Hutchinson-Gilford Progeria Syndrome
A new treatment strategy and the role of prelamin A in oncogenesis

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“The most terrifying fact about the universe is not that it is hostile but that it is indifferent; but if we can come to terms with this indifference and accept the challenges of life within the boundaries of death — howevermutable man may be able to make them — our existence as a species can have genuine meaning and fulfillment. However vast the darkness, we must supply our own light.“

– Stanley Kubrick, 1968
Prelamin A, a CaaX-protein is a key structural protein of the inner nuclear lamina, a meshwork lining the inner nuclear envelope. Farnesylated prelamin A is cleaved just upstream of the farnesylcysteine residue to produce mature lamin A. We generated Zmpste24 knockout mice and documented a striking accumulation of farnesylated and methylated prelamin A in cells. Zmpste24 knockout cells exhibit premature senescence and misshapen cell nuclei. Zmpste24 knockout mice show slow growth, hair loss, micrognathia, bone fractures, muscle weakness, and premature death. These phenotypes are similar to those in HGPS. HGPS is caused by a LMNA point mutation that leads to the deletion of 50 amino acids in the carboxyl terminus of prelamin A (eliminating the ZMPSTE24 cleavage site and preventing formation of mature lamin A). Consequently, a mutant farnesylated and methylated prelamin A accumulates at the nuclear rim in HGPS cells, interfering with the nuclear lamina and causing misshapen cell nuclei.

Specific Aim and Results of Paper 1: To define the importance of ICMT in the pathogenesis and treatment of progeria. In this project we bred Zmpste24 knockout mice with mice harboring a hypomorphic (reduced expression) allele of Icmt. We found that these mice where protected from most aspects of progeroid disease. They had an increased survival, lack of osteoporosis, and increased strength. Icmt inhibition in cells derived from Zmpste24 KO mice and cells from human progeria patients also showed increased proliferative and somatrophic activity, without affecting the frequency of nuclear shape abnormalities which is one of the hallmark phenotypes of progeria.

Specific Aim and Results of Paper 2: To test the hypothesis that prelamin A is a tumor suppressor. We bred Zmpste24 knockout mice with mice expressing a Cre-inducible endogenous oncogenic K-RAS and B-Raf alleles (K-RASLSL/+ and B-RafCA). Groups of mice where then allowed to inhale a Cre- adenovirus to activate the expression of oncogenic K-RASG12D/+ and B-RafV600E in lung cells (these mice normally develop lung adenomas to adenocarcinoma without metastases). 10 and 8 weeks post inhalation mice were euthanized and lungs where prepared for routine histology. Surprisingly, Zmpste24-deficiency had no impact on the development K-RASG12D/+ and B-RafV600E driven tumor except for a reduction in grade. Furthermore, fibroblasts derived from the same mice could be readily transformed and proliferated at the same rate as Zmpste24 competent cells. Finally, K-RASG12D/, B-RafV600E Zmpste24-fibroblasts had significantly reduced basement membrane invasiveness.

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II. Ibrahim MX, Sayin VI, Bergo MO. Prelamin A inhibits K-RAS and B-Raf induced invasion but is dispensable for tumorigenesis. Manuscript.
LIST OF PAPERS
This thesis is based on the following studies, referred to in the text by their Roman numerals.


II. **Ibrahim MX**, Sayin VI, Bergo MO. Prelamin A inhibits K-RAS and B-Raf induced invasion but is dispensable for tumorigenesis. *Manuscript*. 


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Introduction

The understanding of organismal aging, the predominant risk factor for the development of neoplastic, metabolic, cardiovascular and neurodegenerative disorders (1), has improved markedly in the last decade (2). From a view of aging as a completely stochastic process of slow deterioration, to a detailed understanding of the molecular underpinnings of both its causes and its regulation.

Humans and mice do not die of aging (3); they die of diseases that correlate with factors that increase in incidence and severity over time. Be it acquired or hereditary, these factors predispose most species to a range of events linking a failure of highly interconnected multi-system mechanisms leading to, for instance, a stroke caused by atherosclerotic cerebral artery, different types of cancer, or cognitive decline. This thesis aims to describe two diametrical aspects of human disease. From Progeria, a disorder occurring in only 1 in 4 million live births, to cancer, a disease so common it is globally responsible for more than a tenth of all recorded deaths each year (4, 5). What is our current collective knowledge of premature aging? How has this understanding led to encouraging leaps in possible new therapies for the treatment of this dramatic disorder? Could perhaps the harnessing of the molecular mechanisms causing premature aging lead to a new potential therapy for the amelioration of cancer? The knowledge of the molecular underpinnings of these disorders, through the use of model systems, has profoundly increased our knowledge of each and enabled the development of therapies. However, both cancer and premature aging harbor considerable complexities and a number of scientific questions remain unsolved. Questions that this thesis aims to give context to and, together with the enclosed papers, answers for.
The nuclear envelope

The highly ordered and defining feature of any eukaryotic cell is its nucleus (Figure 1). Roughly, it is divided into two major parts. The outer nuclear membrane (ONM) that is continuous with the endoplasmic reticulum (ER) and on the opposite side of the lipid-bilayer that separates the nucleoplasm from the surrounding cytoplasm lies the inner nuclear membrane (INM). The INM part of the nucleus is made up of an intermediate filament (IF) meshwork composed mostly of proteins called Lamins (6-9). The lamins have the important role of providing structural support to the nucleus as a whole, regulate cell division and aspects of the replication/transcriptional machinery (10-15).

A major part of the intermediate filament network is the lamina. The lamin proteins, first recognized as components of the nuclear matrix, include two protein families, namely A and B type lamins (16) and are encoded by three different genes. Lamins of the A type are encoded by the gene LMNA which is located on chromosome 1q21.2(17) and consists of 12 exons. Prelamin A (the immature form of Lamin A) and C are two alternative splicocoforms of the LMNA gene product. They share significant sequence homology with 566 common amino acids and the c-terminus being differentiating between the two (98 unique a.a. for lamin A and 6 a.a. for lamin C). The second, B type lamin family, are encoded by LMNB1 or LMNB2 and are located on chromosome 5 and 18, respectively.

![Figure 1. The nuclear envelope](image-url)
Figure 2. Schematic of Lamin protein maturation and final destination at the nuclear membrane.

During interphase, the lamins form higher order structures by polymerizing primarily in a lamin-lamin dimer structure through interactions between their conserved head, tail and central α-helical rod domains (Figure 2). These dimers further polymerize into head-to-tail secondary structures that make up the intermediate filament network (18). Important functions of the nuclear lamins are their ability to regulate the size of the nucleus (19), spacing and incorporation of nuclear pores (20-23), and its shape (24).

During cell division, the architecture within the nuclear wall has to be disassembled for proper mitosis to proceed. This is done through
phosphorylation at specific sites, immediately preceding mitosis. These sites are targeted by Cdk1 and PKC (25-27) and the phosphorylation event triggers a depolymerization of the higher order lamin structures.

1.1 The CaaX motif

The A and B type lamins have a common c-terminal CaaX-motif. It consists of a cysteine-aliphatic-aliphatic-any aminoacid sequence, which is recognized and modified by a series of enzymatic reactions. The first posttranslational modifications that lead to attachment of a 15-carbon farnesyl lipid by Farnesyl Transferase, a process called isoprenylation. This is followed by endoproteolytic cleavage of the remaining –aaX (28, 29) by two different enzymes, RAS-Converting Enzyme 1 (RCE1), or in the case of prelamin A, also ZMPSTE24. The newly exposed cysteine is then carboxymethylated by isoprenylcysteine carboxylmethyltransferase (ICMT)(Reaction 1)(30).

\[
S \rightarrow \text{adenosyl} \rightarrow \text{L} \rightarrow \text{methionine} \\
+ \\
\text{protein C} \rightarrow \text{terminal} \ S \rightarrow \text{farnesyl} \rightarrow \text{L} \rightarrow \text{cysteine} \\
\downarrow \\
S \rightarrow \text{adenosyl} \rightarrow \text{L} \rightarrow \text{homocysteine} \\
+ \\
\text{protein C} \rightarrow \text{terminal} \ S \rightarrow \text{farnesyl} \rightarrow \text{L} \rightarrow \text{cysteine methyl ester}
\]

**Reaction 1. Carboxymethylation common to Lamins A, B1 and B2**

After carboxymethylation, the newly methylated c-terminus is again cleaved by Zmpste24 (31), 15 amino acids upstream of the c-terminal cysteine (32, 33), producing the fully mature Lamin A. Prelamin A is the only lamin that is cleaved in this way (34, 35). Why this process has been conserved in evolution has been greatly debated. One possibility could be that the loss of the permanently farnesylated and methylated c-terminus leads to an increased solubility during mitosis, enabling an easier disassembly of the nucleus (30). Other, far more extensive studies into the reasons for this
elaborate processing has been carried out by interrogating the c-terminus of prelamin A (36). Mice with complete knockout of LMNA, producing neither Lamin A nor C, display a severe muscular dystrophy. Knockouts of either lamin A or C are completely viable without any clear phenotype. Mice producing mature Lamin A directly, bypassing post-translational processing, and the prelamin A intermediary show no apparent phenotype. The role for the last 15 amino acids cleaved of from prelamin A when the mature lamin A is produced is also unknown.

1.2 STE14p
Brewer’s yeast, S. cerevisiae, one of the most well studied model organisms, can stably persevere with either haploid or diploid genomes. Yeast with a diploid genome can reproduce by budding/mitosis, whereas haploid yeast cells require sexual reproduction. Yeast have two “sexes”, cells that express mating pheromones called α- or α-factor. These factors are CaaX proteins and are processed in much the same way as its human homolog prelamin A (29, 37). It is methylated by prenylcysteine carboxyl methyltransferase Ste14p and cleaved by Ste24p (38) which are well conserved homologs of ICMT and Zmpste24 respectively.

1.3 Laminopathies
Mutations in either LMNA or ZMPSTE24 cause a family of diseases called laminopathies. Depending on where the mutation is located within LMNA (33) or how much residual Zmpste24 protease activity (39) is left determines disease symptoms and severity. These diseases can be classified in 4 different groups.
Hutchinson-Gilford Progeria Syndrome

Table 1. The most frequently observed laminopathies.

<table>
<thead>
<tr>
<th>Class</th>
<th>Disease</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striated muscle diseases</td>
<td>Autosomal dominant EDMD</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Autosomal recessive EDMD</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Cardiomyopathy dilated 1 A</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Limb-girdle muscular dystrophy type 1 B</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Congenital-type muscular dystrophy</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Heart-hand syndrome (with limb defects)</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>X-linked EDMD</td>
<td>EMD</td>
</tr>
<tr>
<td>Partial lipodystrophy syndromes</td>
<td>FPLD2</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Lipoatrophy with diabetes, hepatic steatosis, hypertrophic cardiomyopathy, and leukomelanodermic papules</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Mandibuloacral dysplasia (also has features of progeria)</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Acquired partial lipodystrophy (Barraquer-Simons syndrome)</td>
<td>LMNB2</td>
</tr>
<tr>
<td>Progeria</td>
<td>Hutchinson-Gilford Progeria Syndrome (HGPS)</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Atypical Werner syndrome</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Mandibuloacral dysplasia (also has partial lipodystrophy)</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>Restrictive Dermopathy (RD)</td>
<td>ZMPSTE24</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td>Charcot-Marie-Tooth disorder type 2B1</td>
<td>LMNA</td>
</tr>
</tbody>
</table>

Adapted from Worman, HJ. et. al. 2009 (40)

How these mutations, in genes that are nearly ubiquitously expressed, lead to highly tissue-specific disorders remains unclear (41). The LMNA mutations account for the largest spectra of disorders of any known human gene (Table 1). The complexity of disease symptoms suggest that the correct assembly of the nuclear lamina is crucial for normal cell function.
There are three reigning schools of thought for why this is. First, in muscle (vascular smooth, heart and skeletal), the mechanical stress exerted by repetitive contraction and general strain affects fragile or stiff nuclei leading to a progressive decline in function, in essence physically “breaking” the nuclear membrane macroarchitecture that has been weakened by an incorrectly assembled lamina. The second, a “gene-regulation” hypothesis suggests that the interaction between nuclear lamins and the genome is perturbed in such a way that the normal function of organ/tissue specific gene expression is changed detrimentally. The third hypothesis, which is of particular interest for this thesis, proposes that nuclear envelope mutations lead to impaired function in the stem cell niche (42). \textit{LMNA} mutations have been shown to impair the survival, proliferation and differentiation of stem cells (43, 44), with current evidence showing a depletion of stem cells mainly mesenchymal lineage (myoblasts, vascular smooth muscle cells, adipocytes, osteoblasts, fibroblasts).

1.3.1 Hutchinson-Gilford Progeria Syndrome

The first clinical description of Hutchinson-Gilford Progeria Syndrome (HGPS) was published in the year 1886 in the Royal Medical and Chirurgical Society journal by Hutchinson (45), and again in 1904 by Hastings Gilford (46). The clinical features associated with HGPS has a strong resemblance to physiological aging, only greatly accelerated. The earliest symptoms show during infancy. Individuals with segmental aging (i.e. displaying some but not all phenotypes of aging) disorders such as this exhibit a distinct failure to thrive, limited subcutaneous fat depositions, sclerotic skin and prominent veins, joint contractures, severe osteoporosis, alopecia and micrognathia (5). Most have suffered at least one stroke event before the age of 8 (47) and the patients almost exclusively die from the occlusion of a coronary/cerebral artery during their early teens.

The causative point mutation, a \textit{de novo} C-to-T substitution at nucleotide 1824 in \textit{LMNA} (48, 49), spontaneously arise in the germ cell of a parent. Due to this and the fact that the HGPS patients never reach secondary sexual maturity and thus never reproduce, the disease is exceedingly rare with a calculated incidence of only 1 in 4 million live births and around 100 reported cases of children living with HGPS worldwide. This single mutation leads to the activation of a cryptic splice site and a subsequent deletion of 50 a.a. transcribed from exon 11 of \textit{LMNA} (Figure 2, right panel). This deleted sequence contains the cleavage site for the protease, Zmpste24, that carries out the final processing step of prelamin A maturation. Due to the loss of this
cleavage, truncated prelamin A, now called Progerin, retains its CaaX-motif and thus undergoes the same posttranslational processing as prelamin A and accumulates irreversibly at the nuclear rim (50) and causes all the described disease symptoms. Fibroblasts derived from patients with HGPS show a characteristic high frequency of disruption of the nuclear membrane structure (48, 49). Progerin thus acts in a dominant negative manner and disrupts normal nuclear membrane function.

Restrictive Dermopathy (RD) is another progeroid syndrome and is as rare as HGPS. Most patients die perinatally and show signs of thin, tightly adherent and translucent skin, superficial blood vessels and severe joint contractures (ankylosis) (51). RD is caused by a homozygous null mutations in ZMPSTE24 (52, 53). This leads to the loss of ZMPSTE24 protease activity and the proper maturation of prelamin A fails. Thus, permanently farnesylated and methylated prelamin A accumulates at the nuclear rim and causes severe toxicity.

1.4 Mouse models of Progeria

To date, animal model systems are designed for the specific purpose of recapitulating a human disease state and the subsequent treatment thereof. Many different animal models have been created to study progeroid syndromes (34, 35, 50, 54-58).

Through apparent serendipity, the first two models of HGPS were developed independently of each other for completely other reasons than to study progeria. Pendás et. al. (34), their Zmpste24 KO mouse was developed to study the importance of proteases in general and Zmpste24 in particular. In two other papers, Leung et. al. (59) and Bergo et. al. (35) developed the Zmpste24 KO mouse to study the in-vivo effects of the removal of the enzyme that cleaves S. cerevisiae a/α-factor in higher order animals. Both groups detailed several phenotypes that show great resemblance to the human HGPS, although this was not until the following year when the exact mutation was located (48, 49) at the LMNA locus. Zmpste24 KO mice show severe growth retardation, osteoporosis, histological skin and muscle abnormalities and death before 20 weeks of age. Cells derived from these mice also showed increased levels of prelamin A. In these cells, proper maturation was partially dependent upon the presence of FTase and ICMT but not RCE1.
The discovery of the HGPS mutation enabled the development of new, targeted mutations that aimed to recapitulate the disease more closely by expressing progerin (the toxic human LMNA transcript) in mice.

Yang et al. developed the first progerin knock-in model showing striking similarities to HGPS in cells (50) and in mice (57). The second model, by Varga et al.(55), introduced a bacterial artificial chromosome expressing Progerin mice. This model showed no apparent phenotypes except for non-lethal vascular involvement with a loss of cells in the vascular wall. In order to study how the expression of Progerin affects specific tissues or cell types, Sagelius et al. (54) developed a tetracycline inducible mouse model that specifically expressed progerin under the keratin-5 promotor showing HGPS-like defects in the skin and teeth. More recently, Osorio et al.(58) developed another Progerin expressing model, the first to express progerin spliced from the endogenous mouse LMNA allele. These mice showed the most comprehensive HGPS manifestations with involvement of bone, skin, vascular and cellular phenotypes leading to a lifespan similar to the initial Zmpste24 KO models.

In an elegant study, the authors knocked in wildtype Zmpste24 into the Hprt locus on the X chromosome (Zmpste24 is normally on chromosome 4) (60). Mice are diploid for the X chromosome but go through stochastic inactivation of one allele in all cells. They then crossed them with progeroid Zmpste24 KO mice and due to the extra Zmpste24 allele on the X chromosome they in essence created mosaic mice that had progeria in
roughly half of all the cells. The major finding, which has exiting implications for future therapies, is that these mosaic mice lacked all phenotypes of progeria. This indicates that the penetrance threshold for any therapy might be quite low and the potential rewards can be major.

![Figure 4](image)

*Figure 4. Prelamin A at the nuclear membrane, show characteristic folds (1) blebs (2) and micronuclei (3).*

### 1.5 Cellular phenotypes

In a recently published review, the authors specify nine cooperating factors that determine the progression and severity of aging; genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (3). All these aspects, having their origin at a subcellular level and their consequences at an organism level, is what we determine to be aging. In progeria, all these aspects are in full effect. Cells derived from HGPS/RD patients or from the many different mouse progeroid models accumulate either prelamin A or rogerin within the nuclear lamina leading to the appearance of characteristic blebs, folds and eventually micronucleation (Figure 4). This change in nuclear architecture causes segregation and ultimate loss of genomic material, and is likely of great importance in the pathogenesis of progeria.

In the defense of the genome the cell has a number of means in its war chest. The cell is constantly bombarded by external and internal insults like reactive oxygen species, chromosome instability, nutrient deprivation and DNA damage and cell risks permanent changes in essential genes and their transcriptional regulation. When this occurs, the cellular machinery detects this stress and most often takes one of two decisions; either it goes into programmed cell death, apoptosis, or prevents cell division, a process called senescence. Senescence is a process where the cell permanently escapes
the cell cycle and is a prominent feature of cells with progeria. Evidence of the senescence process being active has been widely published. The transcription factor p53, the guardian of the cell, has been shown to be activated in mouse models of progeria (61, 62) and an inability to recruit 53BP1 (63), indicating a pronounced and chronic DNA damage response.

Telomeres are repeating nucleotide regions which cap chromosome ends. Telomere shortening in physiological aging is a means for cells to protect itself from chromosome degradation. Through mitosis, the telomeres erode due to polymerases not being able to replicate these long repeating sequences. The telomeres thus become shorter at each division and can only be replenished by the expression of the Telomerase enzyme. Progeria is accompanied by enhanced telomere shortening (64) despite their inability to divide as much as healthy cells.
Progeriod cells and mice have a somatrophic signaling defect (65), preventing the cell from sensing, or reacting to the binding of growth factors. One pathway that this signaling can be mediated through is the PI3K-AKT-mTOR pathway which has a central importance for cell homeostasis, controlling metabolism, proliferation, growth and glucose uptake, and mediates signals triggered by ligands such as IGF1, insulin and VEGF (66). Increased signaling from upstream receptors stimulate AKT phosphorylation leading to increased kinase activity and phosphorylation of downstream targets such as the mTOR/RAPTOR complex, increasing growth and protein 
translation. AKT also signals to cyclin-dependent kinase inhibitors by directly phosphorylating p21Cip1 and p27Kip1, leading to their export from the nucleus and subsequent degradation, which promotes cell cycle progression.
Figure 6. Current treatments targeting the formation and maturation of Progerin
Current treatment paradigms

2.1 Farnesyltransferase inhibitors

The rapid progress from identifying the mutation causing HGPS to the first clinical trial, 2.5 years in total, enabled by large pharmaceutical companies already having developed drugs targeting the prelamin A/progerin maturation pathway. These compounds, called farnesyl transferase inhibitors (FTIs), where originally developed for the treatment of cancer driven by RAS mutations (67). FTIs are well tolerated but have shown limited efficacy in clinical trials for the treatment of solid tumors (68). The understanding, that FTIs target the CaaX-motif, led to initial studies of FTIs on cells (50), showing a reduction of toxic prelamin A at the nuclear membrane and a significant reduction in the frequency of nuclear shape abnormalities. Mouse models of progeria showed surprisingly that reducing the expression of LMNA by 50% leads to a complete rescue if disease symptoms (69). This suggested that FTIs would mislocalize progerin/prelamin A from the nuclear membrane and thus improve disease phenotypes. Indeed, the first trial of FTIs in vivo was extremely encouraging (70), showing a significantly improved survival, strength and weight of progeroid/Zmpste24 deficient mice. Follow-up studies by others further validated the hypothesis (71, 72).

2.2 Statins and Amino Bisphosphonates

In 2008, Varela et al. hypothesized that the inhibition of the mevalonate/cholesterol synthesis pathway could prove efficacious in the treatment of progeria by inhibiting the synthesis of geranylgeranyl- and farnesyl-pyrophosphate (73). This would in turn inhibit the prenylation of prelamin A/progerin. Indeed, the treatment of cells and mice with a cocktail of the HMG-CoA synthase inhibitor pravastatin and a farnesyl pyrophosphate synthase inhibitor Zoledronate improved nuclear shape, reduced level of toxic protein in the nuclear membrane and affect the overall survival of Zmpste24-deficient mice significantly.

2.3 The FTI clinical trial

The ready availability of an FTI compound, Lornafarnib, quickly led to the first open label clinical trial in patients with HGPS. Twenty five patients were enrolled and followed for 2 years (74). Results however where mild.
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Lornafarnib-treated patients showed signs of improved vascular health, shown by decreased pulse wave velocity in the carotid artery, which is a surrogate marker for blood pressure and vessel stiffness. Also, the treatment led to some improvement in bone parameters in bone mineral density and rigidity.

A new trial with 45 patients enrolled (roughly half the global patient population), have been started on a treatment combination with lornafarnib, together with pravastatin and zoledronate, the results of which have yet to be published.

2.4 Splicing directed therapies

As early as 2005, Scaffidi and Misteli (75) presented a technique where utilized small molecule morpholinos that bound to the aberrant, Progerin expressing, DNA sequence and prevented mRNA transcription leading to a reduction in total Progerin levels. This led to a rescue of the cellular phenotypes associated with progeria, i.e. reduction in nuclear shape abnormalities, increased proliferation and gene expression. In 2011, Osorio et al. took this further and administered aberrant-splicing directed molecules to mice expressing progerin and were also able to increase their survival, body weight and gene expression (58).
Progeria and Cancer

The nuclear lamina is a major player in the stability and regulation of the genome. The predisposition to develop cancer has been tightly associated with diseases that have elements of premature aging. For instance, patients with Werner Syndrome, also called atypical progeria, present with clear premature aging phenotypes but of milder severity than HGPS. In contrast to HGPS, patients with mutation in the Werner Syndrome gene (WRN) exhibit diminished ability to repair DNA damage and have an increased risk of developing many different cancers (76). This difference is puzzling since cells and mice with HGPS show clear genomic instability (5, 61, 63, 77-79). Interestingly, an increasing number of tumors have been shown to down regulate the expression of A-type lamins (80, 81) leading us to believe that perhaps almost complete absence of cancer diagnoses (82) is because that A-type lamins tumor suppressors.

De La Rosa et al., studied Zmpste24 KO mice that where mosaics for the wild type Zmpste24 allele (60). Besides showing that mice with toxic prelamin A accumulation in 50% of all cells lacked any discernable phenotype, they also tested how the mice would respond to tumor development. They induced tumors by administering the mutagenic 7,12-dimethylbenz[a]anthracene (DMBA)–12-o-tetradecanoylphorbol-13-acetate (TPA) or urethane, and observed no difference in tumor development except for a reduction in grade. Additionally, the in vitro invasion of several cancer cell lines was reduced when Zmpste24 was inhibited.

3.1 The MEK/ERK pathway and cancer

In the MEK/ERK pathway, growth factors bind and activate receptors to initiate a signaling cascade that results in an increased cell division, migration and differentiation. Important players in this signal cascade are the family of RAS proteins. RAS proteins, also CaaX proteins, are part of a larger group of signaling proteins called small GTPases. In quiescent cells, they are bound to guanosine diphosphate (GDP) and are inactive. Through the actions of guanosine exchange factors (GEFs), RAS proteins gain an additional phosphate group leading to the formation of guanosine triphosphate (GTP). By a conformational change, similar to loading a spring, that leads to its activation. This activation then leads to a propagation of the signal by binding of GTP bound RAS to a number of downstream effectors. Once this is done the GTP is hydrolysed by guanosine activating proteins (GAPs) (83) and the RAS structure is relaxed and thus inactivated (84).
One of the effectors that RAS complexes with is Raf. The activation of Raf leads to the phosphorylation of MEK1 and MEK2, which in turn activates ERK1 and ERK2 that then translocates into the nucleus, increasing the transcription of genes involved in a diverse set actions like angiogenesis, cell proliferation, survival and migration (85-87).

K-RAS, or V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, an isoform in the RAS family (i.e. K-RAS4A, K-RAS4B, N-RAS and H-RAS) is the most frequently mutated gene in cancer (88). K-RAS has been found to be mutated in 30% of non–small cell lung cancers (NSCLC), 50% of colon cancers and as much as 95% of all pancreatic ductal adenocarcinomas (PDAC). The most frequent mutation is a non-synonymous (i.e. a change in nucleotide sequence leading to a change in amino acid sequence) at residue G12, changing a glycine to aspartic acid, preventing RAS interaction with GAPs and thus its persistent activation.

B-RAF, a signaling effector directly downstream of RAS, is a highly conserved serine/threonine kinase and is also frequently mutated in many human tumors such as melanomas, colon and thyroid carcinomas being the predominant types (89, 90). The most common mutation is a miss-sense substitution at codon 600, leading to a change from valine to glutamic acid (i.e. V600E). Codon 600 is right within B-Raf’s kinase domain and the mutation leads to an impaired kinase activity causing, via C-Raf, an activation of the MEK/ERK pathway (91).
Aim

The aim of this thesis has been to investigate two different aspects of Lamin protein biology and its potential implications on the treatment of Hutchinson-Gilford Progeria Syndrome and Cancer.

Specific aims:

Paper 1: To define the importance of ICMT in the pathogenesis and treatment of progeria.

Paper 2: To test the hypothesis that prelamin A is a tumor suppressor
**Experimental strategy**

Central to my studies has been the use of transgenic mouse models. I will in this section specify the properties of and the reasoning behind the use of these specific mouse models. The HGPS mouse model used has been described in detailed in the introduction and for more detailed descriptions of any other techniques and experimental setups, see the methods and materials in each of the enclosed papers.

4.1 **Transgenic mice and the Cre-LoxP system**

Despite striking similarities between genomes of many model organisms, they do not develop the same genetic diseases. By utilizing gene targeting techniques, researchers can introduce engineered/foreign DNA constructs with mutations/modifications enabling controlled changes in expression. The most used technique is the Cre-LoxP system which allows the controlled expression of a transgene. This is done by retooling a bacterial endonuclease called Cyclic-Recombinase (Cre) and expressing it in prokaryotic/eukaryotic systems. The Cre enzyme carries out a site specific recombination by binding to consensus sequences (LoxP). If two LoxP sites are close enough together in the genome, Cre binds to LoxP and removes the LoxP encompassed sequence.

4.2 **Targeting CaaX protein processing**

The development of transgenic mice with targeted mutations against the entire post-translational processing pathway has enabled comprehensive studies on the role of these enzymes in vivo (92-95). Because prelamin A and progerin are prenylated and methylated, the targeting of these enzymes have gathered considerable interest in the possible treatment of Progeria (96), though all experimental focus has been towards farnesyl transferase due to the existence of specific and bioavailable small molecule inhibitors and important clinical trials. In this thesis, both Fntb and Icmt have been targeted to define their therapeutic potential in the treatment of progeria.
4.3 Oncogenesis

To study the initiation and progression of cancer both in vivo and in vitro we’ve used 2 transgenic lines that expresses oncogenes at physiological levels only after activation of Cre Recombinase. This allows us to both temporally and spatially control the development of tumors and enable the detailed study of cellular and in vivo significances of the following oncogenes:

**K-RAS<sup>LSL-G12D</sup>**

This transgenic mouse carries an allele with an activating mutation, K-RAS<sup>G12D</sup>, immediately preceded by a LoxP flanked STOP codon (97). This is a null mutation (no oncogenic protein is translated and the mouse is effectively haploinsufficient for wildtype K-RAS) and the mouse is unaffected by its presence. When Cre is expressed, K-RAS<sup>G12D</sup> is expressed and tumors are formed. In vitro, this leads to transformation, an increase in proliferation and a loss of contact inhibition. When Cre is expressed in the lung it causes an increase in endothelial hyperplasia which progresses in grade to adenomas and adenocarcinomas (Figure 8).

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**Figure 7.** Conditional allele constructs used in this thesis and relative mRNA expression of transgene.
**Hutchinson-Gilford Progeria Syndrome**

**Figure 8.** H/E stained lung sections showing normal (left) and tumor (right) tissue

**B-Raf**

Similar to the LSL-K-RAS mouse detailed earlier, this mouse oncogenic expresses oncogenic $\text{B-Raf}^{\text{V600E}}$ (98) only after Cre expression. This however is not a null mutation and B-Raf expression is completely normal (called $\text{B-Raf}^\text{CA}$; CA for Conditional Allele) in the absence of Cre.

**Figure 9.** H/E stained lung sections showing normal (left) and tumor (right) tissue
Results and Discussion

Paper 1: Targeting isoprenylcysteine methylation ameliorates disease in a mouse model of progeria.

Both prelamin A and progerin retain their carboxyl-terminal CaaX motif which triggers farnesylation by FTase (Figure 2). Inhibiting isoprenylation with inhibitor of Farnesyl Transferase reduces the frequency of misshapen nuclei and increases survival of Zmpste24 deficient mice in not only model systems of progeria but also in HGPS fibroblasts (40, 50, 57, 70, 72, 99, 100).

The effects of specific genetic inactivation of ICMT has been studied in normal cells as well as in RAS and RAF driven oncogenesis (92, 101, 102). The loss of ICMT expression leads to the mislocalization of RAS proteins from cellular membranes and an amelioration of cancer. However, the most intriguing aspect of prelamin A/progerin maturation was its partial dependence on the presence of ICMT. Initial studies showed an accumulation of prelamin A in ICMT KO cells (35), leading to the hypothesis that c-terminal methylation of prelamin A is necessary for efficient cleavage by Zmpste24 (99, 103, 104). This led us to hypothesize that inhibiting ICMT would prevent proper prelamin A/Progerin maturation and thus prevent the development of disease associated phenotypes.

Indeed, ICMT deficiency reduced the efficiency of prelamin A maturation, leading to its accumulation (figure 10). Interestingly, a 70% reduction of icmt
expression was sufficient to augment prelamin A maturation, indicating that total *Icmt* knockout was not required to affect prelamin A processing.

We thus interbred progeroid *Zmpste24* deficient mice with mice expressing a hypomorphic *Icmt* allele (figure 7; *Icmt* allele construct).

![Image](image_url)

**Figure 11.** *Icmt* deficiency prevents *Zmpste24* dependent progeroid phenotypes

Indeed, by decreasing the efficiency of prelamin A methylation we showed marked improvement in strength, prevention of weight decline, improved bone phenotypes and survival. Next, we determined to what extent the bone had been modified by *Icmt*-deficiency. To do this we performed histology and bone histomorphometry on L2 vertebra. This revealed a striking change in the bone composition, with reduced bone mineral density and an almost complete absence of osteoblasts in *Zmpste24*-deficient mice. Contrary, bone in *Zmpste24*/*Icmt*<sup>hm/hm</sup> mice showed high presence of osteoblasts and evidence for ongoing bone formation.
5.1 Bone disease and the cell of origin

Bone is a dynamic organ where a balance is kept between cells that build, osteoblasts, and cells that resorb, osteoclasts. Physiological aging and the female sex are the highest risk factor for the pathogenesis of bone disease, or osteoporosis. This happens mainly through estrogen dependent mechanisms in post-menopausal women and the reduction of estrogen levels after menopause prevents an androgen driven inhibition of osteoclasts, leading to a shift in the balance between osteoclasts and osteoblasts towards increased resorption. In progeria, there are clear evidence for osteoporosis. Patients show symptoms of skeletal dysplasias like acrosteolysis (degradation of bone at distal pharynx), clavicular resorption, slender diaphysis of long bones with pronounced demineralization (5, 105). However, there is no clear experimental evidence for what cell type is responsible for this phenotype. A study in Zmpste24 deficient mice (106) showed significant reductions in both osteoclasts but more strikingly a decimation of osteoblasts numbers and a loss of bone cellularity in general. Our own results confirm these findings and we were able to recapitulate the loss of both bone forming and bone degrading cells.

As of today, the inhibition of FTase has been shown to be the best treatment of HGPS. In cells, mice and in the lornafarnib clinical trial the drugs have been able to improve disease phenotypes. In all in vivo contexts, the drug has been able to improve bone parameters, with reductions in osteolytic lesions in mice and improved bone mineralization and strength in HGPS patients. To investigate which cell type mediates this effect, we bred mice with a conditional FTase allele under the control of three different tissue specific Cre strains with the Zmpste24-deficient progeroid mouse model.
We started with an interferon-inducible transgenic mouse expressing Cre under the Mx1 promoter. In Mx1-Cre mice, Cre is under the control of a type I interferon inducible promoter (107) that is expressed in many cell types with an origin in the bone marrow i.e. cells of both mesenchymal and hematopoietic origin including both osteoclasts and osteoblasts (figure 12). At sacrifice, the Zmpste24−/− Fntbfl/fl Mx1Cre mice had significantly reduced osteolytic lesions alongside the spinal column. To narrow down the exact cell type that metabolizes bone we bred the Zmpste24 deficient mice with Cre expressed in either osteoblasts (108) or osteoclasts (109). Indeed, knocking out FTase in osteoblasts was more efficient than in osteoclasts. This further supports the notion that Zmpste24/Progeria pathogenesis is, at least in part, driven by malfunctioning cells of a mesenchymal origin (110). However, regardless of how much improvement of the bone disease we were able to attain, these mice all had to be euthanized at the same rate as mice with no intervention, i.e. no improvement over Zmpste24-deficiency alone. This result signals a word of caution, as improvements in bone phenotype led to no apparent increase in survival.

5.2 Nuclear membrane architecture

Prevailing dogma in progeria is that the accumulation of prelamin A or progerin at the nuclear rim leads to an increased frequency of misshapen
nuclei. To determine if \textit{Icmt} deficiency had any effect on the accumulation and/or localization of prelamin A, we performed conventional and sub-diffraction-limit/super resolution microscopy.

\textbf{Figure 13.} Inhibition of \textit{Icmt} expression has no impact on nuclear shape in \textit{Zmpste24}-deficient fibroblasts.

Interestingly, \textit{Icmt} deficiency had absolutely no impact on the frequency of misshapen nuclei of cells derived from \textit{Zmpste24}\textsuperscript{-/-} embryos (Figure 13). As a hallmark of progeria, the aberrant shape of the nuclear membrane has been the measure of treatment success with many studies showing significant reductions in misshapen nuclei correlating with treatment success \textit{in vivo} (96). Our data suggest that nuclear shape aberrations observed by the research field is likely an artifact found in cell culture and that this has little importance in an in vivo setting. Furthermore, we showed that non-methylated prelamin A accumulates within the nucleoplasm, both in wildtype and \textit{Zmpste24}-deficient cells (Figure 14).
Similar to previous studies, we found no reduction in total levels of the toxic prelamin A by interfering with the c-terminus. We hypothesize that the presence of permanently farnesylated and methylated prelamin A/Progerin within the nuclear rim is the main mechanism of action.

Zmpste24-deficient cells senesce prematurely in vitro and go into an irreversible proliferative arrest (61, 63). We derived fibroblasts from embryos of Zmpste24⁻/⁻ Icmtʰʰ/ʰʰ and Zmpste24⁻/⁻ Icmt⁺/+ controls and ran several proliferation experiments. We found that Zmpste24⁻/⁻ Icmtʰʰ/ʰʰ fibroblasts behave no different than wildtype cells. Further cementing the hypothesis that farnesylated but no longer methylated prelamin A has lost its toxic properties. This in contrast to Zmpste24⁻/⁻ FntbΔ/Δ cells, that we’ve shown to have improved nuclear shape, could not show attain any proliferative benefit of farnesyl transferase inhibition.

**Figure 14. Icmt deficiency creates a nucleoplasmic prelamin A pool.**
What is the mechanism for this improvement in phenotype? Other than the reduction in prelamin A dose at the nuclear membrane, what other mediators could possibly be involved in the amelioration of disease phenotypes? Of note, by removing ICMT from the equation we are not only affecting prelamin A maturation but we are also targeting about 150-200 other CaaX-proteins that are also methylated by ICMT. We started our search by running western blots for the signs of increased growth/survival signaling. We found an increase in PI3K-AKT signaling and robust changes in the pathways downstream effectors. Clear increase in targets of mTOR, S6 and 4E-BP1 phosphorylation indicates and increase in protein synthesis and decreased autophagy. Also, nuclear effector targets that are directly phosphorylated by Akt, like cell cycle inhibitors p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1}, were almost completely lost.

**Figure 15.** Icmt-deficiency increases PI3K-Akt-mTOR signaling and abolishes inhibitors of cell cycle progression.

Further, we validated the importance of the pathway by inhibiting key mediators PTEN and Akt by treating Zmpste24\textasciitilde/Lcmt\textasciitilde/\textasciitilde/\textasciitilde/Icmt\textasciitilde/\textasciitilde/\textasciitilde deficient fibroblast with inhibitors of each. Results were clear. The cells were completely addicted of this pathway and needed it to sustain the beneficial effects of Icmt-deficiency. We then went on to replicate these results in cell lines derived from patient with HGPS and managed to show an increase in proliferation, increased PI3K-AKT-mTOR signaling without having an impact on the frequency of nuclear shape abnormalities in fibroblasts.

Key to our study, we found a direct interaction between prelamin A and Akt in Zmpste24\textasciitilde/Lcmt\textasciitilde/+/+ mice that was absent in Wt and lost in Zmpste24\textasciitilde/
Hutchinson-Gilford Progeria Syndrome

$Icm^{hm/hm}$ mice. This effect fits well into our overall hypothesis that PI3K-Akt-mTOR signaling is impaired, by the sequestration of Akt directly at the nuclear membrane. It is then released by means of Icmt inhibition. A loss of the inhibitory interaction in progeria leads to a rescue of the signaling required for cellular proliferation, survival and growth.
Paper 2: Prelamin A inhibits K-RAS and B-Raf induced invasion but is dispensable for tumorigenesis.

The paucity of cancer in HGPS is puzzling. The accumulation DNA damage paired with a persistent repair response should result in the opposite and the literature has abundant evidence for why cells and patients with HGPS should be increasingly susceptible to tumor initiation (111-116).

Thus, we decided to elucidate the causes for this apparent contradiction. We did this by crossing the \textit{Zmpste24}-deficient mice with 2 different Cre-inducible oncogenes, \textit{K-RAS}^{LSL-G12D} and \textit{B-Raf}^{CA}.

\begin{enumerate}
  \item We started with determining the impact of \textit{K-RAS}^{LSL-G12D} and \textit{B-Raf}^{V600E} driven oncogenesis in cells. Normal and healthy fibroblasts proliferate highly
\end{enumerate}
during early passages. They gradually lose this ability and approach what is called the Hayflick limit (117). The Hayflick limit specifies that in vitro cultured cells have a finite number of cell divisions before they reach a quiescent, or senescent, state. In Zmpste24-deficient cells, the accumulation of toxic prelamin A is a barrier to normal proliferation and they enter quiescence prematurely. Surprisingly, after B-Raf- or K-RAS-induced transformation, Zmpste24-deficient cells displayed a robust increase in proliferation. This despite high/unchanged levels of toxic prelamin A and irrespective of inducing oncogene. This increase of proliferation was accompanied by a decrease in p21 protein levels in Zmpste24-deficient cells indicating a senescence escape.

De La Rosa et al. showed that the mosaic mice have a clear invasion defect in Zmpste24-deficient cells and tumors positive for prelamin A accumulation (60). To test to see if this was reproducible in our model systems, we measured cellular invasion capacity in real time. Indeed, Zmpste24-deficient cells, regardless of oncogene, were unable to invade through a matrigel basement membrane (Figure 17). This result conforms well to the findings in the De La Rosa et al. study, and the hypothesis that Zmpste24-deficiency leads to cell autonomous defects that prevents degradation of surrounding extra-cellular matrix and ultimately invasion.
To determine the *in vivo* impact of \( \text{Kras}^{G12D} \) and \( \text{B-Raf}^{V600E} \) driven oncogenesis in \( \text{Zmpste24}-\text{deficient} \), we inhaled mice with the same adenoviral Cre vector used on the cells. At sacrifice, 10 or 8 weeks after Cre inhalation for \( \text{Kras}^{\text{LSL-G12D}} \) and \( \text{B-Raf}^{V600E} \) respectively, the lungs showed pronounced multifocal tumors of differing grades from slight epithelial/adenomatous hyperplasia to severe adenomas (Figure 17, 18). The tumors were then counted and graded (Figure 18).

**Figure 18.** \( \text{Zmpste24}-\text{deficiency has no impact on K-Ras}^{G12D} \) and \( \text{B-Raf}^{V600E} \) driven lung tumor development
Surprisingly, the accumulation of prelamin A had no impact on tumor burden (Figure 17) additionally confirming that progeria, at least in mice, is not a barrier for cancer development. However, even though the total tumor area was unaffected, the tumor grade was reduced in Zmpste24-deficient mice compared to Zmpste24 wildtype (Figure 18). The pathogenesis of the progeroid phenotypes have been shown to be p53-dependent (61). As malignant progression of tumors have been shown to be associated to both the presence and activity (i.e. loss/null mutations increases malignancy) of p53 (118-121) this reduction in grade could be because of the increased p53 activity (part of an orchestrated response against pervasive stress) reducing the frequency of high grade adenomas/adenocarcinomas.

**Figure 19.** Zmpste24-deficiency lead to a decrease in tumor grade.
Conclusions

In Paper 1

We knew that permanently farnesylated and methylated progerin/prelamin A is toxic. We knew that these molecules accumulate at the nuclear rim, leading to a destruction of its normal function. We understand that this leads to a powerful response in defense of the genome that has profound consequences for almost the entire organism and that targeting of the c-terminal processing machinery has clear merit.

Indeed, by directly targeting the c-terminal methylation, we’ve likely interfered with the pathogenic assembly of prelamin A/progerin at the nuclear membrane and relocated it into the nucleoplasm. This led to complete amelioration of bone disease, increased weight and hair development.

Clearly, we have only started to understand the role of the lamin proteins at the nuclear membrane. We have shown an intriguing interaction between AKT and prelamin A, a likely inhibitory interaction that can be broken by inhibition of c-terminal methylation. We have some indications that this interaction is occurring at the nuclear membrane and that the increased dose of prelamin A/progerin is sequestering Akt, preventing it from carrying out its normal signaling.

We have several ongoing collaborations to develop new inhibitors of ICMT and hopefully we’ll be able test any new molecule that becomes available.

In Paper 2

Though certain types of progeria show significant increases in tumor development, patients with HGPS do not. The lack of data in the literature on why this is led us to test the hypothesis that Zmpste24-deficiency, and the resulting prelamin A accumulation would act as a barrier for tumor development.

First, we activated either K-RAS$^{G12D/+}$ or B-Raf$^{V600E}$ in Zmpste24-deficient fibroblasts and saw a robust increase in proliferation. This despite high levels of prelamin A. Further, K-RAS or B-Raf transformed Zmpste24-deficient cells had reduced ability to invade through an extra cellular matrix mimic. Next, we wanted to see if prelamin A accumulation could have a role
in vivo by activating the same oncogenes the lungs of wildtype and Zmpste24-deficiency. The activation of K-RAS and B-Raf had no impact on tumor development. Interestingly, the tumor grade was significantly reduced compared to Zmpste24 wildtype.

We hypothesize that in progeria, this is due to a robust p53 activation at baseline and that adding further oncogene induced stress only serves to activate p53 further, leading to a prevention of tumor grade progression.

Both these phenotypes could potentially be harnessed and Zmpste24 could be targeted for the treatment of invasive and malignant tumors.
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