscript{33} Functional studies have provided more evidence for this theory. For example, lipodystrophy related \textit{LMNA} mutations did not result in defective lamin A targeting or prevention of the lamin A-emerin interaction, as is the case for EDMD.\textsuperscript{34} However, Vigouroux et al. showed NE disorganization in cells having a FPLD mutation,\textsuperscript{35} suggesting mechanical defects in these cells. Besides this, laminopathies show considerable disease heterogeneity and some individuals may be unaffected despite the presence of a disease related mutation in \textit{LMNA}. FPLD patients, for example, can have clinical features of other laminopathies, like cardiomyopathy and atrial fibrillation.\textsuperscript{19,33} Some phenotypic variability may be explained by the influence of modifier genes or environmental factors.

1.6 Lamin A versus lamin C

Recently, new evidence concerning the distinct roles of lamin A and C in disease has been provided. Fong et al. (2006) showed unexpectedly that lamin A and prelamin A are dispensable in the nuclear lamina. They developed an elegant model to test the necessity of lamin A in mice. Mice having only lamin C, called lamin C-only mice, appeared to show an entirely normal development. By generating lamin C-only/\textit{Zmpste}\textsuperscript{-/-} mice it was also illustrated that the presence of the lamin C allele, while lamin A is absent, partly rescues the \textit{Zmpste}\textsuperscript{-/-} phenotype. This supports the idea that lamin A function is redundant and that lamin A and prelamin A are dispensable in mice.\textsuperscript{36} However, this is contradicted by earlier results published by Vaughan et al. (2001). They illustrated that both emerin and lamin C depend on lamin A for their correct localization.\textsuperscript{37} Strangely, lamin C-only cells target their emerin appropriate to the NE. The same holds true for lamin C, which was normally localized at the NE in these cells.\textsuperscript{36} Besides the results presented by Vaughan et al., another observation doubts the dispensability of lamin A; dilated cardiomyopathy causing mutations that affect only lamin A and not lamin C have been reported.\textsuperscript{19} It is certainly possible that mutations affecting only lamin A are toxic to cells and disease is not the consequence of dysfunctional lamin A. The inconsistencies in literature might be explained by interspecies differences in
lamin functioning or cell type specific variations of A-type lamin functions. While the group of Vaughan used two human cell lines (one cervix carcinoma and one adrenal cortex carcinoma), Fong et al. exclusively used murine cells. Clearly, more studies have to be performed to clarify the relative roles of lamins A and C.

1.7 Two hypotheses explaining lamin A/C functioning
Researchers are currently trying to solve the paradox that mutations in a single gene can cause multiple phenotypically distinct diseases. Insights into lamin structure and function led to several models that are not necessarily mutually exclusive.

1.7.1 The mechanical stress hypothesis
The mechanical stress hypothesis states that lamin mutations disrupt the structural integrity of the nucleus. The nucleoskeleton in cooperation with the cytoskeleton protects the cell from physical damage. Most vulnerable cell types are cells that are subjected to mechanical stress and have a low cell turnover, e.g. cardiac and other muscle cells that have to endure mechanical forces during contraction. Changes in cellular stability may result in damage after years of strain on the cell.

Researchers have approached the mechanical hypothesis by making use of various methods. In order to provide direct evidence, cells were subjected to mechanical strain. Also, more indirect evidence has shown its usefulness in explaining the reduced strength seen in lamin null cells. A selection of papers supporting the mechanical hypothesis is discussed below. In the first place, a reduction in resistance to detergent extraction of mutated lamins suggested altered mechanics of lamins as a pathologic mechanism in laminopathies. This is supported by the finding that laminopathy mutations result in increased mobility of lamins in the normal non-dynamic interphase lamina as measured by fluorescence loss of intensity after photobleaching (FLIP). Recently, direct evidence illustrating the importance of A-type lamins in determining cell stability was supplied. Lamin knockout cells were more easily deformed in response to stretching in comparison with wild type cells. Supporting this observation, lamin knockout mouse embryonic fibroblasts (MEFs) appeared to have reduced mechanical stiffness and a lower bursting force, as measured with a cell compression device, when compared to wild type MEFs. Besides this, the nuclei of knockout cells had an isotropic deformation, despite an anisotropic deformation of the cell as a whole, while wild type nuclei deformed anisotropically.
The altered nuclear deformation when A-type lamins are absent may be caused by a defective mechanical link of the nucleoskeleton to the cytoskeleton. The linker of nucleoskeleton and cytoskeleton (LINC) complex has been described as the molecular link between nucleoskeleton and cytoskeleton. This complex is composed of lamins, actin, and several proteins bound to the NE. Lamins are thought to be physically linked to actin via lamin binding SUN domain proteins. Giant nesprins located at both the INM and ONM link to SUN domain proteins via their KASH (KLarsicht, ANC-1, and SYne (nesprin) homology) domain and can bind actin in the cytoplasm.\textsuperscript{41} A newly defined nesprin family member, nesprin 3, might link lamins with other cytoskeletal proteins, the intermediate filaments, via a different linker protein (plectin).\textsuperscript{42} The importance of this link is illustrated by observations of a disorganized cytoskeleton in the vicinity of the nucleus when A-type lamins are absent.\textsuperscript{39} This implies that lamin A/C mutations not only impair nucleoskeletal integrity, but also affect cytoskeletal organization. Apparently, cellular strength can only be maintained if the proteins forming interconnections between the cellular membrane and the nucleus are intact.\textsuperscript{43} Indeed, muscle dystrophy can also be caused by mutations in proteins that are assumed to be linked indirectly to lamins; e.g. desmin, sarcomeric proteins, and proteins of the dystrophin-glycoprotein complex at the plasma membrane of muscle cells. Lack of dystrophin for example makes cells more susceptible to contraction induced rupture.\textsuperscript{44}

Finally, it is important to remark that the mechanical hypothesis is most relevant to just a subgroup of laminopathies. Whereas some mutations in the lamins A and C may alter their structural and mechanical properties or their linkage with proteins in the above described LINC complex, others may affect their role in gene regulation and perhaps the functioning of some currently unknown proteins.

\textit{1.7.2 The gene expression hypothesis}

The mechanical hypothesis can not completely account for the pathogenesis of all laminopathies. For example lipodystrophic laminopathies are more likely to result from altered interactions of the mutated lamins with a tissue specific lamin binding protein. This in turn may affect the expression of differentially regulated genes. There are several ways in which lamins possibly affect gene expression. One way of gene regulation is by binding transcription factors and hence influencing their behavior. Lamins could also be needed to target transcription factors to the nucleus. An alternative mechanism might be regulation of DNA accessibility for transcription factors and the transcription machinery (e.g. regulation of chromatin state). Indeed, lamins appeared to interact with a number of transcription factors
either directly or indirectly for example via emerin. Among these are the retinoblastoma protein (Rb)\textsuperscript{45}, a repressor of the E2F transcription factor complex\textsuperscript{46} and c-Fos, a component of the activation protein 1 (AP-1) transcription factor complex.\textsuperscript{47} Lamins inhibit the proteasomal degradation of Rb by tethering Rb at the nucleus.\textsuperscript{48} A-type lamins have a negative regulatory effect on the transcription factor c-Fos: the c-Fos-lamin A/C interaction suppresses AP-1 activity and decreases proliferation.\textsuperscript{47} Supportive for a role of lamins in proliferation are the observations of lower or absent lamin expression in some fast proliferating cells, such as low differentiated and cancer cells.\textsuperscript{49,50} Other transcription factors that interact with A-type lamins are germ cell-less (GCL), an E2F repressor; sterol response element-binding protein 1 (SREPB1), an adipocyte differentiation factor;\textsuperscript{51} and the zinc finger protein MOK2;\textsuperscript{52}

As seen in previous sections there are many unknown aspects about lamin functioning and several discrepancies in literature that need elucidation. Until now, the outcome of alterations in A-type lamin expression is only compared in genetically different cell lines, preventing a correct interpretation of lamin function. Since the effect of A-type lamin loss or \textit{LMNA} mutations differs among cell types and even changes with increasing passage number,\textsuperscript{35} it is of great importance to compare cells that have the same genetic background and similar passage number. This allows researchers to find more subtle alterations caused by changes in lamins A/C expression levels. The aim of this study was to test the effect of A-type lamins loss on the cell by an \textit{in vitro} model that permits controllable down- and upregulation of A-type lamin expression. For this purpose, we have transduced a human cell line with a tetracycline regulated lamin A/C RNAi system. By controlling the down- and upregulation of lamins A/C expression in genetically identical cells we are obtaining a better understanding of the functions of A-type lamins.

A second line of research was focused on lamin B2. This is the least studied lamin subtype, although it is as widely expressed as lamin B1. Insights in lamin B2 behavior can be established by several methods. Generally used for detection of lamin B2 are antibodies. However, immunofluorescence analysis is limited to fixed cells. A more precise manner to visualize proteins is by tagging them with a fluorescent label. To study lamin B2 localization a lamin B2-EGFP fusion construct was generated and transfected into mammalian cells.
2 Methods

Controllable knockdown of A-type lamin transcripts was achieved by transducing a human cancer cell line with two vectors, one containing a short hairpin RNA (shRNA) under control of an *E. coli* tet operator that inactivates the *LMNA* transcript, the other constitutively expresses the tetracycline repressor (TR). TR is able to downregulate shRNA expression by binding to tet operator sequences at the shRNA promoter location. ShRNA expression can be rescued by the addition of tetracycline, which prevents TR from interacting with the tet operators. This mechanism is also called the ‘Tet-On’ system, because tetracycline is able to induce shRNA expression. The transduced cells were used to test several aspects of lamin A/C knockdown.

To generate a lamin B2-EGFP fusion protein two incomplete parts of the lamin B2 cDNA that had partly overlapping sequence were fused using cloning vectors. Next, the complete cDNA was inserted into an EGFP expression vector which was used to transfect Chinese Hamster Ovary (CHO-K1) cells. The lamin B2-EGFP transfected cells were used to study lamin B2 behavior during mitosis.

2.1 Cell culturing

HCT116 colon cancer cells were grown on McCoy’s 5A medium containing L-glutamine (Gibco, Paisley, Scotland, UK), supplemented with 10% fetal calf serum (FCS) and 50μg/ml Gentamycin (Eurovet, Bladel, NL). CHO-K1 cells were cultured in Ham F-12 medium containing L-glutamine (Gibco) supplemented with 10% FCS and 50μg/ml Gentamycin (Eurovet). Culture flasks and dishes were kept in a humidified 5% CO₂ incubator at 37°C. Two times a week, cells were passaged by splitting at a 1:5 to 1:15 ratio for HCT116 cells and a 1:8 ratio for CHO-K1 cells using trypsin digestion (0.125% trypsin/0.02 M ethylenediaminetetraacetic acid (EDTA)/0.02 % glucose solution in phosphate-buffered saline (PBS)).

2.2 Generation of cells with controllable lamin A/C knockdown

The human colon cancer cell line HCT116 was transduced with the pLenti4-GW/H1/TO-lamin<sup>shRNA</sup> and pLenti6/TR vectors (Invitrogen Inc, Carlsbad, CA, USA) using the BLOCK-iTTM Inducible H1 Lentiviral RNAi System (Invitrogen). HCT116 cells were first transduced with the pLenti4-GW/H1/TO-lamin<sup>shRNA</sup> vector. After single cell subcloning and selection of
clones with reduced A-type lamin expression, cells were transduced with the pLenti6/TR vector. Transductions were performed as described in the manufacturer’s instructions, except that instead of the addition of polybrene, the cells were centrifuged in 6 well culture dishes (Corning, Corning, NY, USA) for 90 minutes (min) at 2750 G and 25°C (Hettich Rotanta/TRC, Hettich, Tuttlingen, D). pLenti6/TR transduced cells were selected using blasticidin (Invitrogen, 5 μg/ml) for two weeks. ShRNA expression in double transduced cells (transduced with the shRNA and the TR vectors) was induced by culturing for 6 days with tetracycline (Invitrogen, 1 μg/ml).

2.3 Subcloning of shRNA transduced cells
HCT116 cells subjected to pLenti4-GW/H1/TO-lamin^{shRNA} (Invitrogen) transduction were subcloned to single cell colonies in a 96 well culture dish (Corning) by limited dilution. One week later, the plate was screened for the presence of colonies and the number of colonies per well was evaluated. Colonies were propagated in 24 well culture dishes (Corning) and subsequently in 25 cm^2 culture flasks (Corning). Each clone was screened for A-type lamin expression using immunofluorescence (see section ‘immunofluorescence staining’). After transduction with the pLenti6/TR vector (Invitrogen) monoclonal double transduced cell lines were obtained using the same procedure.

2.4 Immunofluorescence staining
For immunofluorescence purposes, cells were plated onto 18 mm glass coverslips (Menzel-Gläser, Braunschweig, D) in a 12 well culture dish (Corning) and grown for two days before fixation in methanol (-20°C, 10 min) or 3.7% formaldehyde in PBS (room temperature (RT), 10 min). After formaldehyde fixation, cells were permeabilized using 0.1% Triton X-100 for 10 min at RT. Before and after fixation and/or permeabilization, cells were washed with PBS. Thereafter, cells were incubated with primary antibodies (depicted in the table 1) for 1 hour (hr) at RT. All antibodies used for immunofluorescence microscopy were diluted in PBS containing 1.5-3% bovine serum albumin (BSA, Roche, Basel, CH) and a total of 50 μl antibody solution per coverslip was applied.

After washing in PBS, secondary antibodies were incubated for 1 hr at RT. Fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse Ig (RaM-FITC, DAKO, Glostrup, DK) diluted 1:100, or Texas Red (TxR) conjugated goat anti-mouse Ig (GaM-TxR; Southern Biotechnology Associates, SBA/ITK, Birmingham, AL, USA) diluted 1:80 was used to detect
murine primary antibodies. TxR-conjugated goat anti-rabbit Ig (GαR-TxR; SBA/ITK) diluted 1:80 was used to detect rabbit primary antibodies.

Finally, slides were washed with PBS and mounted in 90% glycerol, 0.02 M Tris-HCL pH 8.0, 0.8% Na3, 2% 1,4 diazoabicyclo (2,2,2)-octane (DABCO, Merck, Darmstadt, D) containing 0.5 μg/ml diamidino-2-phenylindole (DAPI, Sigma, St Louis, MO, USA) or 1 μg/ml propidium iodide (PI, Calbiochem, San Diego, CA, USA) for DNA staining.

Table 1. Antibodies used for immunofluorescence analysis and immunoblotting

<table>
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<th>Antibody</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Jol-2</td>
<td>A mouse monoclonal antibody (MoAb) IgG1 that reacts with both lamins A and C (1:50 dilution). Jol-2 was kindly provided by Dr C.J. Hutchison (Durham, UK).</td>
</tr>
<tr>
<td>133A2</td>
<td>133A2 (mouse MoAb, IgG3), was a kind gift from Dr Y. Raymond (Montréal, Canada). This antibody binds specifically lamin A and was diluted 1:100.</td>
</tr>
<tr>
<td>RαLC</td>
<td>Polyclonal rabbit anti-lamin C (RαLC, dilution 1:50, MuBio Products BV, Maastricht, NL), recognizes only lamin C, and not lamin A.</td>
</tr>
<tr>
<td>RαLB</td>
<td>Polyclonal rabbit anti-lamin B1 (RαLB, 1:200) was kindly provided by Dr J.C. Courvalin (INSERM, Paris, France).</td>
</tr>
<tr>
<td>NCL-Emerin</td>
<td>Mouse MoAb NCL-emerin (IgG1) to the nuclear membrane protein emerin from Novocastra (Newcastle upon Tyne, UK) was diluted 1:60.</td>
</tr>
<tr>
<td>GJA1-ab113669</td>
<td>A mouse MoAb (IgG1) that binds connexin 43 (Abcam, Cambridge, UK). GJA1-ab113669 was used at a 1:500 dilution.</td>
</tr>
<tr>
<td>Anti-p62</td>
<td>A mouse MoAb directed against nucleoporin p62 (IgG2b, Transduction Laboratories, San Diego, CA, USA), was used at a dilution of 1:300.</td>
</tr>
<tr>
<td>C-1821</td>
<td>C-1821 (Sigma) is a mouse MoAb IgG1 that recognizes all cadherins. A 1:500 dilution was used.</td>
</tr>
<tr>
<td>6F9</td>
<td>6F9 from MuBio Products is a mouse monoclonal IgG1 specific for epithelial cadherin (E-cadherin), and was diluted 1: 100.</td>
</tr>
<tr>
<td>LN43</td>
<td>An antibody specific for lamin B2 (mouse monoclonal IgG1, 1:5 diluted) was generously provided by professor E.B. Lane (Dundee, UK).</td>
</tr>
<tr>
<td>p21</td>
<td>A mouse MoAb (IgG1) directed at p21 was bought from BD Transduction Laboratories and used at a 1:100 dilution.</td>
</tr>
<tr>
<td>X67</td>
<td>An antibody specific for lamins A and C, kindly provided by Dr G. Krohne (Wuerzburg, Germany)</td>
</tr>
</tbody>
</table>

2.5 Quantification of lamin A/C expression by gel electrophoresis and immunoblotting

To quantify A-type lamin expression, lamin protein levels were analyzed by gel electrophoresis and immunoblotting.

2.5.1 Sample preparation

Cells were counted and lysed in lysisbuffer (20*10⁶ cells/ml, 250 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma) pH 7.0, 5mM EDTA, 1% NP-40) on ice for 30 min. Part of the lysate was centrifuged at high speed in a refrigerated Sigma 2K15 centrifuge (15 min 10000 G, 4°C). The supernatant was removed and 100 μl lysisbuffer (250 mM NaCl, 50 mM HEPES (pH 7.0), 5mM EDTA, 0.1% NP-40) was added to the pellet. The sample was sonicated using a Soniprep 150 sonicator (MSE Scientific
Instruments, Beun-De-Ronde) for 5 seconds (amplitude: 12 micron). Next, an equal volume of sample buffer (62.5 mM Tris-HCL, 10% glycerol, 2.3% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 0.05% bromophenol blue) was added to this suspension for gel electrophoresis.

2.5.2 Gelelectrophoresis and immunoblotting
Samples (containing the equivalent of $10^7$ cells/ml) were boiled for 5 min and afterwards centrifuged for 3 min in a microfuge (Sigma). Samples (25 μl) were electrophoresed (1 hr at 200 V) in a 10% SDS polyacrylamide electrophoresis gel using a Mini-Protean II system (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and blotted onto nitrocellulose sheets (Protran BA85, Schleicher and Schuell Bioscience, Dassel, D) for 1 hr at 100 V (transfer buffer: 25 mM Tris, 190 mM glycine, 20% v/v methanol).

Nitrocellulose membranes were stained with Ponceau S (Fluka BioChimica, Buchs, CH) to visualize proteins, which was afterwards washed away with PBS. Membranes were blocked for 1 hr with blocking buffer (PBS/0.05% Tween 20/5% non-fat dry milk (NFDM)). X67, diluted 1:250 in blocking buffer, was incubated for 1 hr at RT. Thereafter, membranes were rinsed in PBS/0.05% Tween 20 (3 x 10 min). Secondary antibody (peroxidase conjugated rabbit anti-mouse, RαM-PO) was diluted 1:10000 in blocking buffer, and incubated for 60 min. Membranes were washed with PBS/0.05% Tween 20 (3 x 10 min, 60 min). Peroxidase activity was visualized using an enhanced chemiluminescence (ECL) kit (West Dura; Pierce Biotechnology, Rockford, IL, USA) according to the supplier’s instruction and exposure to X-ray film (Fuji Photo Film Europe, Düsseldorf, D).

2.6 Vital imaging and fluorescence microscopy
Phase contrast vital images of cells were acquired on a Leica DM-IRBE inverted microscope. Immunofluorescence and EGFP expression were evaluated using confocal and fluorescence microscopy. A Bio-Rad MRC600 confocal microscope (Bio-Rad Laboratories Ltd) equipped with an air-cooled Argon-Krypton mixed gas laser and mounted onto an Axiophoto microscope (Zeiss) was used as described previously.39 Alternatively, images were recorded with the Metasystems Image pro System (black and white CCD camera, Sandhausen, D) mounted on a Leica DM-RE fluorescence microscope. Images were recorded with either automated integration times or fixed integration times to compare fluorescence intensities.
All immunofluorescence images were recorded with a 40x oil lens. Vital imaging and immunofluorescence photographs were processed using Photoshop 7.0 (Adobe systems, San Jose, CA, USA).

2.7 Proliferation assay
A proliferation assay was set up to compare the proliferative capacity of HCT116 cells with reduced and normal lamin A/C expression. We tested the following cell populations: wild type, lamin A/C knockdown, and shRNA and TR double transduced cells cultured with and without tetracycline. Cells were seeded at three different densities (200,000, 100,000, and 50,000 cells/well) in a 6 well culture dish (Corning) in duplicate and the total number of cells was determined 48 hr post seeding. To determine significance of obtained results, a Student’s T-test was used (two-tailed, unequal variances).

2.8 Apoptosis assay and sensitivity to apoptosis inducing agents
HCT116 wild type and lamin A/C knockdown cells were seeded at 3*10^5 cells/well in a 12 well culture dish (Corning). The next day, cells were incubated for 8 hr at 37°C with or without the presence of an apoptosis inducing agent (roscovitine, 50 μM, or etoposide 50 μM). Roscovitine was a kind gift from Dr L. Meijer (Station Biologique CNRS, Roscoff, France). Etoposide was bought from Calbiochem. The supernatant was collected and cells were washed once with PBS. Cells were trypsinized and resuspended in culturing medium. The cell suspension and supernatant were mixed and centrifuged. The pellet was washed with PBS and fixed in cold (-20°C) methanol. After washing with PBS/BSA (1 mg/ml, Roche), cells were incubated for 1 hr at RT with M30 CytoDEATH (66 ng, IgG2b, Boehringer Mannheim GmbH, Mannheim, D). This monoclonal antibody detects apoptotic cells by binding to caspase cleaved cytokeratin 18. After washing twice with PBS/BSA, cells were incubated overnight at 4°C with FITC-conjugated F(ab)2 fragments of rabbit anti-mouse Ig (F0313; DAKO A/S, ITK Diagnostics, Uithoorn, NL) in a 1:10 dilution. After rinsing in PBS/BSA, cells were resuspended in PBS/BSA/100 μg/ml RNase/20 μg/ml PI (Calbiochem). Fluorescence was analyzed using a FACSort flow cytometer (Becton-Dickinson, Sunnyvale, CA) and Cell Quest Software (Becton-Dickinson). Excitation was done at 488 nm, and emission was detected using a 600 nm long pass emission filter for PI signal and a 515-545 nm band pass emission filter for FITC signal.
2.9 Measurement of DNA-content

The flow cytometer was used to measure DNA content and analyze cell cycle stage of wild type and lamin A/C knockdown HCT116 cells. Approximately $10^6$ cells were rinsed with PBS and fixed in methanol (-20°C) for 20 min. Thereafter, cells were washed in PBS/BSA (1 mg/ml, Roche) and suspended in PBS/BSA/PI (20 μg/ml, Calbiochem). After a 15 min incubation period, PI signal was recorded by flow cytometric analysis using a FACSort flow cytometer (Becton-Dickinson) as described in the previous section. Cell cycle analysis was carried out by analyzing DNA histograms with ModFitLT 2.0 cell cycle analysis software (Becton-Dickinson).

2.10 Description human lamin B2 RT-PCR product

A PCR product of reverse transcribed T24 (a human bladder carcinoma cell line) mRNA was used for constructing a lamin B2-EGFP expression vector. The sequences of the forward and reverse primers were respectively; 5’ tcc tcc agc tcc cga atg cga 3’ (B2-Sac) and 5’ cc caa gct tta atg gcc acg 3’ (B2-pVecht). Using these primers, a 1035 bp product was generated. The PCR product comprised the lamin B2 cDNA from the HindIII restriction site at bp 88 to the B2-Sac primer site at bp 1035 (reference sequence: NM_032737).

2.11 Construction of a lamin B2-EGFP expression vector

To construct a lamin B2-EGFP expression vector, two cloning vectors (pGEM-T Easy, Promega (Madison, WI, USA) and pUC19, Invitrogen) and one expression vector (pEGFP-C1, Clontech, Palo Alto, CA, USA) were used. Vectors and sample DNA were cut with restriction endonucleases (0.3 μl of each enzyme, 1.5 μl buffer (supplied with the enzymes), 1 or 2 μl DNA, and adjusted with sterile dimineralized water (milliQ) to a volume of 15 μl) for 90 min at 37°C. Used enzymes were: EcoRI (Gibco BRL), HindIII (Gibco BRL), and SphI (Invitrogen). Digestion products were separated on a 1 or 1.2% agarose gel next to a DNA ladder (250 bp or 1 kb, Invitrogen; 100 bp, New England BioLabs). DNA was mixed with loading buffer and visualized using GelStar (Cambrex Bio Science Rockland, Rockland, Maine, USA). Appropriate bands were excised and purified with the QIAquick gel extraction kit (Qiagen, Valera, CA, USA) per manufacturer protocol and used for ligation.

Ligation of the pGEM-T Easy vector with the lamin B2 PCR product was performed as described in the manufacturer’s instructions. pUC19 and the lamin B2 3’ part were ligated at a 1:2 ratio (4 μl vector DNA, 8 μl sample cDNA, 0.8 μl ligase, 2 μl buffer, 4.2 μl sterile milliQ). Both the pUC19-lamin tail vector with the PCR product and pEGFP-C1 vector with the lamin
B2 cDNA were ligated using 4 μl vector DNA, 8 μl sample cDNA, 1.2 μl ligase, 2 μl buffer, 4.8 μl sterile milliQ. Ligations were performed for 180 min at RT.

Before transformation of chemically competent cells (DH5α, Invitrogen), the DNA was purified with the QIAquick gel extraction kit (Qiagen) per manufacturer protocol. Competent cells were removed from -80°C and thawed on ice. The ligation products were added to 100 μl competent cells and incubated for 20 min. Cells were heat shocked 60 seconds at 42°C and afterwards kept on ice for 5 min. After the addition of 700 μl Luria Betani (LB) medium, cells were incubated for 90 min in a shaking incubator at 37°C. Next, the bacteria were transferred to LB agar plates containing ampicillin (100 μg/ml) as a selective agent and grown overnight at 37°C. The next day, single colonies were inoculated into 8 ml LB medium containing ampicillin (100 μg/ml) and expanded overnight in a shaking incubator at 37°C. Blue-white screening was used to select pUC19 transformed colonies. White colonies indicate the presence of an insert. Per agar plate, 100 μl 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 20 μl 50 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) was added and plates were screened for white colonies the next day. Alternatively, IPTG and X-Gal were added directly to the medium to an end concentration of 0.05 mM IPTG and 80 μg/ml X-Gal. Single white colonies were expanded for 24 hr in 8 ml LB medium containing ampicillin (100 μg/ml).

Vectors were isolated from bacteria using the Wizard Plus SV Minipreps DNA purification spin protocol (Promega) according to the manufacturer’s instructions.

2.12 Restriction analysis

Restriction analysis was used to determine whether the correct fragments were inserted into the vectors and whether their orientation was correct. Used enzymes are depicted in table 2. 0.3 μl of each restriction enzyme, 1.5 μl buffer, and 2 μl DNA was used and adjusted to a volume of 15 μl with sterile milliQ. Restriction was performed for 90 min at 37°C. The endonuclease restriction products were electrophoresed in a 1% agarose electrophoresis gel, containing GelStar (Cambrex). A 250 bp and a 1 kb DNA ladder (Invitrogen) were used to estimate band sizes of the restriction products. DNA bands in the gel were visualized under UV trans-illumination (Mighty Bright UVTM-25, Hoefer, San Francisco, USA) and photographed using a Kodak DC40 camera (Kodak, Rochester, NY, USA).
Table 2. Endonuclease restriction analysis

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Restriction enzymes</th>
<th>Expected fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamin B2 cDNA bp 88-1035 in pGEM-T Easy</td>
<td>SacI (Fermentas)</td>
<td>191, 719, 3073</td>
</tr>
<tr>
<td></td>
<td>EcoRI + HindIII (Gibco BRL)</td>
<td>975, 2997</td>
</tr>
<tr>
<td>Lamin B2 cDNA bp 340-STOP in pUC19</td>
<td>SacI</td>
<td>572, 3671</td>
</tr>
<tr>
<td></td>
<td>PstI (Gibco BRL)</td>
<td>81, 294, 3868</td>
</tr>
<tr>
<td>Complete lamin B2 cDNA in pUC19</td>
<td>EcoRI + HindIII</td>
<td>1810, 2635</td>
</tr>
<tr>
<td></td>
<td>XhoI (Fermentas)</td>
<td>922, 3523</td>
</tr>
<tr>
<td>Complete lamin B2 cDNA in pEGFP-C1</td>
<td>XhoI</td>
<td>287, 922, 5325</td>
</tr>
<tr>
<td></td>
<td>SacI</td>
<td>106, 719, 5709</td>
</tr>
<tr>
<td></td>
<td>DraIII (New England BioLabs)</td>
<td>784, 5750</td>
</tr>
</tbody>
</table>

Vectors containing the different lamin B2 cDNA fragments were digested with restriction endonucleases. Expected sizes of the restriction fragments are given in the third column.

2.13 Transfection of mammalian cells with a lamin B2-EGFP expression construct

CHO-K1 cells were plated in a 6 well culture dish (Corning) and transfected after two days of culturing with the lamin B2-EGFP expression vector using GeneJammer (Stratagene, La Jolla, CA) following the manufacturer’s instructions. DNA concentration was measured with the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). 1.6 μg DNA per well was used with 5 μl GeneJammer (Stratagene). Transfected cells were selected using geneticin (5μg/ml, Gibco BRL) for 20 days. EGFP expression was evaluated using an inverted fluorescence microscope. Thereafter, two rounds of subcloning were performed to obtain a monoclonal lamin B2-EGFP expressing cell line. First, cells were subcloned in a 96 well culture dish (Corning) at a density of approximately 10 cells/well. After screening for lamin B2-EGFP expressing colonies, these cell populations were subcloned in 96 well culture dishes (Corning) to single-cell colonies by limited dilution. Single colonies showing EGFP fluorescence in the nucleus were selected to maintain in culture and used for further experiments.

2.14 Live fluorescence imaging of lamin B2 during mitosis

Lamin B2 dynamics during cell division were studied by vital imaging. Cells were grown on 20 mm diameter coverslips (Menzel-Gläser) in 12 well culture dishes (Corning) and mounted on slides with round wells (16-18 mm diameter and 0.6-0.8 mm in depth) containing 20 μM HEPES (Sigma) and 100 μl culturing medium. Excessive medium was removed and slides were immobilized with nail polish. Cells were imaged using a confocal scanning laser microscope (see section 2.6) and dividing cells were photographed every 30 seconds starting at metaphase. Slides were kept at a constant 36-37°C with the use of a fan.
3 Results

Lamin A/C knockdown was achieved by stably transducing HCT116 colon cancer cells with a \textit{LMNA} transcript targeting shRNA vector. The transduction with a second vector encoding the tetracycline repressor rescued lamin A/C expression by preventing shRNA transcription. ShRNA expression was induced again by adding tetracycline (Tet-ON system).

3.1 Reduced A-type lamin expression by RNAi

Cells transduced with the lamin A/C targeting shRNA vector were immunofluorescently stained for A-type lamins, lamin A, and lamin C to evaluate the reduction in A-type lamin expression. In figure 1A it is shown that HCT116 cells treated with A-type lamin targeting shRNA (shRNA treated, arrow) had a reduced A-type lamin expression (green) compared to wild type HCT116 cells (wt). This reduction was seen in a substantial proportion of the cells but was very heterogeneous; cells with and without reduced A-type lamins were present and the fluorescence levels varied.

To obtain a population of cells that shows a stably repressed expression of A-type lamins, the transduced cells were subcloned in a 96 well culture dish to single-cell colonies by limited dilution. After evaluation of the number of colonies/well, wells containing only one colony were screened for their A-type lamin expression by immunofluorescence. Several colonies with reduced A-type lamin expression were chosen to maintain in culture. Figure 1A shows a monoclonal cell line with an increased proportion of low lamin A/C expressing cells (lamin A/C knockdown, kd) compared to the originally transduced cell population (shRNA treated, arrowhead; a single cell expressing higher lamin A/C levels). This figure also illustrates that A-type lamin expression is still heterogeneous, indicating a high variability in the response to the shRNA in genetically identical cells. A second round of subcloning was performed to ensure monoclonality of cells. This did not result in less heterogeneity (data not shown).

To analyze the expression of lamins A and C separately, wild type and knockdown cells were stained with lamin A and lamin C specific antibodies. Figure 1B shows an almost complete loss of lamin A expression (red) in the lamina in lamin A/C knockdown cells (kd) compared to wild type cells (wt). A diffuse staining is seen in the nucleoplasm of both wild type and knockdown cells. To see whether this diffuse signal is specific, MEF cells were stained. In these cells (MEF), less nucleoplasmic lamin A is seen and a higher expression in the lamina is
observed. Lamin C expression (green) is reduced compared to wild type cells (wt) in lamin A/C knockdown cells (kd), represented in figure 1D.

**Figure 1.** Comparison of A-type lamin and emerin expression in wt HCT116 cells and HCT116 cells treated with lamin A/C targeting RNAi. A) Immunofluorescent detection of lamins A and C with an A-type lamin recognizing antibody (green) reveals a reduction in lamin A/C expression in shRNA treated cells (shRNA treated, arrow) compared to wild type cells (wt). Nuclei were counterstained with PI (red). Subcloning of shRNA treated cells resulted in cell populations (hereafter referred to as ‘knockdown’) that have an increased proportion of cells with reduced A-type lamin expression (kd). However, expression is still heterogeneous (higher expression in a single cell, arrow). B) Lamin A expression was analyzed with an antibody that specifically recognizes lamin A (red). Knockdown cells (kd) lose their lamin A expression in the lamina, while a diffuse nucleoplasmic staining is maintained. Wild type cells (wt) have both a nucleoplasmic and lamina staining. MEF cells have less nucleoplasmic and a stronger staining in the lamina (arrowhead). C) Emerin expression (green) in lamin A/C knockdown cells (kd) was comparable to that in wild type cells (wt). D) Lamin C expression is
reduced in knockdown cells (kd) compared to wild type cells (wt) as detected with a lamin C specific antibody (green). All images were visualized with a 40x oil lens.

The cells were also immunofluorescently stained for emerin, because in previous studies it was demonstrated that this NE protein depends on A-type lamins for its correct localization. These studies indicate that emerin is translocated to the ER when no lamin A/C is present. In figure 1C it is illustrated that cells with reduced lamin A/C expression (kd) have no visibly altered localization of emerin (red) compared to wild type cells (wt).

3.2 Regulated A-type lamin expression by the tet-ON system
To rescue lamin A/C expression in knockdown cells, they were transduced with a vector that expresses TR. Again, we used immunofluorescence to monitor A-type lamin expression. As seen in figure 2A the lamin A/C expression (green) can be restored to normal levels by introducing TR in these cells (kd versus kd + TR). By adding tetracycline, the expression can be reduced again (kd + TR + tet), because tetracycline binds TR and prevents the TR-DNA interaction. For comparison we also stained wild type cells (wt).

To quantify A-type lamin expression in shRNA transduced cells a western blot was carried out. In figure 2B it is shown that lamin A/C expression was reduced in knockdown cells (kd and kd + TR + tet) compared with wild type (wt) and rescued cells (kd + TR).

Figure 2. Immunofluorescence (A) and western blot (B) analysis of A-type lamin expression reveal controllable knockdown of lamins A and C in HCT116 cells. Lamin A/C expression can be reduced by siRNA (kd), and rescued by inhibiting shRNA transcription with TR (kd + TR). Knockdown A-type lamin levels can be achieved again by adding tetracycline (kd + TR + tet).
3.3 Cellular changes after lamin A/C knockdown

3.3.1 Altered morphology of cells with low lamin A/C levels
From figure 3 it can be concluded that cells with reduced A-type lamin expression are morphologically distinct from wild type HCT116 cells. Whereas the former (kd) have a flattened shape and form groups of adhering cells, the latter (wt) are spindle-shaped or have multiple extensions and seem to adhere less to each other. Morphology of double transduced cells cultured with or without tetracycline resembled knockdown morphology.

![Figure 3](image.png)

**Figure 3.** Morphology of wild type (wt) and knockdown cells (kd) HCT116 cells photographed after 72 hr culturing (10x magnification, 20x magnification for the insets). Wild type cells have a spindle-shaped appearance and adhere less to each other, while knockdown cells form epithelial like monolayers.

3.3.2 Changes in other protein expression levels of cells with low lamin A/C levels
To investigate whether the morphological changes into the formation of epithelial like monolayers of knockdown cells was dependent on increased expression of adhesion molecules, cells were stained for cadherins and connexin 43. The cell-cell adhesion molecule E-cadherin is normally present in wt HCT116 cells, and is the major adhesion molecule in normal colon epithelia. This, however, could not be confirmed in this study. Immunofluorescence detections of cadherins with pan-cadherin (which binds all cadherins) and E-cadherin (specific for epithelial cadherin) were negative in both wild type and A-type lamin knockdown HCT116 cells (data not shown). Both antibodies were shown to be specific for human cadherins by staining other cell lines (HT29, and a human fibroblast cell line, data not shown).

In figure 4B the results of immunofluorescence detection of the gap junction protein connexin 43 are given. Connexin 43 (green) was absent in the majority of wild type HCT116 cells (wt), while virtually all lamin A/C knockdown cells (kd) cells express this protein. Connexin 43 is
also expressed in double transduced cells cultured with (kd + TR + tet) or without tetracycline (kd + TR).

Figure 4. Expression of gap junction protein connexin 43. Immunofluorescence analysis shows the induction of connexin 43 expression (green) in lamin A/C knockdown HCT116 cells (kd). In wild type cells (wt), almost no connexin 43 specific signal is detected. In shRNA and TR transduced cells connexin 43 is expressed when cultured with (kd + TR + tet) or without (kd + TR) tetracycline. Nuclei were counterstained with PI (red). Images were recorded with a confocal scanning laser microscope.

3.3.3 Lamin A/C knockdown increases cell proliferation
We measured cell proliferation by seeding cells at different densities in 6 well culture dishes and counting those 48 hours later. The lamin A/C knockdown cells showed a marked increase in cell numbers after two days of culturing compared to wild type cells (figure 5). At all seeding densities the number of knockdown cells was significantly increased in comparison with wild type cells (p-value < 0.001) after two days of culturing. Double transduced cells (kd + TR) proliferated significantly faster than wild type cells (p-value < 0.001). Addition of tetracycline to the culturing medium of these rescued cells (kd + TR + tet) resulted in significantly lower proliferation at two seeding densities compared with double transduced cells cultured without tetracycline (p-value < 0.01). At the lowest seeding density, double transduced cells proliferated similar to knockdown cells, while culturing in the presence of tetracycline slowed the proliferation rate in rescued cells to wild type levels.

To establish whether the increase in cell numbers of knockdown cells compared to wild type cells was the result of decreased apoptosis in knockdown cells, we stained cells with M30 CytoDEATH and determined the percentage positive cells using flow cytometry. The results are represented in figure 4B. It was found that lamin A/C knockdown cells even had a higher number of apoptotic cells than wild type cells. After induction of apoptosis with two apoptosis inducing agents (roscovitine and etoposide), this difference was maintained.
Figure 5. Increase in proliferation in lamin A/C knockdown cells. A) Analysis of cell proliferation indicates that A-type lamin knockdown cells have a significant increase in cell numbers after two days of culturing compared to wild type cells. Double transduced cells (kd + TR) proliferated significantly faster than wild type cells. When these cells were grown in the presence of tetracycline (kd + TR + tet) no difference was observed at the highest seeding density, while at the other densities there was a decrease in proliferation. B) The increase in cell number of knockdown cells compared with wild type cells was not the result of decreased apoptosis in knockdown cells. Wild type cells had even a lower percentage of apoptotic cells than knockdown cells measured with M30 CytoDEATH (control). Also, after induction of apoptosis with two apoptosis inducing agents (roscovitine and etoposide), knockdown cells had a higher percentage of apoptotic cells.

3.3.4 Reduction of A-type lamin levels results in altered cell cycle progression

The implications of an increase in cell proliferation in HCT116 cells with reduced A-type lamin expression motivated us to analyze cell cycle progression. For this purpose HCT116 wild type and knockdown cells were stained with PI and the DNA content per cell was
quantified by flow cytometric analysis. Figure 6 shows a reduction in G1 phase cells and an increase in G2/M and S phase cells in lamin A/C knockdown cells compared to wild type cells. This suggests a shorter G1 phase duration and an accelerated cell cycle progression.

Figure 6. Flow cytometric analysis of cell cycle progression in HCT116 wild type and knockdown cells. DNA staining with PI and flow cytometric detection of PI shows that the number of cells in the different cell cycle stages (G1, S, and G2/M) is distinct between wild type (wt) and lamin A/C knockdown (kd) cells. Lamin A/C knockdown cells have more cells in G2/M and S phases.

3.3.5 Decreased expression of the cyclin dependent kinase inhibitor p21 by lamin A/C knockdown

To find an explanation for the increased proliferation rate in A-type lamin knockdown cells, we examined expression of p21. Wild type HCT116 cells (wt) normally express the cyclin dependent kinase inhibitor p21 as shown in figure 7. Knockdown cells, however, have no detectable p21 expression by immunofluorescence analysis (kd).

Figure 7. P21 expression in HCT116 wild type and knockdown cells. While wild type cells express p21 (green), knockdown cells did not show any detectable p21 staining. Nuclei were counterstained with PI (red).
3.4 Generation of a lamin B2-EGFP expression vector

Until recently, only an incomplete 3’ human lamin B2 cDNA was cloned and published. In order to obtain the full-length cDNA, the 5’ missing part of the cDNA was predicted from a comparison between the mouse and human genomic DNA and human EST sequences. Two primers were chosen, the 5’ primer immediately upstream the predicted start codon and the 3’ primer in a region partly overlapping with the known 3’ cDNA sequence. This fragment consisted of bp 88 to 1035 from the reported lamin B2 cDNA sequence (reference sequence: NM_032737) and was obtained by reverse transcription PCR (RT-PCR) of T24 mRNA. The PCR product was cloned into pGEM-T Easy. The other fragment, comprising the 3’ end of the lamin B2 cDNA starting at bp 340, was kindly provided by Dr Y. Raymond, and was cloned into pUC19. The PCR fragment was chosen to contain an unique restriction site (SphI at bp 960) of the lamin B2 sequence which was also present in the other fragment such that digestion with SphI and fusion of the resulting fragments would lead to a complete lamin B2 coding sequence. Both fragments are schematically represented in figure 8A. Agaroses gel electrophoresis results of these fragments are represented in figure 8B.

Figure 8. A) Schematic illustration of the lamin B2 fragments that were used to construct a lamin B2 expression vector. Fragment 1 represents the PCR product of reverse transcribed lamin B2 mRNA. Fragment 2 represents...
the fragment obtained from Dr Y. Raymond. Restriction sites used for cloning and the B2-Sac primer site are placed in italics; the numbers represent the bp number in the lamin B2 mRNA (NM_032737). The SphI restriction site was used to fuse the two fragments. The lamin B2 transcript (fragment 3) is given to illustrate the restriction sites that were used to confirm the presence of the proper cDNA in the plasmids. B) Agarose gel electrophoresis results of both lamin B2 fragments (1 → PCR product, 947 bp; 2 → 3’ end, 1550 bp) and (3) the complete lamin B2 cDNA (1802 bp). The 3’end (arrow) was cut out of the pUC19 vector (upper band) with EcoRI.

In order to fuse the overlapping sequences, the 5’ part of the PCR product was excised out of the pGEM-T Easy vector with HindIII and SphI endonucleases and the vector (pUC19) containing the other fragment was digested using the same restriction enzymes. The vector and the PCR product fragment were ligated to generate a vector inserted with the complete lamin B2 coding sequence. The cDNA was excised from the pUC19 vector using the HindIII and EcoRI sites and ligated into an EGFP expression vector (pEGFP-C1). When transfected into mammalian cells, this vector expresses an EGFP tagged lamin B2 protein. A schematic illustration of the generation of the lamin B2-EGFP expression vector is given in figure 9. By placing the cDNA in-frame with the EGFP coding sequence, a fusion transcript can be generated resulting in an EGFP-tagged lamin B2 protein. A schematic diagram of the lamin B2-EGFP fusion protein is represented in figure 10.
Figure 9. Schematic representation of the construction of the lamin B2-EGFP expression vector. Two fragments of the lamin B2 cDNA were first inserted into two cloning vectors; the 5' part (lamin B2 PCR product) in the pGEM-T Easy vector and the 3' in the pUC19 vector. The PCR product was ligated with the pUC19-lamin tail vector using the HindIII and SphI sites. The complete lamin B2 cDNA was excised from the pUC19 vector and inserted into an EGFP expression vector using the EcoRI and HindIII restriction sites.
Restriction analysis was used to confirm correct ligations of the lamin B2 cDNA fragments into the vectors. The restriction products were gel electrophoresed and visualized by UV trans-illumination (figure 11). The PCR product in the pGEM-T Easy vector was digested with SacI (A1) or EcoRI and HindIII (A2). The bands are of expected sizes, namely 191 (A1, arrow), 719, and 3073 bp for digestion with SacI, and 975 and 2997 bp for digestion with EcoRI and HindIII (figure 11A). In figure 11B the restriction analysis results are represented for the 3’ end of the lamin B2 cDNA in pUC19 and the total lamin B2 cDNA in pUC19. The lamin B2 cDNA 3’ end (bp 340 to the STOP codon) was digested with PstI (B1) or SacI (B2). Again, product bands are of expected sizes, namely 294 (B1, arrow) and 3868 for digestion with PstI (the 81 bp band is not visible on this picture due to the small quantity of DNA), and 572 and 3671 bp for digestion with SacI. The upper band in B1 is uncut vector DNA because of incomplete digestion. The complete lamin B2 cDNA in the pUC19 plasmid was digested with XhoI (B3) or EcoRI and HindIII (B4). Sizes of the restriction fragments are as expected (922 and 3523 for digestion with XhoI and 1810 and 2635 for digestion with EcoRI and HindIII). Restriction analysis of the final product (lamin B2 cDNA in pEGFP-C1) is represented in figure 11C. Digestion of this product with XhoI (C1) or SacI (C2) resulted in fragments that are approximately 287, 922, and 5325 bp in size for XhoI digestion and 106 (C2, arrow), 719 and 5709 bp for SacI digestion as expected. The generated construct probably contains the STOP codon as deduced from fragment sizes, but misses the 3’ untranslated region. The region is not relevant in this case because it is non-coding.
Figure 11. Restriction analysis of integrity of lamin B2 cDNA fragments in cloning vectors. A) The PCR product in pGEM-T Easy was cut with SacI (1) or EcoRI and HindIII (2). B) The 3’ end of the lamin B2 cDNA in pUC19 was digested with PstI (1) or SacI (2). The complete lamin B2 cDNA in the pUC19 plasmid was digested with XhoI (3) or EcoRI and HindIII (4). C) The complete lamin B2 cDNA in pEGFP-C1 was cut with XhoI (1) or SacI (2).

3.5 Lamin B2-EGFP expression in mammalian cells

The lamin B2-EGFP expression vector was used to transfect CHO-K1 cells. Transfected cells appeared to have a variable expression level of lamin B2-EGFP and a heterogeneous expression pattern. Cells with a diffuse nucleoplasmic staining and low expression in the lamina (figure 12A) were seen most frequently. Sometimes, these cells had a higher expression in the lamina. Cells with pronounced fluorescent spots in the nucleoplasm (figure 12B) or in both nucleoplasm and cytoplasm (figure 12C) were also observed. Another expression pattern was a diffuse fluorescence in both nucleoplasm and cytoplasm (figure 12D).

Transfected cells were subcloned twice to obtain monoclonal lamin B2-EGFP expressing cell lines. Clones were selected for their correctly lamin B2-EGFP targeting to the nucleus i.e. absence of cytoplasmic fluorescence and “normal” nuclear labeling. As shown in figures 12 and 13, lamin B2-EGFP expression remained variable after subcloning, with several cells showing lamin B2-EGFP so low that GFP signal was not detectable in these cells when recording high lamin B2-EGFP expressing cells. However, the presence of lamin B2-EGFP in these cells was confirmed by immunofluorescent staining with anti-lamin B2 antibodies that only bind human lamin B2 and not hamster lamin B2 (figure 12 E and F). Lamin B2 detection using immunofluorescence resulted in a staining of the lamina (12 E, arrowhead), while the EGFP was detected as a diffuse nucleoplasmic signal.
Figure 12. Lamin B2-EGFP expression (green) in CHO-K1 cells resulted in various expression patterns. Nuclei were counterstained with DAPI (blue). A) In most cells a diffuse nucleoplasmic fluorescence was detected. B) Some cells had pronounced nucleoplasmic fluorescent spots of various sizes (arrows). C) Very few cells show besides nucleoplasmic spots, also similar spots in the cytoplasm (arrowhead). D) Another expression pattern was a diffuse fluorescence of both nucleoplasm and cytoplasm (arrow). The presence of lamin B2 in cells that had non-detectable lamin B2-EGFP signal was confirmed by staining with an anti-lamin B2 antibody that only binds to human lamin B2. Transfected cells are positive for human lamin B2 (red, E), and show a lamin B2 staining in the lamina (arrowhead). Wild type hamster lamin B2, in contrast, can not be detected with this antibody (F).

3.6 Lamin B2 overexpression induces nuclear abnormalities

Analysis of EGFP-tagged lamin B2 expressing cells revealed many nuclear alterations in cells that express high levels of lamin B2-EGFP (green). Nuclei were bigger (figure 13A, B, C) than normal CHO-K1 nuclei (D) and often had protrusions (figure 13A and B, arrowheads). Some nuclei vesiculated, shown in figure 13C (arrows). Normal CHO-K1 nuclei are represented in figure 13D, and were stained with anti-lamin B1 antibodies (red). Nuclei were counterstained with DAPI (blue).

Figure 13. Nuclear irregularities in lamin B2-EGFP transfected CHO-K1 cells. Various abnormal nuclei were observed in lamin B2 transfected cells; increase in size (A, B, C, compare with wt (D)), nuclear protrusions (A,
B, arrowheads), and vesiculation (C, arrows). Normal CHO-K1 cells stained with a lamin B1 specific antibody (red) are shown in (D). The EGFP fluorescence is shown in green. Nuclei were counterstained with DAPI (blue).

3.7 Immunofluorescent staining of nuclear proteins

Several proteins located near the INM (lamin B1, nucleoporin p62, lamins A/C, emerin) were stained to observe whether their localization was altered after transfection. These proteins did not seem to be mislocalized in lamin B2-EGFP expressing cells.

![Figure 14. Immunofluorescent staining of nuclear proteins in CHO-K1 wild type (B) and lamin B2-EGFP expressing cells (A). Lamin B2-EFGP expression is shown in green. P62, emerin, lamin A/C, and lamin B1 are shown in red. None of these proteins seemed to be significantly altered localized in lamin B2-EGFP transfected cells. Not all lamin B2-EGFP expressing cells have detectable EGFP, because of low levels of expression.](image)

3.8 Lamin B2 reassembly occurs after cytokinesis has completed

Lamin B2 behavior during mitosis was studied by recording lamin B2-EGFP in dividing cells using a confocal scanning laser microscope. Image recordings were started at metaphase and every 30 seconds a confocal recording was taken. In figure 15, live fluorescence recordings of a cell at different mitotic stages are shown. Time in minutes is indicated in the lower right corner of each frame. The first image (t = 0) shows a cell in metaphase, the condensed chromosomes are positioned at the center of the cell, seen as a dark stripe. In the next frame, the cell has started cell division, deduced from its slightly elongated shape. After 6.5 minutes (frame 6) cell division is complete. It takes approximately 4 minutes to observe an accumulation of lamin B2-EGFP in the nucleus (frame 7, closed arrowhead) after completion of cell division. After 14 minutes, all lamin B2-EGFP signal is detected in the nucleus, whereas it has disappeared from the cytoplasm (last frame, open arrowhead).
Figure 15. Time-lapse series of a lamin B2-EGFP transfected CHO-K1 cell undergoing mitosis. The EGFP signal was detected using confocal scanning laser microscopy. Recordings were started at metaphase and ended when there was no detectable EGFP signal in the cytoplasm left. Time in minutes is indicated in the lower right corner of each frame, starting at the first frame. In the first frame a cell in the metaphase of the cell cycle is shown. The condensed chromosomes are aligned at the equatorial plate and are seen as a dark stripe (arrow). The lamin B-EGFP can be seen as a diffuse signal throughout the whole cell. After 2.5 minutes (frame 2) the first signs of cytokinesis are seen. 4 minutes later (frame 6) cytokinesis has completed (double lined arrow) and lamin B2-EGFP is sill dispersed throughout the cell. It takes another 4 minutes before lamin B2-EGFP starts to reassemble in the vicinity of the chromosomes (frame 7, closed arrowhead). About 3.5 minutes are needed before the EGFP signal is no longer detectable in the cytoplasm (last frame, open arrowhead).
4 Discussion

Although A- and B-type lamins belong to the same protein family, each has their unique functions in cells and organisms. B-type lamins participate in essential cellular events and are constitutively expressed. A-type lamins, in contrast, are not expressed in poorly differentiated tissues and are well known for their role in genetic disorders. These differences have led to the general opinion that A-type lamins have a regulating function in adult tissues and provide mechanical stability to the cell, whereas B-type lamins not only stabilize the cell, but are also necessary for basal cellular needs.

Until now, in most A-type lamin research genetically distinct cells are used for comparison of normal and absent or abnormal lamin A/C expression. Possible acquired or genetic differences between these cell populations can not always be taken into account when studying lamin function and may therefore lead to an incorrect interpretation of obtained results. As a better alternative model, genetically identical cells should be used. Ideally, the effect of alterations in lamin expression is studied in the same cell population. To achieve this, we have used a relatively new technique. HCT116 colon cancer cells were transduced with a shRNA that targets the \textit{LMNA} transcript and an additional vector (encoding TR) that regulates shRNA expression according to the Tet-On system. In relation to earlier studies, this allows the comparison of low versus normal A-type lamin expressing cells that have an identical genetic background and thus eliminates the effects of inter-cell line variability.

4.1 Lamins A and C

Lamin A/C expression was monitored with immunofluorescence using A-type lamins, lamin A, and lamin C specific antibodies and immunoblotting. Two rounds of subcloning resulted in cell populations with heterogeneous but strongly reduced lamin A/C levels. Apparently, the effect of RNAi and/or the shRNA expression varies even in genetically identical cells, since some cells still showed a rather strong lamin A/C expression. The heterogeneity in lamin A/C expression complicates the use of these cells in for example cell compression studies, because in these single cell experiments information about the lamin expression level per cell is needed to correctly interpret results. Although not suitable for single cell experiments, the lamin A/C knockdown cells can be used for many other purposes. On shRNA, TR double transduced cells, lamin A/C expression was increased when compared to knockdown cells and
could be lowered by culturing these cells in the presence of tetracycline. A monoclonal cell line with controllable A-type lamin expression thus could be generated.

A remarkable difference between lamin A and C expression was found by analyzing immunofluorescence data. Whereas lamin C was reduced but still noticeably present in the lamina, a diffuse lamin A signal was maintained inside the nucleoplasm of knockdown cells, while its signal in the lamina was no longer detectable. This may be due to epitope specificity and the accessibility of the epitope for the antibody. Another explanation is the cell type specific distribution of the various lamin subtypes. MEF cells, for example, did not have this characteristic nucleoplasmic lamin A staining.

Cells were also immunofluorescently stained for emerin, because this protein is thought to depend on A-type lamins for its correct nuclear localization. Lamin A/C knockdown cells, however, had a similar emerin distribution as wild type cells. These findings support the observation that emerin localization does not always depend on lamins A and C. However, the absence of mislocalized emerin may also be due to the less than total knockdown. It is certainly possible that emerin only requires low levels of A-type lamins to be targeted correctly at the INM.

It has long been assumed that A-type lamins play an active role in regulating cell proliferation. Already in 1985 A-type lamins were called statins because of the presumed inverse correlation between lamins and proliferation. Cell cycle progression, proliferative capacity, and apoptosis were studied in order to get insights into the effect of A-type lamin expression on cell proliferation. An important observation in this study was the higher number of knockdown cells after two days of culturing compared to wild type cells when seeded at identical densities. Also, in cell cultures growing under ideal conditions, a higher percentage of knockdown cells were in G2/M- and S-phases of the cell cycle, indicating that lamin A/C knockdown results in a shorter G1 duration. Besides this, the cyclin-dependent kinase inhibitor p21 was non-detectable in knockdown cells, while present in a part of the wild type cells. One of the p21 functions is inhibition of cell-cycle progression. Taken together, the results from these experiments support the theory that A-type lamins negatively influence proliferation by a decreased progression through the cell cycle. The possibility of reduced apoptosis in lamin A/C knockdown cells was excluded by detection of even more spontaneous or induced (with roscovitine or etoposide) apoptosis in knockdown cells than in wild type cells. The negative effect of A-type lamins on proliferation is supported by data from many previous studies. First, lamin A/C expression is low or absent in poorly differentiated cells.
and increases upon differentiation.\(^9\) An additional indication for a role of A-type lamins in proliferation is the reduced or absent lamin A/C levels in many malignancies.\(^{50}\) Since low differentiated cells and cancer cells have a higher proliferative capacity, it is tempting to speculate that this may be related to lamin A/C expression. However, the correlation of lamin A/C expression and tumorigenesis is not systematic. Lamins A/C levels are even increased in some cancer cell lines.\(^{50}\) Additional evidence for a causal relationship between lamin A/C expression and proliferation was provided by Ivorra et al. They showed a reduced cell cycle progression in cells overexpressing lamin A. The authors argued that A-type lamins induced growth inhibition, that might be dependent on the release of the lamin A/C mediated negative regulation of the AP-1 complex.\(^{47}\) The AP-1 transcription regulator complex is known for its involvement in cell proliferation, survival, differentiation, and transformation. AP-1 is a dimeric complex mainly composed of fos and jun proteins, which have either a positive or a negative regulatory effect on AP-1 target genes. C-fos expression has been implicated in cell transformation and invasion of tumor cells and negatively regulates p21.\(^{62,63}\) C-jun is a positive regulator of cell proliferation.\(^{63}\) Ivorra et al. showed that lamin A overexpression reduces c-fos/c-jun heterodimerization and suppresses AP-1 DNA-binding and transcriptional activity. Besides this, the co-expression of c-fos in lamin A/C overexpressing cells resulted in a reduced percentage of cells in G0/G1-phase and accumulation of cells in S-phase, indicative for a faster cell cycle progression.\(^{47}\)

An interesting observation after the treatment of HCT116 cells with lamin A/C targeting shRNA was the altered morphology of knockdown cells in comparison to wild type cells. HCT116 wild type cells have a mesenchym-like appearance; they are spindle shaped or form extensions and do not adhere much to each other. Lamin A/C knockdown cells in contrast, had cells with flattened cuboidal-like shape forming epithelial-like monolayers. Colon epithelial cells normally form monolayers and have extensive cell-cell contacts. In the process of transformation cell adhesion is often reduced,\(^{64}\) like in the colon cancer cell line we have used. The differences in morphology between wild type and knockdown cells may thus be caused by an increased expression of adhesion molecules. Similar changes in morphology of HCT116 cells have been described in literature. In these publications, morphology was altered by interfering with several pathways that are important in transformation.\(^{65,66}\) To further study cell-cell adhesion, the expression of two kinds of adhesion molecules was analyzed; one gap junction protein (connexin 43) and members of the adherens junction protein family (cadherins). Gap junctions form continuous aqueous channels between
neighboring cells and consist of connexins. Gap junctions not only physically link cells, but also allow electrical and chemical coupling between cells. Cadherins form a continuous adhesion belt in epithelial sheets and are intracellularly linked indirectly to a bundle of actin filaments. HCT116 cells and non-transformed epithelial colon cells normally express E-cadherin, but are deficient in connexin 43 protein and have non-detectable connexin 43 mRNA levels. In this study, the almost complete absence of connexin 43 in wild type HCT116 cells was confirmed by immunofluorescence analysis. Transducing HCT116 cells with the lamin A/C targeting shRNA vector, however, resulted in connexin 43 expression in nearly all cells. This could be due to the effect of lamins on gene expression. Support for this hypothesis comes from previous experiments. First, connexin 43 mRNA levels are non-detectable in wild type HCT116 cells, which suggests a deregulation of connexin 43 expression at the transcriptional level. Second, expression of connexin 43 is among others regulated by AP-1 through AP-1 binding sites in the connexin 43 proximal promoters. AP-1 acts as a transcriptional activator on the connexin 43 gene. Furthermore, A-type lamins have been shown to interact with AP-1 and suppress its DNA binding and transcriptional activity. A reduction in lamin A/C levels may therefore result in induction of connexin 43 expression by release of AP-1 suppression.

Possible changes in cadherin expression could not be observed in this study, because of the lack of immunofluorescent detection of cadherins in both wild type and knockdown cells. Integrity of the antibodies that were used was confirmed by staining other cell lines. The absence of specific binding may be dependent on epitope specificity of the used antibodies or epitope masking in these cells. Other cadherin antibodies might be used to analyze E-cadherin expression. In addition, cell-matrix adhesion proteins could be analyzed to investigate whether there are alterations in cell-matrix interactions.

To provide more conclusive data for the suggested role of lamins in proliferation, proliferation rates were measured in shRNA/TR double transduced cells cultured with or without tetracycline. To our surprise, no difference in proliferation was observed between these populations at a high seeding density and cells cultured without tetracycline proliferated faster than those cultured with tetracycline at low seeding density. Closer examination showed that all double transduced cells had a higher proliferation rate than wild type cells, and similar to knockdown cells. At least four different explanations for this finding are possible. First there is no clear relationship between A-type lamin expression and proliferation rate in these cells. Second, the double transduced cells had a high increase in abnormally shaped nuclei.
(about twice as much as wild type and knockdown cells), possibly causing an abnormal growth behavior. Third, only one rescued clone was used. In order to draw any firm conclusions it would be better to repeat these experiments with other clones from double transduced cells that retain their nuclear morphology. Fourth, the restoration of lamin A/C expression did not (yet) rescue the phenotype as seen in lamin A/C containing cells, i.e. lamin expression was restored but not (yet) the corresponding cellular behavior. This last hypothesis was supported by the morphology studies, which showed that the morphology of shRNA transduced cells was not reversed to wild type shape by transduction with TR. Also, addition of tetracycline had no effect. Moreover, the levels of connexin 43 could not be diminished in double rescued cells nor upregulated by tetracycline in double transduced cells; both populations had a high expression of connexin 43.

In conclusion, lamin A/C knockdown seemed to affect cell functioning on several levels; morphology, proliferation and adhesion molecule expression were altered. These effects, however, could not be regulated by tetracycline in shRNA/TR double transduced cells. This emphasizes the dangers of comparing non-identical cells for studying the effects of lamin expression and supports the use of controllable lamin A/C expressing cells in future experiments. However, some prudence is in order, because only one double transduced cell line was tested. Besides this, the double transduced cells had high levels of nuclear abnormalities. These experiments thus need confirmation with other newly generated double transduced HCT116 cells.

Of interest is the transduction of other cell lines with this controllable lamin A/C expression system. Comparing the effect of A-type lamin down- and upregulation in different cell lines may lead to knowledge of cell-type specific functions of lamins. Most relevant cell types to use are probably those commonly affected in laminopathies, such as muscle cells or adipocytes. Results from such experiments should be compared with knowledge learned from lamin A/C knockout or mutant cells. Particularly interesting would be the controllable knockdown of lamin A or lamin C separately. This year, Fong et al. demonstrated that expression of lamin C without lamin A is sufficient in mice for normal functioning. It is still questionable whether this holds true for humans. Knowledge of regulating functions of lamins A and C, will probably lead to further understanding of laminopathy pathogenesis and lead to development of target directed therapies.
4.2 Lamin B2

Although lamin B2 is as widely expressed as lamin B1,\(^9\) it has been studied less extensively. The absence of concrete information about lamin B2 motivated us to make a lamin B2-EGFP fusion protein. This probably gives more detailed information about the exact localization and dynamics of lamin B2. Similar constructs have already been made for A-type lamins and lamin B1\(^{17,70}\). A lamin B2-EGFP expressing vector was constructed by fusing two fragments of the lamin B2 cDNA. Correct insertion was confirmed by restriction endonuclease analysis. Transfection of CHO-K1 cells with this vector resulted in a diffuse green fluorescent signal inside the nucleus and therefore likely stems from correctly localized lamin B2. Expression levels and patterns were variable despite of subcloning cells. After subcloning, a substantial proportion of cells had no visible EGFP signal by visualization with a fluorescence microscope but lamin B2-EGFP could be detected by immunofluorescence staining. Anti-lamin B2 antibodies resulted in a nuclear rim labeling rather than the diffuse EGFP signal. This illustrates the preference of these antibodies to detect lamins in the vicinity of the NE. Other nuclear proteins (emerin, p62, lamin A/C, and lamin B1) were normally localized in the majority transfected cells. This provides support for the assumption that the lamin B2-EGFP is correctly localized and does not negatively affect other nuclear proteins. Restriction endonuclease digestion results suggest that the complete lamin B2 open reading frame was present in the construct. Sequencing is needed to confirm this assumption.

The lamin B2-EGFP expression pattern most commonly seen was a diffuse nucleoplasmic staining. This is distinct from other lamin subtypes, which have a relatively high expression at the lamina when fluorescently tagged\(^{17,70}\). Therefore, lamin B2 might constitute a separate lamin pool that has features distinct from the other lamins. This is supported by the observation that lamin B2 had different interactions with itself and the other lamins when compared to the other lamin subtypes\(^{71}\). Next to a diffuse nucleoplasmic signal, several other expression patterns were observed. Strong nucleoplasmic and/or cytoplasmic spots were seen in a minor fraction of the cells. These expression patterns were only observed in cells that had high lamin B2-EGFP expression and may therefore be an artifact of overexpression. Intranuclear channels, like those observed in cells transfected with fluorescently tagged A-type lamins, were not seen\(^{70}\). This might be related to differences in interactions with INM proteins between A-type lamins and lamin B2.

Lamin B2-EGFP overexpression resulted in profound nuclear abnormalities like increased size, nuclear protrusions and blebbing. The increase in size likely results from the lamin overexpression. It has been shown that the lamin CaaX motif causes nuclear growth. Lamin
C, which lacks the CaaX box, does logically not induce nuclear growth. Overexpression of lamin B2 may result in a more fragile nucleoskeleton, since lamin B2 forms weaker interactions with itself and the other lamin subtypes, which could be the cause of the observed nuclear abnormalities.

Especially interesting to investigate is the organization and behavior of lamins in mitosis. Fluorescent tagged lamins have been useful to illustrate that A-type lamins and lamin B1 behave dissimilar during M-phase. Lamin B2 likely behaves differently from lamin A/C and lamin B1, because it forms weaker lamin-lamin interactions. To study the dynamic behavior of lamin B2 behavior during mitosis and nuclear reassembly in daughter cells, time-lapse series of dividing cells starting at metaphase were analyzed. It was illustrated that lamin B2 reassembled after cytokinesis was completed. It took about 4 minutes for lamin B2 to start concentrating at the perichromosomal region. Another 3.5 minutes are needed for the complete disappearance of lamin B2 from the cytoplasm.

The high speed with which the nucleus reforms after mitosis has complicated the investigation of the order of events that are required for NE reassembly. Live imaging, however, enables to determine the localization of proteins during mitosis to some extent. Similar experiments like those in this study have illustrated their usefulness for getting insights in the behavior of other lamin subtypes during mitosis. In 1999, Broers et al. showed with the use of GFP-tagged A-type lamins that lamins A, AΔ10, and C only reform a lamina after cytokinesis has completed. About 3 minutes pass by after mitosis before A-type lamins aggregate around the chromosomes. Others have shown that lamin B1 reorganized before or during cytokinesis, at late anaphase to mid-telophase. The early lamin B1 reorganization has led to the suggestion that lamin B1 is needed for chromosome decondensation and NE assembly. Other studies contradict this hypothesis, by stating that lamin B1 reassembly occurs at late telophase/early cytokinesis. At this stage the NE is already encircling the chromosomes. In the present study it was shown that lamin B2 even seems to repolymerize after A-type lamins start reassembling. Lamin B2 reassembly started about 4 minutes after cell division was completed, while A-type lamins already reassemble after 3 minutes. These results doubt the active participation of lamin B2 in NE reassembly like lamin B1. Reassembly of A- and B-type lamins is probably differentially regulated. Lamin A appeared to be dependent on BAF and emerin for its assembly, while lamin B reassembly was independent of BAF and emerin. Besides this, inhibition of B-type lamin polymerization at the end of mitosis did not interfere with lamin A/C reassembly.
SiRNA of lamins has shown that both knockdown of lamin B1 and lamin B2 resulted in apoptosis indicating that both proteins are essential for cell survival and have non-redundant functions.\textsuperscript{2} Contrasting results have been provided recently. Lamin B1 was truncated in mice by an insertional mutation in the last rod domain. This resulted in a protein which lacked part of the fourth rod domain and the tail domain containing the NLS and the CaaX box. These mice died shortly after birth and exhibited severe abnormalities.\textsuperscript{10} Their survival until birth may support the notion that lamin B2 can partly compensate for loss of lamin B1 function. However, preservation of functionality of the truncated lamin B1 cannot be excluded. In accordance to the lamin B1 knockdown results from Harborth et al,\textsuperscript{2} the latter possibility is more likely. Despite the knowledge about the dysfunctionality of this truncated lamin B1 protein, it can be stated that proper functioning lamin B1 is needed for normal development. The striking differences between lamin B1 and B2 behavior during mitosis supports the hypothesis that these lamins have distinct functions. While lamin B1 seems to be significant for NE assembly after mitosis, lamin B2 might play another role in mitosis by providing a mitotic spindle matrix as shown in a previous study.\textsuperscript{20}

In conclusion, a full length lamin B2 transcript was constructed and fluorescently tagged. This construct was transfected into mammalian cells and appeared be translated into a correctly localized lamin B2. Visualization of lamin B2 during mitosis revealed that lamin B2 translocates to the nucleus after cytokinesis has completed. Certainly this lamin B2-EGFP construct can be used for many purposes, such as in depth study of lamin B2 localization and dynamics. The other main lamin subtypes (A, B1, and C) have already been studied by tagging them with a fluorescent protein. Investigating the dynamic properties of these by fluorescence recovery after photobleaching (FRAP) and FLIP revealed that these lamins are present as two distinct pools. Inside the nucleoplasm lamins are more mobile than at the nuclear lamina. Lamin C seemed to be the most mobile studied lamin subtype. It would be interesting to perform similar experiments with the lamin B2-EFGP transfected cells. Furthermore, it might be useful to double transfec cells with lamin B2-EGFP and fluorescent tagged lamin B1, A, or C. By studying mitosis in such double transfected cells, more insights in the temporal organization of lamin subtype disassembly and reassembly will be obtained. At last, it will be useful to make fluorescent tagged lamin B2 deletion mutants to study the function of the different lamin B2 protein domains.
5 References


71. Schirmer EC, Gerace L. The stability of the nuclear lamina polymer changes with the composition of lamin subtypes according to their individual binding strengths. The Journal of Biological Chemistry 2004;279(41):42811-42817.


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