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Generation and characterisation of laminopathy-specific antibodies

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Z jezikom smo ali nismo,

z jezikom bomo ali ne bomo.

- Florjan Lipuš -

Thanx to

Egon Ogris

Stefan Schüchner

Ogris-lab members

friends

familiy

Abstract

Missense and nonsense mutations in proteins account for over 50% of all annotated and documented mutations causing monogenic diseases. If the disease-causing mutation is autosomal-dominant inherited, the wild-type and the mutant protein are potentially expressed side-by-side in a cell. Therefore, with currently available antibodies the special molecular features of the mutant, disease-causing protein cannot be investigated in a patient cell, because these antibodies cannot distinguish between the wild-type and mutant protein. The knowledge about the disease-causing functions of the mutant protein relies entirely on studies using epitope tagged mutant protein.

Laminopathies are a group of diseases caused mainly by mutations in A-type lamins. The vast majority of these mutations cause single amino acid substitutions inherited in an autosomal-dominant way. The molecular mechanisms of laminopathies and their etiology, however, are still poorly understood, mostly because of the lack of specific research tools to study the mutant proteins in patient cells. Based on the finding from the Ogris lab that antibodies against protein phosphatase 2A (PP2A) subunit isoforms can discriminate between isoforms differing only in one amino acid at the respective epitope, we thought that it would be feasible to develop antibodies recognizing specifically the wild-type or the mutant A-type lamin proteins, which are differing only in one amino acid. These antibodies would be extremely helpful for a detailed characterization of the pathological conditions and the elucidation of the disease pathogenesis at the cellular level.

Therefore we aimed to develop mouse monoclonal antibodies specific for autosomal-dominant Emery Dreifuss Muscular Dystrophy (EDMD), Familial Partial Lipodystrophy (FPLD), and Dilated Cardiomyopathy (DCM) causing point-mutations in the A-type lamins, which would allow to distinguish between proteins differing in a single amino acid, and thus to study the special molecular features of the wild-type and the mutant proteins, respectively.

To obtain such antibodies we made use of three different approaches of antigen presentation. These include the use of short peptides coupled to the keyhole limpet hemocyanin (KLH) protein, recombinant proteins consisting of the lamin A/C C-terminal Ig-fold domain fused to the Hepatitis B virus core protein (HBcAg), and recombinant proteins consisting of short peptides corresponding to the desired lamin A/C epitopes fused to HBcAg.

We were successful in developing an EDMD relevant point-mutations specific antibody against the lamin A/C mutant R453W by the KLH-peptide approach. In addition, a lamin A/C

Ig-fold specific monoclonal antibody was developed. Importantly, we could also show that a recombinant HBcAg-Lamin A/C Ig-fold R482W fusion protein elicited a humoral immune response resulting in the production of lamin A/C R482W point-mutant specific antibodies, indicating that immunizations with a whole protein domain can elicit a point-mutant specific immune response.

Importantly, experiments using primary EDMD patient cells showed that the newly developed anti-lamin A/C R453W antibody could be used to specifically immunoprecipitate the mutant proteins, and to detect the mutant proteins in immunofluorescence and immunoblotting. The anti-lamin A/C R453W antibody will be used to examine the functions of the mutant A-type lamins in primary cells from EDMD patients.

Zusammenfassung

Missense und nonsense Mutationen in Proteinen sind für über 50% aller dokumentierten Mutationen verantwortlich, die eine monogene Krankheit verursachen. Wenn die krankheitsverursachende Mutation autosomal-dominant vererbt wird, werden das Wildtyp und das mutante Protein potenziell zusammen in der Zelle exprimiert. Deshalb können die speziellen molekularen Eigenschaften vom mutanten, krankheitsverursachenden Protein mit zurzeit erhältlichen Antikörpern in Patientenzellen nicht studiert werden, da diese Antikörper nicht zwischen dem Wildtyp und dem mutanten Protein unterscheiden können. Das Wissen über die krankheitsverusachenden Funktionen vom mutanten Protein beruht daher gänzlich an der Forschung an einer rekombinanten Version des mutanten Proteins.

Laminopathien sind eine Gruppe von Krankheiten die hauptsächlich von Mutationen in den A-typ Laminen verursacht werden. Die meisten dieser Mutationen verursachen einen Austausch von nur einer Aminosäure, die meistens autosomal-dominant vererbt werden. Die molekularen Mechanismen von Laminopathien und deren Krankheitsursache, sind aufgrund des Fehlens von spezifischen Forschungswerkzeugen, die das Studieren von den mutanten Proteinen in Patientenzellen erlauben würden, hingegen nur wenig bekannt. Basierend auf dem Ergebnis, dass die im Ogris Labor entwickelten Antikörper gegen Isoformen von Protein Phosphatase 2A (PP2A) Untereinheiten zwischen Isoformen unterscheiden können, die sich nur in einer Aminosäure des entsprechenden Epitops unterscheiden, meinten wir, dass es

möglich wäre Antikörper zu entwickeln, die spezifisch das Wildtyp oder die mutanten A-typ Laminproteine, die sich nur in einer Aminosäure unterscheiden, erkennen würden. Solche Antikörper wären für die detailierte Charakterisierung des pathologischen Zustandes und zur Aufklärung der Krankheitsenstehung auf zellulärer Ebene sehr hilfreich.

Deshalb hatten wir die Absicht, monoklonale Antikörper spezifisch für autosomal-dominant Emery Dreifuss Muskuläre Dystrophie (EDMD), familiäre partielle Lipodystrophie (FPLD), und dilatierte Kardiomyopathie (DCM) verursachende Punktmutationen in den A-typ Laminen zu entwickeln, die es erlauben würden, zwischen Proteinen, die nur in einer einzigen Aminosäure verschieden sind, zu unterscheiden, um so die speziellen molekularen Eigenschaften des Wildtyp oder des mutanten Proteins zu untersuchen.

Um zu solchen Antikörpern zu gelangen wurden drei Ansätze der Antigenpräsentation angewandt. Diese inkludierten den Gebrauch von kurzen Peptiden gekoppelt an das Keyhole limpet hemocyanin (KLH) Protein, rekombinante Proteine bestehend aus der Lamin A/C C-terminalen Ig-fold Domäne fusioniert mit dem Hepatitis B Virus Core Protein (HBcAg), und rekombinante Proteine bestehend aus kurzen Peptiden, mit den gewünschten Lamin A/C Epitopen, fusioniert an HBcAg.

Die Entwicklung eines EDMD relevanten punktmutation-spezifischen Antikörpers gegen die Lamin A/C Mutante R453W war mit dem KLH-Peptid Ansatz erfolgreich. Zusätzlich wurde ein Lamin A/C Ig-fold spezifischer monoklonaler Antikörper entwickelt. Bedeutend ist auch, dass Immunisierungen mit dem rekombinanten HBcAg-lamin A/C Ig-fold R482W Fusionsprotein zu einem punktmutations-spezifischen Antikörper führten. Das deutet darauch hin, dass Immunisierungen mit einer ganzen Proteindomäne eine punktmutation-spezifische Immunantwort auslösen können.

Bedeutend ist, dass Experimente mit primären EDMD Patientenzellen zeigten, dass der neu entwickelte anti-Lamin A/C R453W Antikörper spezifisch die mutanten Proteine immunpräzipitieren, und die mutanten Proteine in der Immunfluoreszenz und im Immunblotting detektieren konnte. Mit dem anti-lamin A/C R453W Antikörper werden Funktionen der mutanten A-typ Lamine in primären Zellen von EDMD leidenden Patienten untersucht.

1. Introduction

Human genetic variation

The human genome codes for approximately 20,000 – 25,000 genes, which are spread along 3 billion pairs of nucleotides on 24 distinct chromosomes. The protein-coding DNA sequence comprises about 2% of the whole genome. The completion of the Human Genome Project was announced in April 2003 and suggested that the DNA sequence across the entire human population is 99.9% identical. However, recent studies revealed that this number is most likely too high and that the DNA sequence of two individuals is just about 99.5% identical (Levy et al., 2007). Due to alternative splicing, the human genome encodes roughly 100,000 proteins (http://www.eurasnet.info/education/alternate-splicing/what-is-alternate-splicing), which execute most of the essential functions of a living cell. The function of proteins depends on their correct folding and three-dimensional (3-D) structure. It is therefore not surprising that the function of the protein can be impaired if a mutation affects the 3-D structure.

The National Center for Biotechnology Information (NCBI) single nucleotide polymorphism (SNP) database (dbSNP) has annotated more than 12 million human SNPs (Wheeler et al., 2008). SNPs represent variations in one nucleotide at a defined position and occur on average at a rate of one SNP per 100 – 300 nucleotides. The Human Genome Project defines a nucleotide variation at a defined position only then as a SNP, if it is present in more than 1% of the population (http://www.ornl.gov/sci/techresources/Human_Genome/faq/snps.shtml). In the case of lower frequency the variant should be called a mutation. Throughout the literature, however, authors, as will I, do not stick to this definition and use one or the other term for variants, regardless of the frequency of those. Even in the dbSNP some of the annotated SNPs occur at frequencies lower than 1%.

SNPs might be responsible for about 90% of all genetic variation in humans (Collins et al., 1998). Although, this could be an overestimation, as only 78% of the differences between the entire DNA sequence of an individual and a reference sequence are accountable to SNPs (Levy et al., 2007).

SNPs occur throughout the human genome. In case they are located in the coding region of a gene two types of SNPs are distinguished due to the degenerate genetic code. While synonymous SNPs (sSNPs) do not change the amino acid sequence, non-synonymous SNPs (nsSNPs) can cause either non-sense mutations by introducing a stop codon, or single amino

acid substitutions, which can affect the 3-D structure and, consequently, the function of the protein. NsSNPs can cause a certain disease (e.g. sickle cell anaemia, laminopathies), alter the predisposition of individuals to develop a disease (e.g. cancer, cardiovascular diseases), they can be neutral and have no effect on an individual's health, or they can be even advantageous for an individual.

Effects of missense mutations on protein function

With more than 45000 entries in the Human Gene Mutation Database, missense and nonsense mutations, caused by nsSNPs, account for over 50% of all annotated and documented DNA mutations, which cause a monogenic disease (Stenson et al., 2003). Non-synonymous SNPs represent about half of all coding SNPs, which – statistically - is an under-representation. Statistically about two thirds of all random mutations in a coding sequence should cause a change in the amino acid. The under-representation of nsSNPs can be explained by the fact that detrimental mutations, which result in amino acid changes of huge physico-chemical divergence, are lost during evolution as they cause lethal phenotypes (Cargill et al., 1999). Although it was shown that most amino acids in a protein are replaceable without impairing a protein's function (Frillingos et al., 1998), highly conserved amino acids are almost never affected by disease-associated SNPs, as their mutations are expected to cause lethal phenotypes and are thus not inherited to next generations (Ferrer-Costa et al., 2002).

Mutations in buried regions of a protein have a high potential to alter the 3-D structure of the protein and thus to affect its function. This is the case when changes in the physico-chemical properties of the amino acids involved, mainly the hydrophobicity and the volume, are severe enough and therefore lead to strong structural alteration and instability of the protein (Ferrer-Costa et al., 2002). Also mutations on solvent-exposed sites have the ability to alter the structural integrity of the protein and can thus lead to diseases, but it is significantly more likely that a missense-mutation in the protein interior causes a disease than a mutation on the protein surface (Ferrer-Costa et al., 2002; Vitkup et al., 2003; Wang and Moult, 2001).

About 70% of disease-associated nsSNPs occur in buried residues. On the other hand only 34% of neutral nsSNPs, which are by definition non-disease causing, occur in buried residues. 30% of disease-associated nsSNPs and 66% of neutral nsSNPs occur in exposed residues (Ferrer-Costa et al., 2002; Sunyaev et al., 2000).

It is estimated that 80% of disease-associated nsSNPs are causing a protein structure destabilisation (Wang and Moult, 2001; Yue et al., 2005), and 10% are thought to cause a disease due to quaternary structure destabilization. For the remaining 10% other features of

the protein, such as the solubility of the protein, or its interaction with substrates, ligands, or other binding partners, are assumed to be impaired (Ferrer-Costa et al., 2002).
The huge influence of mutations on protein stability can be explained by the fact that most amino acids are involved in maintaining the structural integrity of the protein. Therefore most single point-mutations associated with a monogenic disease alter the protein function by affecting protein structure stability.

Amino acid mutations involved in disease

The amino acid with the highest mutation rate is arginine (Cooper and Youssoufian, 1988). Four out of six codons coding for arginine contain a CpG dinucleotide. In mammals, in 70-80% of CpG dinucleotides the cytosine is methylated to form 5-methylcytosine, which is prone to deaminate to thymidine. Thus, the methylated CpG dinucleotide mutates to TG (and CA, respectively), if it is not repaired (Coulondre et al., 1978). In the human genome, with a GC content of 42% the dinucleotide, CpG should statistically appear at a frequency of 4.41%, but the observed frequency is at about 1%. This low frequency of CpG dinucleotides could be due to the accumulation of transitional mutations by deamination of 5-methylcytosin to thymine during evolution (Scarano et al., 1967; Simmen, 2008). Besides arginine, glycine, serine and valin also show high mutation rates. The estimation of the mutation rate of those amino acids varies between the databases, namely the one for disease-associated mutations in OMIM (Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim), or the one for nsSNPs in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), which include, in contrast to OMIM, also nsSNPs not associated with a disease (Steward et al., 2003).
Mutations of arginine are responsible for 15% and glycine for 12% of all disease-associated missense mutations (Ferrer-Costa et al., 2002; Vitkup et al., 2003; Wang and Moult, 2001). Due to their physico-chemical properties, such as size, charge and hydrophobicity, arginine and glycine are often changed to amino acids with significantly different physico-chemical features. On the other hand, mutations of tryptophan and cystein, which are the most highly evolutionary conserved amino acids (Dayhoff, 1978), i.e. those amino acids in proteins, which change at the slowest rate during evolution, have the highest potential to cause a disease, followed by glycine and arginine (Vitkup et al., 2003). It is presumed that tryptophan and cystein have important functional roles in proteins. The higher the evolutionary conservation of a mutated amino acid, the higher is the probability that mutation of such an amino acid will cause a genetic disease (Ferrer-Costa et al., 2002; Vitkup et al., 2003; Wang and Moult,

2001). Proteins can execute their function only in their proper 3-D structure. Since amino acids are unequally distributed among a protein's secondary structure, Khan and Vihinen investigated the location and distribution of disease-associated mutations in secondary structures. By studying 44 different proteins, which contain altogether 2,413 mutations (one missense mutation every 5.2 amino acids), they found that 80% of the disease-causing mutations occurred in secondary structures. They found out that mutations occur at similar frequencies in all secondary structures, but different groups of amino acids (e.g. hydrophobic, positively charged, or negatively charged) have higher or lower mutation rates depending on the secondary structure they occur in (Khan and Vihinen, 2007). Combining all secondary structures, arginine is by far the most mutated amino acid, followed by glycine, cystein, and tryptophan. This is true for alpha-helices, where arginine is followed by cystein, glycine, methionine and tryptophan, as well as beta-structures, where arginine is followed by glycine, histidine and cystein. Also in turns and bends arginine is followed by glycine. The amino acids not mentioned are under-represented among mutated residues in these secondary structures (Khan and Vihinen, 2007).

SNPs and the pharmaceutical industry

It is becoming increasingly clear that some SNPs can not only affect a person's predisposition for developing a certain disease, but can also affect a person's response to a drug therapy. One of the major aims of the pharmaceutical industry is the development of individualized drug therapies. However, certain SNPs, e.g. in drug metabolizing enzymes, drug transporters, or enzymes targeted by the drug, can affect the drug efficacy in and toxicity for individuals (Monzo et al., 2008; Twyman, 2004; Voisey and Morris, 2008). Personal treatment regimens can therefore only be established upon a far more complete understanding of the complex phenotypical consequences of certain SNPs.

The nuclear lamina and laminopathies

Eukaryotes have a compartimentabilized cellular interior. These biochemically distinct compartments are also called organelles and include e.g. mitochondria, the Golgi apparatus, the endoplasmic reticulum (ER), endosomes, lysosomes, and the nucleus. Organelles are enclosed by membranes and perform specialized biochemical tasks. The nucleus is the largest organelle and harbours almost the entire genetic material. It is the place of DNA replication,

transcription, mRNA splicing and ribosome assembly. The nucleus is separated from the cytoplasm by the nuclear envelope, which consists of an inner nuclear membrane (INM) and an outer nuclear membrane (ONM). The ONM is continuous with the rough ER (rER). The INM and the ONM are joined at and interrupted by nuclear pore complexes (NPC), which regulate the selective import and export of molecules larger than ~40 kDa. The main feature distinguishing the metazoan nuclear envelope from those of other eukaryotic nuclei is the presence of a nuclear lamina (figure 1).

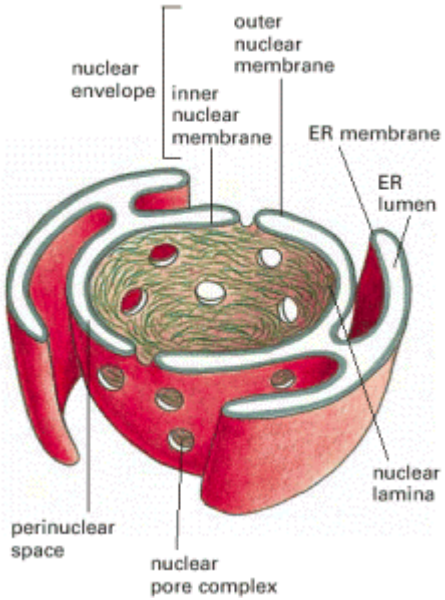

Figure 1. The nuclear envelope (Alberts, 2002). ER = endoplasmatic reticulum.

The nuclear lamina is a 20-50nm thick meshwork lining the INM, which consists of nuclear lamin proteins and INM proteins. Lamins are type V intermediate filaments, and they are subdivided into A-type and B-type lamins depending on sequence differences, expression patterns, post-translational modifications and biochemical properties. All A-type lamins are encoded by one gene, *LMNA*, giving rise by alternative splicing to the main products, lamin A and lamin C. The other splicing variants are lamin AΔ10, which is expressed in cell lines of colon, lung and breast carcinomas, and lamin C2, which is expressed in testis during spermatogenesis. B-type lamins are encoded by two genes, *LMNB1* and *LMNB2*, giving rise to lamin B1 and lamin B2 and the rare splice variant of *LMNB2*, lamin B3. At least one B-type lamin is expressed in all vertebrate cells. B-type lamins are essential for embryonic development and are expressed throughout development. Mice lacking functional lamin B1, the major B-type lamin, survive embryogenesis but die at birth (Vergnes et al., 2004). In contrast A-type lamins are expressed in differentiated cells (Broers et al., 1997; Constantinescu et al., 2006; Stewart and Burke, 1987; Vergnes et al., 2004) and have essential

roles in tissue organization and homeostasis. The embryogenesis of *lmna*$^{-/-}$ mice is normal, but the postnatal growth is retarded and mice suffer from muscular dystrophy and die at 7-8 weeks after birth (Capell and Collins, 2006; Sullivan et al., 1999). A disease phenotype is seen only in mice homozygous for mutations in *lmna*, whereas in humans most laminopathies are triggered in a heterozygous state (Arimura et al., 2005). This suggests that the molecular mechanisms leading to the pathological phenotype might be different in mice and humans (Capell and Collins, 2006).

Structure of A-type lamins

All lamins have a common structure: a short N-terminal head domain, a long alpha-helical rod domain required for formation of coiled-coil di- and multimers, and a C-terminal globular tail domain. All lamins have a nuclear localization signal at their C-terminal end, and all but lamin C, have a C-terminal CaaX motif, which is required for post-translational modification and proteolytic maturation. B-type lamins are stably farnesylated and are thus more tightly associated with the INM than A-type lamins, where the farnesyl group is removed during proteolytic maturation.

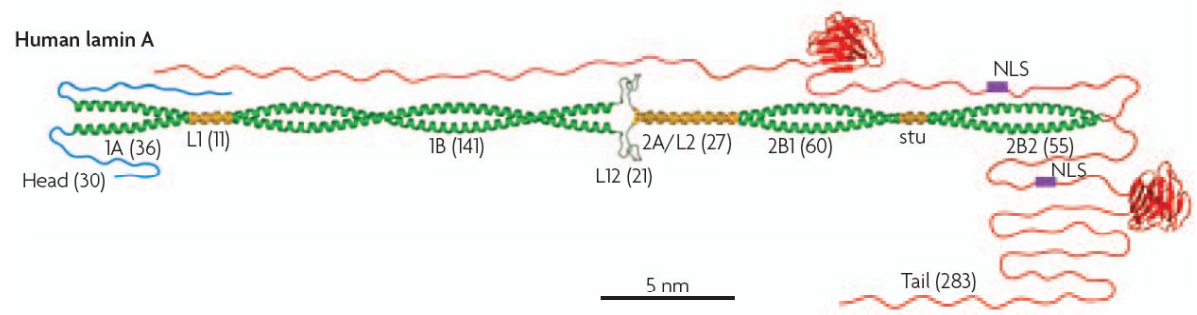

Figure 2. Cartoon of a human lamin A dimer. The schematic structure was obtained from a collection of structure predictions, NMR and X-ray crystallography data. The alpha helical rod domain, in green, is interrupted by linker segments, shown in orange, which connect the alpha-helical segments. The N-terminal head domain is shown in blue, and the C-terminal tail, containing the Ig-fold domain, is shown in red. The number of amino acids in each domain is indicated in brackets. NLS (nuclear localization signal) (Herrmann et al., 2007).

At the C-terminal end lamin A and lamin C share an identical Ig-fold like domain of 116 amino acids (amino acids 430 – 545) (figure 3). The domain is predicted to serve as a structural scaffold and to mediate intermolecular interactions. The mutations in this domain are predicted to either affect the affinity to binding partners such as to the sterol regulatory element binding protein (SREBP1), and others (Vlcek and Foisner, 2007b) or disrupt the 3-dimensional structure of the Ig-fold (Dhe-Paganon et al., 2002; Krimm et al., 2002).

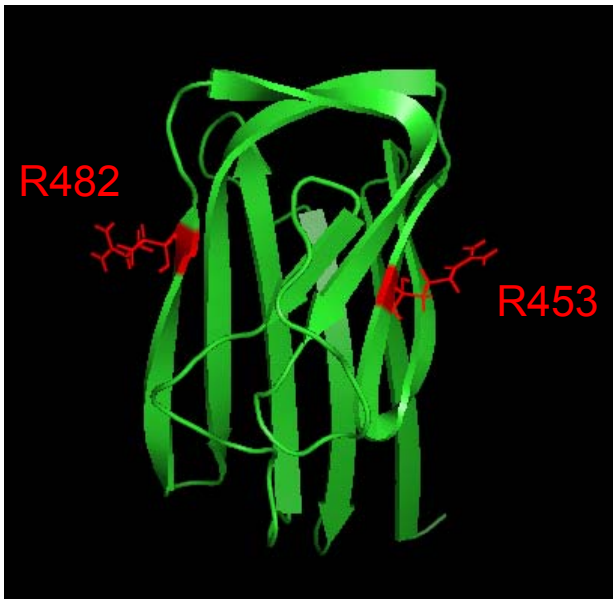

Figure 3: Structure of the lamin A/C Ig-fold. The NMR structure was obtained from Krimm and colleagues (Krimm et al., 2002) and was modified with the PyMOL Molecular Viewer program. The position and the side chains of the amino acids Arg453 and Arg482 are highlighted in red.

Functions of lamins

For a long time it was thought that lamins and lamin-associated proteins have only structural roles providing the cell nucleus with mechanical stability and maintaining the nuclear shape. However, the last decade has brought new insights into many novel roles of lamins and their interactions with various proteins of diverse functions. Besides their structural functions, it was shown that lamins are also involved in chromatin organization, DNA replication, RNA transcription, cell cycle regulation and differentiation, nuclear migration and positioning, nuclear (re-)assembly, NPC anchorage, regulation of gene expression, signal transduction, apoptosis and others (Gruenbaum et al., 2003; Vlcek and Foisner, 2007a).

Lamins and lamin-associated proteins interact with a variety of nuclear proteins. These proteins are involved in a wide variety of cellular processes, including heterochromatin organization, cell cycle regulation and differentiation, and DNA repair and genome stability (Vlcek and Foisner, 2007a; Vlcek and Foisner, 2007b). The INM proteins, lamin associated polypeptide 2β (LAP2β) and Lamin B receptor (LBR), interact with the histone deacetylase HDAC3, or with the heterochromatin protein 1 (HP1), respectively, which are both involved in organization and maintenance of a transcriptionally silent chromatin. For instance, loss of heterochromatin is found in patients suffering from Hutchinson-Gilford progeria syndrome

(HGPS), an accelarated aging syndrome caused by mutations in A-type lamins (Scaffidi and Misteli, 2005; Shumaker et al., 2006).

The lamin A/C-LAP2α complex seems to regulate the activity of the cell cycle regulatory retinoblastoma protein (pRb) in the nucleoplasm. Results by Naetar and colleagues lead to a model in which LAP2α sequesters peripheral lamin to the nucleoplasm, where the lamin A/C-LAP2α complex is required for the repression of pRb/E2F regulated cell cycle regulatory genes. If this complex formation is impaired, for example by mutations in lamin A/C or LAP2α, cell cycle regulatory genes can become constitutively expressed. This leads to the hyper-proliferation of lineage-commited progenitor cells, whereas the differentiation of these cells seems to be normal. This leads to an abnormal ratio of proliferation versus differentiation, as the ratio is shifted towards more proliferation at a constant differentiation rate (Naetar et al., 2008; Vlcek and Foisner, 2007a). Lamins are also known to interact with and modulate the activity of the SREBP1, an adipocyte specific transcription factor. Finally, there are also indications that lamins are involved in DNA repair, but the molecular mechanisms are still unclear (Vlcek and Foisner, 2007b).

Laminopathies

Laminopathies represent a group of rare human hereditary diseases that are caused by mutations in the LMNA gene coding for lamin A/C, or in the genes encoding lamin binding proteins (emerin, LAP2α, MAN1, LBR). Recently, also mutations in the *LMNB1* and *LMNB2* genes have been linked to rare cases of laminopathies (Hegele et al., 2006; Padiath et al., 2006). So far, 218 mutations have been identified in the *LMNA* gene. These mutations are spread along the whole gene (Worman and Bonne, 2007). The vast majority of the mutations are single point missense mutations, thus leading to an exchange of just one amino acid. Most of these mutations are inherited in an autosomal dominant way, thus leading to the expression of the mutated and the wild-type protein, side by side in a cell.

The first laminopathy discovered was linked to the INM protein emerin, the mutation of which can cause X-linked recessive Emery-Dreifuss Muscular Dystrophy (EDMD) (Bione et al., 1994). In 1999 the concept of lamins of having only a role in the maintenance of the nuclear structure integrity had to be reviewed as mutations in the *LMNA* gene were found to cause autosomal dominant EDMD (AD-EDMD) (Bonne et al., 1999).

Patients suffering from laminopathies have a large variety of symptoms including skeletal and/or cardiac muscular dystrophy, lipodystrophy with insulin-resistance, dysplasia, dermopathy, neuropathy, leukodystrophy, and progeria. Laminopathy causing mutations are

associated with at least 13 known diseases, EDMD, Dunnigan-Type Familial Partial Lipodystrophy (FPLD), Dilated Cardiomyopathy with Conduction Defect Type 1A, Limb Girdle Muscular Dystrophy 1B, Charcot-Marie-Tooth Disorder Type 2B1, Mandibuloacral Dysplasia, and Hutchinson-Gilford progeria syndorme. Generally, symptoms develop after birth, typically during childhood or adolescence. Till now it can be just poorly explained how mutations in a ubiquitous protein can have such diverse, tissue-restricted pathological phenotypes.

Laminopathy disease models

Four models have been proposed to explain the multifaceted features of mutant lamina proteins causing laminopathies. The "Structural Model" suggests that the impaired stability of lamin complexes would lead to an increased nuclear fragility, and the disturbance of nuclear anchorage and positioning. The "ER-retention Theory" suggests that some INM proteins are dislocalized to the ONM and the ER due to a reduced affinity of mutant A-type lamins to these INM proteins. These incorrectly localized proteins might interact with ER proteins, thereby affecting their function (fatty acid metabolism, Ca^{2+} release during muscle contraction). The "Gene Expression Model" is based on the fact that lamins bind to DNA and chromatin and thus regulate the epigenetic chromatin organization, which could be impaired in laminopathies. Finally, the "Proliferation-Cell Cycle-Ageing Model" suggests a role of A-type lamins in the control of cell proliferation and differentiation in the adult organism, as manifested e.g. in their binding to Rb and other cell cylce regulators. A new model, "tissue regeneration", was proposed recently, which tries to combine all of the four disease models. This latest hypothesis proposes that impaired tissue homeostasis and tissue regeneration rather than the degeneration of differentiated tissues is responsible for the disease phenotype. Such a mechanism would also explain the fact that laminopathies manifest in childhood or puberty where defects in e.g. skeletal muscles have accumulated, providing a reasonable explanation for the late onset of e.g. myopathies. The "tissue regeneration" model is favoured over the "differentiated tissue degeneration" model, as A-type lamins seem to be involved in cell-cycle regulation. Thus, e.g. in myopathies the cell-cycle regulation of satellite cells may be affected, causing an impairment of regeneration of damaged tissue. The disease manifests after accumulation of muscle damage, because the impaired satellite cells cannot repair the tissue to the level required. Nevertheless, the reasons for tissue-restricted phenotypes in laminopathies remain unclear (Gotzmann and Foisner, 2006; Vlcek and Foisner, 2007a).

Hutchinson-Gilford Progeria Syndrome

The most severe and striking laminopathy phenotype is associated with progeroid syndromes, such as Hutchinson-Gilford progeria syndrome (HGPS), atypical Werner syndrome (WS) and restrictive dermopathy (RD).

HGPS (OMIM #176670) is by far the best-studied laminopathy. By studying HGPS researchers aim to understand molecular mechanisms of pathological as well as normal human aging. Children born with HGPS appear normal, but accelerated aging is manifested within the first year. Symptoms include loss of hair, wrinkled skin, diminished subcutaneous fat, cardiovascular disease and skeletal abnormalities. Children usually die in the second decade of their life due to heart attack or stroke.

At the cellular level, HGPS is associated with blebbing of the NE, thickening of the nuclear lamina, loss of peripheral heterochromatin, clustering of nuclear pores (Goldman et al., 2004), aberrant chromosome segregation and bi- and multinucleation at the cellular level (Cao et al., 2007), as well as increased genomic instability (Liu et al., 2005).

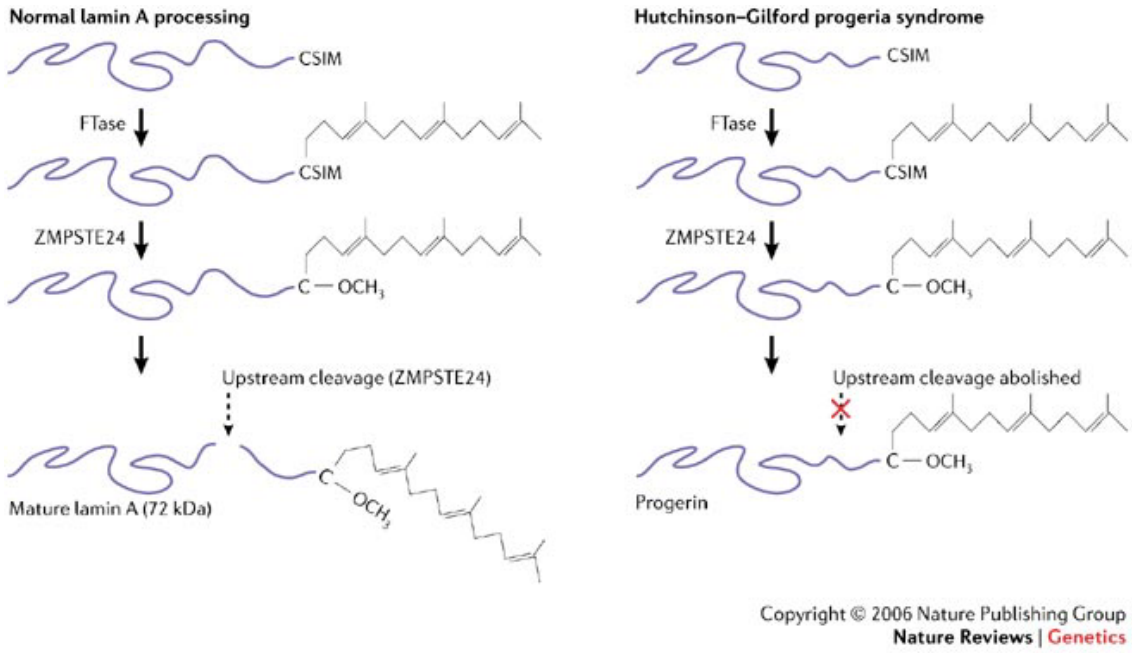

Figure 4: Post-translational processing of lamin A and progerin. In HGPS cells one allele gives rise to correctly spliced prelamin A mRNA, and one allele yields an aberrant progerin mRNA, with an internal deletion of 150nt from exon 11, due to activation of a cryptic splice site by the 1824C>T mutation. After translation, the C-terminal amino acid sequence CSIM signals for farnesylation of cystein by the enzyme farnesyltransferase (FTase). Upon farnesylation, the three C-terminal amino acids are cleaved by the ZMPSTE24 endoprotease. The farnesylated cystein is then carboxymethylated. ZMPSTE24 cleaves prelamin A a second time and removes the terminal 15 amino acids containing the farnesyl group, producing mature lamin A. The second cleavage by

ZMPSTE24 is inhibited in progerin, as the cleavage site was removed by the aberrant splicing (Capell and Collins, 2006).

About 90% of HGPS cases are due to a dominant 1824C>T mutation in the *LMNA* gene. This mutation does not alter the encoded amino acid (G608G), but activates a cryptic splice site, which is also used at a very low frequency in wild-type cells (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003; Scaffidi and Misteli, 2006). The protein product is termed progerin, an incompletely processed prelamin A protein containing a 50 amino acid internal deletion, which is stably farnesylated, as the signal for the second proteolytic cleavage was eliminated by the aberrant splicing caused by the mutation (figure 4). Due to the stable farnesylation, progerin remains tightly associated with the nuclear membrane and disrupts in this way the normal lamina function.

It was shown that the elimination of progerin by correction of the aberrant splicing form in HGPS patient cells rescues the disease phenotype in these cells (Scaffidi and Misteli, 2005). Combining this finding with the fact that overexpression of wild-type lamin A in HGPS cells does not restore normal cellular phenotype, and that ectopic expression or microinjection of progerin triggers a disease phenotype in wild-type cells indicates that the disease phenotype in HGPS patients is due to a dominant-negative effect of progerin on lamin A, interfering with essential lamina functions, such as the maintenance of nuclear shape or regulation of gene expression (Fong et al., 2006; Goldman et al., 2004; Scaffidi and Misteli, 2005; Scaffidi and Misteli, 2006). The involvement of progerin in normal human aging was demonstrated by inhibition of the *LMNA* aberrant splice site in skin fibroblast cell lines from old individuals, which could reverse age-related defects in nuclear structure (Scaffidi and Misteli, 2006). This finding shows that lamin A is involved in normal physiological aging and that the cryptic splice site is also used sporadically in cells from healthy individuals, in this way possibly contributing to normal tissue aging. Based on these findings and their own experiments, Haithcock and colleagues suggest that HGPS patients are suffering from an accelerated aging process (Haithcock et al., 2005; Varela et al., 2005).

Emery-Dreifuss Muscular Dystrophy

Emery-Dreifuss Muscular Dystrophy (EDMD) (OMIM #310300) is a relatively common laminopathy. 60% of all laminopathies affect striated muscle tissue. About 50% of all EDMD patients have mutations in either the *lmna* or the *emd* gene, coding for A-type lamins or emerin, respectively. The remaining patients have mutations in other, not yet identified genes

(Stewart et al., 2007). The disease is associated with contractures (shortening of muscles and/or tendons), muscle weakness and wasting, and cardiac conduction defects. Three types of EDMD are distinguished, depending on the pattern of inheritence. X-linked EDMD is associated with mutations in the gene coding for the INM protein emerin (mainly nonsense mutations), autosomal dominant and autosomal recessive EDMD are associated with mutations in the *LMNA* gene coding for lamin A/C (mainly missense mutations).

The pathophysiology of EDMD is mostly unknown. It is generally thought that mutations in A-type lamins, which cause myopathies may be due to an impaired ability of the mutant lamin A and lamin C proteins to form stable filaments, or an altered affinity of mutant lamin A/C to certain binding partners (Broers et al., 2005; Holt et al., 2003; Krimm et al., 2002).

The hot spot mutation R453W in the Ig-fold of lamin A/C (figure 3) is responsible for 16% of all AD-EDMD cases and is the cause of 11% of all laminopathies (Gotzmann Josef, 2004). This mutation is predicted to destabilize and alter the 3-D structure of the C-terminal Ig-fold of lamin A/C by disrupting a salt bridge (Dhe-Paganon et al., 2002; Krimm et al., 2002).

It was shown that lamin A R453W impairs myogenic differentiation in almost all cells expressing lamin A R453W, by inhibiting expression of myogenin, a muscle-specific transcription factor required for myogenesis, by maintaining a suppressive chromatin state on the *Myog* promoter. In addition, genome-wide epigenetic defects, such as rearrangement of pericentric heterochromatin and dissociation of H3K27me3 (tri-methylation of lysine 27 on histone 3), a mark for repressed genes or heterochromatin, from the imprinted X-chromoseome (Xi), were observed in cells expressing lamin A R453W (Hakelien et al., 2008). The observed effects caused by lamin A R453W may describe a molecular basis for AD-EDMD, as the presence of lamin A/C R453W in satellite cells of an individual suffering from AD-EDMD may impair the differentiation of myoblasts to form muscle fibers, leading to impaired tissue regeneration.

Dunnigan-Type Familial Partial Lipodystrophy

Dunnigan-Type Familial Partial Lipodystrophy (FPLD) (OMIM #151660) is also a relatively common disease associated with mutations in *LMNA*, affecting approximately 15% of all laminopathy patients (Gotzmann Josef, 2004). FPLD is characterized by partial lipodystrophy and insulin resistance. In puberty, subcutaneous fat is relocated from the upper and lower extremities and the gluteal and truncal regions to the face, neck and intra-abdominal regions.

Most FPLD causing mutations known are located at the C-terminus of lamin A and lamin C.

85% of patients diagnosed with *LMNA*-linked FPLD have heterozygous mutations of the amino acid R482, leading to a substitution by either tryptophan, glutamine, or leucine. The amino acid R482 is solvent exposed and located at the C-terminal end of the "d" β-strand of the lamin A/C Ig-fold domain (figure 3). The R482W mutation causes a loss of a positive charge, which is conserved in vertebrate lamins at this position. The mutation is not predicted to affect the 3-D structure of the protein (Dhe-Paganon et al., 2002; Krimm et al., 2002). It seems that R482 is required for A-type lamins to interact with the adipocyte specific transcription factor, SREBP1, and DNA, as the mutant protein shows a decreased affinity to SREBP1 and DNA. The reduced affinity of the mutant A-type lamins to SREBP1 could interfere with adipocyte differentiation (Capeau et al., 2005; Lloyd et al., 2002). However, the exact molecular mechanism leading to an adipocyte-restricted disease phenotype is still unknown.

Dilated Cardiomyopathy with Conduction Defect Type 1A

Dilated Cardiomyopathy with Conduction Defect Type 1A (DCM) (OMIM #115200) is caused by autosomal dominant mutations in *LMNA*. The disease phenotype consists of a dilated cardiomyopathy and conduction system defects. The joints and skeletal muscles are not affected.

Lamin A and lamin C share the same N-terminal alpha-helical rod domain containing amino acids 31 - 381. The point mutation N195K in the rod domain causes DCM. The rod domain consists of a heptad repeats, which mediate the formation of coiled-coiled dimers. Considering the heptad repeat pattern in the rod, where hydrophobic amino acids interact with their counterparts of the corresponding heptad repeats of the neighbouring protein to form the so-called "coiled-coil" structure, N195 should not occupy a position at the interface of two coiled-coil domains, but is predicted to be solvent exposed (Raharjo et al., 2001).

The mutation N195K is associated with a high rate of sudden cardiac death, and progressive heart failure necessitating cardiac transplantation (Fatkin et al., 1999; van Tintelen et al., 2007). $LMNA^{N195K/N195K}$ mice diplay cardiac conduction defects, but no other tissue type is affected, and they die due to arrhythmia at about 12 weeks after birth. Fetal genes, required for reorganization of the heart, are found to be upregulated in hearts of $LMNA^{N195K/N195K}$ mice, compared to wild-type mice. It is assumed that the upregulated fetal gene products, required for reorganization of the heart, try to compensate the decreased conduction efficiency of the heart of $LMNA^{N195K/N195K}$ homozygous mice. In addition, sarcomer organization seems

to be disturbed in hearts of mutant mice. The phenotype of the mouse model resembles the phenotype of DCM patients (Mounkes et al., 2005).

Antibodies

The function of a huge portion of the proteome is still unknown. One of the most important, if not crucial, tools for the investigation of protein function are antibodies. As mentioned above, point mutations leading to changes in the primary amino acid sequence can predispose individuals to certain diseases and can also lead to monogenic inherited diseases, some of which are inherited in a dominant way. If autosomal dominantly inherited, the wild-type protein and the disease-causing mutant protein are present side by side in the cell. Genetic abnormalities like point mutations can be detected by and screened with Polymerase Chain Reaction (PCR) in individuals, but PCR can not reveal any information about the encoded mutant protein like its expression, localization within the cell, interaction with ligands or other binding partners, stability and (catalytic) activity. Hence, in many cases the altered biological function of the mutant protein leading to a diseased phenotype remains unclear. Specialized tools, such as antibodies, are required, which allow to study the molecular functions of those mutant proteins in patient cells or tissue sections in detail.

History of antibodies

Antibodies were discovered by the German scientist Emil von Behring in 1890. At this time, it was known that if an animal survives an infection with a pathogen, it remains resistant to a re-infection to this particular pathogen for a lifetime (e.g. *Corynebacterium diphtheriae*, *Clostridium tetani*, *Bordetella pertussis*), but it did not develop immunity against a different pathogen. Behring and his co-worker, Shibanasaru Kitasato, found out that the blood serum from animals exposed to bacterial extracts could transfer resistance against the bacterium and its toxins to an animal previously not exposed to this pathogen. They concluded that the transfer of resistance was due to soluble “antitoxins” present in the blood serum and that this resistance was specific for the one pathogen. Soon after, the terms antibody, also called immunoglobulins, and antigen were born. With experiments done e.g. by Paul Ehrlich and Karl Landsteiner it became clear that almost any biological and chemical compound can serve as an antigen to which antibodies can specifically bind. The perception of such a seemingly unlimited number of potential antigens subsequently led to the so called “antibody problem”.

In 1944 Oswald Avery and his co-workers discovered that the DNA was the carrier of genetic information and was inherited from one generation to the next. Subsequently, a series of experiments established the "one gene – one protein" dogma. Since there are vast numbers of immunogenic antigens, the number of genes coding for antigen-specific antibodies would need to be enormous. Two models were proposed to tackle this "antibody problem". The "instructional theory" proposed that the antigen instructs the antibody to bind to it, which would require only one antibody gene. The "clonal selection theory" (Burnet, 1957; Jerne, 1955) argued that an antibody serves as a receptor on B-lymphocytes, which are activated upon binding of the corresponding antigen. In this model, the number of genes coding for antibodies is limited and the large amount of antibodies is due to the rearrangement of gene segments and somatic hypermutation. The first evidence supporting the "clonal selection theory" was an experiment which showed that a given B-lymphocyte expresses only one given antibody (Nossal and Lederberg, 1958). To further confirm the validity of the "clonal selection theory", to trace somatic mutations in antibody genes, an antibody population of predefined specificity against a defined epitope was required. In 1975, Cesar Milstein and his co-worker Georges Köhler were successful in developing the first stable hybridoma cell line producing mouse monoclonal antibodies of predefined specificity (anti-sheep red blood cell) (Kohler and Milstein, 1975). Since B-lymphocytes from immunized animals, in most cases mice, cannot proliferate indefinitely *in vitro*, Köhler and Milstein fused them with myeloma cells to obtain immortal antibody-secreting cells, the so-called hybridoma cells. With the help of this novel technique, mechanisms such as the somatic hypermutation in the immunoglobulin variable genes as well as the affinity maturation of antibodies were confirmed. However, this invention did not only help to answer the questions of the "antibody problem", but revolutionized research in life sciences in general (Alkan, 2004; Milstein, 2004).

Antibody structure and function

Antibodies are glycoproteins and, together with the major histocompatibility complex (MHC) molecules and the T cell antigen receptor, are used by the adaptive immune system to recognize foreign, potentially harmful antigens (bacteria, viruses, protein antigens) to target them for destruction and thus eliminate them from the body. Antibodies are produced by B-lymphocytes, first in a membrane-bound form to serve as specific receptors and later, after the activation of B-lymphocytes, in a secreted form to perform effector functions. Depending on the nature of the antigen and the antibody classes (isotypes) and subclasses (in humans IgA1-

2, IgD, IgE, IgG1-4, IgM) different effector mechanisms, strategies, are applied to eliminate the antigen.

All antibodies have the same basic structural characteristics, a symmetric core structure composed of two identical light chains and two identical heavy chains, but they display high variability in the antigen-binding region, located in the N-terminus of the light and heavy chains (so called complementary-determining regions, CDRs). There are up to 10^7 – 10^9 different antibody molecules in an individual and up to 10^{10} different antibodies could be made in theory. Antibodies do not only recognize naturally occurring antigens, but can also specifically detect and bind to non-naturally occurring chemicals (synthetic chemicals coupled to a carrier protein - haptens) (Abbas, 2003).

Monoclonal antibodies

Antibodies - in particular monoclonal antibodies - display high specificity for a certain protein. Thus, they are essential tools for studying protein function and molecular pathways of biological processes and are used in different molecular biological techniques, like immunoblotting, immunoprecipitation, immunofluorescence, or cell-sorting. Besides the use in research, antibodies are in clinical use since their discovery. Passive immunization, i.e. the treatment of infectious diseases by administration of antibodies to patients, is in use since 1890. Infectious diseases such as botulism, diphtheria, rabies and many others are still cured by direct administration of specific antibodies. Furthermore, there is an increasing interest from the pharmaceutical industry in using antibodies for therapeutic interventions of a wide variety of other diseases. Till now 21 therapeutical antibodies have been approved by the U.S. Food and Drug Administration (FDA), which aim to cure cancer, infectious diseases, allergies, asthma, autoimmune diseases, or transplant rejection. Examples of successful therapeutic antibodies are Herceptin (Genentech) for the treatment of a certain subset of breast cancers with highly elevated levels of EGFR; Rituxan, Mabthera (IDEC Pharmaceuticals) and Bexxar (Corixa) for the treatment of Non-Hodgkin lymphoma; Lucentis (Genentech) for the treatment of macular degeneration; Avastin (Genentech) for the treatment of colorectal cancer; and Xolair (Genentech) for the treatment of allergic asthma. Thus, given their promising potential to cure a wide range of diseases, therapeutic antibodies are a very fast growing segment in the pharmaceutical industry.

Aim of diploma thesis

The aim of my diploma thesis was the generation of mouse monoclonal antibodies specific for mutant forms of lamin A/C, namely the R453W mutant, which is associated with AD-EDMD, the R482W mutant, which causes FPLD, and the N195K mutant, which causes dilated cardiomyopathy (DCM), and to test these antibodies for their usability in different molecular biological techniques, such as e.g. immunoblotting, immunoprecipitation, and immunofluorescence. Besides the generation of point-mutation specific antibodies we also aimed to generate complementary wild-type specific antibodies with the ultimate goal to generate pairs of antibodies directed towards the respective epitopes encompassing the same amino acid region, but differing in a single amino acid. As mentioned before, all of these three laminopathies, AD-EDMD, FPLD, and DCM, are inherited in a dominant fashion, most likely resulting in the expression of both the wild-type proteins and the point-mutants side by side in the same cell. One major obstacle for a more complete understanding of the molecular mechanisms underlying the development of laminopathies is the lack of research tools, which can distinguish between the wild-type and the mutant protein, e.g. none of the currently available antibodies is specific for either form.

The project was started based on observations in the Ogris lab that antibodies raised against protein phosphatase 2A (PP2A) subunit isoforms are able to discriminate between isoforms differing only in one amino acid at the respective epitope (Sontag et al., 2004). This observation evoked the idea to generate either wild-type or disease-relevant point-mutation specific antibodies. Until now only one point-mutation specific antibody, raised against the R426W point-mutation in the Alexander disease-causing glial fibrillary acidic protein, has been reported (Der Perng et al., 2006). The newly developed, specific antibodies against the lamin A/C mutants – and the corresponding wild-type epitopes – shall be employed to examine and characterize in patient cells the molecular properties and functions of the mutant lamin A/C proteins, and the differences therein compared to the wild-type proteins.

Approaches

To generate point-mutation specific antibodies against the disease-causing point mutations N195K, R453W and R482W in lamin A and lamin C, two different approaches were employed for the presentation of the respective antigens. First, short peptides of 9 amino acids in length and bearing the respective mutations or the corresponding wild-type epitopes were coupled to keyhole limpet hemocyanin (KLH) as immunogenic carrier protein. Second, fusion proteins of the Hepatitis B virus core antigen and the C-terminal Ig-domain of lamin A and lamin C (in the wild-type sequence, and with the point-mutations R453W and R482W) were used as antigens to stimulate a specific immune response in mice. The point mutation N195K was not inserted into the HBcAg protein for reasons discussed below. Besides the generation of point-mutation specific antibodies we were also interested of generating complementary wild-type specific antibodies with the ultimate goal to generate pairs of antibodies directed towards the wild-type and mutant epitopes, which are differing in one amino acid. In this way the antibodies would allow to distinguish between wild-type and mutant proteins. All the mentioned mutations are inherited in an autosomal dominant way. The wild-type and the mutant protein are expressed side-by-side in a cell. A pair of antibodies recognizing specifically the mutant protein and the wild-type protein, would allow to investigate the special features of the two protein variants separately.

Approach A: Immunization of mice with KLH-coupled peptides

The administration of KLH-coupled peptides as antigens to mice or rabbits is a traditional and well-documented approach to stimulate the production of specific antibodies against short peptides. This approach has the advantage that the immune response can be directed very specifically to a chosen epitope. This feature is on the other hand also a disadvantage, namely in the case that the chosen peptide is not immunogenic. Another disadvantage could be that peptides coupled to KLH are not displayed in an identical manner as in the full-length protein. In many circumstance these peptide-induced antibodies do not recognize the full-length protein. Due to their small size peptides are generally not immunogenic and require the aid of a carrier protein to stimulate a humoral immune response. KLH is the most widely used carrier protein. KLH is a heterodimer with a molecular weight of about 740 kDa. The huge size of KLH is one big advantage, as it contains many, potent T-cell epitopes, which support the stimulation of the immune system. The other advantage is the abundance of lysine residues on the protein surface, because these lysine residues are required for the coupling of

the peptide of interest to KLH. Thus, a high number of lysines available for coupling results in many copies of the peptide linked to one carrier molecule.

To direct the immune response to the epitope containing the point-mutation, short peptides were coupled to KLH. The peptides used for immunisation of mice are displayed in Materials and Methods (please see section 2.3.5.), and were injected sub-cutaneously into mice as outlined in the immunisation schedule for mice (please see section 2.5.4.). We decided to raise antibodies against short peptides corresponding to the lamin A and lamin C sequences containing amino acids 191-199, with the mutation N195K, and amino acids 449-457, with the mutation R453W. The peptides corresponding to the point mutation R482W were already used for immunizations by Veronika Huber, and were not used again for immunizations, as the results from the immunizations were not promising (for data see diploma thesis of Veronika Huber).

Approach B: Immunization of mice with HBcAg recombinant proteins

The immune response elicited to a KLH-coupled peptide antigen can vary a lot. To overcome this problem the use of the hepatitis B virus capsid as a carrier for some of the antigens was chosen.

The Hepatitis B virus is an icosahedral DNA virus of the hepadnavirus family and is the causative agent of B-type hepatitis in humans. The viral capsid is built up by multiple copies of the 183 amino acid long capsid protein, the so-called Hepatits B core antigen (HBcAg). A strong, long-lasting humoral immune response is elicited in people infected with Hepatitis B virus. This humoral immune response is mainly directed against the immunodominant epitope c/e1, which is located at the spike of the capsid surface, encompassing amino acids 78-83 of HBcAg (Pushko et al., 1994; Salfeld et al., 1989). Besides this very strong immunogenic epitope, HBcAg also contains potent T helper cell epitopes, which support the humoral immune response against a proteinogenic antigen. On the other hand, HBcAg can also act as a T-cell independent antigen due to its ability to form multimers, which allow the simultaneous cross-linking of sufficient surface B-cell receptors to activate the naive B-lymphocyte without the help of T-lymphocyte. These characteristics make the HBcAg protein a strong immunogen (Kratz et al., 1999).

Kratz and colleagues have shown that it is possible to insert a whole protein, namely the Green Fluorescent Protein (GFP), into the HBcAg capsid protein at the position of the c/e1 immunodominant epitope. The use of HBcAg recombinant protein as an antigen is thought to require that the inserted protein, or protein domain, has its N- and C-terminal ends in close

spatial proximity, which should facilitate proper folding of the carrier protein, HBcAg, and the inserted protein or protein domain. As shown by Kratz and colleagues, the HBcAg – GFP fusion protein was properly folded, as the recombinant protein formed fluorescent particles. In addition a GFP-specific humoral immune response was generated (Kratz et al., 1999).

Since the structure of the lamin A/C Ig-fold revealed that the N- and C-termini of the Ig-fold domain are in close spatial proximity (Dhe-Paganon et al., 2002; Krimm et al., 2002), we reasoned that the insertion of the whole lamin A/C Ig-fold in either the wild-type sequence, or containing the point-mutations R4543W or R482W, into the HBcAg protein could be a suitable alternative to the traditional KLH-peptide approach to elicit a point-mutation specific, and a complementary wild-type specific immune response. In addition, the lamin A/C Ig-fold R453W mutant is predicted to cause a disruption of the native globular structure of the Ig-fold domain (Dhe-Paganon et al., 2002; Krimm et al., 2002). Therefore we thought of generating a lamin A/C Ig-fold R453W conformational specific antibody.

We generated HBcAg recombinant proteins containing the Ig-fold in either the wild-type, R453W or R482W sequence. In agreement with Kratz and colleagues we used a truncated version of HBcAg lacking amino acids 150 - 183, which are required to bind to DNA, but are not required for the formation of the proper, spike-like 3-D structure and the formation of di- and multimers of HBcAg. The point-mutation N195K lies in the alpha-helical rod domain of lamin A/C in which the N- and C-termini of this domain are predicted to be at opposite ends. Hence, we decided not to insert the domain into the HBcAg protein, as the linear structure of this rod domain most likely would not allow a proper folding of the carrier protein.

“HBcAg linker –peptide” recombinant proteins

Finally, short peptides of the desired epitopes were also inserted into the HBcAg protein instead of the c/e1 epitope (for design of constructs please see section 2. 1. 13. 2.). Using these recombinant proteins I tried to combine advantages from the KLH-peptide approach, namely its high specificity, and the HBcAg recombinant protein approach, namely the strong immunogenicity of HBcAg. In combining these advantages, I expected to elicit a strong and specific immune response against the amino acid sequences. The peptides corresponding to the mutations N195K, R453W and R482W, as well as the corresponding wild-type peptides, were inserted into the HBcAg protein. Some of these HBcAg linker-peptide recombinant proteins were then used for immunisations of mice.

Recombinant HBcAg (C3d-P28)$_2$ proteins

C3d is a component of the complement system of the immune system. C3d has the ability to bind to antigens, which enables the C3d bound antigen to simultaneously activate signalling cascades from the B cell receptor and the complement receptor 2 (CR2) of the B lymphocyte. This leads to a strong activation of the B cell and thus elicits a strong humoral immune response. The antigenicity of a protein is significantly increased when coupled to C3d (Abbas, 2003). Through expression of the strong immunogens HBcAg and C3d in the recombinant protein HBcAg (C3d-P28)$_2$, the immune response should be increased in mice upon immunization of HBcAg (C3d-P28)$_2$, compared to immunizations with the immunogen HBcAg.

2. Materials and Methods

2. 1. Working with DNA

2. 1. 1. Buffers and solutions

Miniprep solutions

Solutions I (lysis buffer)

50mM Glucose; 25mM Tris pH 8.0; 10mM EDTA pH 8.0. Filter sterilize and store at 4°C.

Solution II (alkaline SDS buffer)

0.2M NaOH; 1% (w/v) SDS. Store at RT.

Solution III (high salt buffer)

3M Potassium acetate; 2M Acetic acid. Store at 4°C.

50x TAE

484g Tris; 114ml acetic acid; 200ml 0.5M EDTA pH 8.0. Dissolve and fill up to 2l with H_2O and store at RT.

1x TAE (working solution)

40mM Tris acetate; 2mM EDTA.

RNAse A

Dissolve 10mg/ml RNAse A (Sigma #R-5125) in 10mM sodium acetate pH 5.2. Heat to 100°C for 15min and cool down to RT. Adjust the pH by adding 0.1 volumes of 1M Tris-Cl pH 7.4. Store 1ml aliquots at -20°C.

10x Annealing buffer

100mM Tris pH 7.5; 1M NaCl; 10mM EDTA

Lambda DNA Marker

Dilute the concentrated marker (Fermentas #SM0111) with TE pH 8.0 to a final concentration of 0.1µg/µl. Heat the solution to 65°C for 5 minutes, cool on ice and store aliquots at -20°C.

6x DNA loading dye (Fermentas #R0611)

5x Sequencing buffer

100mM Tris pH 9.0; 10mM $MgCl_2$

Big Dye Terminator v3.0 (Applied Biosystems #4390253)

PCI, Phenol:Chloroform:Isoamyl alcohol (25:24:1) (AppliChem #A0889,0500)

2. 1. 2. Restriction digest

For digesting 1 µg plasmid DNA use 1-5 U of appropriate restriction enzyme and prepare the reaction mix as suggested by the supplier. The total volume of a reaction mix was either 100µl for preparative purposes or 30µl for control digests. Incubate the sample 3 hours at the recommended temperature. Stop the reaction by adding 6x DNA loading dye.

Analyze the restriction digest on a 1% - 2% agarose gel (in 1x TAE), depending on expected sizes of fragments.

2. 1. 3. CIP treatment of digested plasmid

Add 20 U of alkaline phosphatase, calf intestinal (CIP; New England Biolabs, NEB #M0290S) in a reaction mix as suggested by the supplier to the restriction digest and incubate for 1h at 37°C. Stop the reaction by adding 6x DNA loading dye. Analyze the CIP-treated restriction digest on a 1% - 2% agarose gel (in 1x TAE), depending on expected sizes of fragments.

2. 1. 4. Elution of DNA fragments

Use the Wizard® SV Gel and PCR Clean-Up System (Promega #A9283) to isolate DNA from agarose gels. Excise desired DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube. Follow the manufacturer´s instructions. Store DNA at –20°C. Check 1µl – 5µl of elution on an agarose gel.

2. 1. 5. Annealing of oligos

Anneal 4pmol/µl of corresponding forward and reverse oligo in a mix containing annealing buffer in a total volume of 50µl. Incubate for 5 minutes in a baker with boiling water. Turn off heater and let cool down to room temperature. Store DNA at –20°C.

2. 1. 6. Phosphorylation of annealed oligos

Phosphorylate oligos with 20U of T4 PNK (Polynucleotidekinase, NEB #M0201L) in a reaction mix containing 1mM ATP and T4 PNK Buffer (NEB) for 3 hours at 37°C.

2. 1. 7. Purification of DNA by PCI extraction/EtOH precipitation

Extract DNA by adding ½ volume of PCI to 1 volume of the DNA solution, vortex and spin 30-60 sec. Remove the upper phase containing DNA into a clean 1.5ml microcentrifuge tube. Repeat this step once.

Add 1/10 volume of 3M Na-Acetate pH 5.2 to the DNA solution. Add 2.5 volumes of cold 96% ethanol (stored at –20°C). Precipitate DNA over night at –20°C. Centrifuge the solution 30 minutes at 14000rpm at 4°C. Wash DNA pellet once with 70% ethanol. Air dry pellet and resuspend DNA in 50µl H_2O. Store DNA at –20°C.

2. 1. 8. Ligation of DNA fragments

Mix ~50-100ng of plasmid DNA and insert DNA at a molar ratio of 1:3 in a final volume of 20µl containing2µl T4 ligation buffer (containing 10mM ATP) and 0.5 U T4 DNA ligase (NEB). Incubate the mixture at 16°C over night. Take 10µl of mixture DNA to transform heat – shock competent E. coli.

2. 1. 9. Alkaline lysis plasmid DNA MINI preparation

Grow bacteria o/n in LB+ appropriate antibiotics. Take 1ml – 2ml of bacterial over night culture and centrifuge 5 minutes at 5000rpm. Resuspend bacterial pellet in 100µl solution 1 and add 200µl of cold solution 2. Shake and lyse for 5 minutes at room temperature. Add 150µl of solution 3 and shake vigorously. Spin the mixture for 10 minutes at 14000rpm. Take the supernatant and add 300µl isopropanol and mix. Spin 10 minutes at 14000rpm. Wash the DNA pellet with 70% ethanol. Air dry DNA pellet and redissolve in 50µl Tris-EDTA + RNAse.

2. 1. 10. Plasmid MINI preparation using the QIAprep® Spin Miniprep Kit (Qiagen #27106)

For small amounts of plasmid DNA preparation the QIAprep® Spin Miniprep Kit (Qiagen #27106) kit was used. Follow the guidelines provided by the supplier's protocol.

2. 1. 11. Plasmid MIDI preparation using the PureYield™ Plasmid Midiprep System (Promega #252219)

For large amounts of plasmid DNA preparation the PureYield™ Plasmid Midiprep System (Promega #252219) kit was used. Follow the guidelines provided by the supplier's protocol.

2. 1. 12. Sequencing of DNA

Use 0,5-1µg of DNA for a sequencing reaction in a total reaction volume of 20µl, containing 4µl sequencing buffer, 2µl Big Dye Terminator v3.0 and 4pmol of primer (1pmol/µl). The sequencing reaction was performed with the following program:

96°C	1min	
96°C	30sec	
50°C	10sec	
60°C	4min	30 cycles
4°C		

2.1.12.1. Sequencing primer used

pB'His_fw_new (#523)
5'-CTCTAGAAAGGAGGTACGATC-3'

2. 1. 13. Cloning of contructs

All constructs generated were derivates of the N-terminally His-tagged HBcAg linker protein. The maps of the generated constructs are listed in the Appendix.

A simplified composition of the recombinant proteins generated:

N-terminus of HBcAg (aa 1-78) – Gly-linker (aa GGGGSGGGGT) – inserted peptide or protein domain – Gly-linker (aa GGGGSGGGG)– C-terminus of HBcAg (aa 81-149)

2. 1. 13. 1. Cloning of pB'His HBcAg Ig-fold WT, pB'His HBcAg Ig-fold R453W, and pB'His HBcAg Ig-fold R482W

The cloning of the constructs was started during my Wahlbeispiel and finished by Veronika Huber.

The DNA sequence coding for the N-terminus of HBcAg including the first linker (aa GGGGSGGGGT) was cut with NcoI and KpnI from pET28a-c149GFP (a gift from Michael Nassal) (fragment size 257 bp). The pB´His-NP was cut with NcoI (5´-end of multiple cloning site, MCS) and SphI (3´-end of MCS). The N-terminus of HBcAg including the first linker, cut with NcoI and KpnI, and the NcoI and SphI cut pB'His-NP were electrophoretically separated in an agarose gel, eluted from the gel and used together with the HBcAg Adapter for ligation to give pB´His HBcAg N-term. The HBcAg Adapter is the new multiple cloning site in the construct (the MCS is flanked by the N-terminus of HBcAg and by the C-terminus of HBcAg in pB' His HBcAg linker).

HBcAg Adapter fw (#591):

5´- CTACGTAGCATGCATGTACAACGAAGCTTACATG -3´
KpnI SnaBI SphI BsrGI HindIII destr. SphI

HBcAg Adapter rev (#592):

5´- TAAGCTTCGTTGTACATGCATGCTACGTAGGTAC -3´
HindIII BsrGI SphI SnaBI KpnI

3 factor ligation gives pB'His HBcAg N-term (Gly-rich linker is in red, MCS is in blue):

start 6x His-tag NcoI
5´-ATGCACCACCACCACCACCACTCCATGGATATCGATCCTTATAAAGAATTCGG
AGCTACTGTGGAGTTACTCTCGTTTCTCCCGAGTGACTTCTTTCCTTCAGTACGAG
ACCTTCTGGATACCGCCAGCGCGCTGTATCGGGAAGCCTTGGAGTCTCCTGAGCA
CTGCAGCCCTCACCATACTGCCCTCAGGCAAGCAATTCTTTGCTGGGGGGAGCTC
ATGACTCTGGCCACGTGGGTGGGTGTTAACCTCGAGGATGGTGGAGGTGGCTCCG
GAGGGGGTGGTACCTACGTAGCATGCATGTACAACGAAGCTTACATGC----3xStop
KpnI SnaBI SphI BsrGI HindIII

The DNA sequence coding for the C-terminus of HBcAg including second linker (aa GGGGSGGGG) was cut with BsrGI and HindIII from pET28a-c149GFP (fragment size 235 bp). pB´His HBcAg N-term was cut with BsrGI and HindIII. The C-terminus of HBcAg including the second linker, cut with BsrGI and HindIII, and the BsrGI and HindIII cut

pB'His HBcAg N-term were electrophoretically separated in an agarose gel, eluted from the gel and used for ligation to give pB´His HBcAg linker (Gly-rich linker is in red, the multiple cloning site is in blue):

start 6x His-tag NcoI

5´-ATGCACCACCACCACCACCACTCCATGGATATCGATCCTTATAAAGAATTCGG
AGCTACTGTGGAGTTACTCTCGTTTCTCCCGAGTGACTTCTTTCCTTCAGTACGAG
ACCTTCTGGATACCGCCAGCGCGCTGTATCGGGAAGCCTTGGAGTCTCCTGAGCA
CTGCAGCCCTCACCATACTGCCCTCAGGCAAGCAATTCTTTGCTGGGGGGAGCTC
ATGACTCTGGCCACGTGGGTGGGTGTTAACCTCGAGGATGGTGGAGGTGGCTCCG
GAGGGGGTGGTACCTACGTAGCATGCATGTACAAGGGTGGAGGTGGCTCCGGAG
GGGGTGGATCTAGAGACCTGGTAGTCAGTTATGTCAACACTAATATGGGTTTAAA
GTTCAGGCAACTCTTGTGGTTTCACATTAGCTGCCTCACTTTCGGCCGAGAAACA
GTTATAGAATATTTGGTGTCTTTCGGAGTGTGGATCAGAACTCCTCCAGCTTATAG
GCCTCCGAATGCCCCTATCCTGTCGACACTTCCGGAGACTACTGTTGTTTAGTGAA
GCTT - 3´ stop
HindIII

The amino acid sequence of recombinant 6xHis HBcAg linker protein is (the Gly-rich linker is in red):

MHHHHHHSMDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHCSP
HHTALRQAILCWGELMTLATWVGVNLEDGGGGSGGGGTYVACMYKGGGGSGGGG
SRDLVVSYVNTNMGLKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAP
ILSTLPETTVV

The coding sequence of the lamin A Ig-fold (WT, R453W, R482W; amino acids 430 – 545 from lamin A/C) was amplified by PCR from pSVK3-LaminA (obtained from Sylvia Vlcek) with primers LaminA_Igfold_fw (#593) and LaminA_Igfold_rev (#594). The PCR product was cut with KpnI and BsrGI (fragment size 351 bp). pB' His HBcAg linker was cut with KpnI and BsrGI. The cut PCR product and the cut pB´His HBcAg linker were electrophoretically separated in an agarose gel, eluted from the gel and used for ligation to give pB'His HBcAg Ig-fold WT, pB'His HBcAg Ig-fold R453W, and pB'His HBcAg Ig-fold R482W. These constructs were cloned by Veronika Huber. The maps of the constructs are shown in the appendix.

LaminA_Igfold_fw (#593):

5´- CACATTGGTACCTTCTCACAGCACGCACGC -3´
KpnI

LaminA_Igfold_rev (#594):

5´- GAAATTGTACATGCGCACCAGCTTGCGCATGG -3´
BsrGI

2. 1. 13. 2. Cloning of pB'His HBcAg linker – N195/-K195/-R453/-W453/-R482/-W482

Short oligonucleotides corresponding to the respective parts of human lamin A and lamin C were inserted into the HBcAg cDNA at the position of the c/e1 epitope.

The plasmid pB'His HBcAg linker corrected Cl. #1 (generated by Veronika Huber) was cut with KpnI and BsrGI and the following oligos coding for short peptides corresponding to the respective parts of human lamin A and lamin C were inserted:

Lamin A - N195

LA191-202N195fw (#735)

5'-C GTG GAT GCT GAG AAC AGG CTG CAG ACC ATG AAG GAG AT-3'
KpnI N195 BsrGI

LA191-202N195rev (#736)

5'-GTA CAT CTC CTT CAT GGT CTG CAG CCT GTT CTC AGC ATC CAC GGT AC-3'
BsrGI KpnI

This corresponds to the amino acid sequence (N195 is in bold)

^{191}VDAE**N**RLQTMKE^{202}

Lamin A - K195

LA191-202K195fw (#737)

5'-C GTG GAT GCT GAG AAA AGG CTG CAG ACC ATG AAG GAG AT-3'
KpnI K195 BsrGI

LA191-202K195rev (#738)

5'-GTA CAT CTC CTT CAT GGT CTG CAG CCT TTT CTC AGC ATC CAC GGT AC-3'
BsrGI KpnI

This corresponds to the amino acid sequence (K195 is in bold)

^{191}VDAE**K**RLQTMKE^{202}

Lamin A - R453

LA448-457R453fw (#739)

5’-C GAG GGC AAG TTT GTC CGG CTG CGC AAC AAG AT-3’
KpnI R453 BsrGI

LA448-457R453rev (#740)

5’-GTA CAT CTT GTT GCG CAG CCG GAC AAA CTT GCC CTC GGT AC-3’
BsrGI KpnI

This corresponds to the amino acid sequence (R453 is in bold)

^{448}EGKFV**R**LRNK^{457}

Lamin A - W453

LA448-457W453fw (#741)

5’-C GAG GGC AAG TTT GTC TGG CTG CGC AAC AAG AT-3’
KpnI W453 BsrGI

LA448-457W453rev (#742)

5’-GTA CAT CTT GTT GCG CAG CCA GAC AAA CTT GCC CTC GGT AC-3’
BsrGI KpnI

This corresponds to the amino acid sequence (W453 is in bold)

^{448}EGKFV**W**LRNK^{457}

Lamin A - R482

LA478-487R482fw (#743)

5’-C TTG CTG ACT TAC CGG TTC CCA CCA AAG TTC AT-3’
KpnI R482 BsrGI

LA478-487R482rev (#744)

5’-GTA CAT GAA CTT TGG TGG GAA CCG GTA AGT CAG CAA GGT AC-3’
BsrGI KpnI

This corresponds to the amino acid sequence (R482 is in bold)

^{478}LLTY**R**FPPKF^{487}

Lamin A - W482

LA478-487W482fw (#745)

5’-C TTG CTG ACT TAC TGG TTC CCA CCA AAG TTC AT-3’
KpnI W482 BsrGI

LA478-487W482rev (#746)

5’-GTA CAT GAA CTT TGG TGG GAA CCA GTA AGT CAG CAA GGT AC-3’
BsrGI KpnI

This corresponds to the amino acid sequence (W482 is in bold)

^{478}LLTY**W**FPPKF^{487}

pB’His HBcAg linker -N195 was sequenced and was correct. The other constructs were tested by control digest and seemed to be correct.

2. 1. 13. 3. Cloning of pB’His HBcAg linker w/o stop

I wanted to replace the stop codon present in the HBcAg nucleotide sequence with the BamHI restriction site, to be able to insert a nucleotide sequence after the 3’ end of the HBcAg coding sequence to express the inserted sequence as a fusion protein with HBcAg.

The plasmid pB’His HBcAg linker corrected Cl. #1 (generated by Veronika Huber) was cut with SalI and HindIII and the following annealed oligo was inserted (#779 and #780) to substitute the stop codon present in the HBcAg sequence of pB’His HBcAg linker corrected Cl. #1 with a BamHI restriction site (underlined), which is required to insert a nucleotide sequence at the 3’ end of HBcAg. The nucleotide sequence coding for the HBcAg C-terminus is in red (aa sequence is in blue).

HepLinkw/ostopfw (#779)

5’ – TCG ACA CTT CCG GAG ACT ACT GTT GTT GGA TCC AGC A – 3’
SalI BamHI HindIII

aa S T L P E T T V V

HepLinkw/ostoprev (#780)

5’ – AGCTTGCTGGATCCAACAACAGTAGTCTCCGGAAGTG – 3’
HindIII BamHI SalI

pB’His HBcAg linker w/o stop was not sequenced, but was tested by control digest and seemed to be correct.

2. 1. 13. 4. Cloning of pB'HisHBcAg(C3d-P28)$_2$

The plasmid pB'His HBcAg linker w/o stop Cl. #1 was cut with BamHI and HindIII. The insert was derived by PCR of pDC1474 (plasmid received from Tedd Ross) using primers #781 and #782. The PCR product of ~300bp length was cut, containing two copies of the complement receptor 2 (CR2) binding domain of C3d, termed C3d-P28, with BglII (present at the 5'end of the C3d-P28 ORF) and HindIII. The fragment was inserted into the BamHI and HindIII cut pB'His HBcAg linker w/o stop.

Gly-P28(C3d)fw (#781)

5' – CGCGAATTCGCCCTTGCCCCCACC – 3'

Gly-P28(C3d)rev (#782)

5' – ACCACCAAGCTTCTA GGC GTA GGA TGT GGC C – 3'

HindIII stop

aa A Y S T A

The inserted fragment is 255nt long, and has the following coding sequence, obtained by sequencing the insert with the primer pB'His_fw_new (#523):

destroyed BamHI/BglII

5'- GA TCT GGT GGA GGT GGA AGC AGT GGT GGA GGT GGA AGC AGT AAG TTT CTG AAC ACA GCC AAA GAT CGG AAC CGC TGG GAG GAG CCT GAC CAG CAG CTC TAC AAC GTA GAG GCC ACA TCC TAC GCC GGA TCT GGT GGA **N**GT GGA AGT AGT GGT GGA GGT GGA AGC AGT AAG TTT CTG AAC ACA GCC AAA GAT CGG AAC CGC TGG GAG GAG CCT GAC CAG CAG CTC TAC AAC GTA GAG GCC ACA TCC TAC GCC TAG A – 3'

stop HindIII

The identity of the nucleotide N (bold and underlined) was investigated by visual inspection of the sequencing chromatogram and identified as G. Thus the respective codon codes for glycine, which is the correct amino acid.

The sequenced sequence corresponds to the following amino acid sequence (C3d-P28 sequence is underlined; the Gly-rich linker, which is from the vector pDC1474, is shown in red):

5'- GSGGGGSSGGGGSSKFLNTAKDRNRWEEPDQQLYNVEATSYAGSGGGGSS GGGGSSKFLNTAKDRNRWEEPDQQLYNVEATSYA – 3'

2. 1. 13. 5. pB'His PreScission HBcAg linker

In order to cleave off the native HBcAg linker protein from the Ni-NTA beads, a PreScission protease cleavage site was introduced between the 6x His tag and the HBcAg linker sequence. The plasmid pB'His HBcAg linker corrected Cl. #1 (generated by Veronika Huber) was cut with NcoI and treated with CIP (calf intestinal phosphatase, NEB #M0290S). The annealed oligo (#794 and #795) was inserted. Note that the NcoI site on the 5'-end of the coding strand is destroyed and that a cleavage site for the PreScission protease was introduced.

pB'HisPresciscode (#794)

5' – CATGCTGGAAGTTCTGTTCCAGGGGCCCTC – 3'
destroyed NcoI NcoI

pB'HisPrescisnoncod (#795)

5' – CATGGAGGGCCCCTGGAACAGAACTTCCAG – 3'
NcoI destroyed NcoI

The amino acid sequence of the PreScission cleavage site is (PreScission cleaves between Q and G, indicated with an "|"):

N- LEVLFQ|GP -C

The plasmid was sequenced and the sequence was correct.

2. 2. Working with bacteria

2. 2. 1. Solutions and Media

LB-medium

5.0g Bacto tryptone; 2.5g Bacto yeast extract; 2.5g NaCl.

Dissolve in 500ml H_2O, autoclave immediately and store at RT.

LB-agar plates

5.0g Bacto tryptone; 2.5g Bacto yeast extract; 2.5g NaCl; 7.5g Bacto agar. Dissolve in 500ml H_2O, autoclave immediately, cool to 50°C and add the appropriate antibiotics and pour plates. Store the plates at 4°C.

100x Ampicillin-stock, Gerbu #1046

Dissolve 10mg/ml of Ampicillin in H_2O. Filter sterilize through a membrane filter with a pore size of 0.2µm. Store 5ml aliquots at -20°C.

Bacterial strains:

XL1blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F'*proAB lacI*^{q}*ZΔM15 Tn10* (Tet^r)]

The bacterial strain XL1blue is nalidixic acid resistant and tetracycline resistant.

Origami(DE3)pLysS: *Δ(ara-leu)7697 ΔlacX74 ΔphoA Pvu*II *phoR araD139 ahpC galE galK rpsL* F'[*lac*$^+$ *lacI*^{q} *pro*] (DE3) *gor522*::Tn*10 trxB* pLysS (Cam^R, Str^R,Kan^R Tet^R)

The bacterial strain is chloramphenicol, streptomycin, kanamycin, and tetracycline resistant.

Tuner(DE3)pLysS: F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm lacY1*(DE3) pLysS (Cam^R)

The bacterial strain is chloramphenicol resistant.

Rosetta(DE3)pLysS: F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE (Cam^R)

The bacterial strain is chloramphenicol resistant.

Rosetta^{TM}(DE3): F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE (Cam^R)

The bacterial strain is chloramphenicol resistant.

2. 2. 2. Growth of bacteria

Grow bacteria in LB-medium or on LB-agar plates containing 100µg/ml Ampicillin at 37°C.

2. 2. 3. Freezing / thawing of bacteria

For freezing, add 200µl of glycerol to 800µl bacterial over night culture. Mix thoroughly and store the bacterial stock at -80°C.

For thawing, scrape with a pipette tip on the freezing stock and inoculate 5ml – 50ml of LB medium containing the appropriate antibiotics with the bacteria sticking on the tip over night at 37°C

2. 2. 4. Transformation of temperature sensitive bacterial strains

Thaw an aliquot of temperature sensitive bacteria on ice. Add 10µl of ligation or ~ 50ng of plasmid DNA to the bacteria and incubate for 15min on ice. Incubate the bacteria for 1min at 42°C. Cool them down for 2 minutes on ice. Add 1ml of LB (without antibiotics) and incubate the bacteria for 1 hour at 37°C. Spin bacterial culture shortly and streak on LB-agar plates containing selective antibiotics. Incubate over night at 37°C.

2. 2. 5. Bacterial expression of fusion proteins

Inoculate 5ml LB-Amp with bacteria from a freezing stock or by picking a bacterial clone from an LB-agar plate and incubate over night at 37°C. Dilute over night culture in 200ml LB-Amp and incubate at 37°C until the culture reaches an OD^{550} of ~0.8-1. Induce expression of the recombinant protein with 1mM IPTG for 3.5h at 37°C or RT. Centrifuge bacterial culture for 10min at 5000rpm at 4°C. Resuspend bacterial pellet in 2ml per 100ml bacterial culture 25mM Tris pH 8.4 (containing Aprotinin and PMSF). Lyse bacteria by sonication (3x 30sec with 30sec breaks between the sonication steps) on ice.

Take a 60µl aliquot after sonication, centrifuge for 10min at 14000rpm at 4°C. Add 30µl 3x GSD to the supernatant and resuspend the pellet in 90µl 1x GSD. Boil samples for 5min and load an aliquot on a SDS-PAG to check expression of the desired protein.

2. 2. 5. 1. Bacterial expression of HBcAg Ig-fold R453W (see 3. 3. 10.)

Different bacterial strains (Origami, Tuner, Rosetta (-), and Rosetta (+)) were tested for the expression and solubility of HBcAg Ig-fold R453W by Veronika Huber. All strains showed similar results. Veronika Huber recommended to use Origami or Rosetta (+) (in 2. 2. 5. 2.) for the large scale expression of the antigens.

Induce one litre of bacterial culture (Origami) with 0.1 mM IPTG for 24 hours at room temperature (RT). Continue as described in 2. 2. 5.

2. 2. 5. 2. Bacterial expression of HBcAg Ig-fold R482W (sse 3. 4. 1.)

Induce 150ml bacterial culture (Rosetta (+)) with 1mM IPTG for 4 hours at RT. Take 50ml bacterial culture for the un-induced control. Continue as described in 2. 2. 5.

2. 2. 5. 3. Bacterial expression of HBcAg linker N195 in four different bacterial strains (see 3. 5. 1.)

For each of the four bacterial strains (Origami, Tuner, Rosetta (-), and Rosetta (+)), induce 25ml of bacterial culture with 1mM IPTG for 3 hours at 37°C. Use 25ml of bacterial culture for the non-induced control. Continue as described in 2. 2. 5.

2. 2. 5. 4. Bacterial expression of HBcAg linker R453, HBcAg linker W453, HBcAg linker R482, HBcAg linker W482, and HBcAg linker (see 3. 5. 1.)

Express the antigens HBcAg linker R453, HBcAg linker W453, HBcAg linker R482, HBcAg linker W482, and HBcAg linker in the bacterial strain Rosetta (+) (Rosetta (+) showed high expression and solubility of HBcAg linker N195). Incubate 500ml of bacterial culture at 37°C for 5.5 hours and do not induce with IPTG, as experiments have shown that large amounts of the recombinant protein are expressed without induction with IPTG. Continue as described in 2. 2. 5.

2. 2. 5. 5. Bacterial expression of HBcAg (C3d-P28)$_2$ (see 3. 6.)

Add also Complete to the bacterial lysis buffer (25mM Tris pH 8.4, containing PMSF and Aprotinin), to avoid degradation of the recombinant HBcAg (C3d-P28)$_2$ protein by proteinases, as the recombinant protein was degraded in a previous experiment. Induce 150ml bacterial culture (Rosetta (+)) with 1mM IPTG for 3.5 hours at room temperature. Use 50ml bacterial culture as an un-induced control.

2. 3. Working with proteins / antigens

2. 3. 1. Solutions

30% Acrylamide

292g acrylamide; 8g Bisacrylamide. Fill up to 1l with H_2O. Add mixed-bed, ion-exchange resin (BioRad AG 501-X6) to the final solution and store in dark at 4°C.

1M Tris pH 8.8

Dissolve 242.3g Tris in H_2O. Adjust pH to pH 8.8 and fill up to 2l with H_2O, autoclave and store at 4°C.

1M Tris pH 6.8

Dissolve 60.5g Tris in H_2O. Adjust pH to pH 6.8 and fill up to 500ml with H_2O, autoclave and store at 4°C.

20% SDS

Dissolve 40g SDS (Amresco #0227) in H_2O, stir and slightly heat, fill up to 200ml with H_2O. Store at RT.

10% APS

Dissolve 1g Ammoniumperoxodisulfate (Merck #1201) in 10ml H_2O and store at 4°C.

TEMED (N,N,N',N'-Tetramethylenediamine, Fluka #87689)

10x Running buffer

250mM Tris; 2M Glycine; 35mM SDS. Dissolve in 5l H_2O and store at RT.

Transfer buffer with Methanol

25mM Tris; 190mM Glycine; 20% (v/v) Methanol. Dissolve Tris and Glycine in H_2O and add Methanol, store at 4°C.

Ponceau S stock solution (10x)

2g Ponceau S (Serva #33429); 30g Trichloroacetic acid (AppliChem #A1431); 30g Sulfosalicylic acid (Merck #1.00691). Dissolve and fill up to 100ml with H_2O and store at RT.

Ponceau S working solution

Dilute Ponceau S stock solution 1:10 with H_2O.

20% Sodium-azide (Merck #67188)

Dissolve 2g sodium-azide in 10ml H_2O. Store in the dark at 4°C.

GSD stock (3x)

335mM DTT (Gerbu #1008); 230mM SDS; 4.5M Glycerol (Merck #1.04092); 20ml H_2O. Add a bit bromphenolblue (Amresco #0312) and a few drops 1M Tris pH 6.8 until solution appears blue. Store 2ml aliquots at -20°C.

To obtain 1x GSD, dilute the stock with H_2O.

1% Thimerosal (Sigma #T-5125)

Dissolve 0.5g Thimerosal in 50ml H_2O. Store in the dark at 4°C.

Prestained Protein Molecular Weight Standards (Biorad #161-0373)

Store at -20°C. Use 3µl or 10µl for one lane on a small or large SDS-polyacrylamide gel, respectively.

10x PBS

1.37M NaCl; 27mM KCl; 43mM Na_2HPO_4; 14mM KH_2PO_4. Dissolve in H_2O and autoclave.

1x PBS (working solution)

137mM NaCl; 2.7mM KCl; 4.3mM Na_2HPO_4; 1.4mM KH_2PO_4.

3% milk (blocking solution)

15g Non fat dry milk (NFDM); 50µl 20% sodium azide. Add PBS + 0.05% Tween 20 to 500ml and store at 4°C.

0.5% milk (for incubation)

2.5g Non fat dry milk (NFDM); 500µl 1% Thimerosal. Add PBS + 0.05% Tween 20 to 500ml and store at 4°C.

Coating buffer (for coating of Protein A Sepharose)

5ml 1M Tris pH 8.0; 10g BSA (Sigma # A-9647); 100µl 20% Azide. Add H_2O to 100ml and store at 4°C.

Protein G Sepharose 4 fast flow (Amersham Pharmacia #17-0618-01, 5ml)

Protein A Sepharose CL-4B (GE Healthcare #17-0780-01, 1.5g)

Ni-NTA beads (Qiagen #30410)

PMSF – stock (100x)

Dissolve 0.697g PMSF (Roche #837091) in 20ml isopropanol and store aliquots of 1ml at room temperature, protected from light.

Aprotinin – stock (200x)

Store aliquots of 1ml Aprotinin (Sigma #A-6012) at 4°C. ???

Complete (25x)

Dissolve one Complete™ Protease Inhibitor cocktail tablet (Boehringer Mannheim #1836145) in 2ml IP buffer and store at 4°C.

RNAse A (Sigma #R-5125)

See Chapter 2.1.1.

DNAse I form bovine pancreas (Sigma #DN25)

Dissolve 10mg/ml Dnase I in H_2O and store at –20°C.

0.2M Borate buffer

Dissolve 40.24g Di-Sodiumtetraborate (anhydrous) in 1l H_2O (heating required) and adjust the pH to 9.0. Store at RT.

Dimethylpimelimidate Dihydrochlorid (Sigma D8388)

Store at -20°C.

0.2M Ethanolamine buffer

Dilute 6.25ml ethanolamine in 400ml H_2O and adjust to pH 8.0. Add H_2O to 500ml.

0.1M Glycine buffer

Dissolve 3.76g glycine in 400ml H_2O. Adjust to pH 3.0 and add H_2O to 500ml. Store at 4°C.

Wash buffer (for Ni-NTA beads)

50 mM NaH_2PO_4; 300 mM NaC; 20 mM imidazole. Adjust pH to pH 8.0.

Elution buffer (for Ni-NTA beads):

50 mM NaH_2PO_4; 300 mM NaCl; 250 mM imidazole. Adjust pH to pH 8.0.

Buffer A (for solubilisation of insoluble His-tagged recombinant proteins)

6M Guanidinium HCl; 0.1M NaH_2PO_4; 0.01M Tris pH 8.0

Buffer B (washing buffer for purification of insoluble His-tagged proteins via Ni-agarose beads)

8M Urea; 0.1M NaH_2PO_4; 0.01M Tris pH 8.0

Buffer C (washing buffer for purification of insoluble His-tagged proteins via Ni-agarose beads)

8M Urea; 0.1M NaH_2PO_4; 0.01M Tris pH 6.3

Coomassie-stock

Dissolve 2.5g Coomassie (Serva #17524) in 500ml methanol, stir over night, add 500ml H_2O and store at room temperature.

Coomassie working solution

30% Ethanol; 30% Coomassie-stock; 10% acetic acid; 30% H_2O.

Destaining solution

30% Ethanol; 60% H_2O; 10% Acetic acid.

Coomassie working solution for electro-elution

10% Coomassie-stock; 5% Acetic acid; 85% H_2O.

Elutionbuffer for electroelution

15mM $(NH_4)_2CO_3$; 0.1% SDS.

IP Wash

10% glycerol; 20mM Tris pH 8.0; 135 mM NaCl. Filter sterilize and store at 4°C.

IP Lyse

IP Wash; 1% NP-40.

RIPA buffer

1% NP-40; 0.1% Deoxycholic acid (Sigma); 0.1% SDS; 0.15M NaCl; 10mM Na-Phosphate pH 7.2. Dissolve in H_2O, filter sterilize and store at 4°C.

Lamina lysis buffer

1% Triton X-100; 50mM HEPES; 4mM $MgCl_2$; 10mM EGTA; 0.1M NaCl; 0.5mg/ml DNase; 0.2mg/ml RNase A; 0.5% SDS.

TBS

137mM NaCl; 2,7mM KCl; 25mM Tris. Dissolve in H_2O, adjust pH to 7.4. Autoclave and store at 4°C.

TMB substrate solution

0.3mg TMB (3,3',5,5'-Tetramethylbenzidine); 0.1ml DMSO; 9.9ml 100mM Na-Acetate pH 6; 3.3µl 30% H_2O_2.

2. 3. 2. Purification of insoluble His-tagged proteins via Ni-agarose beads

Centrifuge bacterial lysate for 30min at 5000rpm at 4°C. Add 1ml Buffer A per 100ml bacterial culture to the pellet. Incubate suspension for 30min at RT and vortex every 5min to

dissolve the pellet. Centrifuge the suspension for 15min at 5500rpm at RT and collect the supernatant (containing the protein). Add again 1ml Buffer A per 100ml bacterial culture to the pellet and resuspend. Incubate suspension for 30min at RT and vortex every 5min to dissolve the pellet. Centrifuge the suspension for 10min at 14000rpm. Combine the supernatants. Add 200µl Buffer A pre-equilibrated Ni-NTA beads (Qiagen #30410) and incubate on a roller over night at RT. Wash the Ni-NTA beads 4x with 1-2 solid bead volumes of Buffer A, 3x with Buffer B and 2x with Buffer C (between steps centrifuge for 1min at 1000rpm). Boil beads 3 times in 1x bed volume with 1x GSD for 5min. Check aliquots of these eluates on an SDS-PAG.

2. 3. 3. Electro-elution of denatured antigens

For further purification, the eluted proteins are loaded on a SDS-PAG to electrophoretically separate it from other proteins. The gel is incubated in "Coomassie working solution for electro-elution" for 10min to stain the proteins. Destain with H_2O. Cut out antigen band with a clean scalpel. Cut the gel slice into small pieces and transfer it to the elution device (S&S ELUTRAP® Electro-Separation System, Schleicher & Schuell). Elute for 3h at 200V, take out first elution fraction, elute again for 3h at 200V. Check efficiency of electro-elution by analysing aliquots of eluates on SDS-PAG.

2. 3. 4. Purification of soluble His-tagged proteins via Ni-agarose beads

Centrifuge bacterial lysate for 30min at 5000rpm at 4°C. Collect the supernatant and add 100µl of Ni-NTA beads, which were equilibrated in 25mM Tris pH 8.4, per 100ml of a bacterial culture with OD_{550}=1 to the supernatant. Shake at 4°C for 1-2h or over night. Wash beads 4x with 25mM Tris pH 8.4 (containing Aprotinin and PMSF), and 5x with washing buffer (containing Aprotinin and PMSF) (between steps centrifuge for 1min at 1000rpm). Elute bound proteins with elution buffer either for 1h at 4°C, RT or 37°C. Check efficiency of elution on an SDS-PAG.

2. 3. 5. KLH-coupled peptides used for immunisation

The following KLH-coupled peptides (coupled via the cysteine, in red) were purchased from piCHEM and used for immunisation of mice:

Lamin A N195: KLH - Ac-C-^{191}VDAE**N**RLQT^{199} - NH_2
Lamin A K195: KLH - Ac-C-^{191}VDAE**K**RLQT^{199} - NH_2
Lamin A R453: Ac-^{449}GKFV**R**LRNK^{457}-C - NH_2 - KLH
Lamin A W453: Ac-^{449}GKFV**W**LRNK^{457}-C - NH_2 - KLH
Lamin A R482: KLH - Ac-C-^{478}LLTY**R**FPPK^{486} - NH_2
Lamin A W482: KLH - Ac-C-^{478}LLTY**W**FPPK^{486} - NH_2

2. 3. 6. Recombinant proteins/antigens used for immunisation

All recombinant antigens were expressed as 6xHis tagged proteins. The following fusion proteins were used for immunizations:

- HBcAg Ig-fold WT
- HBcAg Ig-fold R453W
- HBcAg Ig-fold R482W
- HBcAg linker R453
- HBcAg linker R482
- HBcAg linker W482

2. 3. 7. Determination of protein concentration

Dilute Bradford reagent (Bio-Rad Protein Assay Dye Reagent #500-0006) 1:5 with H_2O. Mix the solution with 1µl – 5µl of cell lysate or protein solution and incubate 10min – 30min at RT. Determine the protein concentration by measuring the absorption at 595nm.

2. 3. 8. SDS-PAGE (SDS-Polyacrylamide gel electrophoresis)

Wash glass plates with dextran and rinse with H_2O and ethanol. Assemble the gel unit. Mix the components of the gel solution (without APS and TEMED) and degas. Add 10% APS and TEMED, stir and pour the gel. Cover separating gel with H_2O. Wait until gel is polymerized, remove H_2O, pour the stacking gel and insert a comb. After gel is polymerized, remove the comb, place the gel unit into the running unit, add running buffer, load boiled samples and run gel.

1x Separating gel				
	7.5%	**10%**	**12.5%**	**15%**
Acrylamide, 30%	10.1ml	13.4ml	16.7ml	20ml
Tris pH 8.8	15ml	15ml	15ml	15ml
SDS 20%	200µl	200µl	200µl	200µl
H2O	15ml	11.7ml	8.3ml	5ml
APS 10%	134µl	134µl	134µl	134µl
TEMED	26µl	26µl	26µl	26µl

1x Stacking gel	
Acrylamide, 30%	1.7ml
Tris pH 6.8	1.25ml
SDS 20%	50µl
H2O	7.1ml
APS 10%	50µl
TEMED	10µl

2. 3. 9. Commassie staining of gels

Stain SDS-PAG with Coomassie working solution for 2h and destain with destaining solution.

2. 3. 10. Western Blotting

Assemble the western sandwich in the following order: support pad, 3 sheets of 3MM Whatman chromatography paper, gel, nitrocellulose membrane (Whatman Protran Nitrocellulose Membrane Filters, Whatman #10402096), 3 sheets of 3MM whatman chromatography paper and a support pad. All components are wetted in transfer buffer. Avoid air bubbles during the assembly. Insert the western sandwich into the transfer unit and transfer proteins at 500mA for 3.5h at 4°C. Wash the membrane with H_2O after the transfer and block the membrane with 3% milk in PBS-Tween (blocking solution) for 1h at room temperature. Wash the blot shortly with PBS-Tween and incubate membrane either over night at 4°C or for 2h at room temperature with the appropriate primary antibody, diluted in 0.5% milk/PBS-Tween. Wash the membrane 3x for 10min with PBS-Tween and incubate with secondary, HRP-coupled antibody, diluted in 0.5% milk/PBS-Tween, for 1h at room temperature. Wash membrane 3x 10min after incubation with secondary antibody. Mix ECL solutions (oxidizing reagent and enhanced luminal reagent, PerkinElmer) in equal amounts and incubate membrane for 1min. Wrap membrane in a foil and expose the membrane to X-ray films.

2. 3. 11. Antibodies used in immunoblot analysis (western blotting)

2. 3. 11. 1. Primary antibodies

Antibody	Clone/Source	Species	Dilution
Anti-lamin A/C	3A6-4C11	mouse monoclonal	1:1000
Anti-lamin A/C R453W	12A-2F5	mouse monoclonal	1:100
Anti-Flag	M2 (Sigma, F1804)	mouse monoclonal	1:10000
Anti-His-tag	(GE Healthcare, #27471001)	mouse monoclonal	1:10000
Anti-beta-actin	AC-74 (Sigma, #A5316)	mouse monoclonal	1:500
Anti-lamin B1	Ab-1 (Oncogene)	mouse monoclonal	1:50
Anti-lamin A/C	N-18 (Santa Cruz, #sc-6215)	goat polyclonal	1:2000

2. 3. 11. 2. Secondary antibodies

Peroxidase conjugated AffiniPure Goat Anti Mouse IgG, Fc_γ Fragment specific (Jackson ImmunoResearch, #115035008)

Peroxidase conjugated AffiniPure Rabbit Anti Goat IgG, Fc Fragment specific (Jackson ImmunoResearch, #305035008)

Use secondary antibodies in 1:5000 dilution (in 0.5% NFDM/PBS-Tween).

2. 3. 12. Immunofluorescence

Seed 200000 HeLa cells (or HeLa cells ectopically expressing Flag-lamin A WT, and Hela cells ectopically expressing Flag-lamin A R453W), or 100000 primary skin fibroblasts in a single well of a 6 well tissue culture plate. Cultivate cells o/n until they reach a confluency of ~50-75%. Suck off medium and wash cells 2x with PBS. Perform all steps at room temperature. Fix cells with 3.7% formaldehyde/PBS for 15min and wash 2x with PBS. Quench any remaining formaldehyde with 50mM NH_4Cl/PBS for 10min. Wash 2x with PBS. Permeabilize cells with 0.2% - 0.5% Triton X-100/PBS (optional: add 0.1% SDS) for 10min and wash 2x with PBS. If 0.1% SDS were used during permeabilization of cells, it is mentioned for each experiment shown in the results. Block cells with 0.2% gelatine/PBS for 1h and wash again 2x with PBS. Incubate cells with primary antibody in 0.2% gelatine/PBS for 2 hours. Wash 3x with PBS and incubate with secondary antibody in 0.2% gelatine/PBS for 1h. Wash cells 2x with PBS and incubate with Hoechst 33342/PBS (750ng/ml) for 10min.Wash 2x with H_2O. Mount cover slips onto slides with Vectashield (Vector

Laboratories). Store slides at 4°C. Take pictures with a Zeiss LSM 510 Meta confocal microscope using the corresponding LSM 510 META software.

2. 3. 13. Antibodies used in Immunofluorescence

2. 3. 13. 1. Primary antibodies

Antibody	Clone	Species	Dilution
Anti-lamin A/C	N-18 (Santa Cruz, sc-6215)	goat ploYclonal	1:200
Anti-lamin A/C	3A6-4C11	mouse monoclonal	1:100
Anti-lamin A/C R453W	12A-2F5	mouse monoclonal	1:10
Anti-Flag	M2 (Sigma, F1804)	mouse monoclonal	1:1000

2. 3. 13. 2. Secondary antibodies

Alexa Fluor® 594 goat anti-mouse IgG (H+L), (Molecular Probes, #A11005)

Alexa Fluor® 488 goat anti-mouse IgG (H+L), (Molecular Probes, #A11029)

Donkey anti-goat IgG (H+L) Texas Red (Jackson ImmunoResearch, #705075003)

2. 3. 14. Lysis of mammalian cells with IP Lyse buffer

Cultivate cells until they are about 80% confluent. Wash cells 1x with cold PBS and 1x with cold IP Wash buffer. Suck off buffer completely. Add 1ml IP Lyse buffer (containing Aprotinin, PMSF and Complete) to a 100mm tissue culture dish and 2ml to a 150mm tissue culture dish. Incubate rotating for 20min at 4°C. Scrape off cells and centrifuge at 14000rpm for 10min at 4°C. Take supernatant and measure protein concentration using the Bradford method. Store cell lysates at -80°C, or use it for SDS-PAGE or immunoprecipitation immediately.

2. 3. 15. Lysis of mammalian cells with Lamina Lysis buffer (lysis with 0.5% SDS)

Cultivate cells until they are about 80% confluent. Wash cells with PBS and trypsinize. Stop trypsinization by adding culture medium. Transfer the cell solution into a tube. Determine the number of cells with a CASY® (Schärfe System) cell counter. Centrifuge cells at 1200rpm for 5 min. Wash cells 2x with PBS. Resuspend cell pellet in Lysis buffer (containing Aprotinin, PMSF and Complete) (take 2ml Lysis buffer for a 150mm culture dish) and incubate for 5min at room temperature. Add SDS to a final concentration of 0.5%. Incubate solution for 15min at room temperature and mix solution from now till then. Dilute SDS concentration to 0.25%

by adding Lysis buffer. Store aliquots of 1ml at -80°C, or use directly for immunoprecipitation.

2. 3. 16. Immunoprecipitation of mammalian cell lysates

Take 2mg of whole cell protein in IP lyse buffer, or cell lysate of a defined number of cells in Lamina lysis buffer and add the desired amount of antibody supernatant. Incubate shaking at 4°C for 1h. Add 15µl of a 1:1 mix of solid BSA-coated protein A and BSA-coated protein G sepharose beads and incubate shaking for 30min at 4°C. Wash beads 1x with 1ml of corresponding buffer and 3x with 1ml of ice-cold TBS. Boil beads in two bead volumes of 1x GSD for 5min and load supernatant on an SDS-PAG for electrophoretic separation.

2. 3. 17. Immunoprecipitation of mammalian cell lysates using Protein A sepharose cross-linked antibodies

Take 15µl solid anti-lamin A/C 3A6-4C11 cross-linked BSA-coated sepharose beads for a lysates containing either 2mg of whole cell protein or a lysate of a defined number of cells and incubate shaking at 4°C for 1h. Wash beads 1x with 1ml of the corresponding buffer and 3x with 1ml TBS. Boil beads in two bead volumes of 1x GSD for 5min and load supernatant on a SDS-PAG for electrophoretic separation.

2. 3. 18. ELISA (Enzyme linked immunosorbent assay)

Coat 96 well ELISA plates with 50µl per well of a 5µg/ml antigen solution in 10mM Na-Phosphate buffer pH 7 over night at 4°C. Wash plates 2x with PBS and block with 3% BSA/PBS for 2h at RT. Wash plates again 2x with PBS and incubate with 50µl of hybridoma supernatant or 50µl of 1:1000 diluted mouse anti-serum for 1h at room temperature. Wash plates 2x with PBS and incubate with HRP-coupled anti-mouse antibody (1:10000 in 1.5% BSA/PBS) for 1h at room temperature. Wash plates 3x with PBS. After the final wash, add 50µl TMB substrate solution to the wells. Stop reaction after 10min – 30min with 50µl of 1M H_2SO_4. Measure absorption at 450nm.

2. 3. 19. Coating of Protein A Sepharose

Suspend 1.5g Protein A Sepharose CL-4B in 50ml coating buffer. Incubate rolling 1 hour at RT. Spin 1min at 1000rpm and wash 6x with H_2O (last wash with H_2O + 0.02% azide). Make 2ml aliquots as a 1:1 slurry and store at 4°C.

2. 3. 20. Cross-linking of anti-lamin A/C 3A6-4C11 and anti-lamin A/C R453W 12A-2F5 antibodies to BSA-coated Protein A Sepharose beads

Take 1ml solid BSA-coated Protein A Sepharose beads and add an appropriate volume of antibody supernatant (1ml of 3A6-4C11 hybridoma supernatant, 5ml of 12A-2F5 hybridoma supernatant). Incubate rolling at room temperature for 2h. Wash beads twice with 10 volumes 0.2M sodium borate pH 9.0. Centrifuge for 1min at 3000rpm. Resuspend the beads in 10 volumes of 0.2M sodium borate pH 9.0 and remove the equivalent of 10µl of beads. Add 10 volumes of 0.2M sodium borate pH 9.0 containing 20mM dimethylpimelimidate. Incubate rolling for 1h at room temperature. Remove the equivalent of 10µl beads. Stop the reaction by washing the beads once in 0.2M ethanolamine pH 8.0 and incubate rolling for 2h at room temperature in 0.2M ethanolamine. Wash beads twice with 10 volumes of 100mM glycine pH 3.0. Immediately wash 5x with 10 volumes of PBS + 0.02% Na-azide. Check efficiency of coupling by boiling samples of beads taken before and after coupling in 1x GSD and run the samples on a 10% SDS-PAG. Stain the proteins in the gel with Coomassie.

2. 3. 21. Peptide competition of antibodies

Prepare the appropriate dilution of the antibody in 0.5% milk. For competition take a 1x – 200x molar excess of peptide over the antibody, and incubate the competition mix over night at 4°C. Check success of competition on a western blot.

2. 3. 22. Lysis of mammalian cells with 1xGSD

Cultivate cells until they are about 80% confluent. Wash cells 2x with cold PBS. Suck off buffer completely. Add 1ml boiled 1xGSD to cells in a 100mm tissue culture dish and 2ml to cells in a 150mm tissue culture dish. Scrape off cells and sonicate for 5 seconds, boil for 5min. Store the lysate at –80°C.

Alternatively, when the exact number of cells has to be known, wash cells 1x with warm PBS and trypsinize. Stop trypsinization by adding culture medium. Transfer the cell solution into a tube. Determine the number of cells with a CASY® (Schärfe System) cell counter. Centrifuge cells at 1200rpm for 5 min. Wash cells 2x with PBS. Resuspend cell pellet in a required volume of boiled 1xGSD. Sonicate the cell lysate for 5 seconds, boil for 5min. Store the lysate at –80°C.

2. 4. Tissue culture

2. 4. 1. Solutions and Media

DMEM (Gibco #31660-083)

Dissolve one package in 5l H_2O and stir for 30min until completely dissolved. Add 30g $NaHCO_3$, stir until completely dissolved and add H_2O up to 10l. Filter sterilize and make aliquots of 450ml. Store at 4°C. Add glutamine, if stored longer than 2 weeks.

CS (newborn calf serum, Gibco #16010-084)

FCS (fetal calf serum, Gibco #4023696J)

Store 50ml aliquots at -20°C.

AB

0.6g Penicillin-G; 1g Streptomycin-sulfate; 10ml 10x PBS. Add H_2O to 100ml, filter sterilize and store 5ml aliquots at -20°C.

Trypsin

Dissolve 250mg trypsin in 25ml 10x PBS and add H_2O up to 245ml. Stir for 2 hours. Add 5ml 1% Na-EDTA pH 7.4, mix and filter sterilize. Store 10ml aliquots at -20°C.

Hygromycin B (Calbiochem #400051)

Dissolve Hygromycin B in H_2O to a final concentration of 50mg/ml, filter sterilize and store 5ml aliquots at -20°C. HeLa Flag-Lamin A WT, HeLa Flag-Lamin A N195K, HeLa Flag-Lamin A R453W, and HeLa Flag-Lamin A R482W cell lines were cultivated in DMEM (Dulbecco's modified Eagle's medium) containing 10% FCS and antibiotics (0.06mg/ml penicillin-G and 0.1mg/ml streptomycin-sulfate), and 175µg/ml Hygromycin B in an incubator at 37°C and 7.5% CO_2.

DMSO (Dimethylsulfoxide, Applichem #A3672.0250)

HAT Medium

400ml DMEM (Sigma, #D5671); 50ml Fetal Cl.1 (HyClone, #SH30080.03); 25ml BM-Condimed H1 (LaRoche, #11088947001); 5ml Penicillin/Steptomycin (P/S) (Sigma, #P4333); 5ml L-Glutamine (Sigma, #G2150); 5ml Na-Pyruvate (Sigma, #S8636); 10ml HAT supplement (Gibco, #21060).

X63 Medium

435ml DMEM (Sigma, #D5671); 50ml FCS; 5ml P/S; 5ml Glutamine (Sigma); 5ml Na-Pyruvate (Sigma).

HT Medium

400ml DMEM (Sigma, #D5671); 50ml Fetal Cl.1 (HyClone, #SH30080.03); 25ml BM-Condimed H1 (LaRoche, #11088947001); 5ml Penicillin/Steptomycin (Sigma, #P4333); 5ml L-Glutamine (Sigma, #G2150); 5ml Na-Pyruvate (Sigma, #S8636); 10ml HT supplement (Gibco, #41065).

Red blood cell lysing buffer, Sigma #R7757

2. 4. 2. Cell lines

The following cell lines were used:

Cell lines		specifics
HeLa	human cervical cancer cell line	
HeLa Flag-Lamin A WT	human cervical cancer cell line	ectopically expressing Flag-tagged Lamin A WT
HeLa Flag-Lamin A N195K	human cervical cancer cell line	ectopically expressing Flag-tagged Lamin A N195K
HeLa Flag-Lamin A R453W	human cervical cancer cell line	ectopically expressing Flag-tagged Lamin A R453W
HeLa Flag-Lamin A R482W	human cervical cancer cell line	ectopically expressing Flag-tagged Lamin A R482W
X63Ag8.653	mouse (Balb/c) myeloma cell line	Ig heavy- and light-chain non-secreting

The HeLa Flag-Lamin A WT, HeLa Flag-Lamin A N195K, HeLa Flag-Lamin A R453W, and HeLa Flag-Lamin A R482W cell lines were obtained from Sylvia Vlcek.

Primary cells		specifics
T.M.wt	Human skin fibroblasts	wt
G-8628	Human skin fibroblasts	heterozygous for the mutation Lamin A/C R453W

These primary cells were a gift from Manfred Wehnert, University of Greifswald, Germany.

2. 4. 3. Cultivation of cells

Cells were grown in DMEM (Dulbecco's modified Eagle's medium) containing 10% FCS and antibiotics (0.06mg/ml penicillin-G and 0.1mg/ml streptomycin-sulfate) in an incubator at 37°C and 7.5% CO_2. Hybridomas were cultivated in HAT selection medium, HT or X63 Medium at 37°C and 5% CO_2.

2. 4. 4. Counting of cells

To determine the cell number, dilute 50µl of cell suspension in 5ml CASY®ton solution and count cells with a CASY® (Schärfe System) cell counter. Alternatively, count cells with a Bürker 0.1mm counting chamber (Optik Labor).

2. 4. 5. Freezing / thawing of cells

Suck off medium, wash cells with PBS and trypsinize for 5min at 37°C. Add 10ml culture medium and transfer to a tube. Centrifuge 5min at 1200rpm. Suck off medium and resuspend cells in 1ml FCS + 10% DMSO. Transfer cell suspension to a cryo-tube and incubate on ice for 10min. Store the cells at -80°C over night and transfer them afterwards to liquid nitrogen.
Thaw freezing vial in a water bath at 37°C. As soon as the cell suspension is thawed, dilute it in 10ml culture medium. Centrifuge cells at 1200rpm for 5min. Suck off medium, resuspend cells in culture medium and seed onto Petri dish.

2. 5. Working with mice

2. 5. 1. Mouse strains used

- BALB/c

Mice suffer from progressive hearing loss, which starts at the age of 10 month. This defect is due to the homozygosity for *Cdh23*^{ahl} (cadherin-like 23, age-related hearing loss).The BALB/c mouse strain is the most widely used inbred strain for hybridoma generation. Upon injection of emulsified antigen suspension they develop plasmacytomas, supporting the generation of monoclonal antibodies (http://jaxmice.jax.org/strain/001026.html).

- RBF/DnJ Rb(8.12)5Bnr

The mice are homozygous for the Robertsonian translocation of chromosomes 8 and 12. Chromosome 8 contains the mouse APRT gene and chromosome 12 contains the murine Ig heavy chain locus. Splenocytes can be fused to APRT$^-$ myeloma cells, thus allowing the survival of just Ig heavy chain locus containing hybridomas in AAT (Adenine-Aminopterin-Thymidine) selection medium (http://jaxmice.jax.org/strain/001802.html).

2. 5. 2. Immunisation

Immunize the mice with 50µg of antigen. Mix the antigen with adjuvants in a ratio of 1:1. Apply this mix subcutaneously. The final immunization (final boost) was carried out without adjuvants and was injected intraperitoneally.

2. 5. 3. Bleeds

Take bleeds from the tail vein. Cut the vein with a sharp scalpel and collect the blood with a glass capillary. Take about 100µl of blood. Incubate the blood at 37°C for 1h. Centrifuge for 5min at 14000rpm. Store over night at 4°C. Centrifuge for 10min at 14000rpm, take cleared serum, add Na-azide (0.02%) and store serum at 4°C.

After the mouse has been killed, the final bleed is taken from the heart.

2. 5. 4. Mouse Immunisation schedule

Day 0: take pre-immune bleed from tail vein

1. immunisation (sc) with ~ 50µg of antigen + complete adjuvants

Day 14: 2. immunisation (sc) with ~ 50µg of antigen + incomplete adjuvants

Day 24: 1. bleed from tail vein

Day 35: 3. immunisation (sc) with ~ 50µg of antigen + incomplete adjuvants

Day 45: 2. bleed from tail vein

Day 56: if titre is satisfactory: final boost for best responder (ip) with ~ 50µg of antigen
All others get 4. immunisation (sc)

Day 59: Fusion of splenocytes from best responder

Alternatively:

Day 56: 4. immunisation (sc) with ~ 50µg of antigen + incomplete adjuvants

Day 66: 3. bleed from tail vein

Day 77: 5. immunisation (sc) with ~ 50µg of antigen + incomplete adjuvants

Day 87: 4. bleed from tail vein

Day 97: 6. immunisation (sc) with ~50µg of antigen + incomplete adjuvants

Day 107: 5. bleed from tail vein

2. 6. Generation of hybridoma

2. 6. 1. Splenectomy / Fusion

Kill the mouse by cervical dislocation and wash it with 70% EtOH. Fix the mouse with needles to a cork board and dissect it. Take final bleed from the heart and remove the spleen. Remove the connective tissue from the spleen and wash it 2x in warm DMEM. Cut the spleen into small pieces, and grind these pieces between two frosted objective slides. Filter the splenocytes through a 100µm cell strainer and wash 3x with DMEM. After the first wash, incubate the splenocytes for 1.5min with 3ml Red blood cell lysis buffer on ice. Count the splenocytes in a Bürker 0.1mm counting chamber (Optik Labor). Wash off the myeloma cells from the culture dish using DMEM and wash cells 3x with DMEM. Count the number of myeloma cells in a cell counting chamber.

Combine splenocytes and myeloma cells at a ration of 3:1 for fusion. Centrifuge for 5min at 1200rpm. Suck off DMEM and detach the cell pellet from the tube walls manually. Hold the

tube in the hand from here on to keep cells warm. Add 1ml of 37°C PEG 8000 (Sigma, #P7181) drop-wise to the cell pellet in 1min under continous shaking. Add 1ml of DMEM drop-wise in 1min, then 5ml of DMEM drop-wise in 2min and finally 10ml of DMEM drop-wise in 3min, always under constant shaking. Incubate the cell suspension for 5min at 37°C and centrifuge for 5min at 12000rpm to get rid of PEG. Resuspend the cell pellet in 50ml HAT selection medium. Plate out one third of the fusion on 24 well plates and 96 well plates. Freeze the rest in 2 cyro-vials.

2. 6. 2. Test of hybridoma supernatant

Change ~80% of medium on hybridoma 4 days after th fusion. Test the supernatants of the hybridomas for presence of antibodies approximately 8-10 days after fusion by immunoblotting assay on whole cell lysates using miniblotter devices from Immunetics (#MN28) or by ELISA using immobilized peptides or recombinant proteins.

2. 6. 3. Minimal dilution of positive hybridoma clones

Take off supernatant and keep as control for further testings. Resuspend hybridoma mix clone in HAT medium and determine the number of cells using the counting chamber. Prepare dilutions of 25 cells/well, 12 cells/well, 6 cells/well, 3 cells/well and 1.5 cells/well on a 96 well tissue culture plate. Freeze the remaining cells, as well as other positive mix clones. Repeat minimal dilutions until a monoclonal hybridoma is obtained.

2. 6. 4. Expansion of monoclonal hybridoma and production of monoclonal antibody

Expand the single hybridoma clone stepwise to the next bigger culture dish. Freeze several vials during these steps. Displace the clone from HAT selection medium by growing in HT medium for three passages before cultivating in DMEM containing 10% FCS w/o selection drugs. Freeze several vials of cells grown w/o selection. Expand the clone onto 5-10p100 cell culture dishes and cultivate without change of medium until all the cells are dead. Harvest the supernatant and store it at 4°C or freeze at -20°C.

2. 6. 5. Heavy and light chain isotyping

The heavy and light chain isotypes of the antibodies were determined using the ELISA-based ImmunoPure® Monoclonal Antibody Isotyping Kit II (Pierce #37502) by following the manufacturer´s instructions.

2. 7. Working with rabbits

2. 7. 1. Rabbit strain used

- New Zealand white rabbit

2. 7. 2. Immunisation

Immunize rabbit with a defined amount of antigen (see below). Proceed like for mouse immunizations. Apply the antigen emulsion intradermaly at neck.

2. 7. 3. Bleeds

Take bleeds from ear vein. Up to 50ml of blood are taken. Incubate the blood at 37°C for 1h. Centrifuge for 5min at 5000rpm. Store over night at 4°C. Centrifuge for 10min at 5000rpm, take serum, add Na-azide (0.02%) and store one aliquot of serum at 4°C, store remaining aliquots at –20°C.

2. 7. 4. Rabbit Immunisation schedule

Day 0:	take pre-immune bleed from ear vein
	1. immunisation (id) with ~ 250µg of antigen + complete adjuvants
Day 14:	2. immunisation (id) with ~ 125µg of antigen + incomplete adjuvants
Day 24:	1. bleed from ear vein
Day 35:	3. immunisation (id) with ~ 75µg of antigen + incomplete adjuvants
Day 45:	2. bleed from ear vein
Day 56, 77:	all following immunisations (id) with 75µg of antigen + incomplete adjuvants
Day 66, 87:	take all following bleeds from ear vein

3. Results

3. 1. Antibodies against lamin A/C N195 and K195

To raise antibodies against the epitope containing amino acid N195 or K195, respectively, we decided to use short peptides. These peptides were either coupled to the carrier protein KLH (please see section 2. 3. 5. for amino acid sequence of used peptides), or were expressed as a recombinant fusion protein with the HBcAg carrier protein (please see section 2. 1. 13. 2. for nucleotide sequence of used oligos).

The pre-immune sera, and sera from 1. and 2. bleeds of mice immunized with KLH-N195 and KLH-K195 were tested by Western blot analysis for the presence of the desired antibodies on whole cell lysates of HeLa cells ectopically expressing the N-terminal Flag tagged lamin A N195K (figure 5). In addition, HeLa cells express endogenous wild-type lamin A and lamin C, which share the identical N-terminal rod domain.

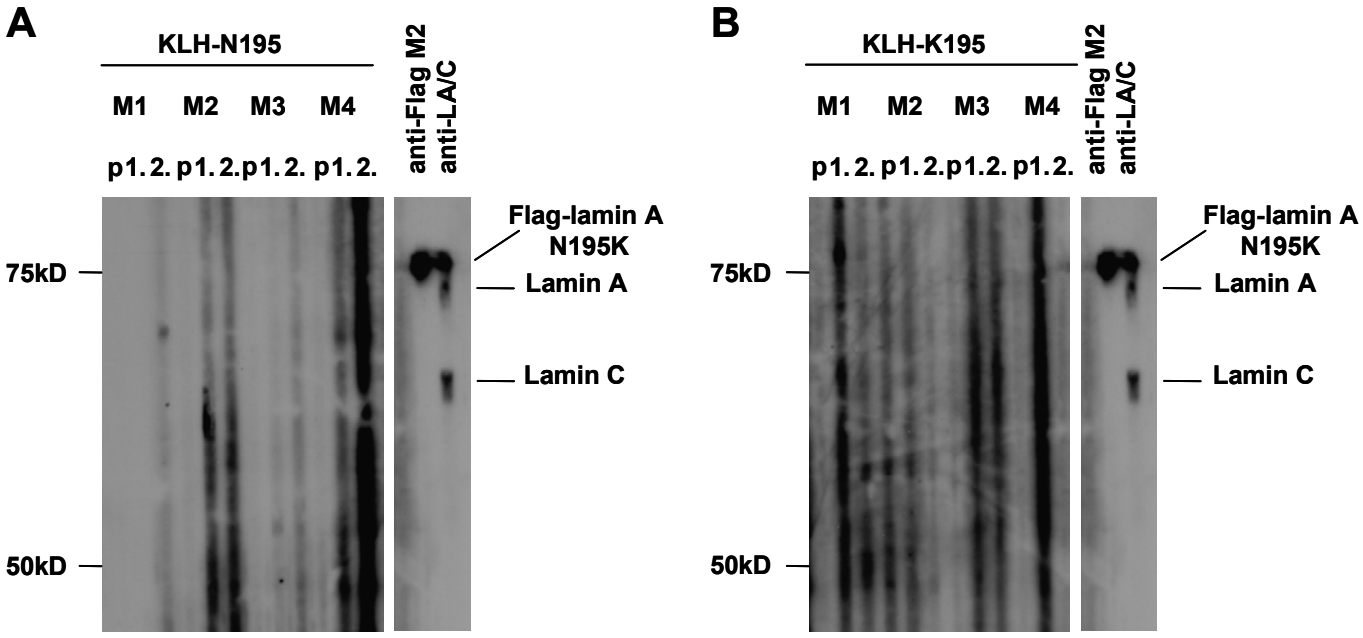

Figure 5: Test of sera of KLH-N195 (A) and KLH-K195 (B) immunized mice. Sera from mouse 1 (M1) – mouse 4 (M4) were tested in a Western blot against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A N195K (10% SDS-PAG). The sera were tested in a dilution of 1:1000. Anti-Flag M2 (1:10000), anti LA/C hybridoma supernatant 4A3. p=pre-immune serum; 1.=serum of 1. bleed; 2.=serum of 2.bleed. M1 = mouse1; M2 = mouse 2; M3 = mouse 3; M4 = mouse 4.

The anti-Flag M2 antibody confirmed that the HeLa Flag-lamin A N195K cell line expressed Flag-lamin A N195K (2nd lane from the right, figure 5A and 5B), and an anti-lamin A/C polyclonal hybridoma supernatant (generated in the lab) confirmed that also endogenous wild-type lamin A and lamin C was expressed in the cells (last lane, figure 5A and 5B). The electrophoretic mobility of Flag-lamin A N195K was just somewhat slower than that of endogenous wild-type lamin A (compare 2nd lane from the right and last lane, figure 5A and 5B).

The immune response against KLH-N195 should yield antibodies recognizing the endogenous wild-type lamins A and C, but not the Flag-tagged lamin A N195K mutant. In case of perfect specificity (i.e. no cross-reactivity to the wild-type epitope), immunization with the mutant peptide KLH-K195, should give rise to antibodies recognizing just the Flag-lamin A N195K mutant, but not the endogenous wild-type lamin A and lamin C.

Incubation with pre-immune sera of mice immunized with KLH-N195, and mice immunized with KLH-K195, with the exception of mouse 2, where the pre-immune serum detected a smear of proteins, did not detect any proteins (figure 5A and 5B). Incubation of the blot with the serum of the 1. and 2. bleeds of mice immunized with KLH-N195 and KLH-K195 resulted in a smear, with differing intensities and no defined protein bands (figure 5A and 5B). Unfortunately, antibodies specific for either the wild-type or the mutant lamin A/C proteins were missing in all sera of bleeds of mice immunized with KLH-N195 or KLH-K195.

As mice immunized with KLH-N195 and KLH-K195 did not show a sufficient immune response they were further immunized and the 3. and 4. bleeds were taken. The sera of these bleeds were tested for presence of desired antibodies again by Western blot analysis against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A N195K (figure 6A and 6B).

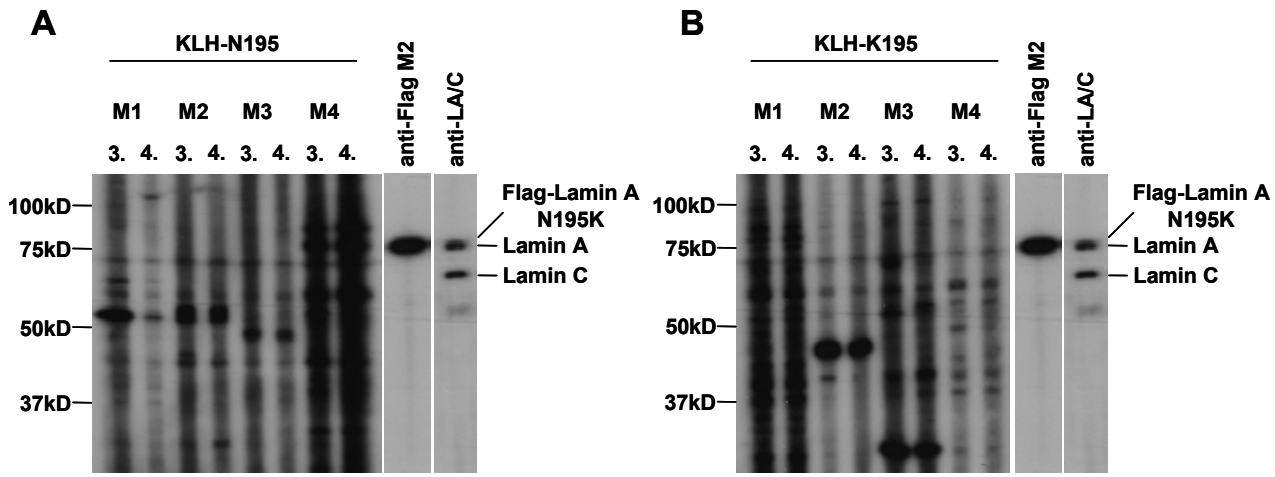

Figure 6: Test of sera of KLH-N195 and KLH-K195 immunized mice. Sera from M1 – M4 were tested in a Western blot against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A N195K (10% SDS-PAG). Sera were tested in a dilution of 1:1000. Anti-FlagM2 (1:40000), anti LA/C hybridoma supernatant 4A3. 3.=serum of 3. bleed; 4.=serum of 4.bleed. M1 = mouse1; M2 = mouse 2; M3 = mouse 3; M4 = mouse 4.

As positive controls anti-Flag M2 antibody, recognizing Flag-lamin A N195K, and a hybridoma supernatant recognizing lamin A and lamin C were used (2nd lane and last lane from the right, figure 6A and 6B). I did not use again the pre-immune sera as a negative control in this experiment, because it did not detect lamin A and lamin C in the experiment seen in figure 5. Again there was no endogenously expressed lamin A and lamin C detected by any serum of KLH-N195 immunized mice (figure 6A). Also no ectopically expressed Flag-lamin A N195K was detected by any serum of KLH-K195 immunized mice (figure 6B). Unlike in the first testing, all sera of the eight mice recognized several different proteins of unknown identity (figure 6A and 6B).

Due to the results presented in figure 5 and figure 6 the immunization of mice with the antigens KLH-N195 and KLH-K195 was stopped, as these antigens did not elicit a specific immune response.

As the KLH-peptide approach was not successful for the peptides containing either amino acid N195 or K195, I generated HBcAg recombinant proteins containing amino acids 191 - 202 either in the wild-type or in the mutated version (for further details please see section 2. 1. 13. 2.). The aim of this approach was to increase the antigenicity of the respective peptides, and to elicit the production of antibodies in mice recognizing the respective proteins in Western blot analysis. However, due to time constraints and the fact that I focused on two

different antigenic pairs, “HBcAg linker N195” and “HBcAg linker K195” have not yet been used for immunizations.

3. 2. Antibodies against the Ig-fold of lamin A and lamin C

As shown in figure 5A, 6A, and 14, none of the wild-type peptides coupled to KLH elicited the generation of antibodies in mice recognizing lamin A and lamin C in Western blot analysis. Therefore HBcAg Ig-fold WT was used for immunizations of mice to generate antibodies against epitopes present in the whole Ig-fold. By using the whole Ig-fold, we expected to generate two sorts of antibodies: first, antibodies, which recognize epitopes that do not encompass R453 or R482 of the Ig-fold. In this case the wild-type, and the point-mutation R453W and R482W containing Ig-folds would be recognized by the generated antibody. Second, we speculated that we might also generate antibodies specific for the wild-type epitope encompassing amino acid R453 or R482, if this protein region is immunogenic. This would allow to discriminate between wild-type and mutant lamin A and lamin C molecules. The expectation of generating a wild-type specific antibody recognizing the epitope containing R482 was raised, as the beta-strand containing amino acid R482 was found to be part of an immunogenic epitope (Manilal et al., 2004).
The immunization of four mice with HBcAg Ig-fold WT had been started by Veronika Huber, and I continued with the immunizations.

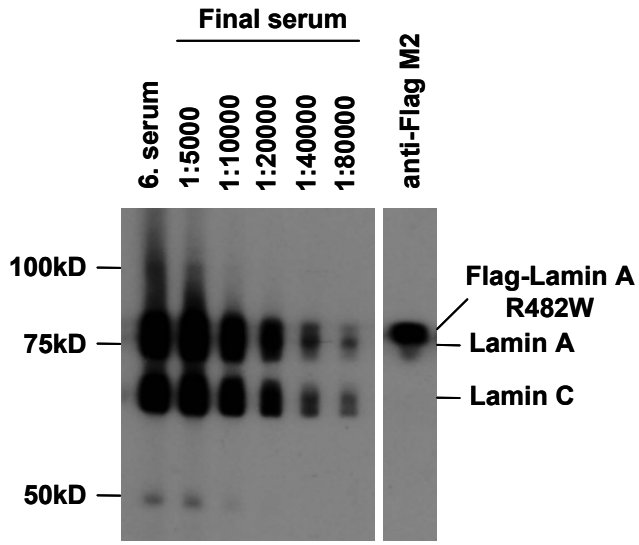

Figure 7. Test of sera of HBcAg Ig-fold WT immunized mouse 1. Sera were tested by a Western blot against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R482W (7.5% SDS-PAG). The serum of the 6. bleed was tested in a dilution of 1:5000, and the serum of the final bleed in a dilution series. Anti-Flag M2 (1:40000).

A strong humoral immune response was elicited in all four mice injected with HBcAg Ig-fold WT (data not shown, please see data from Veronika Huber), demonstrating the strong and consistent immunogenicity of such a fusion protein. The serum of the 6. bleed and a dilution series of the serum of the final bleed of mouse 1 immunized with HBcAg Ig-fold WT were tested by Western blotting (figure 7). Anti-Flag M2 antibody confirmed the expression of the ectopical Flag-lamin A R482W. The serum of the final bleed detected a clear signal corresponding to Flag-lamin A R482W and endogenous wild-type lamin A and lamin C even at a dilution of 1:80000 (figure 7). In contrast, sera of bleeds of KLH-peptide (such as KLH-W453) immunized mice, which elicited a humoral immune response, can rarely be diluted more than 1:10000 without losing the specific signal (e.g. test of dilutions of final bleed of KLH-W453 immunized mouse 3, data not shown).

The splenocytes of mouse 1 were fused with X63Ag8.653 cells and seeded onto four 24 well plates and six 96 well plates. One week after the fusion the supernatants of the hybridoma cells were tested by Western blotting on whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R482W for the presence of lamin A and lamin C specific antibodies (figure 8).

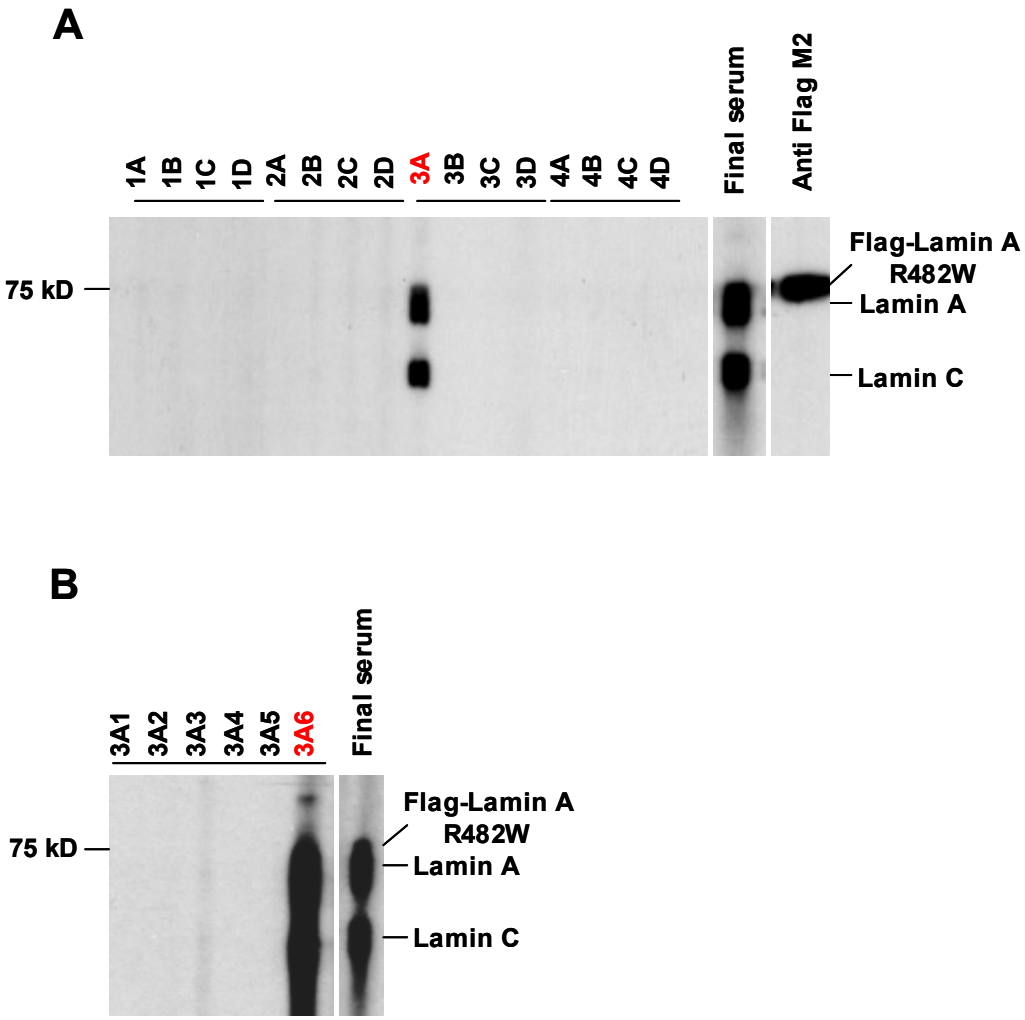

Figure 8: Test of hybridoma supernatants after fusion of HBcAg Ig-fold WT mouse 1. A) In a first round of testing, supernatant pools of 6 wells from a 24 well plate (=4 pools per plate) were tested by Western blot analysis against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R482W (7.5% SDS-PAG). B) The only positive supernatant pool from the first testing round was split into the individual supernatants and each single well supernatant was tested again by Western blot analysis against a HeLa Flag-lamin A R482W cell lysate (7.5% SDS-PAG). Final serum = serum of final bleed (1:20000), anti-Flag M2 (1:40000), hybridoma supernatants were not diluted.

In the first testing round the supernatants were pooled either into pools of six wells (for 24 well plates) or into pools of eight wells (for 96 well plates). Positive pools, in terms of detecting lamin A/C in a Western blot analysis, were split and each single well of this positive pool was retested by Western blot analysis to identify the well containing the desired antibodies-producing hybridoma clone. In total 88 pools were tested, which corresponded to 672 single wells.

Wild-type endogenous lamin A and lamin C, as well as the ectopically expressend Flag-lamin A R482W, were expressed in the HeLa Flag-lamin A R482W cells, as confirmed by the serum of the final bleed of the mouse 1 immunized with HBcAg Ig-fold WT and the anti-Flag M2 antibody (figure 8A and 8B). Only one pool of supernatants, number 3A, which consisted of the supernatants from six 24-wells, contained antibodies that recognized wild-type endogenous lamin A and lamin C, as well as Flag-lamin A R82W (figure 8A). None of the other pools from the 96-well plates showed any specific signals by Western blotting (data not shown). Each of the six single wells of pool 3A was retested (figure 8B), and only well 3A6 was found to contain a lamin A/C specific antibody.

At this stage there were on average about 20 clones present in a single 24-well. The mix clone 3A6 was therefore minimally diluted (please see section 2. 6. 3. for details) to obtain single clones. The minimal dilution was carried out on 96-well plates. The hybridoma cells were seeded at a density of 1.5 cells/well, 3 cells/well, 6 cells/well, and 12 cells/well.

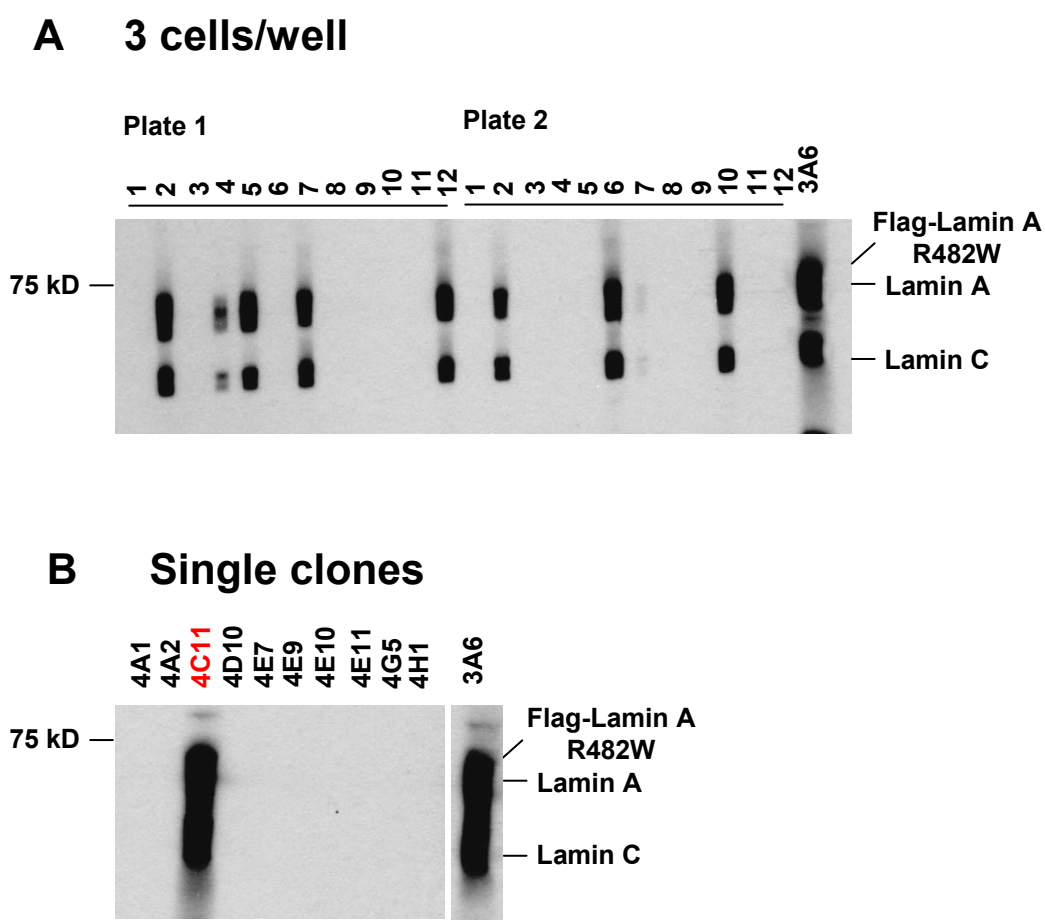

Figure 9: Test of mix and single clones from minimal dilution of mix clone 3A6 producing anti-lamin A/C antibody. Supernatants from pools of eight wells (A) and single clones (B) were tested by Western blot analysis against cell lysates of HeLa cells ectopically expressing Flag-lamin A R482W (7.5% SDS-PAG). The single

clone 4C11, which was chosen for further propagation, is highlighted. 3A6 corresponds to the SN of the mix clone from the well of the 24-well plate. Hybridoma supernatans were not diluted.

The supernatants of the wells from two 96 well plates (in this case the one on which I seeded the cells at a density of 3 cells/well) were pooled into 24 pools, each pool representing the supernatants of eight wells. From these 24 pools eight contained antibodies specific for lamin A/C (figure 9A). However, there was more than one clone visible per well.
Since on several wells of the 1.5 cells/well dilution single clones could be identified by visual inspection under the microscope, only supernatants of those single clones were tested (figure 9B). Of 19 single clones tested (only ten shown in figure 9B), just one, 4C11, was positive (figure 9B). If calculated back, about 5% of all hybridoma cells produced the antibody in the first positive well, 3A6.

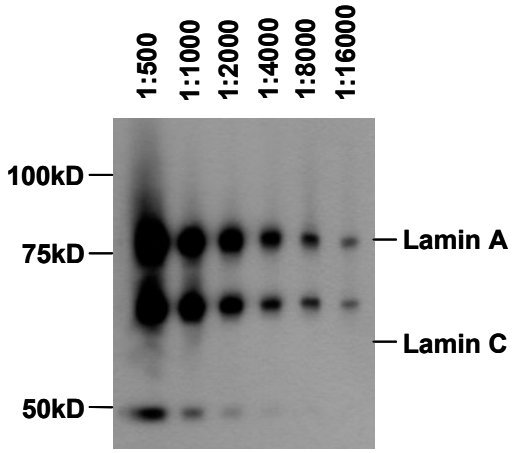

Figure 10: Test of dilutions of the anti-lamin A/C 3A6-4C11 monoclonal antibody. The monoclonal antibody was tested by a Western blot against whole cell lysates of HeLa cells (7.5% SDS-PAG) in dilutions depicted in the figure.

The single clone 3A6-4C11 was expanded for cryopreservation and for the production of larger amounts of monoclonal antibody supernatant. The supernatant from ten p100 plates was harvested after an incubation time of one week and retested by Western blot (figure 10). According to this Western blot the hybridoma supernatant should be best used in a dilution of 1:1000 to 1:4000, depending on the amount of lysate loaded. There was an additional protein recognized in the range of about 50 kDa by the monoclonal antibody (figure 10). This band was not recognized in all lysates by the antibody and only at high concentrations of antibody (see also figure 7). Thus, this signal was lysate dependent and could be due to a degradation of lamin A/C (Goldberg et al., 1999; Orth et al., 1996).

The isotyping of anti-lamin A/C 3A6-4C11 revealed that the antibody consisted of the heavy chain subclass IgG2a and the light chain subclass κ.

As the lamins, and especially the lamin A/C-sepcific Ig-fold, are highly conserved proteins (figure 11), it was next tested if the new antibody could recognize also lamins of other species (figure 12).

```
Homo sapiens    430 FSQHARTSGRVAVEEVDEEGKFVRLRNKSNEDQSMGNWQ
Mus musculus    430 FSQHARTSGRVAVEEVDEEGKFVRLRNKSNEDQSMGNWQ
Rattus norvegicus 429 FSQHARTSGRVAVEEVDEEGKFVRLRNKSNEDQSMGNWQ

Homo sapiens        IKRQNGDDPLLTYRFPPKFTLKAGQVVTIWAAGAGATHS
Mus musculus        IRRQNGDDPLMTYRFPPKFTLKAGQVVTIWASGAGATHS
Rattus norvegicus   IRRQNGDDPLMTYRFPPKFTLKAGQVVTIWASGAGATHS

Homo sapiens        PPTDLVWKAQNTWGCGNSLRTALINSTGEEVAMRKLVR 545
Mus musculus        PPTDLVWKAQNTWGCGSSLRTALINSTGEEVAMRKLVR 545
Rattus norvegicus   PPTDLVWKAQNTWGCGSSLRTALINATGEEVAMRKLVR 544
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Figure 11: Alignment of human, mouse and rat lamin A/C Ig-folds. The alignment was done using the program MegAlign (DNASTAR). Amino acid substitutions of the mouse and rat sequence to the human sequence are highlighted in red.

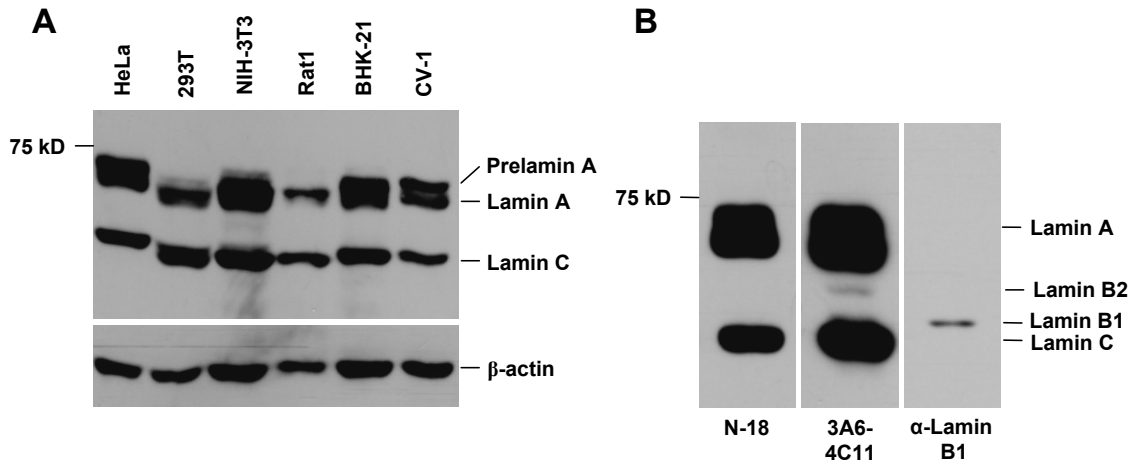


Figure 12: Test of 3A6-4C11 monoclonal antibody cross-reactivity. A) 3A6-4C11 was tested by Western blot analysis (7.5% SDS-PAG) against GSD-lysates of the following cell lines: HeLa (Human cervical cancer cell line), 293T (Human embryonic kidney cell line), NIH-3T3 (Mouse fibroblast cell line), Rat1 (Rat fibroblast cell line), BHK-21 (Syrian hamster kidney cell line) and CV-1 (African green monkey kidney epithelial cell line). Incubation of the blot with anti-β-actin (Sigma) (1:500) served as a loading control. B) Test of cross-reactivity of 3A6-4C11 to Lamin B1 and Lamin B2 by Western blot analysis (7.5% SDS-PAG) against HeLa GSD-lysates. Membrane strips were incubated with the respective antibodies displayed below each strip. Anti-lamin A/C N-18 (1:2000), anti-lamin A/C 3A6-4C11 (1:5000), anti-lamin B1 (1:50).

The species tested included human, mouse, rat, Syrian hamster, and African green monkey. As expected, the antibody recognized lamin A/C in all tested species (figure 12A). The Ig-folds of human and mice lamin A/C differ just in four amino acids in a length of 116 amino acids of the Ig-fold, and the human and rat Ig-folds differ in five amino acids (figure 11). The lamin sequences of the Syrian hamster and the African green monkey were not available in the NCBI gene bank. Since the A-type lamins from all tested species were recognized, the exact epitope recognized by 3A6-4C11 could not be confined. The lamin A band migrates as a doublet (Raharjo et al., 2001), which represents unprocessed prelamin A (upper band) and the mature lamin A (lower band). Lamin C is not proteolytically processed and runs as a single band. Interestingly, the ratios of unprocessed versus mature lamin A differed strongly among the different cell lines tested. Especially 293T and Rat1 seemed to have almost no unprocessed prelamin A (figure 12A).

As there were also faint bands recognized between lamin A and lamin C (see for example NIH-3T3 cells, figure 12A), I tested the cross-reactivity of 3A6-4C11 with lamin B1 and lamin B2 (figure 12B). The human lamin A/C Ig-fold is 52.2% identical to the Ig-fold of lamin B1 and 57.9% identical to the Ig-fold of lamin B2. The Ig-folds of lamin B1 and lamin B2 are 49.1% identical. A mouse monoclonal antibody against lamin B1 was available and showed that 3A6-4C11 probably cross-reacts with lamin B1, whereas the commercially available lamin A/C N-18 antibody (Santa Cruz Biotechnology), which is specific for an N-terminal epitope of lamin A/C, did not cross-react (figure 12B). I assume that the other band recognized between lamin A and lamin C is lamin B2, as this would match with the size of this protein (lamin C 65 kDa; lamin B1 66.4 kDa; lamin B2 67.6 kDa). Unfortunately, neither a lamin B2 specific antibody nor B-type lamin deficient cells were available to further substantiate this assumption. However, although the relative amounts of B-type lamins and A-type lamins expressed in HeLa cells are not known, the amount of B-type lamins detected by 3A6-4C11 is rather low and it is therefore unlikely that the results, for instance from immunofluorescence experiments, are significantly obscured (see figure 13).

3. 2. 1. Characterization of 3A6-4C11 in immunofluorescence and immunoprecipitation

Next I tested the ability of 3A6-4C11 to detect lamin A and lamin C in immunofluorescence experiments and the ability of 3A6-4C11 to immunoprecipitate lamin A and lamin C from cell lysates.

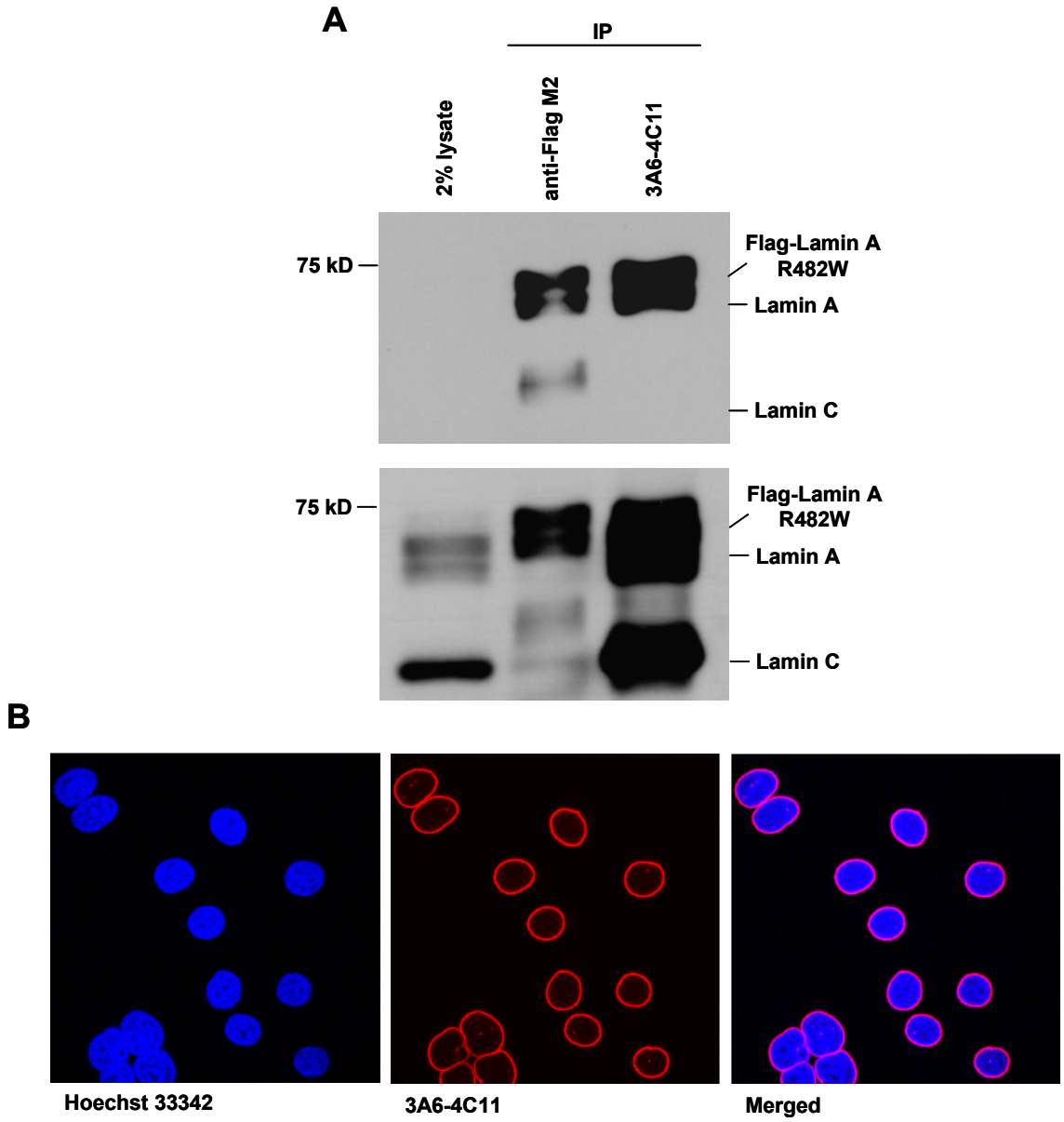


Figure 13. A) Immunoprecipitation of HeLa Flag-lamin A R482W. The cell lysate was made using IP-Lyse buffer. For each immunoprecipitation a total protein amount of 2mg was used. The immunoprecipitation was performed using 20µl anti-lamin A/C 3A6-4C11 hybridoma supernatant or 2µl anti-Flag M2, respectively, with 15µl of solid protein A+G sepharose beads. The immunoprecipitated proteins were electrophoretically separated on a 7.5% SDS-PAG. The membrane was incubated subsequently with anti-Flag M2 (1:10000) (top panel, exposure time: 1 hour) and anti-lamin A/C 3A6-4C11 antibody (1:1000) (bottom panel, exposure time: 10 sec). **B) Immunofluorescence analysis of HeLa Flag-lamin A WT cells.** Cells were grown on cover-slips and fixed with ice cold methanol for 10min at 4°C. Cells were stained with 3A6-4C11 (1:100), and counter-stained with Hoechst 33342.

The monoclonal antibody anti-lamin A/C 3A6-4C11 was very potent in immunoprecipitating lamin A and lamin C, as well as Flag-lamin A R482W (figure 13A). Even in the longest

exposure (1 hour, figure 13A top panel), anti-Flag M2 antibody could not detect Flag-lamin A R482W in 2% of the amount of lysate used for the immunoprecipitation, but it detected strong signals in both immunoprecipitates (figure 13A top panel), confirming the potent immunoprecipitation abilities of both antibodies (anti-Flag M2 and 3A6-4C11). The solubility of lamin A was decreased in IP-Lyse buffer compared to the solubility of lamin A in lysates made with GSD (compare HeLa lane in figure 12A with 2% lysate lane in figure 13A bottom panel). Thus, the lower amounts of lamin A in the lysate seen in figure 13A could be due to the milder lysis of cells using IP-Lyse buffer. In the immunoprecipitation made with the anti-Flag M2 antibody there is an additional band detected, at about 68 kDa, by the anti-Flag M2 and 3A6-4C11 antibodies (figure 13A top and bottom panel), which could be a degradation product of lamin A. However, the nature of this band was not further investigated.

It was also tested, if 3A6-4C11 could stain lamin A and lamin C by immunofluorescence. HeLa cells were fixed with methanol and stained with the 3A6-4C11 antibody. As expected, 3A6-4C11 predominantly stained the nuclear rim (figure 13B), as nucleoplasmic lamins were most probably washed out by the fixation method. A decreased nucleoplasmic staining of A-type lamins was also observed by Vlcek and colleagues upon fixation with methanol when compared to formaldehyde fixation (Vlcek et al., 2004).

In summary, I generated a lamin A/C Ig-fold specific antibody that posseses excellent immunoblotting, immunoprecipitating, and immunofluorescence properties.

3. 3. Antibodies against lamin A/C R453 and W543

To raise antibodies against the epitope containing amino acid R453 and W453, respectively, we decided to use short peptides. These peptides were either coupled to the carrier protein KLH (please see section 2. 3. 5. for amino acid sequence of used peptides), or were expressed as a recombinant fusion protein with the HBcAg carrier protein (please see section 2. 1. 13. 2. for amino acid sequence of used peptides). Besides this, mice were also immunized with the recombinant HBcAg protein containing the full length Ig-fold with the point-mutation R453W, HBcAg Ig-fold R453W.

3. 3. 1. Use of KLH-R453 and KLH-W453 as antigens

The pre-immune sera, and the sera of the 1. and 2. bleeds of mice immunized with KLH-R453, the wild-type peptide, and KLH-W453, the mutant peptide, were tested by Western blot analysis for the presence of the desired antibodies on whole cell lysates of HeLa cells ectopically expressing the N-terminal Flag tagged lamin A R453W (figure 14). In addition, HeLa cells express endogenous wild-type lamin A and lamin C, which share the identical C-terminal Ig-fold domain.

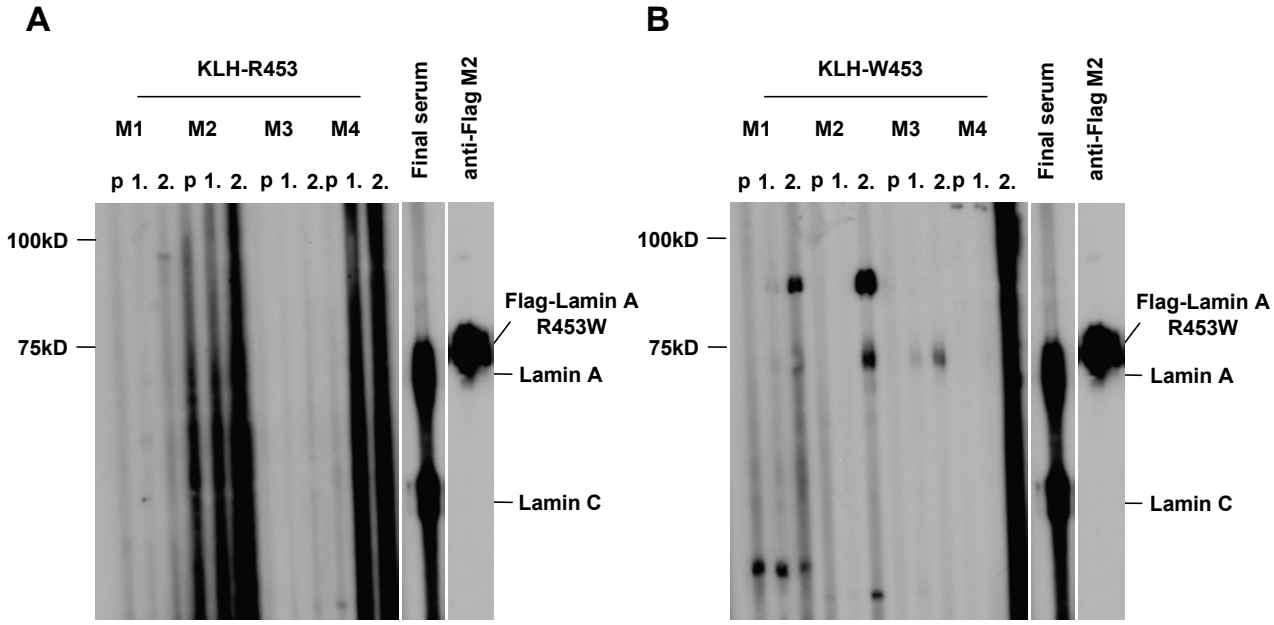


Figure 14: Test of sera of KLH-R453 (A) and KLH-W453 (B) immunized mice. Sera from M1 – M4, either immunized with KLH-R453 (A), or KLH-W453 (B), were tested in a Western blot against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R453W (10% SDS-PAG). Sera were tested in a dilution of 1:1000. Anti-Flag M2 (1:10000); Final serum = serum of final bleed of HBcAg Ig-fold WT immunized mouse 1 (1:5000). p=pre-immune sera; 1.=serum of 1. bleed; 2.=serum of 2.bleed. M1 = mouse1; M2 = mouse 2; M3 = mouse 3; M4 = mouse 4. The proteins loaded on the SDS-PAG were electrophoretically separated for ~20 hours at 20mA, to obtain a better separation between the Flag-lamin A R453W and the endogenous wild-type lamin A. Therefore the 50kDa protein marker is not present on the frame shown.

Experssion of Flag-lamin R453W and endogenous wild-type lamin A and lamin C in HeLa Flag-lamin A R453W cells was confirmed by anti-Flag M2 and the serum of the final bleed of HBcAg Ig-fold WT immunized mouse 1 (recognizes mutant and wild-type lamin A/C, see figure 7), respectively (figure 14A and 14B).

The expected immune response against KLH-R453, the wild-type peptide, should yield antibodies recognizing only the endogenous wild-type lamin A and C, but not the Flag-tagged lamin A R453W mutant. However, wild-type lamin A and lamin C was not detected by any serum of the KLH-R453 immunized mice in Western blot analysis (figure 14A). The immunization of mice with the antigen KLH-R453 was stopped after the 5th immunization, as specific signals were not detected by the sera of bleeds taken after the 4th and 5th immunization in Western blot analysis (data not shown).

In case of perfect specificity (i.e. no cross-reactivity to the wild-type epitope), immunization of mice with KLH-W453, the mutant peptide, should give rise to antibodies recognizing just the Flag-lamin A R453W mutant, but not the endogenous wild-type lamin A and lamin C. In contrast to the wild-type peptide, the peptide with the point mutation R453W, KLH-W453, elicited obviously a point-mutation specific immune response in mouse 2 and mouse 3, as a protein at the position of the Flag-lamin A R453W in the Western blot was recognized (figure 14B). Sera of mouse 1 and mouse 2 recognized also another protein of about 85 kDa. All sera of bleeds of mouse 1 recognized a protein at ~55 kDa. The serum of the 2. bleed of mouse 4 apparently recognized a smear of proteins in the cell lysate (figure 14B).

The apparently specific signals obtained with the sera of mouse 2 and mouse 3 were still weak. Therefore the mice were immunized for an additional time. The sera of the 2. and 3. bleeds of mice immunized with KLH-W453 were then tested again by Western blot analysis against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R453W (figure 15A).

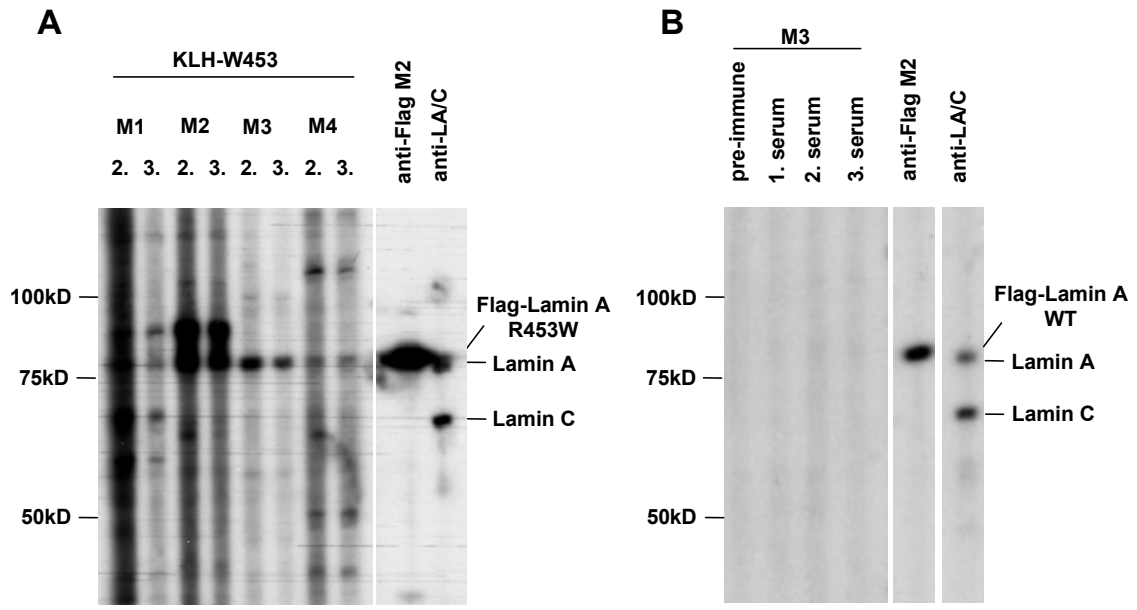


Figure 15: A) Test of sera of KLH-W453 immunized mice, and B) Test of sera of KLH-W453 immunized mouse 3 on specificity. A) Sera were tested in a Western blot against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R453W. B) Sera were tested by Western blot analysis against a HeLa whole cell lysate ectopically expressing Flag-lamin A WT. Cell lysates were separated on a 7,5% SDS-PAG. Sera were tested in a dilution of 1:1000. Anti-Flag M2 (1:10000); anti-LA/C = anti-lamin A/C hybridoma supernatant (mix clone 4A3). 2.=serum of 2. bleed; 3.=serum of 3.bleed. M1 = mouse1; M2 = mouse 2; M3 = mouse 3; M4 = mouse 4.

The sera of KLH-W453 immunized mice tested in this experiment recognized a protein at the size of Flag-lamin A R453W, but with different intensities (figure 15A). The antibody titer decreased after the 4th immunization, as incubation of the blot with all sera of the 3. bleeds gave a weaker signal than the sera of the 2. bleeds. Besides the specific detection of a protein at the size of Flag-lamin A R453W, all sera tested also recognized other proteins. Only the serum of mouse 3 recognized predominantly a protein at the size of Flag-lamin A R453W with almost no detection of other proteins.

We expected that a point-mutation specific antibody would at least slightly cross-react with endogenous wild-type lamin A and lamin C. To confirm and to further substantiate that the antibodies present in the sera of mouse 3 bleeds were specific for the point-mutation R453W in Flag-lamin A R453W and would not cross-react with endogenous wild-type lamin A and lamin C, the bleeds were tested against a whole cell lysate of HeLa cells ectopically expressing Flag-lamin A WT (figure 15B). Expression of Flag-lamin A WT and wild-type endogenous lamin A and lamin C in HeLa Flag-lamin A WT cells was confirmed by anti-Flag M2 and a hybridoma supernatant recognizing lamin A and lamin C (mix clone 4A3),

respectively (figure 15B). The serum of all mouse 3 bleeds did not detect anything in this cell lysate (figure 15B). This demonstrated that the antibody was specific for the mutant lamin A R453W, because it did not cross-react with endogenous wild-type lamin A and lamin C, showing that already the polyclonal serum could distinguish the wild-type and the mutated epitope, which just differ in a single amino acid.

Consequently mouse 3 was immunized a last time and a splenectomy was performed according to the protocol in section 2. 6. 1., and the splenocytes were fused with the myeloma fusion partner X63Ag8.653. The fusion was seeded onto six 24 well plates and four 96 well plates.

3. 3. 2. Test of supernatants from hybridomas of KLH-W453 mouse 3 fusion

The supernatants of hybridomas obtained from the fusion of KLH-W453 mouse 3 were screened for the presence of lamin A R453W specific antibodies by Western blot analysis on whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R453W one week after fusion.

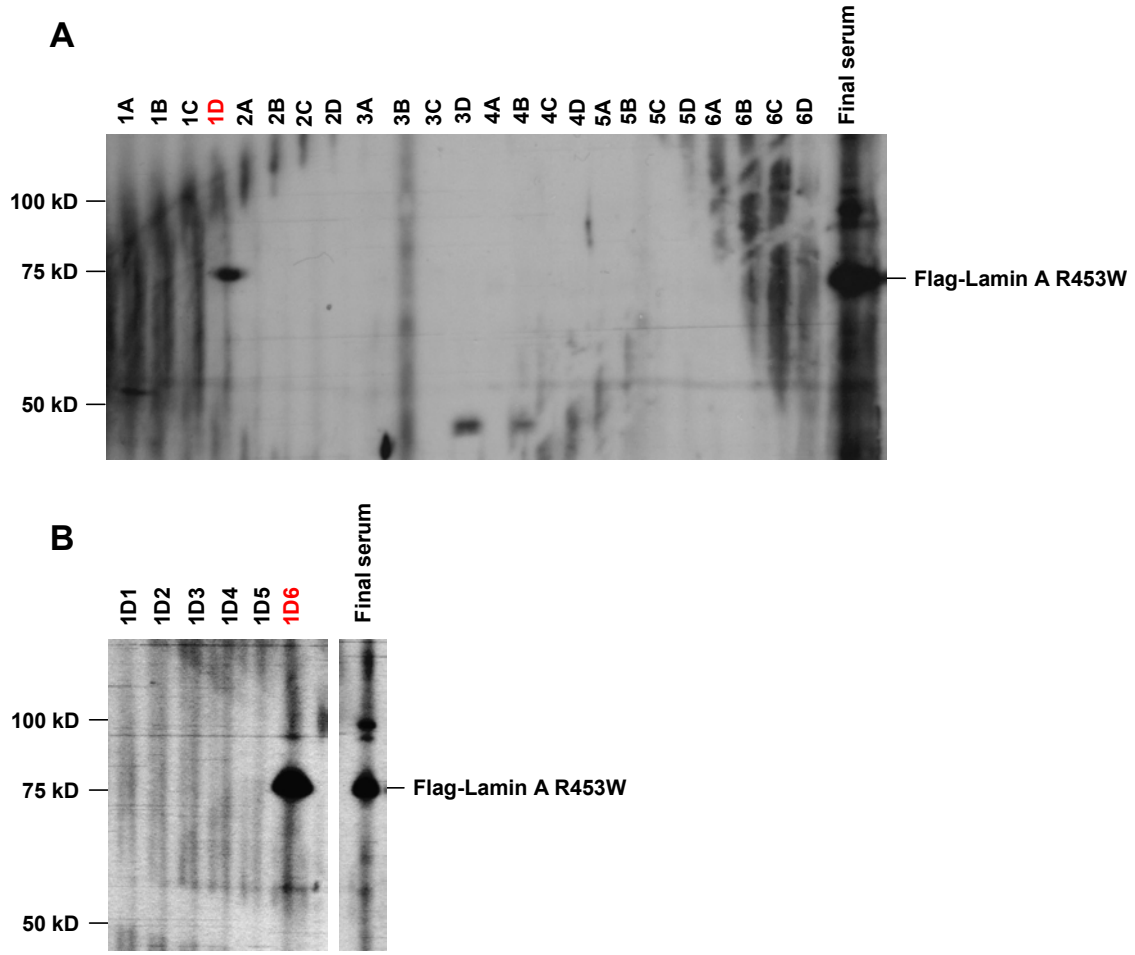


Figure 16: Test of hybridoma supernatants after fusion of KLH-W453 mouse 3 splenocytes. A) In a first round of testing, supernatant pools of 6 wells from a 24 well plate were tested by Western blot analysis against a HeLa Flag-lamin A R453W cell lysate (7.5% SDS-PAG). B) The only positive supernatant pool from the first testing round was split into the individual supernatants and supernatants of single wells was retested by Western blot analysis. Final serum = serum of final bleed of KLH-W453 immunized mouse 3 (1:1000).

I screened 48 supernatant pools, which corresponded to 528 single wells, by Western blot analysis. Only one pool, 1D, consisting of the supernatant from six 24-wells, was tested positive in the first round of testing (figure 16A). None of the other tested pools showed any specific signals by Western blot analysis (data not shown). Each of the six single wells of

pool 1D was retested, and only well 1D6 was found to contain a point mutation specific antibody, recognizing Flag-lamin A R453W (figure 16B).

At this stage there were on average about 20 clones present in a single 24-well. Thus we would expect that around 5% of the hybridoma cells should produce the desired antibody, if we assume an equal cell number per clone (only one 24-well of the fusion contained hybridoma cells producing the point-mutation specific antibody). The mix clone 1D6 was minimally diluted (please see section 2. 6. 3. for details) to obtain specific single clones.

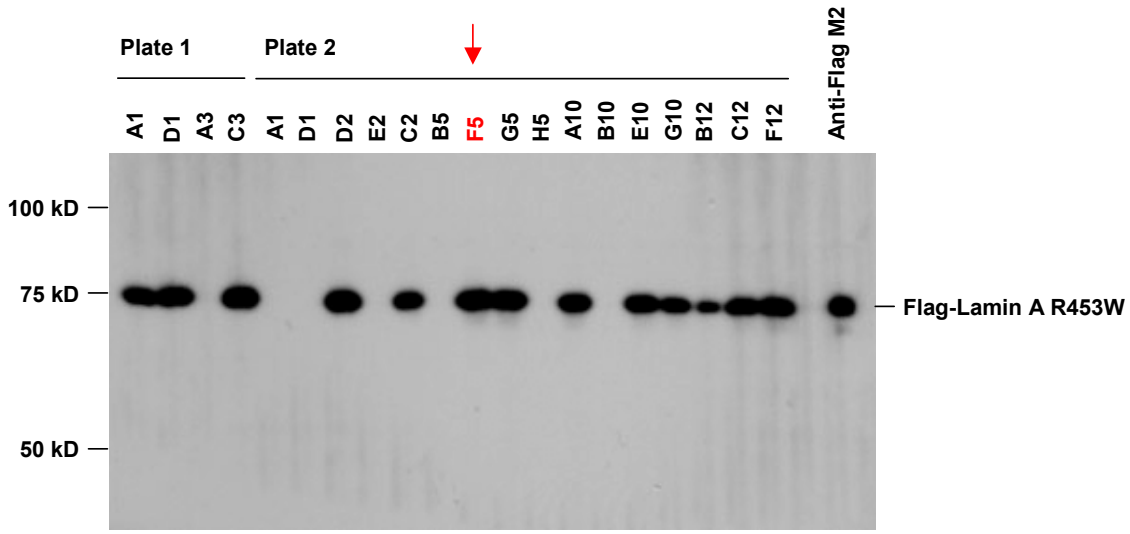


Figure 17: Test of mix and single clones from a minimal dilution of mix clones producing anti-lamin A/C R453W antibody. Supernatants from single and mix clones were tested by Western blot analysis against a HeLa Flag-lamin A R453W cell lysate (7.5% SDS-PAG). The single clone 2F5, which was chosen for further propagation, is highlighted. Anti-Flag M2 (1:40000).

I had to minimally dilute mix clones producing anti-lamin A/C R453W antibodies two times until I could isolate a single clone, thus the name 1D6-12A-2F5 for the anti-lamin A/C R453W monoclonal antibody. The well 2F5 contained the only positive single clone, therefore this clone was chosen for further propagation, and was further used in upcoming experiments (figure 17). The other positive clones were all mix clones, consisting of 2 – 4 clones, and were frozen and were stored in liquid nitrogen.

I tested the usage of this antibody in different techniques including immunoblotting, immunoprecipitation, and immunofluorescence (shown below).

3. 3. 3. Immunoblotting with anti-lamin A/C R453W 12A-2F5 antibody

The single clone 12A-2F5 was expanded for cryopreservation and for the production of larger amounts of monoclonal antibody supernatant. The supernatant from eight p100 plates (~80 ml) was harvested after an incubation time of one week and restested by Western blotting (figure 18).

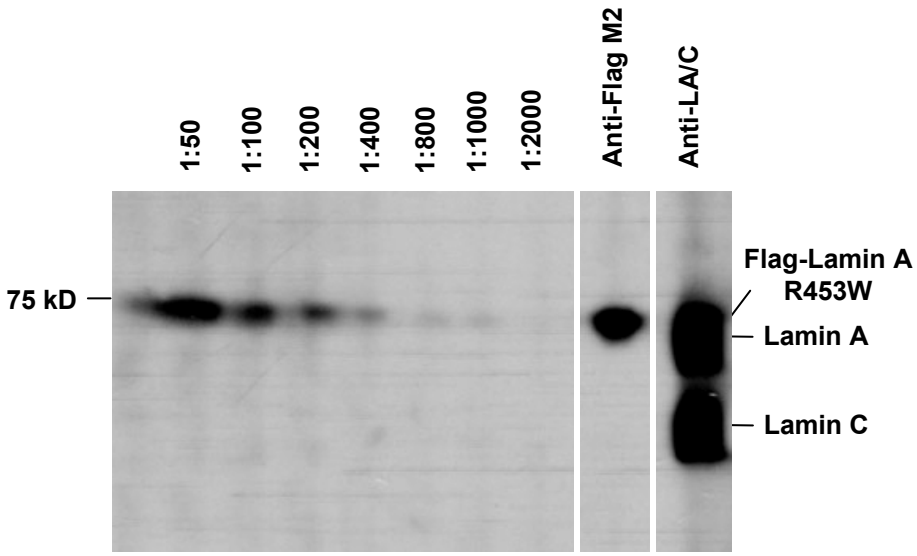


Figure 18: Test of dilutions of the derived anti-lamin A/C R453W 12A-2F5 monoclonal antibody. The monoclonal antibody was tested by a Western blot against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R453W (7.5% SDS-PAG). Anti-Flag M2 (1.40000), anti-lamin A/C 3A6-4C11 (1:1000). Exposure time: 2min.

According to this Western blot the hybridoma supernatant should be best used in a dilution of 1:50 in Western blot analysis (figure 18).
The isotyping of the anti-lamin A/C R453W 12A-2F5 antibody revealed that it consisted of the heavy chain subclass IgG1 and the light chain subclass κ.

3. 3. 4. Lamin A/C R453W recognition by anti-lamin A/C R453W antibody, 12A-2F5, in primary human cells

Further I tested, if the anti-lamin A/C R453W 12A-2F5 antibody could also detect specifically the R453W lamin A/C mutant in primary fibroblasts from an AD-EDMD patient (G-8626). G-8626 cells are heterozygous for the mutation R453W in the gene coding for lamin A and

lamin C. The wild-type and the mutant proteins are expressed side by side in these cells. As a control we used fibroblasts from a healthy individual (T.M.wt). T.M.wt cells have wild-type sequences of lamin A and lamin C in both alleles.

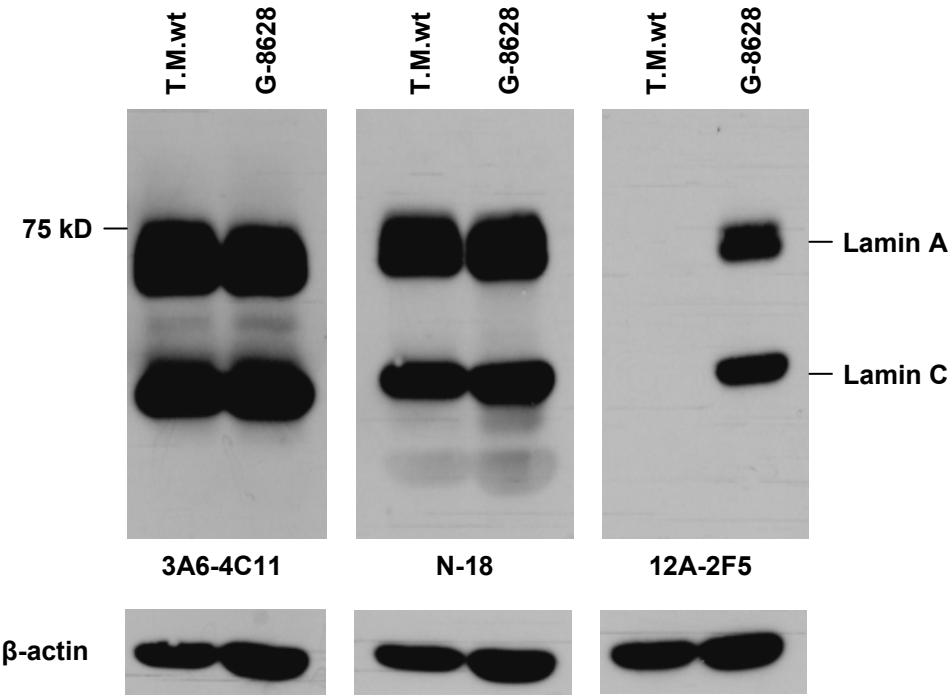


Figure 19: Immunoblot analysis of G-8626 and T.M.wt cells. GSD lysates from T.M.wt cells (primary human skin fibroblasts) and G-8626 cells (primary human skin fibroblasts heterozygous for the mutation R453W in the proteins lamin A and lamin C) were electrophoretically separated on a 7.5% SDS-PAG and blotted to a nitrocellulose membrane. Each lane of T.M.wt and G-8626 contains lysate equivalents of ~1 x 10^5 cells. The membranes were incubated with the antibodies depicted in the figure. Anti-lamin A/C 3A6-4C11 (1:2000); anti-lamin A/C N-18 (1:2000); anti-lamin A/C R453W 12A-2F5 (1:50). Incubation of the blot with anti-β-actin (Sigma) (1:500) served as a loading control.

The lamin A and lamin C antibodies, 3A6-4C11 and N-18, detected lamin A/C in both primary cells (figure 19, left and middle panel), indicating, as expected, that these two antibodies do not allow to differentiate between the wild-type and the mutant lamin A/C proteins. Moreover, as described before (figure 12B) the antibody 3A6-4C11 also cross-reacted with lamin B1 and lamin B2, which are represented by the two faint bands between lamin A and lamin C (figure 19 left panel). The point mutation specific antibody, 12A-2F5, recognized just lamin A and lamin C in the patient primary cells G-8626, which are heterozygous for the R453W mutation in lamin A and lamin C and did not recognize wild-type lamin A and lamin C present in the wild-type primary cells T.M.wt (figure 19 right

panel), again confirming the antibody's specificity for mutant lamin A/C R453W also when tested in primary human cells.

3. 3. 5. Peptide competition of anti lamin A R453W 12A-2F5 antibody

The specificity of the antibody was further confirmed by a peptide competition assay, as described in section 2. 3. 21. The specific peptide W453 (which is the R453W point-mutation containing peptide) and the corresponding wild-type peptide R453 were used to interfere with the binding of the point-mutation specific antibody to Flag-lamin A R453W. To determine the amount of peptide required for competition, the concentration of the antibody in the hybridoma supernatant had to be estimated first by Bradford analysis (for details please see section 2. 3. 7.). The protein content of 1µl and 2µl of hybridoma supernatant was determined. As a reference, the protein content of 1µl and 2µl of DMEM containing 10% FCS was determined, as the hybridomas were cultivated in this medium. The difference of the protein content in the hybridoma supernatant to the plain medium was accounted to the secreted antibodies, and was estimated to be 1.65µg/µl. The mentioned molar ratios of peptide vs. antibody were then calculated.

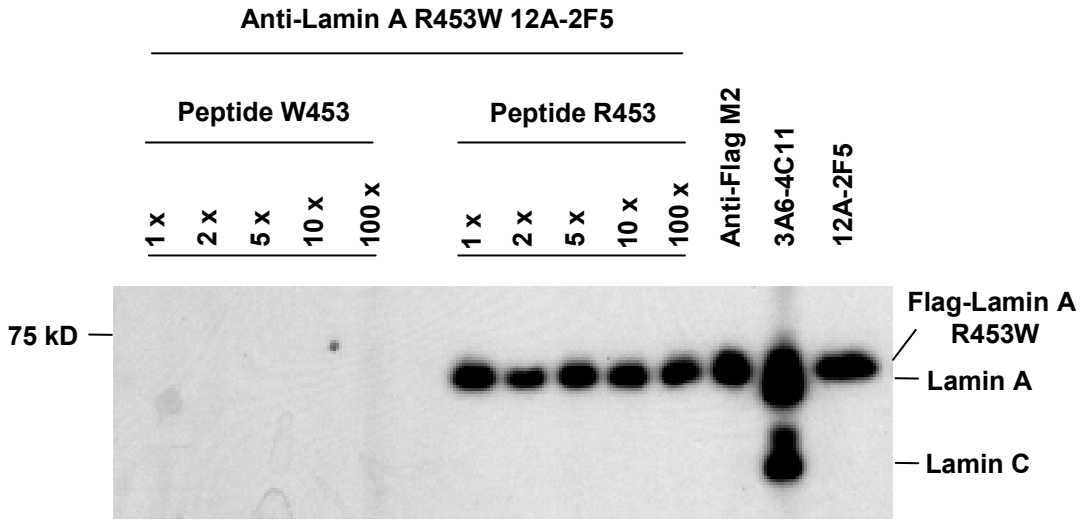


Figure 20: Peptide W453 interfered with binding of 12A-2F5 to Flag-lamin A R453W. 12A-2F5 antibody was competed with peptides W453 and R453 at the indicated molar ratios. The efficiency of competition was tested by immunoblotting on a whole cell lysate of HeLa cells ectopically expressing Flag-lamin A R453W (7.5% SDS-PAG). Exposure time: 1 hour. Anti-Flag M2 (1:20000); anti-lamin A/C 3A6-4C11 (1:1000); anti-lamin A/C R453W 12A-2F5 (1:100).

The results of the peptide competition assay are shown in figure 20. Anti-Flag M2 and anti-lamin A/C 3A6-4C11 confirmed expression of Flag-lamin A R453W and wild-type lamin A/C in these cells (3rd and 2nd last lane from the right). On the far right side of the blot there is the anti-lamin A/C R453W 12A-2F5 control, without peptide incubation (last lane). As seen, all peptide competitions with the wild-type peptide R453 at the indicated molar ratios did not reduce the signal strength (compare to 12A-2F5 control, last lane) (figure 20). On the other hand, the specific peptide W453 was very efficient in competing with the Flag-lamin A R453W in binding to the antibody. Even at the lowest molar ratio no signal could be detected (figure 20). This result once again confirmed the high specificity of the point-mutation specific antibody 12A-2F5.

3. 3. 6. Immunoprecipitation with anti-lamin A/C R453W 12A-2F5 antibody from HeLa cells ectopically expressing Flag-lamin A R453W

Next I tested the ability of 12A-2F5 to immunoprecipitate the mutant Flag-lamin A R453W from lysates of HeLa Flag-lamin A R453W cells.
When I lysed HeLa Flag-lamin A R453W cells with IP-Lyse buffer or RIPA buffer, I could not immunoprecipitate Flag-lamin A R453W with the anti-lamin A/C R453W antibody (data not shown and below). Thus I modified the lysis conditions as follows:

	modified IP-Lyse	**Lysis buffer**	**modified RIPA**
Tris	20mM, pH 8.0		
HEPES		50mM	
Na-Phosphate			10mM, pH 7.2
NaCl	135mM	100mM	150mM
Triton X-100	1%	1%	1%
SDS	0.1%	0.5%	0.1%
NP-40	1%		1%
EGTA	1mM	10mM	
EDTA	1mM		
glycerol	10%		
MgCl2		4mM	
DNase		0.5mg/ml	
RNase A		0.2mg/ml	
Deoxycholic acid			0.1%
Aprotinin, PMSF, Complete	see M&M	see M&M	see M&M
Lysis protocol	2. 3. 14.	2. 3. 15.	2. 3. 14.

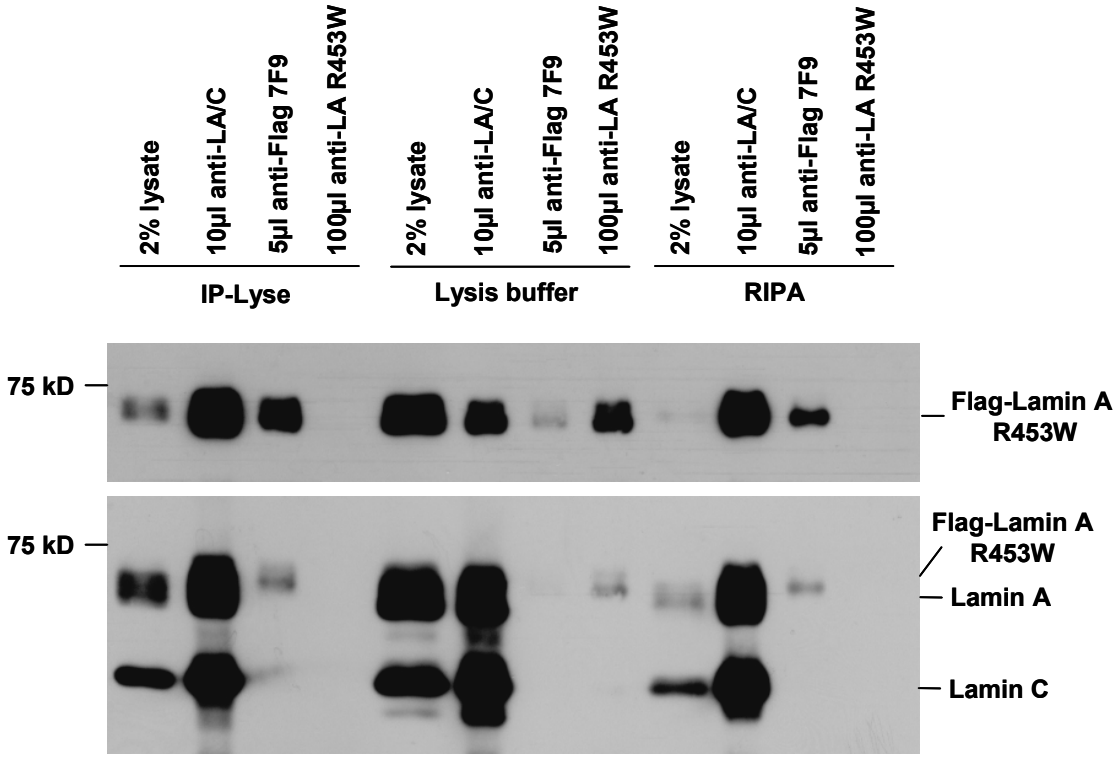


Figure 21: Immunoprecipitation of Flag-lamin A R453W with various mouse monoclonal antibodies. HeLa cells ectopically expressing Flag-lamin A R453W were lysed with 3 different buffers (see table above). Immunoprecipitations were made using the different antibody supernatants in amounts mentioned in the figure. ~2mg protein lysate were taken for each IP. 2% of the different cell lysates and the immunoprecipitations were electrophoretically separated on a 7.5% SDS-PAG. The blot was sequentially incubated with anti-Flag M2 (upper panel, 1:10000) and with anti-lamin A/C 3A6-4C11 antibodies (lower panel, 1:1000).

The cell lysis with Lysis buffer was most efficient in solubilizing Flag-lamin A R453W, and wild-type lamin A and lamin C, followed by the modified IP-lyse buffer and the least efficient modified RIPA buffer, as seen by the amount of those proteins detected in 2% of the lysate in the Western blot analysis (figure 21). The modified IP-Lyse buffer and the modified RIPA buffer contained 0.1% SDS. The Lysis Buffer contained 0.5% SDS, suggesting that a higher SDS concentration enhances solubilization of the A-type lamins. The SDS concentration in the lysate made with the Lysis buffer was reduced to 0.25% to allow binding of the antibodies to their epitopes during the immunoprecipitation.

Anti-lamin A/C 3A6-4C11 immunoprecipitated wild-type lamin A and lamin C, as well as the ectopically expressed Flag-lamin A R453W. The anti-Flag 7F9 (a gift from ALEXIS Biochemicals) and the anti-lamin A R453W 12A-2F5 antibodies immunoprecipitated just the ectopically expressed Flag-lamin A R453W.

Anti-lamin A/C 3A6-4C11 immunoprecipitated Flag-lamin A R453W from all the lysates, regardless of the buffer used, however with different efficiencies. It was most efficient in cell lysates made with the modified IP-Lyse buffer, followed by modified RIPA buffer and least efficient with Lysis buffer (figure 21 upper panel). The efficiencies for immunoprecipitation correlated with the results obtained for the anti-Flag 7F9 antibody, which also immunoprecipitated Flag-lamin A R453W with highest efficiency in the lysate made by the modified IP-Lyse buffer and the modified RIPA buffer, and with just low efficiency in the cell lysate made with Lysis buffer, despite the fact that the cell lysate made with the Lysis Buffer contained most Flag-lamin A R453W (compare 2% lysate lanes for all three cell lysates). The cell lysate made with Lysis buffer was the only cell lysate where Flag-lamin A R453W could be immunoprecipitated by anti-lamin A R453W 12A-2F5 antibody.
The amount of wild-type lamin A and lamin C immunoprecipitated from the cell lysates made with the modified buffer IP-Lyse and the modified RIPA buffer were similar. Higher amounts of wild-type lamin A and lamin C were immunoprecipitated by anti-lamin A/C 3A6-4C11 from the cell lysate made with the Lysis buffer (figure 21 bottom panel).

3. 3. 7. Immunoprecipitation with anti-lamin A/C R453W 12A-2F5 antibody from patient cells

Cell lysates made with the Lysis Buffer were required to successfully immunoprecipitate Flag-lamin A R453W with the anti-lamin A/C R453W 12A-2F5 antibody in cell lysates form HeLa cells ectopically expressing Flag-lamin A R453W (figure 21). Therefore also the primary fibroblast cells were lysed with the Lysis buffer and the cell lysis protocol described in section 2. 3. 15.

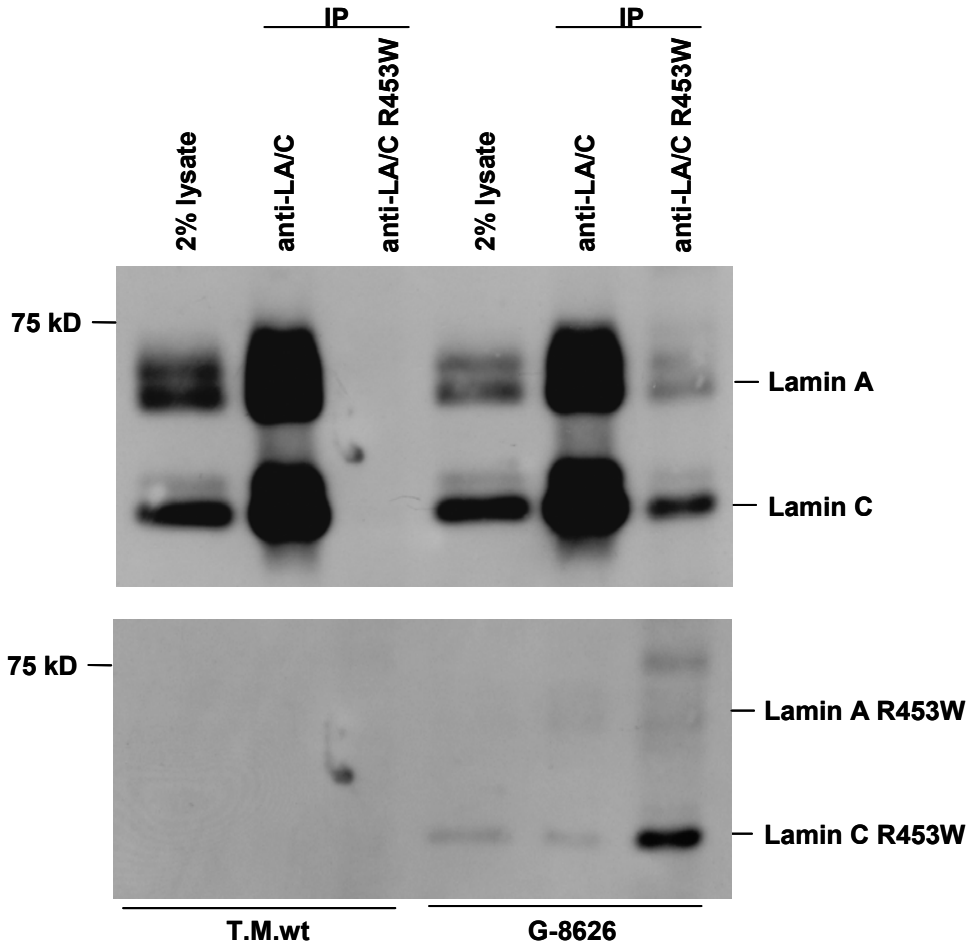


Figure 22: Immunoprecipitation of G-8626 and T.M.wt primary cells. For each immunoprecipitation a cell lysate aliquot equivalent to 3.5 x 10^5 cells was used. 15µl solid anti-lamin A/C 3A6-4C11 cross-linked Protein A sepharose beads or 100µl anti-lamin A/C R453W 12A-2F5 antibody supernatant (+20µl of a 1:1 mixture of solid Protein A and Protein G sepharose beads) were used per immunoprecipitation. The bound proteins were electrophoretically separated on a 7.5% SDS-PAG. The blot was incubated with anti-lamin A/C R453W 12A-2F5 antibody (1:50) (lower panel, exposure time: 1 hour), followed by incubation with anti-lamin A/C 3A6-4C11 antibody (1:500) (upper panel; exposure time: 1 hour).

The anti-lamin A/C antibody, 3A6-4C11, was very efficient in immunoprecipitating lamin A/C from both primary cells. However, 3A6-4C11 could not immunoprecipitate effectively the mutant lamin A R453W and lamin C R453W (compare signals obtained for anti-LA/C immunoprecipitation of G-8626 cell lysates incubated with anti-lamin A/C R453W 12A-2F5 antibody and anti-lamin A/C 3A6-4C11 antibody, figure 22 lower and upper panel). Thus, almost all lamin A and lamin C immunoprecipitated from G-8626 cell lysates by 3A6-4C11 must have been wild-type proteins.

Mutant lamin A and lamin C was immunoprecipitated by the anti-lamin A/C R453W 12A-2F5 antibody from the patient primary cell G-8626 cell lysates. However, the efficiency of immunoprecipitation by the point mutation specific antibody was very low (lower and upper panel of figure 22). In summary, the point-mutation specific anti-lamin A/C R453W 12A-2F5 antibody immunoprecipitated lamin A/C R453W only when the cells were lysed with a buffer containing at least 0.5% SDS.

3. 3. 8. Immunofluorescence with anti-lamin A/C R453W 12A-2F5 antibody of HeLa cells ectopically expressing Flag-lamin A R453W

It was already shown, that ectopically expressed GFP-tagged mutant Ce-lamin R460W, the *C. elegans* equivalent of the human lamin A/C R453W, forms small aggregates in *C. elegans* cells (Wiesel et al., 2008), and that GFP-tagged lamin A/C R453W is having an increased mobility (measured with fluorescence loss of intensity after photobleaching (FLIP) technique), which is an indication for structural instability of lamin polymers, in the nuclei of cells (Broers et al., 2005). Nevertheless, all these studies were done using ectopical and overexpressed recombinant proteins. Thus we wanted to analyze the localization of the mutant A-type lamin proteins in primary fibroblasts from an AD-EDMD patient as well as in HeLa Flag-lamin A R453W cells with our newly generated point-mutation specific antibody.

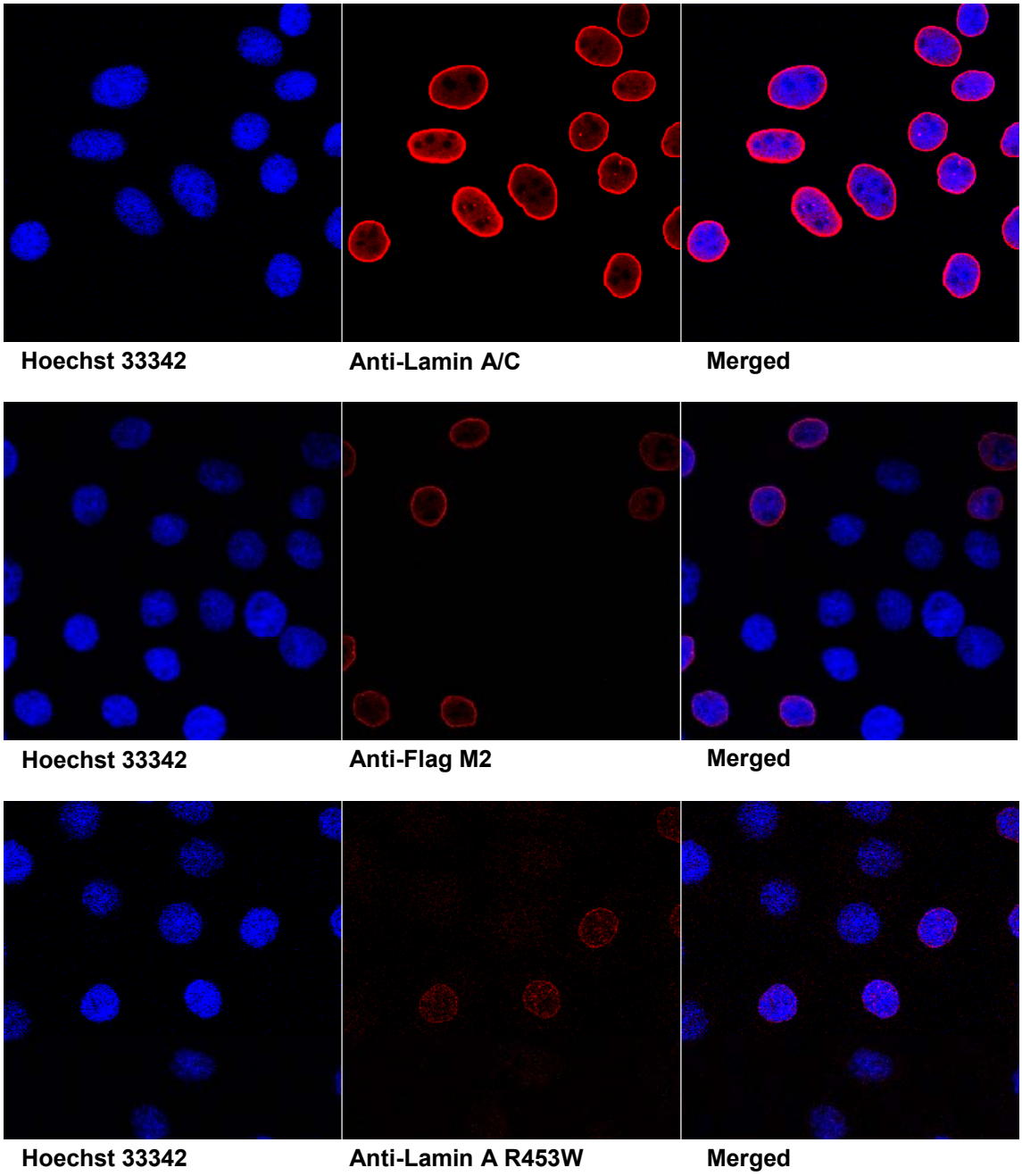


Figure 23: Immunofluorescence images of a mixed population of HeLa + HeLa Flag-lamin A R453W cells. In the top panel, cells were stained with anti-lamin A/C 3A6-4C11 (1:100); in the middle panel, cells were stained with anti-Flag M2 (1:1000); and in the bottom panel, cells were stained with anti-lamin A R453W 12A-2F5 (1:10). Cells were counterstained with Hoechst 33342. Cells were treated with 0.1% SDS during permeabilization.

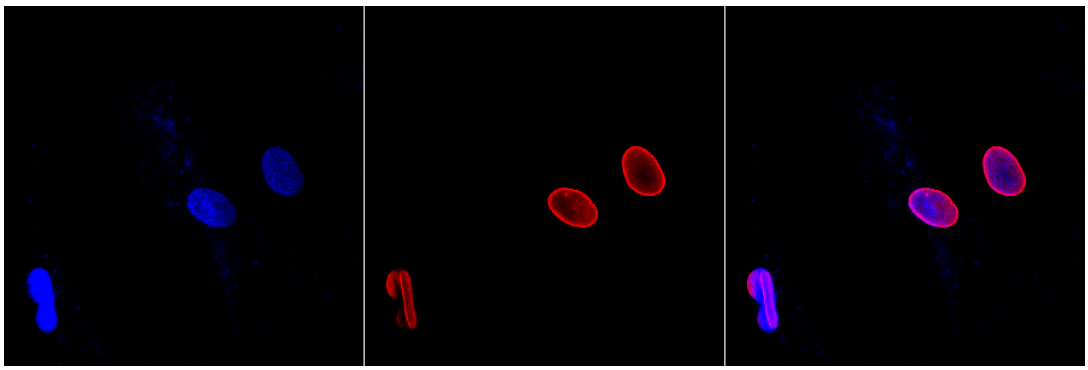
Anti-lamin A/C 3A6-4C11 stained the nuclear rim and also to a lesser extent the nucleoplasm of all HeLa and HeLa Flag-lamin A R453W cells (figure 23 top panel). Anti-Flag M2 stained predominantly the nuclear rim with only slight nucleoplasmic staining in a subfraction of all

cells, which correspond to the HeLa cells ectopically expressing Flag-lamin A R453W (figure 23 middle panel). Note that only eight nuclei out of 20 were stained in the frame shown. Anti-lamin A R453W 12A-2F5 showed a different staining pattern than anti-Flag M2 antibody. In nuclei stained with this antibody it seemed that Flag-lamin A R453W was dispersed more homogenously throughout the nucleoplasm, but with still distinguishable nuclear rim staining (figure 23 bottom panel). Note that three nuclei out of 12 were stained in the frame shown.
I counted cells stained with anti-lamin A R453W 12A-2F5 antibody in the 3:1 mixed cell population. Of 275 cells counted, 67 were stained with the point mutation specific antibody, which is 24%. This result is in line with the amount of cells seeded, as theoretically 25% of all cells in the 3:1 mixed cell population should have been stained either with the point-mutation specific antibody or the anti-Flag M2 antibody. In summary, the antibodies anti-Flag M2 and anti-lamin A/C R453W 12A-2F5 showed a different staining pattern of the Flag-lamin A R453W protein in HeLa Flag-lamin A R453W cells.

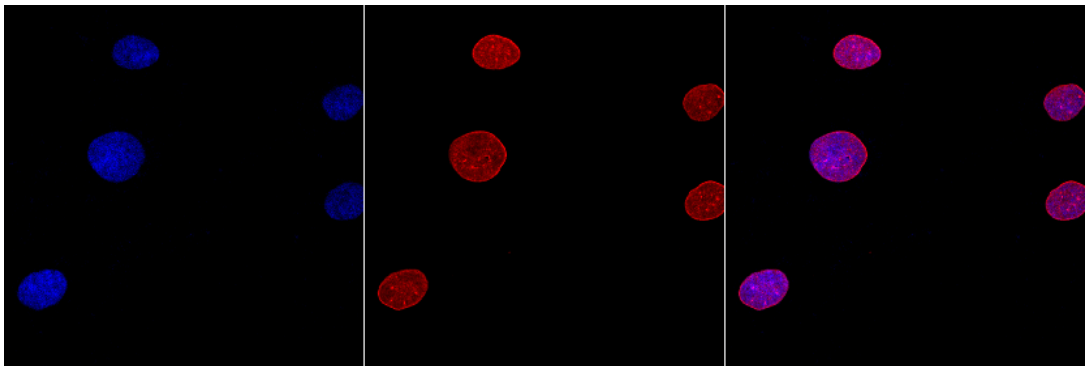
3. 3. 9. Immunofluorescence of T.M.wt and G-8626 primary cells

The wild-type primary fibroblast cells T.M.wt and the primary fibroblast cells G-8626 from an individual heterozygous for the mutation R453W in lamin A and lamin C, were stained with the point mutation specific anti-lamin A/C R453W 12A-2F5 antibody and, as control, with the anti-lamin A/C 3A6-4C11 mouse monoclonal antibody.

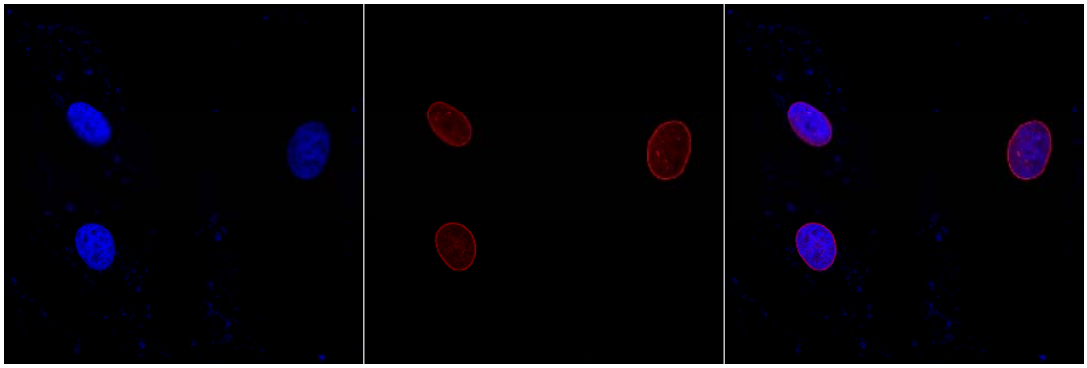
A **G-8626**



Hoechst 33342 | Anti Lamin A/C | Merged



Hoechst 33342 | Anti Lamin A/C R453W | Merged



Hoechst 33342 | Anti Lamin A/C R453W | Merged

Hoechst 33342 | 2^{ary} antibody | Merged

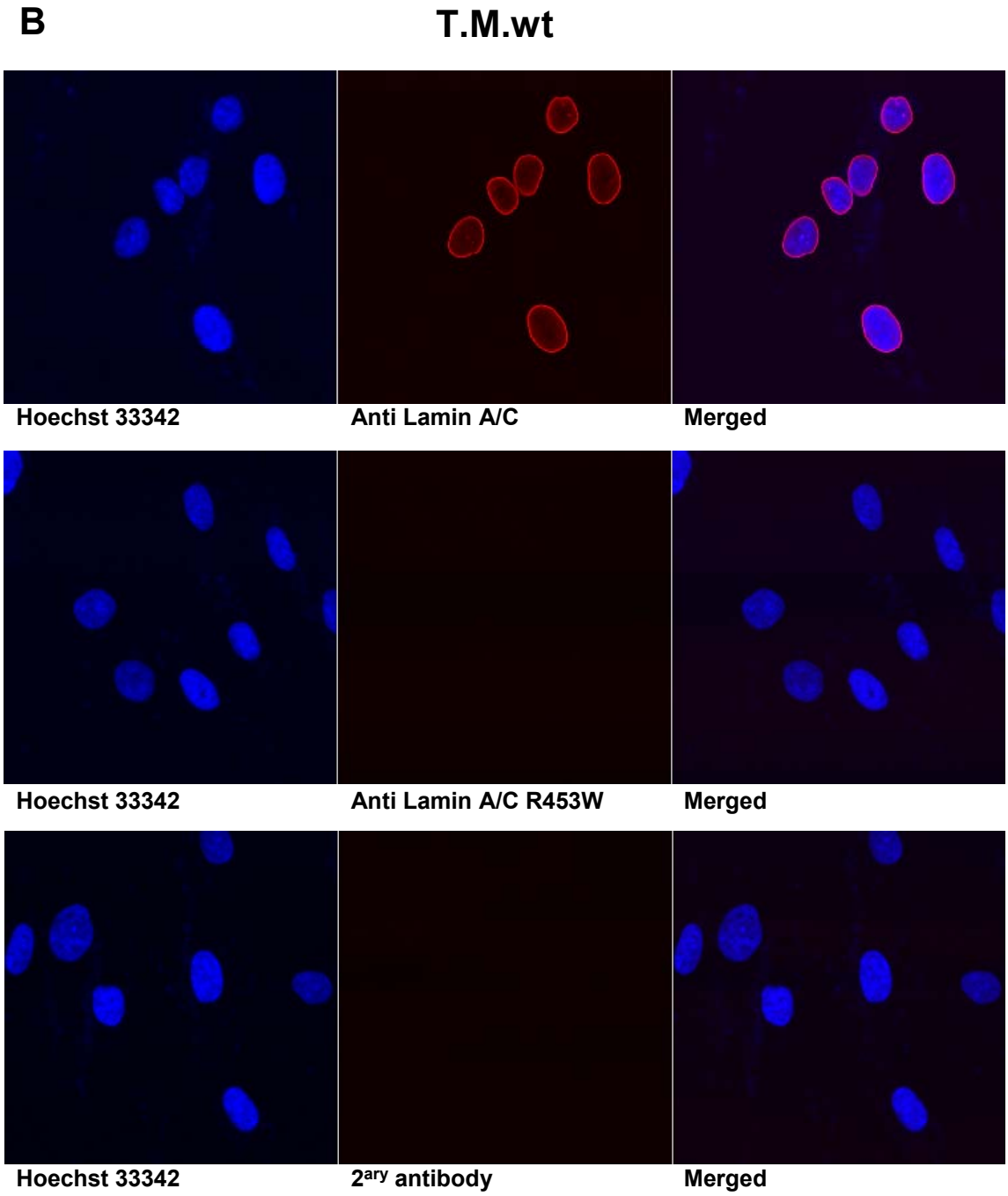


Figure 24: Immunofluorescence of G-8626 EDMD and T.M.wt primary cells with anti-lamin A/C R453W and anti-lamin A/C 3A6-4C11 mouse monoclonal antibodies. A) G-8626 (human skin fibroblasts from patient heterozygous for the mutation R453W in lamin A/C), and B) T.M.wt (wild-type human skin fibroblast) were stained with the respective antibodies depicted in the figure. In A) two pictures of stainings with the 12A-2F5 antibody are shown (2nd panel from the top representing 1/3 of the stainings; 3rd panel from the top representing 2/3 of the stainings). Anti-lamin A/C 3A6-4C11 (1:100); anti-lamin A/C R453W 12A-2F5 (1:10). Cells were counterstained with Hoechst 33342. Cells were treated with 0.1% SDS during permeabilization.

Lamins were stained by anti-lamin A/C 3A6-4C11 in T.M.wt cells and G-8626 cells (figure 24A and 24B). There might have been slightly more nucleoplasmic lamin staining in G-8626 than in T.M.wt cells detected (compare upper panels of figure 24A and figure 24B) by 3A6-4C11. The anti-lamin A/C R453W 12A-2F5 antibody specifically stained patient cells, while it did not give a signal in the wild-type control cells (compare middle panels of figure 24A and 24B). Once again this confirmed that the antibody is indeed specific for the mutant lamins and that it can also be used in immunofluorescence to detect the mutant A-type lamins in primary cells derived from patients suffering from AD-EDMD. The staining with anti-lamin A/C R453W 12A-2F5 revealed a homogenous staining of mutant lamin A/C R453W throughout the nucleoplasm in about one third of the cells (figure 24A, 2nd panel from the top). In about two thirds of the cells, staining with anti-lamin A/C R453W 12A-2F5 showed a prominent rim staining with weak nucleoplasmic staining (figure 24A, 3rd panel from the top). Anti-lamin A/C 3A6-4C11, an antibody recognizing wild-type and mutant lamin A and lamin C in Western blot analysis, stained predominantly lamins at the nuclear rim, with only slight nucleoplasmic staining (figure 24A and 24B, top panel).

3. 3. 10. Expression of HBcAg Ig-fold R453W

After the KLH-approach for the W453 peptide was successful, I wanted to know, if the recombinant protein HBcAg Ig-fold R453W could also elicit a strong immune response, in the best case a “point-mutation” specific immune response, as in the previous immunizations with the various HBcAg Ig-fold antigens. I used the bacterial strain Origami for the expression of HBcAg Ig-fold R453W, as I required more HBcAg Ig-fold R453W antigen for the start of immunization of mice.

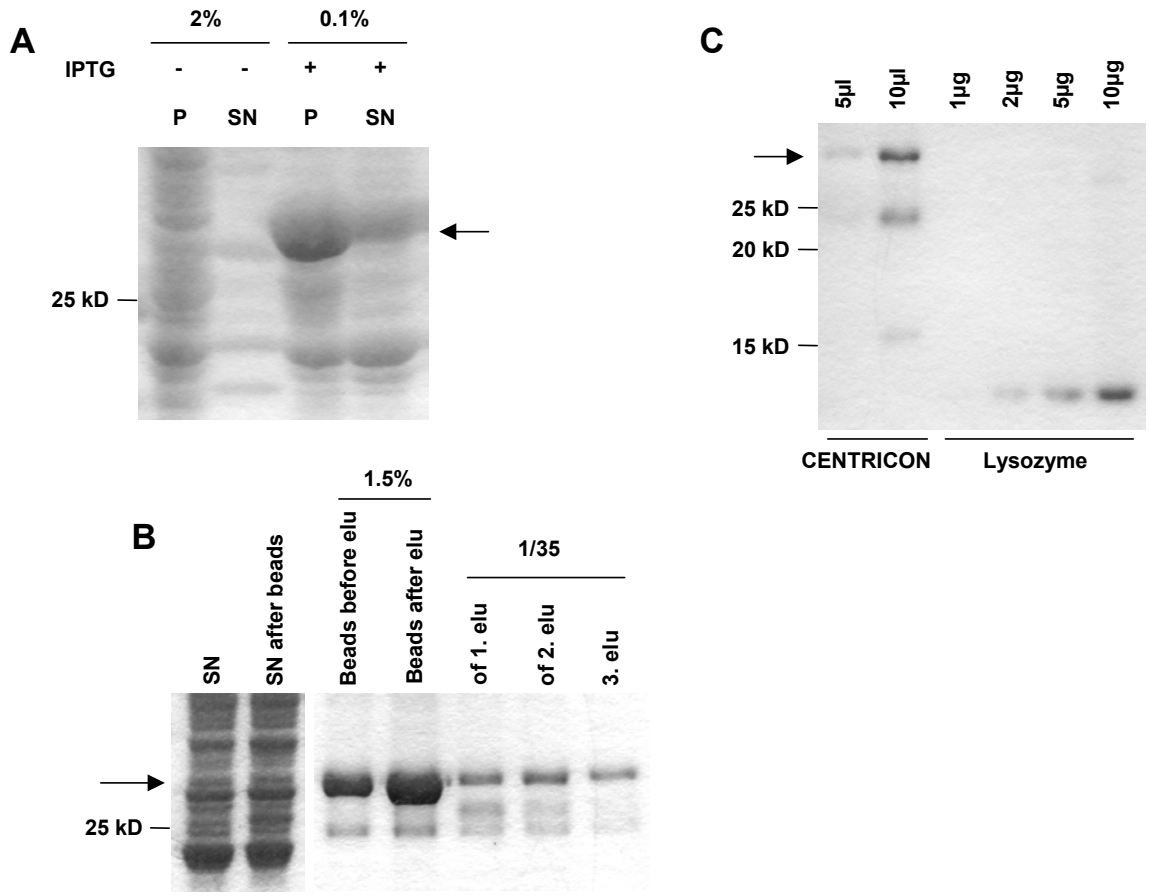


Figure 25. Expression and purification of HBcAg Ig-fold R453W. A) HBcAg Ig-fold R453W was expressed in the bacterial strain Origami. The expression was checked on a 10% SDS-PAG. 2% (non-induced bacterial culture) or 0.1% (induced bacterial culture) of the P and SN fraction of the bacterial lysates were loaded. B) 1.5% of the Ni-NTA beads before and after elution, and aliquots of eluates (1/35 of the eluted amount) from elution of HBcAg Ig-fold R453W under native conditions were checked on a 10% SDS-PAG. C) The concentration of HBcAg Ig-fold R453W after CENTRICON (Millipore) treatment was checked on a 10% SDS-PAG. Defined amounts of lysozyme served as a protein standard. The proteins on all three gels were stained with Coomassie. The HBcAg Ig-fold R453W, having a calculated molecular weight of ~31.7 kDa, protein band is indicated with an arrow in all three figures. SN = soluble fraction; P = insoluble pellet fraction.

Bacteria, from the induced bacterial culture and from the non-induced bacterial culture, were lysed in buffer volumes that were normalized to the optical density of the bacterial cultures. Aliquots of the bacterial lysates (soluble and pellet fraction) were electrophoretically separated on a 10% SDS-PAG. The proteins in the gel were stained with Coomassie (figure 25A). The expression of HBcAg Ig-fold R453W was highly induced upon IPTG addition in the bacterial strain Origami (figure 25A). Unfortunately most of the protein was present in the insoluble pellet fraction (inclusion bodies). The poor solubility of the protein could be due to the already mentioned lower thermostability of this mutant (Krimm et al., 2002).

As for the immunization the protein was required in its native conformation, the relatively small amount of soluble HBcAg Ig-fold R453W protein had to be isolated from the supernatant by Ni-pull down and elution with imidazole according to the protocol (please see section 2. 3. 4.). To check wheter His-tagged HBcAg Ig-fold R453W was purified from the supernatant I eluted the bound proteins from the Ni-NTA beads. Indeed, substantial amounts could be purified (3rd lane, figure 25B), but there was still a huge amount of the protein left in the supernatant (2nd lane, figure 25B). The imidazole elution of the His-tagged HBcAg Ig-fold R453W did not work well (lanes 5-7, figure 25B) as most of the His-tagged protein was still bound to the beads (4th lane, figure 25B). The increased level of protein on the beads after the elution was most probably due to a pipetting artefact.

The concentration of the protein solution after elution from the Ni-NTA beads was low. There was another protein detected at ~25 kDa, which could be a degradation product of HBcAg Ig-fold R453W, or a protein that bound non-specifically to the Ni-NTA beads (figure 25B). Therefore I decided to concentrate the protein by a Centricon Centrifugal Filter Device (Millipore). The cut off range of the filter used was 10 kDa. 0.7% (5µl) and 1.4% (10µl) of the HBcAg Ig-fold R453W protein solution were loaded on the gel to estimate the protein concentration of the protein solution after the use of the Centricon Centrifugal Filter Device (Millipore). The concentration of the protein in the solution after filtration was slightly lower than 1mg/ml (figure 25C). The protein was already fragmented and partially degraded (figure 25C, bands at ~24 kDa and ~16 kDa).

3. 3. 11. Immune response of HBcAg Ig-fold R453W immunized mice

Three mice were immunized with the HBcAg Ig-fold R543W antigen for three times and the pre-immune serum and the sera from the first two bleeds were tested for the presence of Flag-lamin A R453W specific antibodies by Western blot analysis.

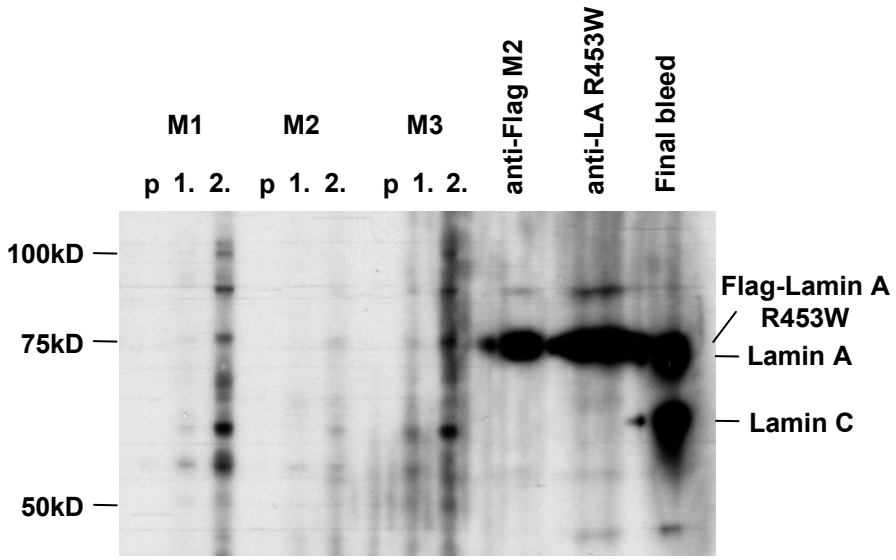


Figure 26. Test of sera of HBcAg Ig-fold R453W immunized mice. Sera were tested in a Western blot on whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R453W (7.5% SDS-PAG). Anti-Flag M2 (1:40000), anti-lamin A/C R453W 12A-2F5 (1:50), final bleed of HBcAg Ig-fold WT immunized mouse 1 (1:20000). p=pre-immune sera; 1.=serum of 1. bleed; 2.=serum of 2.bleed. M1 = mouse1; M2 = mouse 2; M3 = mouse 3; M4 = mouse 4.

The immunization of mice with HBcAg Ig-fold R453W did not elicit a specific immune response in the mice (figure 26). This was surprising, as all immunizations with HBcAg Ig-fold recombinant proteins so far elicited a strong immune response in mice at least against the Ig-fold in general. The antisera of mouse 1 and mouse 3 contained antibodies that recognized weakly a protein at the size of the Flag-tagged lamin A R453W. The identity of the protein recognized at ~75 kDa was not further tested, due to the poor signal strength compared to the controls. Therefore it remained speculative if a point-mutation specific immune response was elicited in mice immunized with HBcAg Ig-fold R453W (figure 26).

3. 4. Antibodies against lamin A/C R482W

3. 4. 1. Expression of HBcAg Ig-fold R482W

To raise antibodies against the FPLD-causing lamin A/C R482W mutant, we immunized mice with HBcAg Ig-fold R482W, as the KLH-approach was not successful (data from Veronika Huber). Veronika Huber had obtained experimental evidence that the immunization of mice

with HBcAg Ig-fold R482W elicited the production of R482W mutant-specific, together with Ig-fold specific, antibodies. Unfortunately, Veronika Huber did not succeed in generating a point-mutation specific monoclonal hybridoma cell line.

The antigen HBcAg Ig-fold R482W was expressed in the bacterial strain Rosetta (+), because high expression of the antigen was achieved independent of transcriptional induction by IPTG.

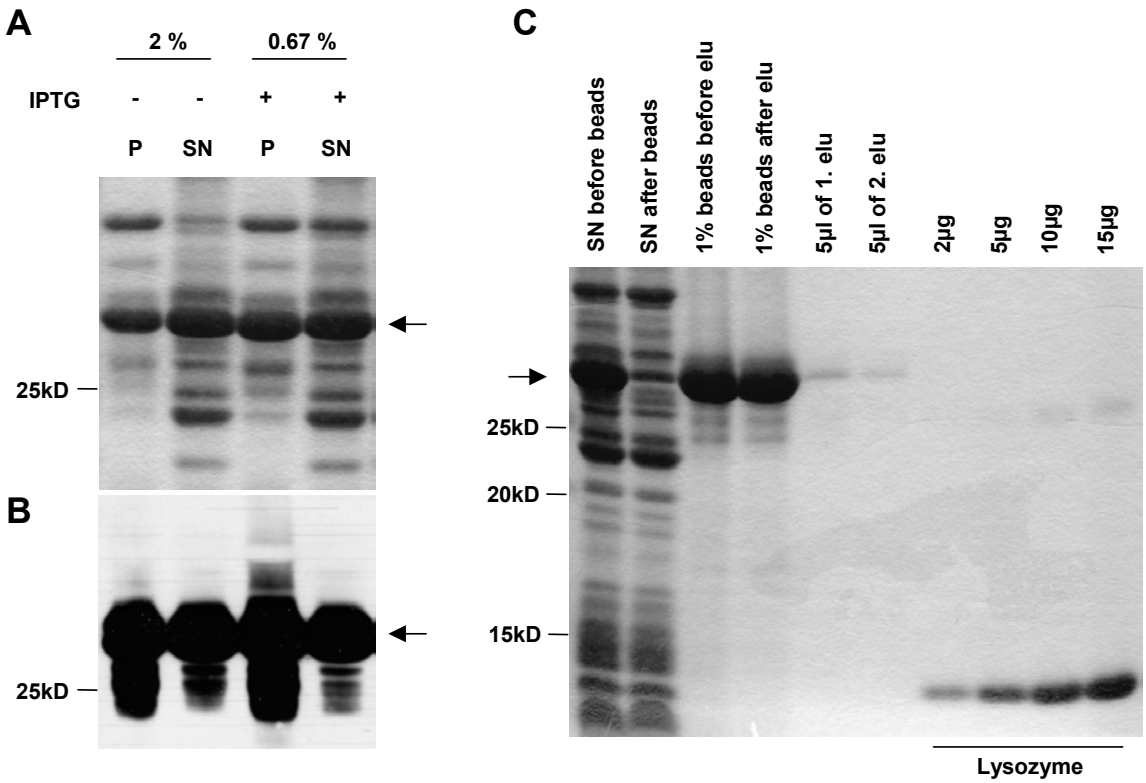


Figure 27. Expression and purification of HBcAg Ig-fold R482W. A) The expression of HBcAg Ig-fold R482W was induced in the bacterial strain Rosetta (+) and checked on two 12.5% SDS-PAG. One gel was stained with Coomassie (A; 2% (non-induced bacterial culture) or 0.67% (induced bacterial culture) of the P and SN fraction of the bacterial lysates were loaded), the other was used for Western blotting (B; 0.7% (non-induced bacterial culture) or 0.22% (induced bacterial culture) of the P and SN fraction of the bacterial lysates were loaded) and incubated with anti His-tag antibody (1:10000). C) Elution of HBcAg Ig-fold R482W from Ni-NTA beads under native conditions. Aliquots of 1% (= 5µl) of the eluates were checked on 12.5% SDS-PAG. The gel was stained with Coomassie. Defined amounts of lysozyme serve as a protein standard. HBcAg Ig-fold R482W (calculated molecular weight of 31.7 kDa) is indicated by an arrow. SN = soluble fraction; P = insoluble pellet fraction.

Large amounts of HBcAg Ig-fold R482W were expressed in Rosetta (+). About 60% of the protein were present in the insoluble pellet fraction (inclusion bodies) and about 40% of the protein were present in the soluble fraction after induction by IPTG (figure 27A and 27B). If the expression of the HBcAg Ig-fold R482W protein in Rosetta (+) was not induced by IPTG,

30% of the protein was present in the insoluble pellet fraction (inclusion bodies) and about 70% of the protein was present in the soluble fraction. Thus I decided to purify the protein from the soluble fraction under native conditions. Elution of the protein from the Ni-NTA beads under native conditions did not work well. Affinity purification of the protein, however, was very efficient from the supernatant (1st lane figure 27C), and almost all of the protein could be isolated from the soluble fraction (2nd lane figure 27C). However, only tiny amounts of protein were eluted from the beads with imidazole (5th and 6th lane figure 27C), as confirmed by presence of the protein on the Ni-NTA beads after elution (4th lane, figure 27C). Several elution methods, including different buffers, different temperatures at elution, different competitors (imidazole, 6xHis peptide), were tested. With none of them I could elute the protein from the affinity matrix (data not shown). Therefore affinity purified HBcAg Ig-fold R482W was directly used for immunizations of mice and a rabbit.

3. 4. 2. Immune response of HBcAg Ig-fold R482W immunized mice and a rabbit

The immunization with HBcAg Ig-fold R482W had been started by a former diploma student in the lab and I continued with the immunizations. The serum of the 6. bleed and a dilution series of the serum of the final bleed of mouse 1 immunized with HBcAg Ig-fold R482W were tested by Western blotting for the presence of the desired antibodies on whole cell lysates of HeLa cells ectopically expressing the N-terminally Flag-tagged lamin A R482W (figure 28A).

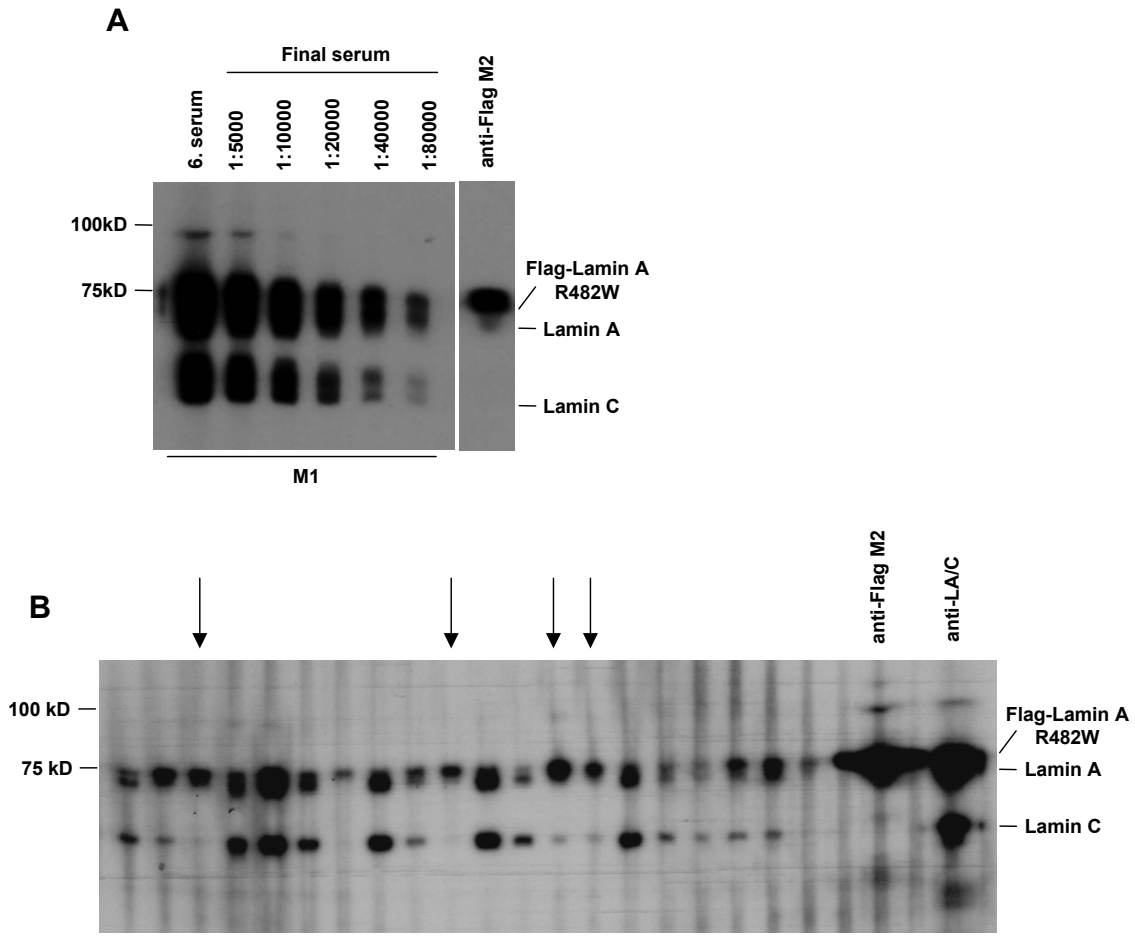


Figure 28. Test of sera of HBcAg Ig-fold R482W immunized mouse 1 (A) and test of hybridoma supernatants after fusion of splenocytes of HBcAg Ig-fold R482W immunized mouse 1 (B). Sera of bleeds were tested by a Western blot against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R482W (7.5% SDS-PAG). 6. serum was tested in a dilution of 1:5000, anti-Flag M2 (1:40000). (B) Test of hybridoma supernatants after fusion of splenocytes of HBcAg Ig-fold R482W immunized mouse 1. Anti-Flag M2 (1:10000), anti-lamin A/C = hybridoma supernatant (mix clone 4A3), hybridoma supernatants were not diluted.

The HBcAg antigen elicited a strong and consistent immune response against the Ig-fold of lamin A/C in all mice (please see data from Veronika Huber). A lamin A/C signal was still detectable at a dilution of 1:80000 (figure 28A). However, due to the fact that the Flag-lamin A R482W protein could not be separated from the endogenous wild-type lamin A by SDS-PAGE, we could not tell whether or not the sera contained point-mutation specific antibodies (figure 28A). The idea was to separate and discriminate the different antibody producing B-lymphocytes of the mouse upon fusion where they would spread into different wells. Therefore the splenocytes of mouse 1 immunized with HBcAg Ig-fold R482W were fused with X63Ag8.653 cells.

One third of the fused cells was seeded onto four 24 well plates and six 96 well plates. One week after the fusion the supernatants of the hybridomas were tested for presence of desired antibodies. There were several supernatants of wells that contained Flag-lamin A R482W specific antibodies, suggesting that indeed a point-mutation specific immune response was triggered in mice immunized with HBcAg Ig-fold R482W (data not shown). Some of these mix clones were minimally diluted. Unfortunately all of hybridoma clones tested after the minimal dilution did not secrete specific antibodies anymore (data not shown). Most probably they lost the ability to produce specific antibodies due to the chromosomal instability of these cells. Thus I tried again to isolate a anti-lamin A/C R482W specific monoclonal antibody and thawed one third of the fused cells and seeded them onto four 24 well plates. One week later, supernatants of single wells of a 24 well plate were tested by Western blot analysis on whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R482W (figure 28B). Again, as mentioned above, a point-mutation specific immune response was triggered in the mouse 1 by the HBcAg Ig-fold R482W antigen (figure 28B). Supernatants of the indicated wells contained R482W specific antibodies, with only weak cross-reactivity to wild-type lamin A and lamin C (figure 28B). This cross-reactivity could also be due to presence of other B-lymphocyte derived hybridoma clones in these wells, which were expressing antibodies recognizing an epitope shared by wild-type endogenous lamin A and lamin C, and the mutant Flag-lamin A R482W. Once again, my attempt to isolate a lamin A/C R482W specific antibody producing single clone failed, most probably due to the already mentioned instability of the hybridoma clones.

To obtain larger amounts of the anti-lamin A/C R482W point-mutation specific antibody, a rabbit was immunized with the antigen HBcAg Ig-fold R482W. The sera of the rabbit bleeds were tested by Western blot analysis for the presence of desired antibodies on whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R482W.

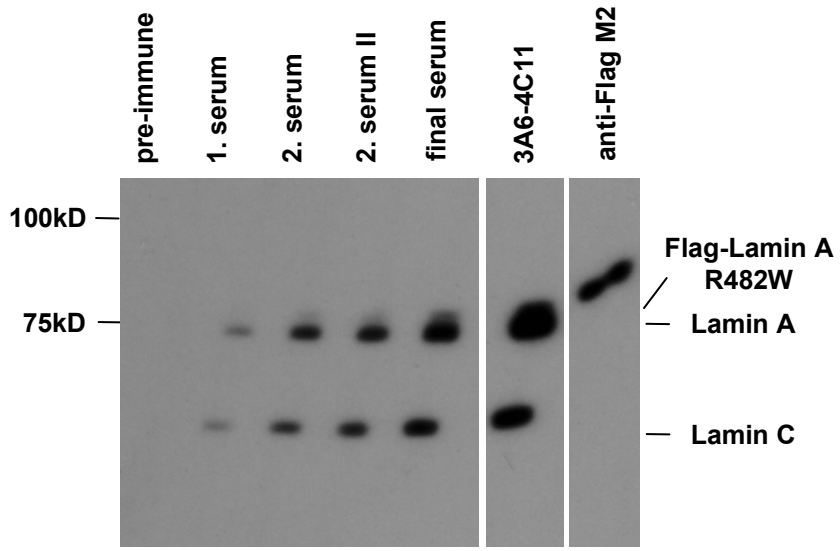


Figure 29. Test of sera of HBcAg Ig-fold R482W immunized rabbit. Sera of bleeds were tested by a Western blot against whole cell lysates of HeLa cells ectopically expressing Flag-lamin A R482W (7.5% SDS-PAG). The serum of the 2. bleed II was taken right before the final immunization. Sera were tested in a dilution of 1:1000, anti-Flag M2 (1:20000), anti-lamin A/C 3A6-4C11 (1:1000).

An immune response, producing antibodies recognizing lamin A/C was elicited in the rabbit and increased during following immunizations (figure 29), but I cannot tell from the Western blot data whether a R482W point-mutation specific immune response was elicited in the rabbit.

3. 5. Method improvements

3. 5. 1. Expression of "„HBcAg linker peptide"" recombinant proteins

As the KLH-N195, KLH-K195 and KLH-R453 antigens did not elicit an humoral immune response in mice producing antibodies being capable of detecting lamin A/C in Western blot analysis, I decided to express the N195, K195, and R453 epitope as a fusion protein with HBcAg, based on the results that immunizations of mice with HBcAg recombinant proteins elicited a strong humoral immune response.

The immunization of mice with "HBcAg linker peptide" recombinant proteins should combine advantages of the KLH-peptide approach, namely the high specificity against a short, well defined peptide, and the HBcAg recombinant protein approach, namely the strong

and consistent immune response elicited. Hence we reasoned that by such an approach, in contrast to the KLH-peptide approach, we might be able of generating antibodies recognizing lamin A/C epitopes in Western blot analysis.

To identify the bacterial strain with the highest expression of soluble "HBcAg linker peptide" protein, I tested the expression of HBcAg linker N195 in the bacterial strains Rosetta (-), Rosetta (+), Origami, and Tuner (for complete names and the genotype of bacterial strains used, see section 2. 2. 1.). The bacterial strain with the best expression profile was then chosen to express the other "HBcAg linker peptide" recombinant proteins.

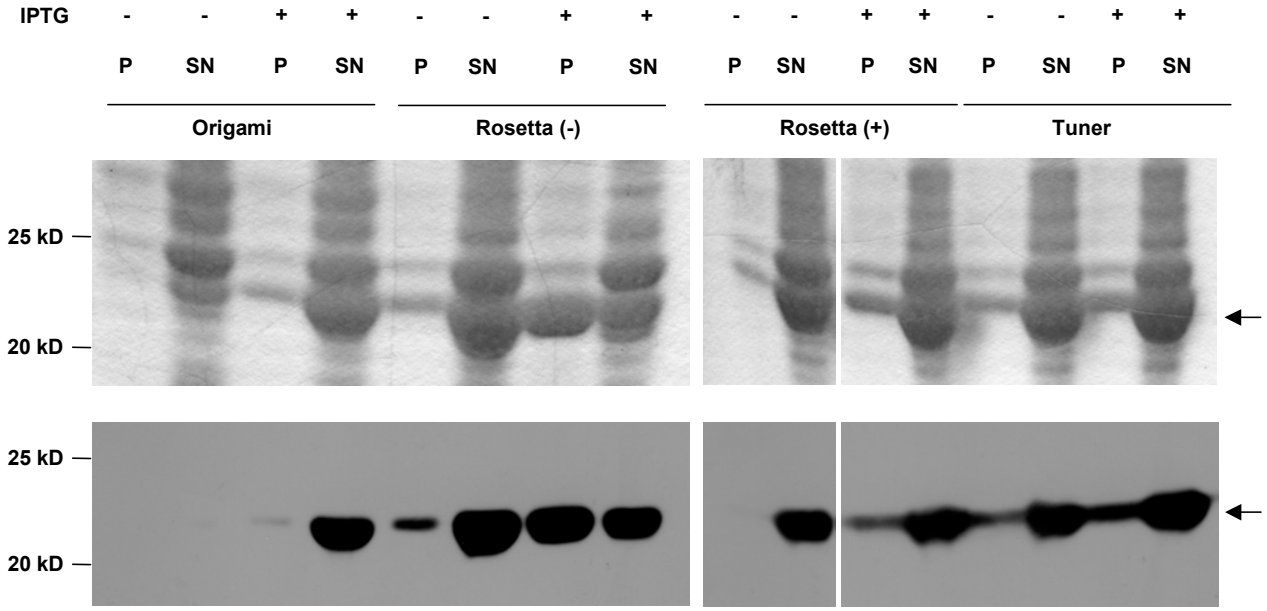


Figure 30. Expression of HBcAg linker N195 in four different bacterial strains. Bacterial lysates were checked for presence of HBcAg linker N195 on four 10% SDS-PAG, two for Coomassie staining (aliquots equivalent to ~1.5% of the pellet and soluble fractions of the bacterial lysates were loaded, top panel) and two for Western blotting (aliquots equivalent to ~0.75% of the pellet and soluble fractions of the bacterial lysates were loaded, bottom panel). HBcAg linker N195 was detected with anti-His-tag antibody (1:10000). The HBcAg linker N195 protein band is indicated with an arrow. + / - = with / without induction of IPTG; P = insoluble pellet fraction (inclusion bodies); SN = soluble fraction (supernatant).

Bacteria, from the induced bacterial cultures and from the non-induced bacterial cultures, were lysed in buffer volumes that normalized the number of bacterial cells in the bacterial lysates. Aliquots of the bacterial lysates (soluble and pellet fraction) were electrophoretically separated on four 10% SDS-PAG. Two gels were stained with Coomassie (figure 30, upper panel). The other two gels were blotted on a nitrocellulose membrane, and then incubated

with the anti-His-tag antibody for detection of the N-terminally His-tagged HBcAg linker N195 (figure 30, bottom panel). The expression of HBcAg linker N195 was very strong in all bacterial strains tested (figure 30). The expression of the protein in the strain Origami was inducible with IPTG, whereas all other strains expressed the protein even without induction by IPTG (figure 30). HBcAg linker N195 was not purified, as I did not have the intention to use it for immunizations of mice as I focused on the point-mutations R453W and R482W.

The bacterial strain Rosetta (+) was chosen to express the antigens HBcAg linker R453, HBcAg linker W453, HBcAg linker R482, HBcAg linker W482 and HBcAg linker (without inserted peptide), because high amounts of recombinant protein were already expressed in an un-induced state, and because the majority of the recombinant HBcAg linker N195 protein was present in the soluble fraction of the bacterial lysates (figure 30). The expression of HBcAg linker W453 in Rosetta (+), Ni-NTA pull down and elution of HBcAg linker W453 under native conditions is shown as an example (figure 31).

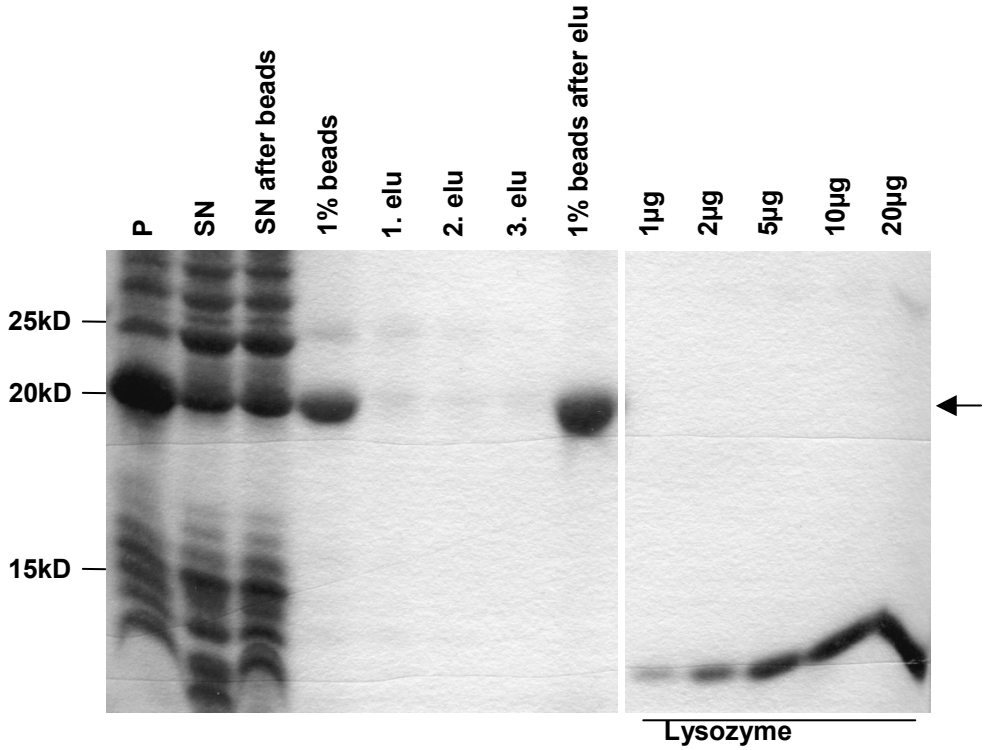


Figure 31: HBcAg linker W453 expression, affinity purification, and elution under native conditions. The amount of HBcAg linker W453 in the bacterial lysate, efficiency of affinity purification, and efficiency of native HBcAg linker W453 elution were checked on a 12.5% SDS-PAG. 0.2% of the pellet fraction (inclusion bodies) and of the supernatant fraction (soluble proteins) of the bacterial lysate before and after elution, 1% of Ni-NTA beads before and after elution, and 1% of all elutions were electrophoretically separated. The gel was stained with Coomassie. Defined amounts of lysozyme served as a protein standard. HBcAg linker W453 (calculated

molecular weight 20.7 kDa) is indicated with an arrow. P = pellet fraction (inclusion bodies); SN = soluble fraction (supernatant); elu = eluate.

Most of the HBcAg linker W453 protein, about 80%, was present in the pellet fraction (inclusion bodies), and the remaining 20% in the soluble fraction (supernatant). The native HBcAg linker W453 protein was purified from the supernatant, as seen by the presence of HBcAg linker W453 on 1% of the Ni-NTA beads (4^{th} lane from the left, figure 31). The native protein was eluted only in tiny amounts, as seen by detection of the protein in 1% of the eluates, from the Ni-NTA beads, using the Elution buffer (for Ni-NTA beads) containing 250mM imidazole. Most of the protein was still present on the Ni-NTA beads after the elutions (1% beads after elu, figure 31). The same problem was already observed with the elution of HBcAg Ig-fold R482W under native conditions (figure 27C). The reason for the failure of eluting native protein bound to Ni-NTA beads remained unknown.

HBcAg linker R453, HBcAg linker R482, HBcAg linker W482, and HBcAg linker without any inserted peptide were also expressed in the bacterial strain Rosetta (+). The His-tagged recombinant proteins were affinity purified from the soluble as well as from the insoluble fraction of the bacterial lysate. None of the mentioned recombinant proteins could be eluted from the Ni-NTA beads under native conditions (data not shown). Therefore we eluted the bound proteins under denaturing conditions. The efficiency of the Ni-NTA pull down of the various antigens present in the pellet fraction (inclusion bodies) as well as in the soluble fraction (supernatant) was checked on a 12.5% SDS-PAG and is shown in figure 32A.

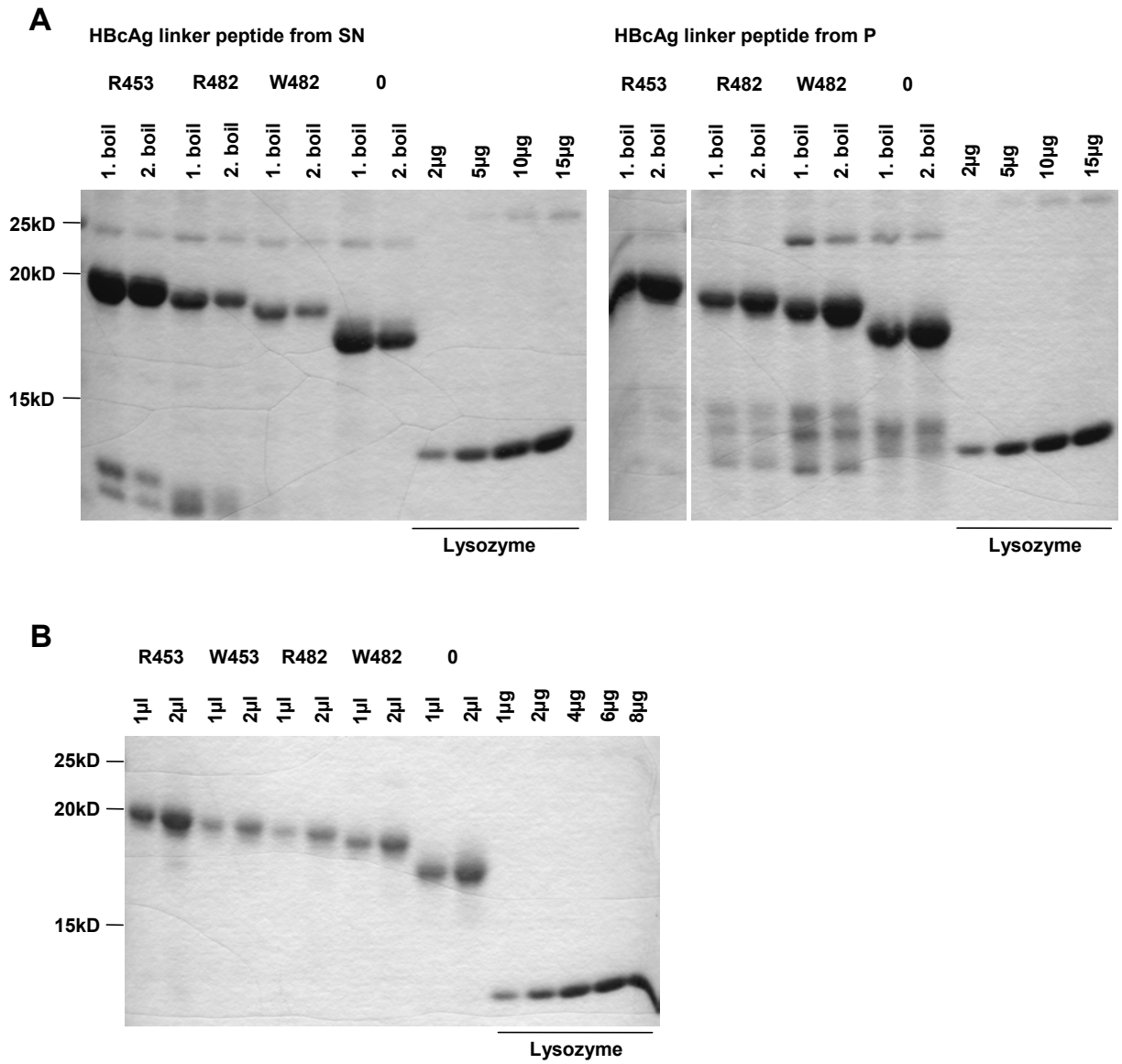


Figure 32: "HBcAg linker peptide" affinity purification and elution under denaturing conditions. A) Efficiency of Ni-NTA pull down of the various recombinant proteins from the soluble fraction (left panel) and from the insoluble pellet fraction (right panel) (1% of each affinity purified protein was loaded) and B) efficiency of electro-elution of denatured antigens was controlled on a 12.5% SDS-PAG (0.25% = 1µl and 0.5% = 2µl of the protein solution were loaded). All gels were stained with Coomassie. Defined amounts of lysozyme serve as a protein standard. 0 = HBcAg linker (without inserted peptide); SN = soluble fraction; P = insoluble pellet fraction.

All antigens were expressed in the bacterial strain Rosetta (+) (data not shown). The proteins were affinity purified from the soluble, as well as the insoluble pellet fraction of the bacterial lysate (figure 32A). The amount of affinity-purified protein differed between the proteins. 1%, the amount loaded on the gel, of affinity purified HBcAg linker R453 from the soluble fraction of the bacterial lysate contained about 100µg of protein, and 1% of affinity purified

protein from the insoluble pellet fraction of the bacterial lysate contained about 50µg of protein (left and right panel, figure 32A). In 1% of HBcAg linker R482 there were about 25µg (soluble fraction) and 40µg (insoluble fraction) protein; in 1% HBcAg linker W482 there were about 10µg (soluble fraction) and 50µg (insoluble fraction) protein; in 1% HBcAg linker there were about 45µg (soluble fraction) and 70µg (insoluble fraction) protein (left and right panel, figure 32A).

Each antigen was then loaded on a 12.5% SDS-PAG and electrophoretically separated for further purification by electro-elution. The electro-elution of denatured antigens was done according to the protocol described in section 2. 3. 3. The concentrations of the antigen solutions after the electro-elution were determined by loading 1µl and 2µl of the eluates on a 12.5% SDS-PAG with a defined amount of the reference protein lysozyme. The concentration of the antigens in the solution was in the range of 1µg/µl to 2µg/µl (figure 32B).

3. 5. 2. Immune response of "„HBcAg linker peptide"" immunized mice

Mice were immunized with the denatured antigens as the native recombinant proteins could not be eluted from the Ni-NTA beads. Following antigens were used for immunization: HBcAg linker R453, HBcAg linker R482 and HBcAg linker W482. HBcAg linker W453 was not used for immunizations, as a point-mutation specific antibody, anti-lamin A/C R453W 12A-2F5, was already generated at this time. HBcAg linker N195 and HBcAg linker K195 were also not used for immunizations, as the focus of my diploma work was oriented towards the point mutations R453W and R482W and their corresponding wild-type epitopes.

The pre-immune sera, and the sera of the 1. and 2. bleeds of mice immunized with HBcAg linker -R453 / -R482 / -W482 were tested for presence of antibodies specific for wild-type lamin A/C or Flag-lamin A R482W, respectively (figure 33A-C).

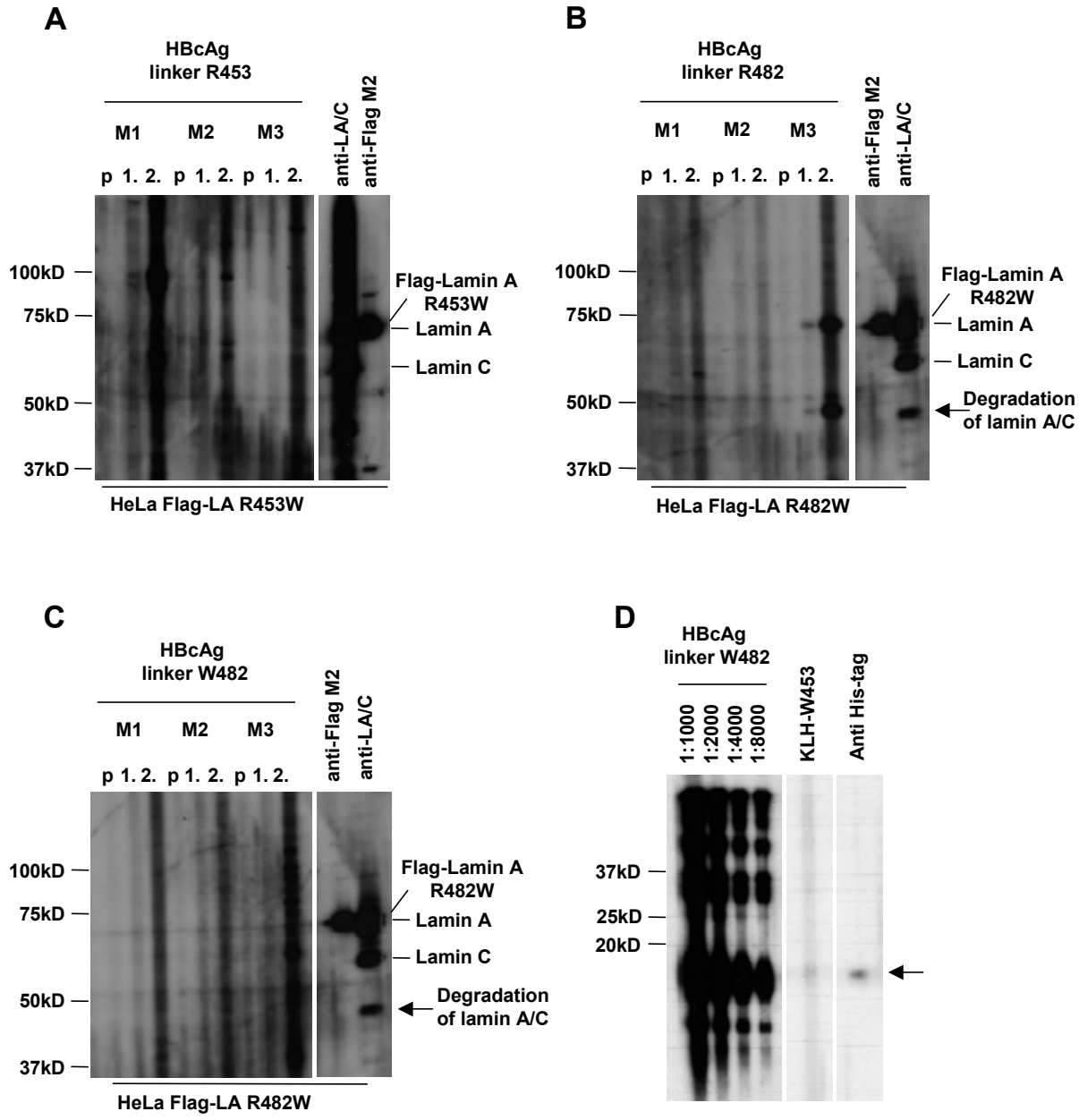


Figure 33. Test of sera of "HBcAg linker peptide" immunized mice. Sera of mice immunized with (A) HBcAg linker R453, (B) HBcAg linker R482, and (C) HBcAg linker W482 were tested by Western blot analysis on whole cell lysates of HeLa Flag-lamin A R453W cells (A) and HeLa Flag-lamin A R482W cells (B, C) (7.5% SDS-PAG). In (D), the serum of 2nd bleed of HBcAg linker W482 immunized mouse 1 was tested in a dilution series against purified HBcAg linker protein (15% SDS-PAG). One lane approximately contains 40ng of HBcAg linker protein. HBcAg linker is indicated with an arrow. In (A, B, C) all sera of bleeds were tested in a dilution of 1:1000. Anti-Flag M2 (1:40000), anti-lamin A/C 3A6-4C11 (1:1000), anti-His-tag (1:10000). p = pre-immune serum; 1. = serum of 1. bleed; 2. = serum of 2. bleed. KLH-W453 = serum of 2. bleed of mouse 3 immunized with KLH-W453 (1:1000). M1 = mouse1; M2 = mouse 2; M3 = mouse 3.

Incubation of the blots with anti-Flag M2 and anti-lamin A/C 3A6-4C11 confirmed the expression of ectopical Flag-lamin A R453W or Flag-lamin A R482W and endogenous wild-type lamin A/C in HeLa Flag-lamin A R453W or HeLa Flag-lamin A R482W cells, respectively (figure 33A, 33B, 33C). The anti-lamin A/C 3A6-4C11 recognized also a band at ~ 50 kDa (figure 33B and 33C), which corresponds to a degradation product of lamin A and lamin C (Goldberg et al., 1999; Orth et al., 1996). No antibodies were present in the sera of mice immunized with the HBcAg linker R453, HBcAg linker R482, or HBcAg linker W482 antigen, recognizing wild-type lamin A/C or mutant Flag-lamin A R482W in Western blot analysis (figure 33A, 33B, 33C).

I wanted to know, if an immune response was triggered in these mice at least against the carrier protein HBcAg linker. One possible explanation for the failure of this approach was the fact that the antigens were injected in a denatured form, where mainly, as assumed, epitopes of the carrier protein HBcAg elicited an immune response. This hypothesis, and whether an immune response was elicited in these mice at all, was checked by testing the serum of the 2nd bleed of HBcAg linker W482 immunized mouse 1 in a dilution series for the ability to recognize purified HBcAg linker protein (figure 33D). The 2. bleed of HBcAg linker W482 immunized mouse contained a high antibody titer for the HBcAg linker protein (figure 33D). The bands recognized at higher molecular weights most probably constituted of di- or multimers of the HBcAg linker protein. The serum of the 2. bleed of the KLH-W453 immunized mouse 3, the negative control, on the other hand did not recognize the N-terminally His-tagged HBcAg linker protein. This result indicated that most probably an immune response was elicited against epitopes of the HBcAg carrier protein, which are not accessible in the native protein (see also data from Kratz et al., 1999).

3. 6. Expression of HBcAg (C3d-P28)$_2$

My idea was to generate a recombinant protein, where the strong immunogen HBcAg was expressed together with C3d (please see section 2. 1. 13. 4. for details). In this way I wanted to "force" the immune system to elicit an immune response against less immunogenic epitopes, which would be inserted into the HBcAg protein in place of the c/e1 epitope. In a normal mouse, C3d has to bind independently to the antigen injected to mount a strong immune response (Abbas, 2003; Bower and Ross, 2006). In case of low affinity of complement proteins for the antigen, no or just a low immune response would be elicited. By

making this recombinant fusion protein this affinity step would be overcome and would elicit an immune response, independent of complement protein affinity to the antigen, and thus would increase the antigenicity of the inserted peptide, protein domain, or protein of the HBcAg $(C3d\text{-}P28)_2$ recombinant protein.

The expression of the recombinant protein HBcAg $(C3d\text{-}P28)_2$ was tested in two Rosetta (+) clones (Cl.#12 and Cl.#13).

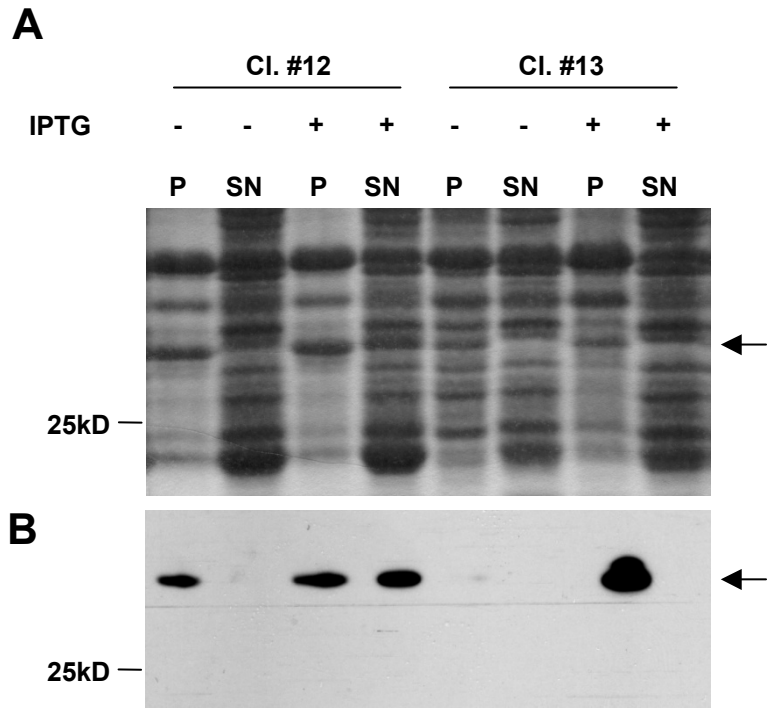


Figure 34. Expression of HBcAg (C3d-P28)2 in Rosetta (+). Expression of HBcAg $(C3d\text{-}P28)_2$ was checked on a 12.5% SDS-PAG. Aliquots equivalent to ~2% of the pellet and soluble fraction of the bacterial lysate (Cl.#12), and aliquots equivalent to ~0.75% of the pellet and soluble fraction of the bacterial lysate (Cl.#13) were loaded on the gel which was stained with Coomassie (A); and 0.33% (for Cl.#12) or 0.12% (for Cl.#13) of the pellet and soluble fractions of the bacterial lysates were loaded on a 12.5% SDS-PAG, electrophoretically separated, blotted to a nitrocellulose membrane and used for Western blotting (B) and incubated with anti-His-tag antibody (1:10000). HBcAg $(C3d\text{-}P28)_2$ (calculate molecular weight 28.4 kDa) is indicated with an arrow.

Without IPTG induction, HBcAg $(C3d\text{-}P28)_2$ was present only in small amounts, compared to other proteins of the bacteria, in the insoluble pellet fraction of Cl.#12. With induction by IPTG, the expression of HBcAg $(C3d\text{-}P28)_2$ doubled and was present in equal amounts in the insoluble and soluble fraction of the Cl. #12 bacterial lysate (figure 34B).

No expression of HBcAg $(C3d\text{-}P28)_2$ in Cl.#13 was detected without induction by IPTG, neither in the insoluble nor the soluble fraction. Expression of HBcAg $(C3d\text{-}P28)_2$ was

induced by IPTG in Cl.#13, but all of the protein was present in the insoluble pellet fraction (figure 34B).

The amount of HBcAg (C3d-P28)$_2$ in the supernatant of Cl.#12 was very low, as was the expression of the protein compared to other proteins in the bacterial lysate (figure 34A). The amount of recombinant protein available in the soluble, native protein lysate was estimated to be not sufficient to be purified by Ni-NTA beads and to obtain enough antigen material for immunization of mice. I did not continue with this approach as I tried to characterize the abilities of the anti-lamin A/C R453W 12A-2F5 antibody.

4. Discussion

The goal of my diploma thesis was to generate antibodies, recognizing specifically either wild-type or mutant lamin A/C proteins. I used three different types of antigen presentation to achieve this goal (KLH-coupled peptides, and HBcAg fusion proteins containing either the Ig-fold domain or short peptides, respectively). The immunization of mice with HBcAg Ig-fold WT led to the generation of antibodies in mice, recognizing lamin A/C in Western blot analysis. The monoclonal anti-lamin A/C 3A6-4C11 antibody, recognizing wild-type and mutant lamin A/C proteins, was generated from this approach. The immunization of mice with HBcAg Ig-fold R482W led to the generation of antibodies in mice, recognizing wild-type and mutant lamin A/C, and also antibodies recognizing specifically Flag-lamin A R482W in Western blot analysis. The immunization of mice with KLH-W453 led to the generation of the point-mutation specific anti-lamin A/C R453W 12A-2F5 antibody. Preliminary characterization experiments of the anti-lamin A/C R453W antibody were done during my diploma thesis. With the anti-lamin A/C R453W 12A-2F5 antibody, the disease-causing characteristics of the mutant lamin A/C R453W can now be revealed in primary cells from patients suffering from AD-EDMD.

Generation of a point-mutation specific antibody recognizing the disease-causing point mutation R453W in the proteins lamin A and lamin C

- **Specificity of 12A-2F5 monoclonal antibody**

The specificity of the point-mutation specific monoclonal antibody 12A-2F5, recognizing the point-mutation R453W in lamin A and lamin C was checked in immunoblotting, peptide-competition assay, immunoprecipitation, and immunofluorescence. In all of these assays the antibody was specific for the single amino acid substitution, and did not show any cross-reactivity with wild-type lamin A and lamin C.

In the peptide competition assay already a 1:1 molar ratio of the competing peptide W453, the mutant peptide, to the 12A-2F5 antibody blocked the recognition of Flag-lamin A R453W by the 12A-2F5 antibody in the Western blot (figure 20). This was somehow surprising and the cause could be slight inaccuracy of the determination of antibody concentration. However this inaccuracy should not be more than double of the actual amount. This assumption and the fact that the wild-type peptide R453 could not compete at all with the antibody 12A-2F5 once again highlighted the high specificity of the antibody for its epitope. A peptide competition

assay with a molar ratio of less than 1:1 of the competing peptide W453 to the 12A-2F5 antibody should have been done.

The high specificity of the antibody is evidence for the fact that the antibody can distinguish between an epitope sequences that differs only in the substitution of an arginine to tryptophan.

- **Immunoprecipitation of Flag-lamin A R453W by 12A-2F5 requires cell lysates made with Lysis buffer containing 0.5% SDS**

Immunoprecipitation of Flag-lamin A R453W by 12A-2F5 worked only in cell lysates made with a buffer that contained 0.5% SDS, but did not work in cell lysates made with a buffer containing 0.1% SDS (figure 21).

The result that 12A-2F5 could immunoprecipitate Flag-lamin A R453W only in the cell lysate made with the Lysis buffer can be explained by the higher degree of protein denaturation in this lysate (caused by 0.5% SDS), which is obviously required to make the epitope encompassing amino acid W453 accessible for the 12A-2F5 antibody. The SDS concentration of cell lysates made with the Lysis buffer was decreased to 0.25% for the immunoprecipitation. Obviously 0.1% SDS in a lysis buffer (modified Lysis buffer, and modified RIPA buffer) was not sufficient to denaturate the proteins to a degree, which would allow the binding of the point-mutation specific antibody 12A-2F5 to its epitope in the Flag-lamin A R453W protein in this immunoprecipitation experiment.

It seems that the immunoprecipitation performance correlated with the SDS concentration. Although the cell lysate made with the Lysis Buffer contained significantly higher amounts of soluble Flag-lamin A R453W and wild-type lamin A and lamin C than the cell lysates made with the modified IP-Lyse and modified RIPA buffer, the anti-lamin A/C 3A6-4C11 and anti-Flag 7F9 antibodies immunoprecipitated less Flag-lamin A R453W in the cell lysate made with Lysis Buffer than in the cell lysates made with the modified buffers (compare signals detected in the upper panel of figure 21). This could be due to an impaired interaction of the antibodies with their respective epitopes, which is caused by the presence of 0.25% SDS in the cell lysate made with Lysis buffer. The interaction of the antibodies to the same epitopes is stronger in the cell lysates containing 0.1% SDS (modified IP-Lyse and modified RIPA buffer).

Krimm and colleagues have shown that the mutation R453W in the Ig-fold domain causes a reduction of the denaturation temperature from 62°C, for the wild-type Ig-fold domain, to 43°C (Krimm et al., 2002). The low amounts of mutant lamin A and lamin C compared to the

amounts of wild-type lamin A and lamin C immunoprecipitated by 3A6-4C11 from G-8626 cells (figure 22), could be due to precipitation of the lamin A R453W and lamin C R453W in the lysate during the immunoprecipitation. Therefore the mutant proteins would not be available for the immunoprecipitation anymore, or just in a small amount. The result shown in figure 22 may also reveal that wild-type and mutant lamin A/C proteins do not form heteropolymers, as lamin A/C dimers should not have been destroyed by the lysis and immunoprecipitation experimental conditions (personal conversation, Roland Foisner).

- **Immunofluorescence of HeLa Flag-lamin A R453W cells**

There was a different staining pattern observed for Flag-lamin A R453W in HeLa Flag-lamin A R453W cells, depending on the antibodies used (anti-Flag M2 and anti-lamin A/C R453W 12A-2F5). The differences in the stainings of HeLa cells ectopically expressing Flag-lamin A R453W by the anti-Flag M2 and the point-mutation specific 12A-2F5 antibodies can be explained by different abilities of the antibodies to recognize the mutant protein depending on the partial denaturation by 0.1% SDS, and/or its subnuclear localization (figure 23). It could be that at a 0.1% SDS concentration the nucleoplasmic fraction of Flag-lamin A R453W is accessible for the 12A-2F5 antibody in the immunofluorescence experiments while the rim fraction of Flag-lamin A R453W is inaccessible, because the R453W epitope at this location is masked, and would require higher SDS concentrations (0.5% SDS, as in immunoprecipitation experiments) to get accessible for the antibody. The N-terminal Flag-tag is accessible for the anti-Flag M2 antibody regardless of the localization and SDS concentration, which was shown also in the immunoprecipitation experiments, were Flag-lamin A R453W was immunoprecipitated by anti-Flag M2 also from cell lysates containing no SDS (data not shown). However, the immunoprecipitation efficiency of anti-Flag antibodies decreased when the SDS concentration was increased (figure 21 and data not shown). Another possible explanation could be that the amino acid W453 is accessible for the 12A-2F5 antibody regardless of the location, whereas the Flag-tag could be hidden in the nucleoplasmic fraction of lamin A by inter- or intramolecular interactions for the anti-Flag M2 antibody, or because the affinity of the anti-Flag M2 antibody to its epitope decreased, when cells were treated with 0.1% SDS for immunofluorescence. To clarify these assumptions, it will be important to repeat the immunofluorescence experiments at different SDS concentrations.

- **Immunofluorescence of G-8626 cells revealed miss-localization of mutant lamin A and lamin C**

The mutant proteins lamin A R453W and lamin C R453W seemed to be miss-localized in primary cells of an AD-EDMD affected individual. The point-mutation specific antibody 12A-2F5 showed a dispersed staining of the mutant proteins throughout the nucleus in about one third of the nuclei (figure 24A, 2nd panel), with no obvious concentration of the mutant lamin A/C proteins at the nuclear rim, as seen with the Ig-fold specific 3A6-4C11 antibody (figure 24A, top panel). Two thirds of the nuclei stained with the anti-lamin A/C R453W 12A-2F5 antibody showed a similar staining pattern than the Ig-fold specific 3A6-4C11 antibody, with stronger rim staining and weak nucleoplasmic staining of lamins (figure 24A, 3rd panel).

One possible explanation for the weak nucleoplasmic staining of lamin A and lamin C in G-8626 by the general lamin A/C antibody 3A6-4C11 could be that the absolute amounts differ for the wild-type and the mutant protein. This suggestion would assume that there are less mutant than wild-type proteins present in the nucleus, as the mutant proteins could be more prone to degradation than the wild-type counterparts. The other possibility would be that wild-type and mutant proteins are present in the nuclei at equal levels, but that the wild-type proteins are mostly associated with the nuclear rim and the mutant proteins are mostly present in the nucleoplasm. In this case, however, the delocalisation of mutant lamin A/C should have been seen, although less pronounced, with the anti-lamin A/C 3A6-4C11 antibody as well.

The differences in the localization of the wild-type and mutant proteins observed by detection with the 3A6-4C11 or 12A-2F5 antibodies, in at least one third of the nuclei, could also be due to the already mentioned possibilities that recognition of mutant proteins associated with the nuclear rim by the anti-lamin A/C R453W 12A-2F5 antibody would require treatment of cells with 0.5% SDS, and/or that both antibodies could have preferences in detecting the proteins in a certain sub-nuclear localization. The differences in the stainings of at least one third of all nuclei made with the anti-lamin A/C R453W 12A-2F5 antibody compared to all stainings made with the anti-lamin A/C 3A6-4C11 antibody, could be also due to the fact that the cells were not synchronized for the immunofluorescence experiments. In this case the distribution of lamin A and lamin C in the nuclei would differ from cell to cell (Moir et al., 2000a; Moir et al., 2000b). However, a cell-cycle effect should have caused also a slight variation in the stainings made with the general lamin A/C 3A6-4C11 antibody.

Wiesel and colleagues have shown that ectopically expressed GFP-tagged Ce-lamin R460W, the *C. elegans* equivalent of the human lamin A/C R453W mutation, caused the formation of

Ce-lamin R460W aggregates in *C. elegans* nuclei, including cells of the intestine, hypodermis and embryo (Wiesel et al. 2008). Currently, experiments are underway to examine the sub-nuclear distribution of wild-type and mutant lamin A/C proteins in immortalized as well as primary human myoblasts with the help of the 12A-2F5 antibody.

HBcAg Ig-fold WT elicited a strong immune response, which led to the generation of the lamin A/C Ig-fold specific antibody 3A6-4C11

The immunization with HBcAg Ig-fold WT of mice elicited a strong immune response, producing antibodies recognizing lamin A/C in Western blot analysis. HBcAg Ig-fold WT recombinant proteins apparently can fold into the native conformation and thus allow oligomerization of the recombinant protein and the presentation of the main epitope, namely the Ig-fold WT, on the tip of the HBcAg protein spike. Both features seem to be essential for the elicitation of a strong humoral immune response. The ability of HBcAg monomers to form multimers causes the presentation of a high density of repetitive epitopes to B-lymphocytes, presented at the tip of the HBcAg particle spike. This leads to the clustering of many surface immunoglobulin receptors, recognizing the epitope, present on B-lymphocytes. This feature allows the activation of naive B-lymphocyte without the help of T-lymphocytes (Milich et al., 1997; Milich and McLachlan, 1986).

As a result of immunization of mice with HBcAg Ig-fold WT, the monoclonal antibody 3A6-4C11, specific for the Ig-fold of lamin A and lamin C, was generated. The anti-lamin A/C 3A6-4C11 antibody showed excellent performance in all techniques tested, including immunoblotting, immunoprecipitation and immunofluorescence. The recognized epitope, however has not been determined. The antibody cross-reacted most probably with lamin B1 and lamin B2 (figure 12B), although the degree of cross-reactivity could not be determined by my experiments. The detection of purified lamin B1 and lamin B2 by 3A6-4C11 could confirm the ability of the antibody to detect lamin B1 and lamin B2. Another pan-lamin antibody, recognizing an epitope identical in all human lamins, would allow to determine the relative amounts of the lamins in cells and would reveal, if 3A6-4C11 recognizes an epitope identical in all lamin Ig-folds, or if it just cross-reacts with a slightly different epitope in the Ig-folds of lamin B1 and lamin B2.

A point mutation specific immune response was elicited in mice immunized with HBcAg Ig-fold R482W

The immunization with native HBcAg Ig-fold R482W, as immunization with native HBcAg Ig-fold WT, of mice elicited a strong immune response, producing antibodies recognizing lamin A/C in Western blot analysis. The R482W mutation in the Ig-fold is not predicted to cause an aberrant structure of the Ig-fold and is therefore not inhibiting the proper folding of the HBcAg protein as well as the Ig-fold R482W domain. Thus it most probably did not interfere with the presentation of the Ig-fold R482W at the tip of the HBcAg protein spike, and the oligomerization of the recombinant protein.

The results of the fusion of mouse 1 immunized with HBcAg Ig-fold R482W confirmed that it is possible to elicit a point-mutation specific immune response in mice by immunization with a protein domain, namely the Ig-fold domain containing the mutation R482W (figure 28B). Further, this result showed that the epitope surrounding the amino acid W482 must be immunogenic, but was only weakly immunogenic in the KLH-coupled peptide approach (see data from Veronika Huber). Of course there was also an immune response elicited by the antigen HBcAg Ig-fold R482W against other epitopes, which are shared by wild-type and mutant lamin A/C Ig-folds (figure 28B). Unfortunately, the hybridoma clones were not stable, and no monoclonal antibodies recognizing lamin A/C R482W were obtained during my time as a diploma student in the lab.

Meanwhile we were successful in generating a stable hybridoma clone producing a lamin A/C R482W point-mutation specific antibody (data not shown).

The antigen HBcAg Ig-fold R453W was not immunogenic

Immunization of mice with the antigen HBcAg Ig-fold R453W showed that the HBcAg recombinant protein approach for eliciting an immune response in mice does not work for all antigens (figure 26). Therefore different approaches might be required to elicit an immune response against a given antigen. One possible explanation for the failure of HBcAg Ig-fold R453W to elicit an immune response is the low thermostability of the mutant Ig-fold domain. The aberrant structure of the Ig-fold R453W could have caused a disruption of the native HBcAg structure. Consequently, the inserted Ig-fold R453W was not presented at the tip of the HBcAg protein spike. In addition, the aberrant structure of the Ig-fold R453W could also have inhibited the formation of HBcAg Ig-fold R453W multimers, which is crucial for the high immunogenicity of HBcAg (Milich et al., 1997; Milich and McLachlan, 1986). The partially denaturated HBcAg Ig-fold R453W antigen would elicit a humoral immune response

against epitopes present on the HBcAg carrier protein, which are not accessible in the native protein (see also data from Kratz et al., 1999). Nevertheless, these are only speculations, which cannot explain why the mice did not generate anti-Ig-fold antibodies, as the inserted Ig-fold contained 116 amino acids.

The antigens KLH-N195, KLH-K195, KLH-R453; HBcAg linker R453, HBcAg linker R482, and HBcAg linker W482 were not immunogenic

The immunization of mice with the KLH-N195, KLH-K195, and KLH-R453 antigens did not elicit the production of antibodies recognizing wild-type lamin A or Flag-lamin A N195K in the Western blot analysis (figure 5, 6 and 14A). However, I could detect antibodies specific for the respective peptides in an ELISA experiment (data not shown), indicating that the generated antibodies were recognizing a peptide unique epitope structure. The structure of a peptide used for immunization, either coupled to KLH or administered as a fusion protein, should mimic the structure of the peptide in the native protein. This, however, cannot be predicted, and if the peptide used for immunizations does not mimic the structure of the peptide in the native protein, the immune system of the mouse cannot produce antibodies recognizing the respective peptide in the protein context.

As the KLH-peptide approach was not successful for the peptides containing the amino acid N195 (wild-type), K195 (mutant), R453 (wild-type), as well as the peptides containing R482 (wild-type) and W482 (mutant) (data from Veronika Huber), I generated HBcAg recombinant proteins containing amino acids 191 - 202, amino acids 448 - 457, or amino acids 478 – 487 of lamin A and lamin C either in the wild-type or in the mutated version (for further details please see section 2. 1. 13. 2.). The aim of this approach was to increase the antigenicity towards the inserted lamin A/C sequence. HBcAg linker R453, HBcAg linker R482 and HBcAg linker W482 were used for immunizations of mice. None of these antigens elicited the generation of antibodies in mice, recognizing wild-type lamin A/C or mutant Flag-lamin A R482W in Western blot analysis (figure 33A, 33B, 33C). One possible explanation for the failure of this approach was the fact that the antigens were injected in a denatured form, where mainly epitopes of the carrier protein HBcAg elicited an immune response (see also data from Kratz et al., 1999). Indeed I detected a strong immune response towards the HBcAg linker by testing the serum of the 2. bleed of HBcAg linker W482 immunized mouse 1 by Western blot analysis against purified HBcAg linker protein (figure 33D). In the native conformation the inserted peptide would be presented at the tip of the HBcAg particle spike, where it would act in place of the immunodominant c/e1 epitope. In the denatured antigen,

the inserted peptide looses its potential to elicit a specific immune response, as it is not presented at the tip of the HBcAg protein spike, which would promote its antigenicity. In the denatured form of the HBcAg carrier protein, epitopes that are not accessible in the native conformation are targeted by the humoral immune system (see also data from Kratz et al., 1999).

Alternative approaches of antigen presentation would include the use of the multiple antigen peptide (MAP) as a carrier for the peptides, recombinant C3d-proteins (Abbas, 2003; Bower and Ross, 2006), the use of the tetanus toxoid protein as a carrier protein for the peptides (Sundaram et al., 2004), or the already mentioned insertion of short peptides into the HBcAg carrier protein, but injected in native conformation to maintain the multimeric structure. The tetanus toxoid protein was successfully used in inducing the generation of specific antibodies against alpha helical coiled-coil structures (Sundaram et al., 2004). The amino acid 195 of lamin A/C is known to be located in the N-terminal rod domain within an alpha-helix important for the formation of coiled-coils. Therefore the tetanus toxoid carrier protein would be a good choice to proceed in the attempt to elicit an immune response against the wild-type and the mutant epitope containing amino acid 195.

Future work

The generation of the point mutation specific monoclonal antibody 12A-2F5, recognizing the point mutation R453W in the proteins lamin A and lamin C presents a new tool for studying AD-EDMD caused by the mutation R453W in lamin A and lamin C. The results could also be extrapolated on other point-mutations in the Ig-fold of lamin A and lamin C causing AD-EDMD, as the mechanisms involved in the generation of a pathological phenotype could overlap. All AD-EDMD causing point-mutations present in the lamin A/C Ig-fold are predicted to destabilize the structure of the Ig-fold domain (Krimm et al., 2002). However, point mutations responsible for AD-EDMD outside of the Ig-fold could have a different pathological mechanism.

Now, with the help of the 12A-2F5 antibody, molecular mechanisms underlying the pathological phenotype of the point mutation R453W in lamin A and lamin C can be elucidated. It is true that the impact of the point mutation R453W in lamin A and lamin C was investigated already. But these experiments all relied on ectopically overexpressed tagged recombinant lamin A or lamin C proteins containing the point mutation R453W, and were done in either nematode cells (using the Ce-lamin R460W, the *C. elegans* equivalent of

human lamin A/C R453W) (Wiesel et al., 2008), or a rodent cell line (Broers et al., 2005). These experiments showed aggregate formation of the mutant proteins (Wiesel et al., 2008), and an increased mobility of mutant lamin A and lamin C containing the point-mutation R453W, indicating loss of structural stability of lamin polymers (Broers et al., 2005) in the nuclei of the studied cells. In both publications GFP-tagged lamin A was used. The GFP-tag could had an effect on the behaviour of the tagged protein and in this way would influence the results. Besides this, the overexpression of the tagged mutant proteins could had caused artefacts, because the expression level of the proteins in the cells studied is different to the expression level of the proteins in patient cells. On the other hand, Wiesel and colleagues investigated the behaviour of ectopically expressed GFP-tagged Ce-lamin with the mutation R460W, the *C. elegans* equivalent of the human mutation R453W in lamin A and lamin C, and found that Ce-lamin R460W formed many small aggregates (Wiesel et al., 2008). *C. elegans* has only one lamin gene coding for one lamin protein, thus simplifying the whole system. The proper localization of mutant lamin A/C in human nuclei could be supported by other factors, such as the presence of a variety of proteins, for example B-type lamins, which are missing in *C. elegans* nuclei. The localization phenotype in human nuclei would be expected to be suppressed to a certain degree by these other factors and therefore would not be as dramatic as in *C. elegans* cells. Also the rodent cell line used by Broers and colleagues could influence the results described due its immortal phenotype (Broers et al., 2005).

The experiments done to examine the molecular behaviour of the mutant lamin A and lamin C had to be done with recombinant tagged proteins in cell lines till now. But through the generation of the point mutation specific antibody recognizing the point mutation R453W in lamin A and lamin C all possible artefacts caused by either the cell lines or the recombinant proteins used can be eliminated as now primary cells from patients suffering from AD-EDMD can be investigated. The molecular mechanisms of the mutant lamin A and lamin C vs. the wild-type protein can now be investigated in primary cells of patients.

This will include co-immunofluorescence of mutant and wild-type lamin A and lamin C proteins of primary myoblasts from patients suffering from AD-EDMD, as these are the cells were a phenotype could be detected most probably, as they are the cells being involved in the disease phenotype.

In addition, it will be important to determine the actual levels of the wild-type vs. mutant lamin A and lamin C in cells derived from patients, as their levels are so far unknown. Krimm and colleagues showed that the mutation R453W in the Ig-fold of lamin A/C causes a

decreased thermo-stability of the mutant protein (Krimm et al., 2002). The protein could likely form aggregates in the nuclei or even be prone to proteolytic degradation.

Lamin A and lamin C are highly phosphorylated proteins. The breakdown and the re-assembly of the nuclear lamina during mitosis are regulated by reversible phosphorylation of lamins. Therefore another area to study would be the phosphorylation pattern of mutant vs. wild-type lamin A and lamin C in two-dimensional (2-D) SDS-PAG. Cenni and colleagues have shown that the phosphorylation of lamin A and lamin C in muscle biopsies form AD-EDMD patients is severely reduced compared to healthy tissues (Cenni et al., 2005), and that the inhibition of lamin A/C phosphorylation by an AD-EDMD causing point-mutation seems to be responsible for the AD-EDMD phenotype (Cenni et al., 2008). Thus, 2-D SDS-PAG could be used to specifically detect the phosphorylation pattern of mutant and wild-type proteins. Further, the affected phosphorylation sites could be determined and their biological role could reveal a better understanding of the pathological molecular mechanism responsible for AD-EDMD.

In summary, we were successful in the development of a point-mutation specific antibody recognizing the AD-EDMD disease-causing mutant lamin A/C R453W, which will help to reveal the pathological mechanisms caused by the mutant lamin A/C R453W proteins in AD-EDMD. The antibody showed high specificity for the epitope containing the mutant amino acid tryptophane, with no cross-reactivity to the wild-type epitope containing the amino acid arginine. Arginine, a positively charged amino acid at physiological conditions, is the amino acid with the highest mutation rate (Cooper and Youssoufian, 1988), and is responsible for 15% of all disease associated missense mutations (Ferrer-Costa et al., 2002; Vitkup et al., 2003; Wang and Moult, 2001). A mutation of arginine to tryptophan causes a replacement with an amino acid with significantly different physico-chemical properties, as tryptophan is a bulky non-polar amino acid, increasing the probability of disturbing the 3-D structure and the function of a protein and thus causing a disease. By results presented we show that antibodies can be developed, which are able to discriminate between a particular epitope containing either arginine or tryptophane. As mentioned, mutations in arginine and tryptophan (Vitkup et al., 2003) have a high potential in causing a disease, and by the development of such point-mutation specific antibodies, recognizing specifically either the wild-type or the mutant epitope, many more common diseases could be studied.

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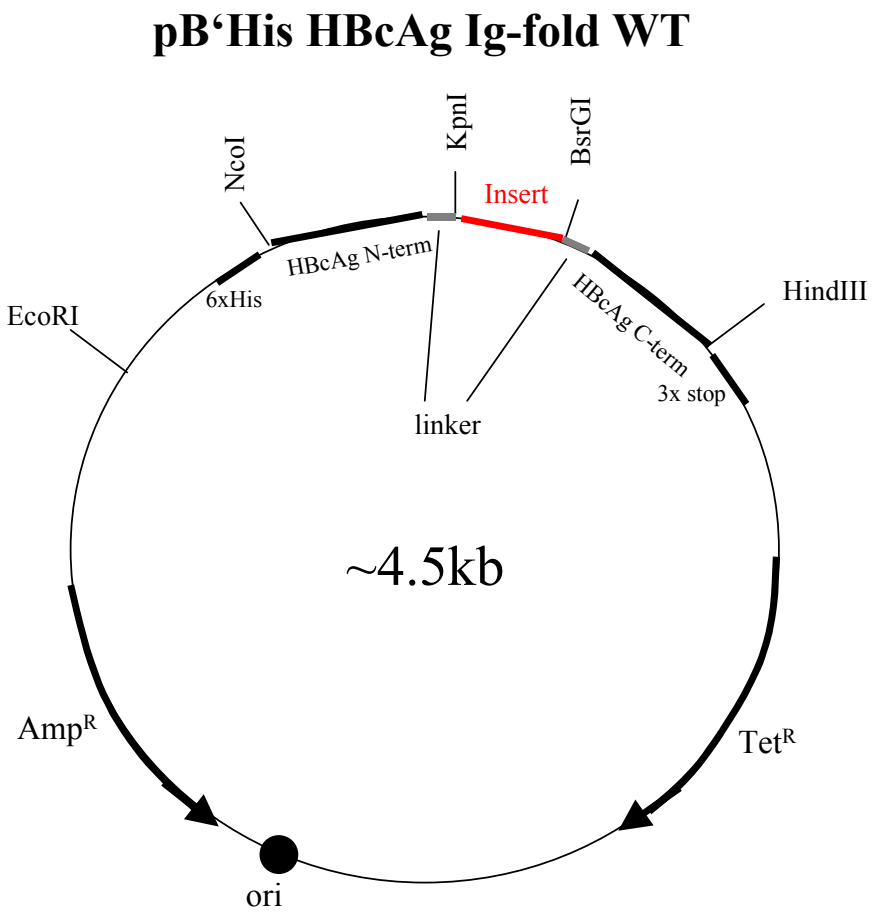
6. Appendix



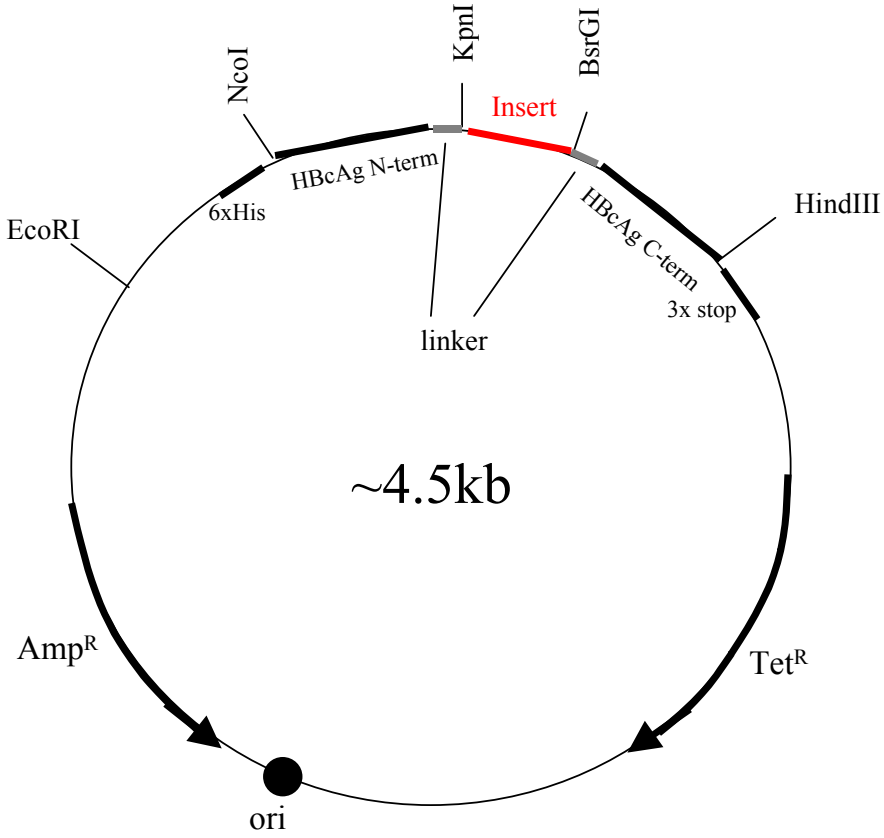
Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with KpnI and BsrGI.

Insert: PCR with #593 & #594 from pSVK3-LaminA (WT). Cut PCR product with KpnI and BsrGI

KpnI
5‘-CTTCTCACAGCACGCACGCACTAGCGGGCGCGTGGCCGTGGAGGAGGTGGATGAGGAGGGC
AAGTTTGTCCGGCTGCGCAACAAGTCCAATGAGGACCAGTCCATGGGCAATTGGCAGATCAAG
CGCCAGAATGGAGATGATCCCTTGCTGACTTACCGGTTCCCACCAAAGTTCACCCTGAAGGCTG
GGCAGGTGGTGACGATCTGGGCTGCAGGAGCTGGGGCCACCCACAGCCCCCCTACCGACCTGG
TGTGGAAGGCACAGAACACCTGGGGCTGCGGGAACAGCCTGCGTACGGCTCTCATCAACTCCA
CTGGGGAAGAAGTGGCCATGCGCAAGCTGGTGCGCAT-3‘
BsrGI

Done by: Veronika Huber

pB‘His HBcAg Ig-fold R453W



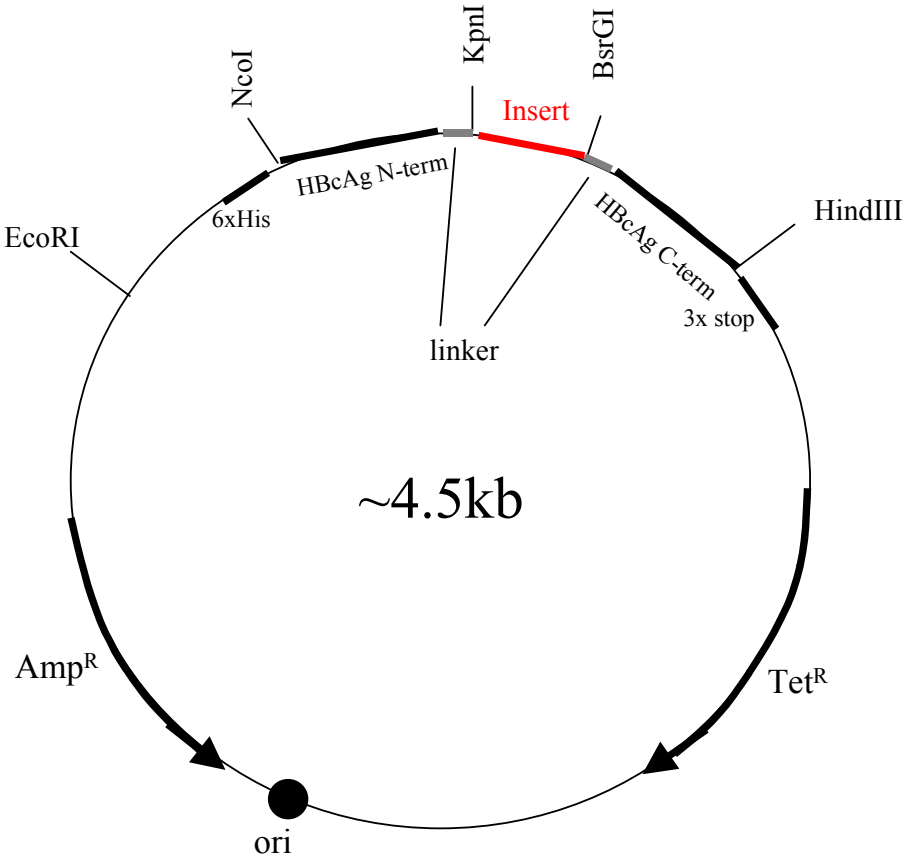
Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with KpnI and BsrGI.

Insert: PCR with #593 & #594 from pSVK3-LaminA R453W. Cut PCR product with KpnI and BsrGI.

KpnI
5‘-CTTCTCACAGCACGCACGCACTAGCGGGCGCGTGGCCGTGGAGGAGGTGGATGAGGAGGGC
AAGTTTGTCTGGCTGCGCAACAAGTCCAATGAGGACCAGTCCATGGGCAATTGGCAGATCAAG
CGCCAGAATGGAGATGATCCCTTGCTGACTTACCGGTTCCCACCAAAGTTCACCCTGAAGGCTG
GGCAGGTGGTGACGATCTGGGCTGCAGGAGCTGGGGCCACCCACAGCCCCCCTACCGACCTGG
TGTGGAAGGCACAGAACACCTGGGGCTGCGGGAACAGCCTGCGTACGGCTCTCATCAACTCCA
CTGGGGAAGAAGTGGCCATGCGCAAGCTGGTGCGCAT-3‘
BsrGI

Done by: Veronika Huber

pB‘His HBcAg Ig-fold R482W



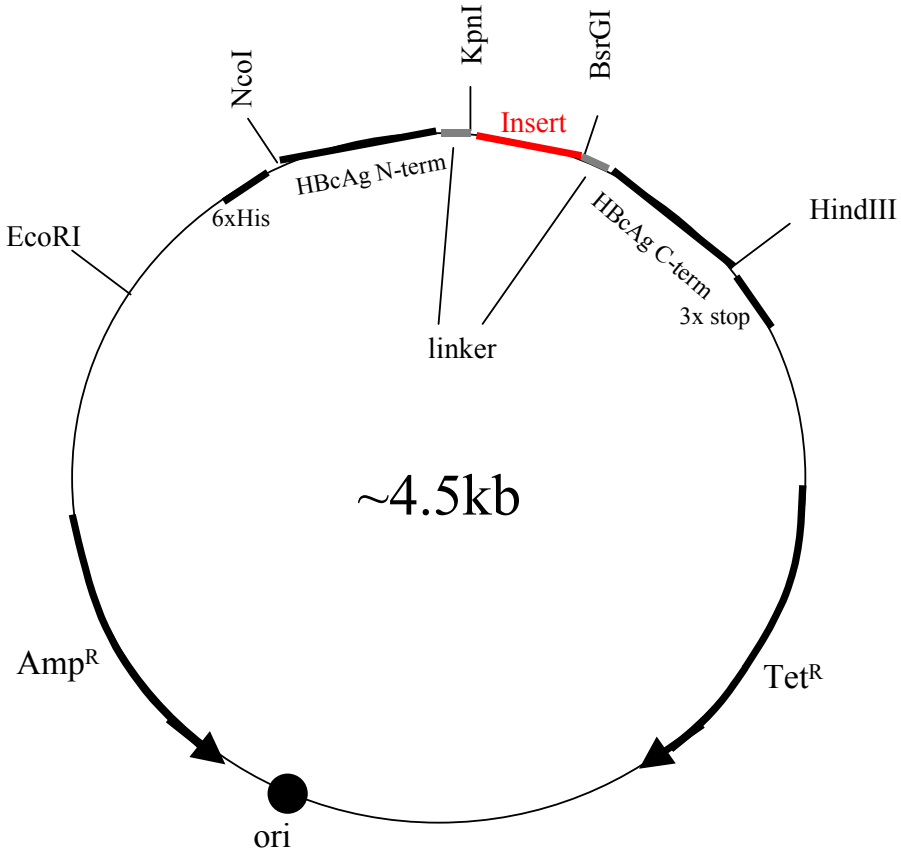
Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with KpnI and BsrGI.

Insert: PCR with #593 & #594 from pSVK3-LaminA R482W. Cut PCR product with KpnI and BsrGI.

KpnI
5‘-CTTCTCACAGCACGCACGCACTAGCGGGCGCGTGGCCGTGGAGGAGGTGGATGAGGAGGGC
AAGTTTGTCCGGCTGCGCAACAAGTCCAATGAGGACCAGTCCATGGGCAATTGGCAGATCAAG
CGCCAGAATGGAGATGATCCCTTGCTGACTTACTGGTTCCCACCAAAGTTCACCCTGAAGGCTG
GGCAGGTGGTGACGATCTGGGCTGCAGGAGCTGGGGCCACCCACAGCCCCCCTACCGACCTGG
TGTGGAAGGCACAGAACACCTGGGGCTGCGGGAACAGCCTGCGTACGGCTCTCATCAACTCCA
CTGGGGAAGAAGTGGCCATGCGCAAGCTGGTGCGCAT-3‘
BsrGI

Done by: Veronika Huber

pB‘His HBcAg linker N195



Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with KpnI and BsrGI.
Gel-elu, 20.11.07, MR

Insert: Lamin A – N195 (oligos #735 & #736)

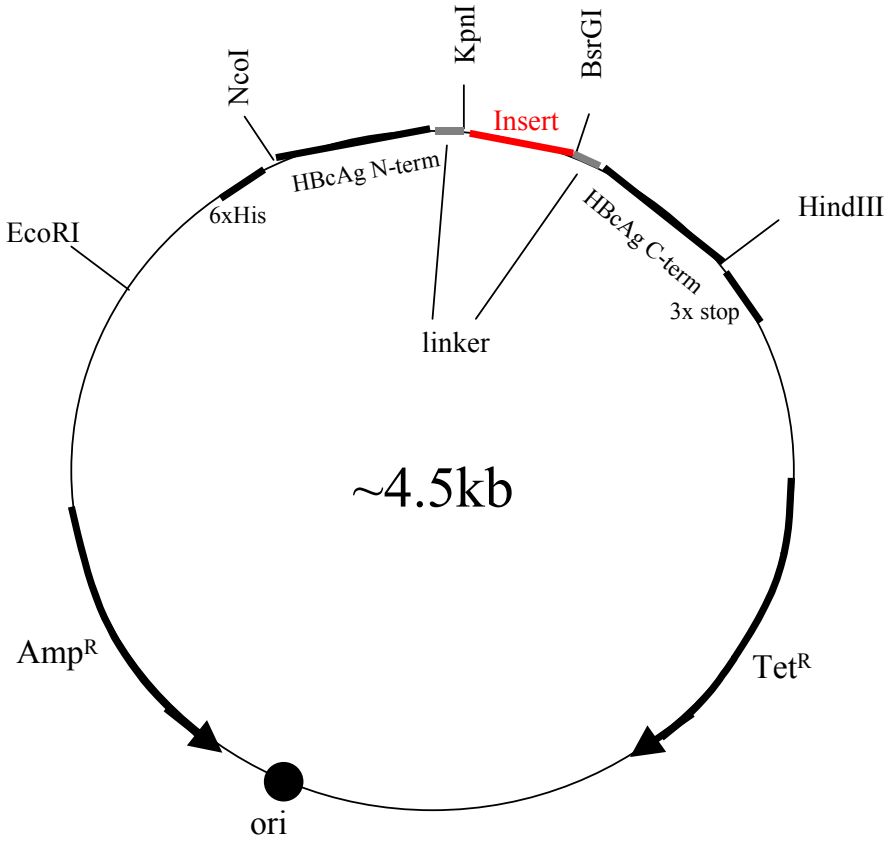
N195

5’-C GTG GAT GCT GAG AAC AGG CTG CAG ACC ATG AAG GAG AT -3’
3’-CA TGG TCT CTA CGA CTC TTG TCC GAC GTC TGG TAC TTC CTC TAC ATG-5’
KpnI BsrGI

Control digest: cl.#1 – cl.#4 with SphI and KpnI

Sequenced clone and bacterial stock: cl.#1

pB‘His HBcAg linker K195



Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with KpnI and BsrGI.
Gel-elu, 20.11.07, MR

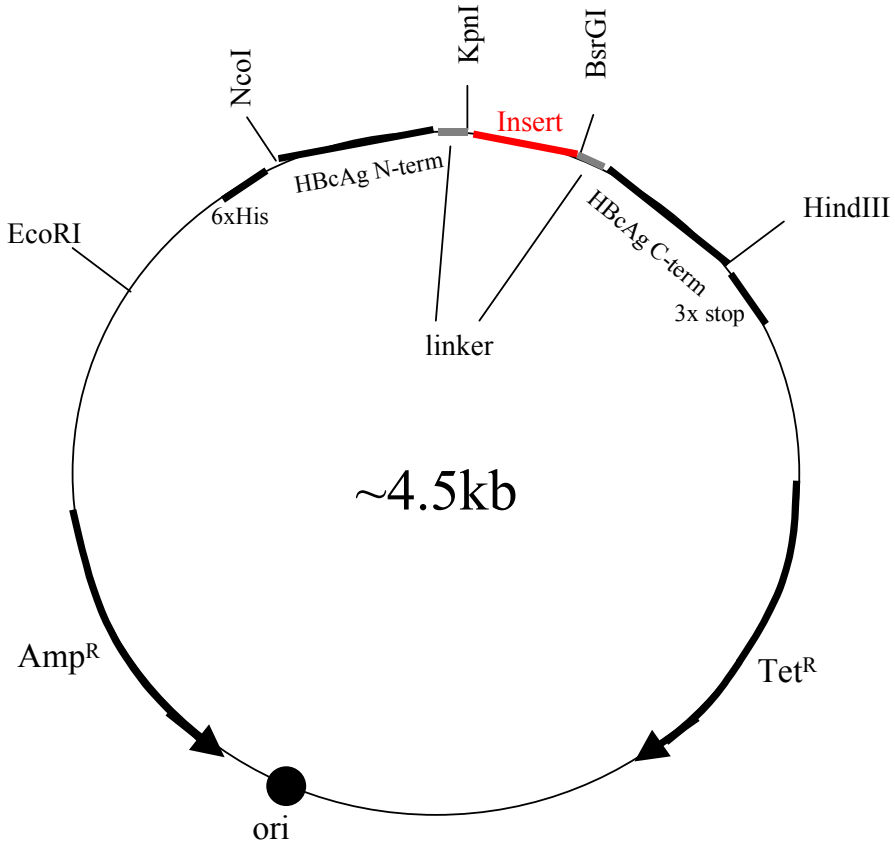
Insert: Lamin A – K195 (oligos #737 & #738)

K195
5’-C GTG GAT GCT GAG AAA AGG CTG CAG ACC ATG AAG GAG AT -3’
3’-CA TGG TCT CTA CGA CTC TTT TCC GAC GTC TGG TAC TTC CTC TAC ATG-5’
KpnI BsrGI

Control digest: cl.#1 – cl.#4 with SphI and KpnI

Bacterial stock: cl.#1

pB‘His HBcAg linker R453



Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with KpnI and BsrGI.
Gel-elu, 20.11.07, MR

Insert: Lamin A – R453 (oligos #739 & #740)

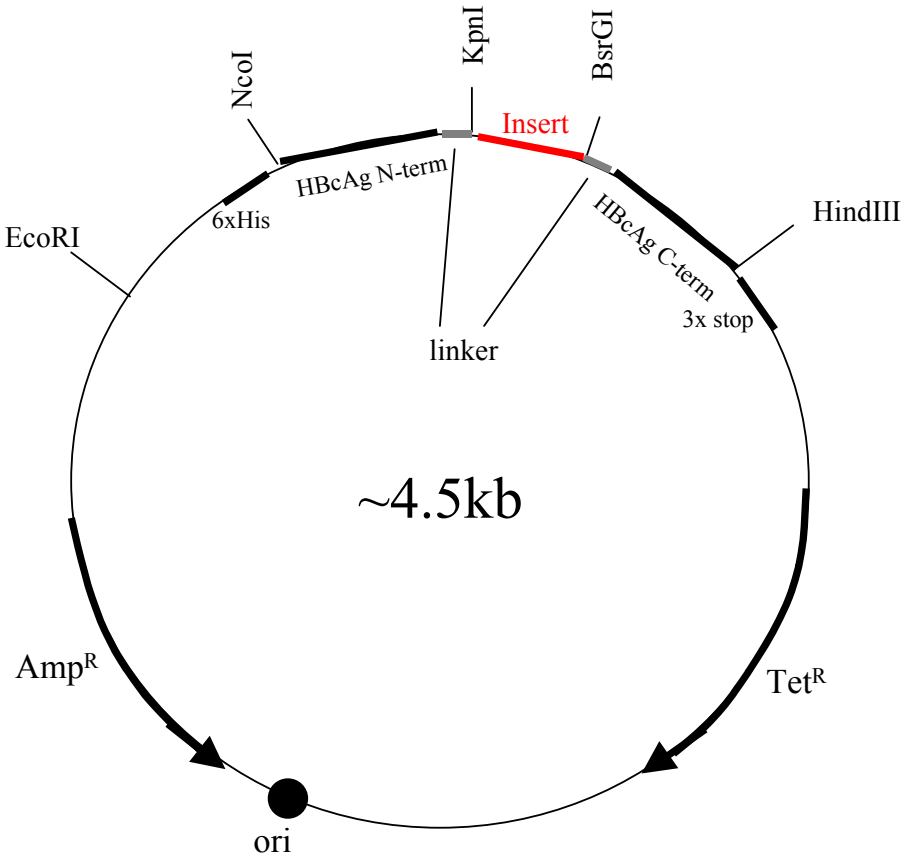
R453

5’-C GAG GGC AAG TTT GTC CGG CTG CGC AAC AAG AT-3’
3’-CA TGG CTC CCG TTC AAA CAG GCC GAC GCG TTG TTC TAC ATG-5’
KpnI BsrGI

Control digest: cl.#1 – cl.#4 with SphI and KpnI

Bacterial stock: cl.#1

pB‘His HBcAg linker W453



Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with KpnI and BsrGI.
Gel-elu, 20.11.07, MR

Insert: Lamin A – W453 (oligos #741& #742)

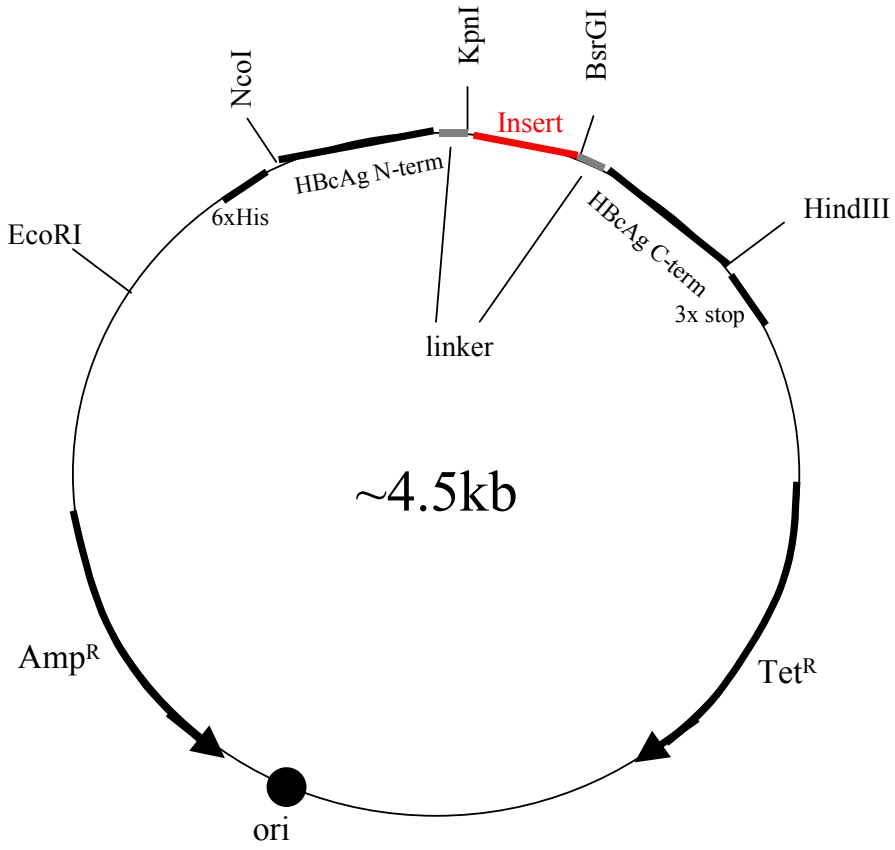
W453

5’-C GAG GGC AAG TTT GTC TGG CTG CGC AAC AAG AT-3’
3’-CA TGG CTC CCG TTC AAA CAG ACC GAC GCG TTG TTC TAC ATG-5’
KpnI BsrGI

Control digest: cl.#1 – cl.#4 with SphI and KpnI

Bacterial stock: cl.#1

pB‘His HBcAg linker R482



Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with KpnI and BsrGI.
Gel-elu, 20.11.07, MR

Insert: Lamin A – R482 (oligos #743& #744)

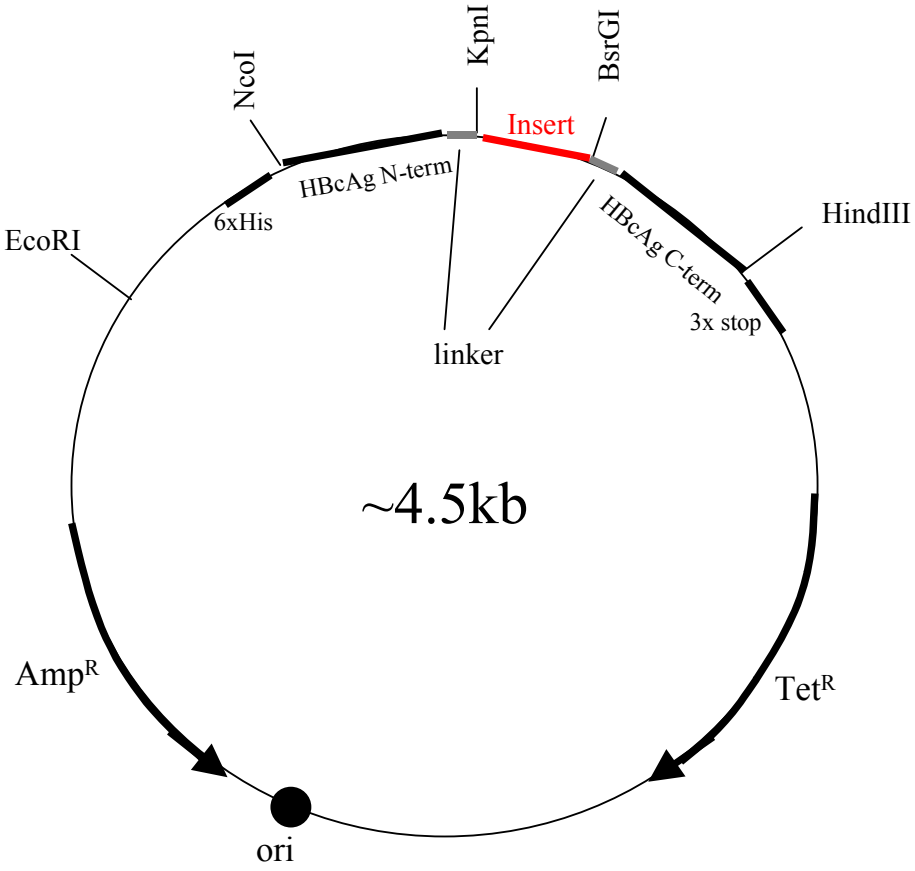
R482

5’-C TTG CTG ACT TAC CGG TTC CCA CCA AAG TTC AT-3’
3’-CA TGG AAC GAC TGA ATG GCC AAG GGT GGT TTC AAG TAC ATG-5’
KpnI BsrGI

Control digest: cl.#1 – cl.#4 with SphI and KpnI

Bacterial stock: cl.#1

pB‘His HBcAg linker W482



Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with KpnI and BsrGI.
Gel-elu, 20.11.07, MR

Insert: Lamin A – R482 (oligos #745& #746)

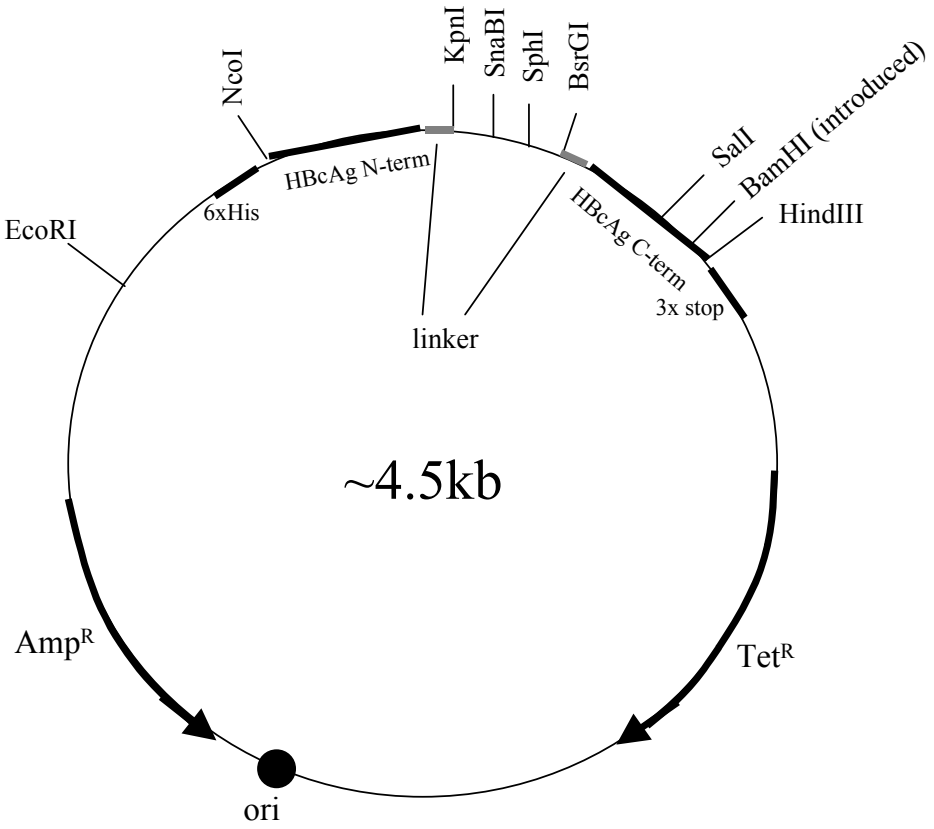
W482

5’-C TTG CTG ACT TAC TGG TTC CCA CCA AAG TTC AT-3’
3’-CA TGG AAC GAC TGA ATG ACC AAG GGT GGT TTC AAG TAC ATG-5’
KpnI BsrGI

Control digest: cl.#1 – cl.#4 with SphI and KpnI

Bacterial stock: cl.#1

pB‘His HBcAg linker w/o stop



Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with SalI and HindIII.
Gel-elu, 19.03.08, MR

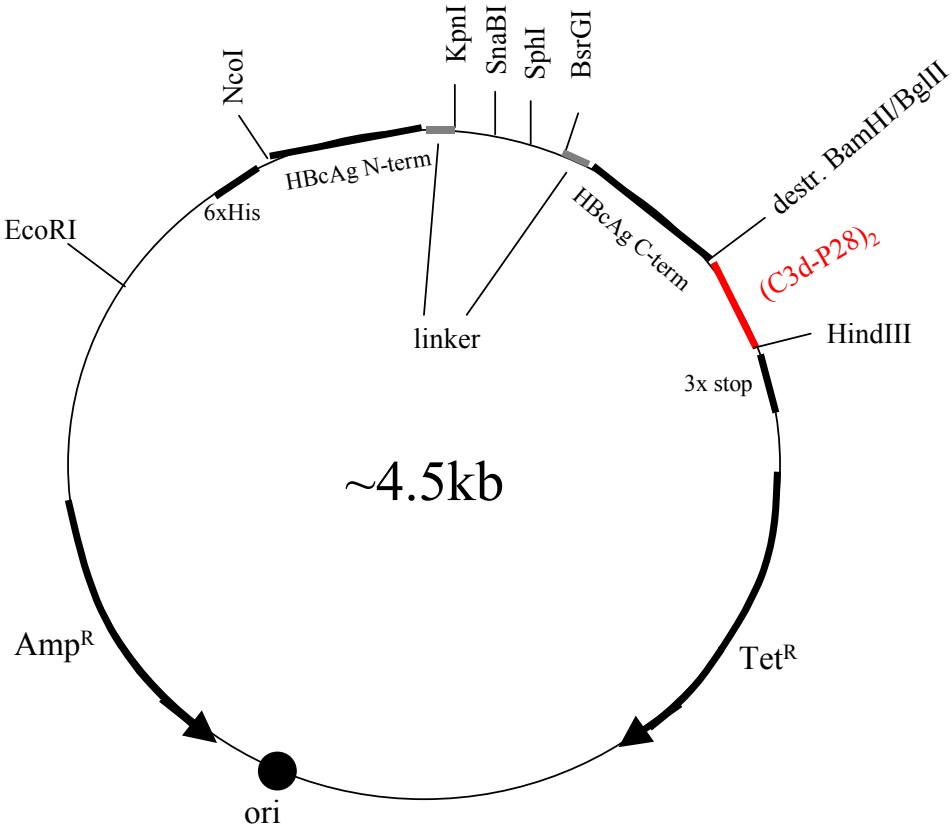
Insert:

5’ – TCGACACTTCCGGAGACTACTGTTGTTGGATCCAGCA – 3’
3‘- GTGAAGGCCTCTGATGACAACAACCTAGGTCGTTCGA-5‘
SalI BamHI HindIII

Control digest: cl.#1 – cl.#5 with BamHI

Bacterial stock: cl.#1

pB‘His HBcAg $(C3d\text{-}P28)_2$



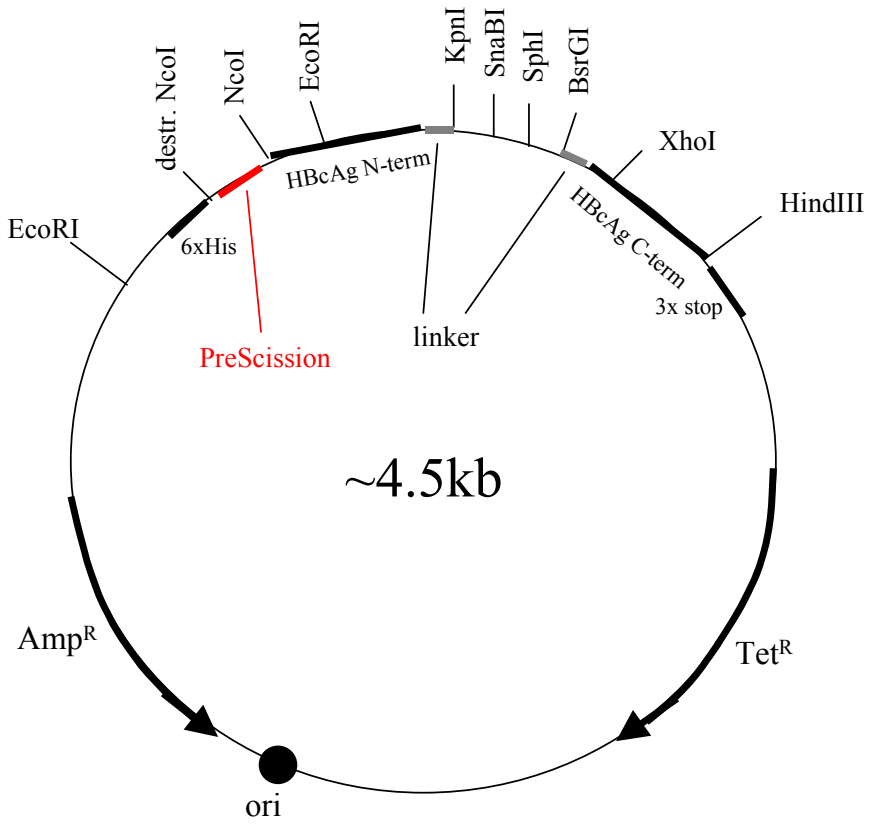
Strategy: cut pB‘ His HBcAg linker w/o stop cl.#1 with BamHI and HindIII. Gel-elu, 1.4.08, MR

Insert: PCR fragment from pDC1474 using primers #781 & #782. Digest PCR fragment of ~ 300bp length with BglII / HindIII (Gel-elu, 1.4.08, MR). Inserted fragment is 255 nt long.

destroyed BamHI/BglII
5’-GATCTGGTGGAGGTGGAAGCAGTGGTGGAGGTGGAAGCAGTAAGTTTCTGAACACAGCCA
AAGATCGGAACCGCTGGGAGGAGCCTGACCAGCAGCTCTACAACGTAGAGGCCACATCCTAC
GCCGGATCTGGTGGANGTGGAAGTAGTGGTGGAGGTGGAAGCAGTAAGTTTCTGAACACAGC
CAAAGATCGGAACCGCTGGGAGGAGCCTGACCAGCAGCTCTACAACGTAGAGGCCACATCCT
ACGCCTAGA – 3’
stop HindIII

Control digest: cl.#11 – cl.#14 with KpnI / HindIII, and BamHI
Sequenced clones: cl.#11 and cl.#14 **Bacterial stock:** cl.#11

pB‘His PreScission HBcAg linker



Strategy: cut pB‘ His HBcAg linker corrected cl.#1 with NcoI.
Treat with CIP. Gel-elu, 26.6.08, MR

Insert: PreScission (oligo #794, #795)
5’ – CATGCTGGAAGTTCTGTTCCAGGGGCCCTC – 3’
3’ - GACCTTCAAGACAAGGTCCCCGGGAGGTAC – 5’
destroyed NcoI NcoI

Control digest: cl.#11 – cl.#15 with NcoI / XhoI

Sequenced clones and bacterial stock: cl.#11 and cl.#13

7. Curriculum Vitae

Personal Information

Name	ROBLEK, Marko
Address	Sele-Borovnica / Zell-Freibach 38, 9170, Borovlje / Ferlach, Austria
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Date of Birth	October 6, 1983
Nationality	Austrian

Education

2002-2009	Study of Molecular Biology at University of Vienna, Graduation
2007	ERASMUS exchange student at University of Oulu, Finland
1994-2002	ZG & ZRG za Slovence v Celovcu – BG & BRG für Slowenen in Klagenfurt