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A-type Lamins in Cell Cycle Regulation

Jaime L. Parman-Ryans
East Tennessee State University

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A-type Lamins in Cell Cycle Regulation

A dissertation
presented to
the faculty of the Department of Biomedical Sciences
East Tennessee State University
In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Biomedical Sciences
with Concentration in Biochemistry and Molecular Biology

by
Jaime Lyn Parman-Ryans
May 2017

Antonio E. Rusiñol, Chair
Douglas P. Thewke
David Johnson
Donald B. Hoover
Dennis M. Defoe

Keywords: Lamin A, Prelamin A, E2F1, Cell Cycle, p21, zmpste24, H2A.Z
ABSTRACT

A-Type Lamins in Cell Cycle Regulation

by

Jaime Parman-Ryans

Proteins of the nuclear lamina provide structural support to the nuclear envelope and participate in a variety of cellular functions, such as chromatin organization and transcriptional regulation. One of these proteins, Lamin A (72kDa), is synthesized as a 74 kDa precursor protein, Prelamin A, which undergoes an unusual maturation pathway that requires two farnesylation-dependent endoproteolytic cleavages. The second cleavage is unique to lamin A in higher vertebrates and is specifically carried out by the endoprotease zmpste24. Although most previous studies have focused mainly on the function of mature Lamin A, recent evidence from our laboratory shows important biological functions for Prelamin A as well. Prelamin A concentration in proliferating cells is very low or undetectable. Conversely, during quiescence induced by mitogen withdrawal or contact inhibition, Prelamin A levels increase dramatically. These variations are directly regulated by changes in expression and enzymatic activity of zmpste24. The central hypothesis of this dissertation is that full-length farnesylated and carboxymethylated prelamin A (FC-PreA) antagonizes both proliferation and apoptosis, therefore playing a role in cellular quiescence/senescence. To accomplish this goal, we studied the transcriptional regulation of zmpste24 and the interaction of FC-preA with proteins that participate in cell cycle control. 1) We identified and characterized a functional site for the E2F1 transcription factor (involved in the control of cell cycle) in the proximal 5’ UTR region of zmpste24. 2) By using proximity-labeling and co-immunoprecipitation-mass spectrometry techniques, we identified a set of proteins that interact preferentially with L467R-Prelamin A
(uncleavable mutant) but not with mature Lamin A. Many of these proteins function to regulate progression through cell cycle.
DEDICATION

This manuscript is dedicated to my family. Without your love and support, I would not have been able to achieve this milestone. To my husband, Benjamin Ryans, there is no way that I would have been able to accomplish this without your unconditional love and support throughout this journey; you have stood by me through the thick and thin. Without your help and encouragement at home as well as at school, I know that I could not have done this without you. This Ph.D. is as much yours as it is mine.

In memory of:

My Mother
Beatrice Ellen Parman Lamons (August 2, 1953 – March 22, 2010)

Nonie
Virginia Kyte (September 10, 1924 – December 15, 2015)

My grandparents
Fred and Sue Parman

Your love and support have been a blessing to me. Thank you.
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LIST OF ABBREVIATIONS

Å ..................Ångström
AEBSF ............Aminoethylbenzenesulfonylfluoride
BAP ...............Barrie-to-autointegration factor
bp .................Base pair
BrdU .............(5-Bromo-2′-deoxyuridine
°C ..................Degrees Celsius
CNS ...............Central nervous system
cm/mm..........Centimeter/millimeter
CMT2 ..........Charcot Marie Tooth disorder type 2
CMV ..........Cytomegalovirus
CRISPR/cas .....Clustered regularly interspaced palindromic repeats
CSIM ..........Cys-Ser-Ile-Met
C-terminus......Carboxyl terminus
DAPI ............4′, 6-diamidino-2-phenylindole
DECODE ........DECipherment Of DNA Elements database
DNA ...............Deoxyribonucleic acid
E2F1 ..............E2F Transcription factor 1
ECL ................Enhanced chemiluminescence
EDMD .............Emery-Dreifuss muscular dystrophy
FACS ............Fluorescence-activated cell sorting
FBS ...............Fetal bovine serum
FC-prelamin A. Farnesylated and carboxylated prelamin A

FPLD .................. Dunnigan-type familial partial lipodystrophy

g/mg/µg/ng .... Gram/milligram/microgram/nanogram

GADD45 .......... Growth arrest and DNA damage gene

Gcl ................. Germ-cell-less

H2A.Z .......... Histone family member Z

HEK293T .......... Human embryonic kidney cells 293, SV40 Large T-antigen

HGPS ............... Hutchinson Gilford progeria syndrome

HRP ............... Horseradish peroxidase

ICMT .............. Isoprenylcysteine methyl transferase

Ig-fold .......... Immunoglobulin fold

kDa .............. Kilodalton

L/ml/µl ............. Liter/milliliter/microliter

L647R .......... Leucine → Arginine mutation in Lamin A at amino acid residue #647

LAPs ............. Lamin-associated polypeptides

LARII ............... Luciferase assay reagent II

LB .................. Luria Bertani

LBR ................. Lamin B receptor

LH .................. Lumenal α-helices

LMNA .............. Lamin A gene
LMNA B1/2.....Lamin B genes

M/mM/µM.....Molar/millimolar/micromolar

MAD ............Mandibular acral dysplasia

MALDI-TOF.....Matrix-assisted laser desorption/ionization- Time of Flight

Mdm2 ..........E3 ubiquitin-protein ligase

MES ............2-N-Morpholino ethanesulfonic acid buffer

MHs .............Metalloprotease helices

mRNA ...........messenger RNA

NLS .............Nuclear localization signal

NP-40 ..........Nonidet P-40 detergent

p21 ..............Cyclin-dependent kinase inhibitor 1

PAGE ............Polyacrylamide gel electrophoresis

PBS .............Phosphate buffered saline

PCR .............Polymerase chain reaction

PIC .............Protease inhibitor cocktail

PLB .............Passive lysis buffer

PVDF ............Polyvinylidene fluoride

qPCR ............Quantitative PCR

Rb ...............Transcription factors retinoblastoma

Rce1 ..........Ras converting enzyme 1

RD ..............Restrictive dermopathy

RIPA ............Radio-immunoprecipitation assay buffer
RNA ..........Ribonucleic acid

rpm ..........Revolutions per minute

SDS-PAGE ........Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOC ..........Super optimal broth with catabolite repression

SREBP-1 ..........Sterol regulatory element binding protein

TBST ..........Tris buffered saline + 0.1% Tween 20

TCA ..........Trichloroacetic acid

TLC ..........Thin layer chromatography

TMH ..........Transmembrane helices

TPCK ..........N-tosyl-L-phenylalanine chloromethyl ketone

WI38 ..........Human lung diploid fibroblast cells

WT ..........Wild type

Zmpste24 ........Zinc metalloprotease Ste24
CHAPTER 1

INTRODUCTION

The Nuclear Lamins

There have been three different lamin genes identified in humans and other mammals: LMNA, LMNB1, and LMNB2. The proteins encoded by these genes constitute the type V intermediate filament proteins that are separated into two classes, A-type lamins and B-type lamins (Goldman et al. 2002). The A-type lamins: A, AΔ10, C, and C2 are spliced variants of the LMNA gene. B-type lamins: B1 and B2-B3 are coded for by two separate genes LMNB1 and LMNB2, respectively (Goldman et al. 2002).

There are two major A-type lamins (lamins A and C) found in mammals, which are produced by alternate splicing of the LMNA gene, and these will be the focus of this work. Lamin A is synthesized first as a precursor prelamin A, and after post-translational modifications it becomes mature lamin A. Both prelamin A and C have identical sequences for the first 566 amino acids then lamin A has a 90- amino acid extension at its carboxyl terminus (Goldman et al. 2002). Lamin C contains sequences from exons 1 to 10 and terminates with six amino acids that are not found in prelamin A (Lin and Worman 1993). Prelamin A has sequences from two additional exons (exons 11 and 12) and terminates with 98 different residues (Lin and Worman 1993).

The nuclear lamins have the tripartite structure of intermediate filament proteins that consists of a highly α-helical central rod domain flanked by a short globular amino-terminal “head” domain and a longer carboxy-terminal “tail” domain (Parry et al. 1986). The central rod
domain is made up of four sub-helical regions consisting of four heptad repeats designated as coil 1A, 1B, 2A, and 2B. Those individual coils are separated from each other by three short linker segments, L1, L12, and L2 (Parry et al. 1986). It appears that the head domain is unstructured, whereas the tail domain contains a highly conserved structural motif similar to a type S immunoglobulin fold (Ig-fold) (Dhe-Paganon et al. 2002; Krimm et al. 2002). All lamins have a nuclear localization signal (NLS) that is located between the carboxy-terminal end of the central rod domain and the Ig-fold, that is required for transport into the nucleus (Figure 1).

**Figure 1: Structure of nuclear lamins.** (A) Schematic drawing of prelamin polypeptide chain. The structure consists of a central α-helical rod domain (red), the NLS (gray), Ig-fold (blue), and the C-terminal –CAAX box (green). (B) Schematic drawings of the C-terminal domains of mature lamins A, C, B1, and B2. Adapted from (Dechat et al. 2008).
Lamin Processing Pathway

The post-translational processing pathway of prelamin A was previously studied by Dr. Michael Sinensky’s laboratory, resulting in the description of a unique set of post translational modifications prior to assembly into the nuclear lamina, out of the seven isoforms of lamin molecules (Sinensky et al. 1994). Before full maturation, lamin A is synthesized as a 74kDa precursor, prelamin A (Figure 2). Both lamin A and lamin B possess a canonical CAAX box (C= cysteine, A= aliphatic amino acid, X= serine, glutamine, methionine, or alanine) at the carboxyl-terminus of the molecule. This CAAX box sequentially undergoes a series of three modifications in both lamin A and B (Sinensky et al. 1994). The first step in processing is the addition of a farnesyl group to the cysteine of the CAAX box, accomplished by a farnesyl protein transferase. Next, the zinc metalloprotease zmpste24 performs an endoproteolytic cleavage termed “AAXing,” which removes the –AAX residues from the C-terminus of prelamin A. Lastly, there is carboxymethylation of the newly exposed cysteine carboxyl group by means of an isoprenylcysteine methyl transferase (ICMT) (Sinensky et al. 1994)(Figure 2). Subsequent modification to lamin A, includes a second endoproteolytic cleavage that removes the post-AAX-processed 15 C-terminal amino acid residues (aa 647-661), which includes the farnesylated cysteine. This second endoproteolytic cleavage is unique to lamin A in higher vertebrates and is specifically accomplished by the zinc metalloprotease zmpste24, which releases mature lamin A (Kilic et al. 1997). Mature lamin A is found at the nuclear lamina and in the nuclear interior (Dobrzynska et al. 2016). Since B type lamins do not undergo the second endoproteolytic cleavage by zmpste24 they are permanently farnesylated. Also, Lamin C does not possess a CAAX box, and therefore is not farnesylated or methylated as previously described.
Figure 2: The maturation pathway of lamins. 1) Farnesyltransferase adds a farnesyl group to the cysteine of the –CAAX box of prelamin A, prelamin B1, and prelamin B2. 2) The last 3 residues (–AAX) are removed by an AAX endopeptidase 3) a carboxylmethyltransferase enzyme methylates the exposed terminal cysteine. 4) In prelamin A, Zmpste24/FACE1 cleaves the terminal 15 amino acids with the farnesyl groups attached. This last cleavage step does not occur in B-type lamins, leaving them permanently farnesylated and carboxymethylated. Adapted from (Dechat et al. 2010).

Lamins: Function

The nuclear envelope is a double membrane structure that maintains the boundaries of the nucleus of a cell. The inner membrane of the nuclear envelope is composed of a complex network of intermediate filament proteins known as the lamins (Aebi et al. 1986). The lamins provide a structural network on the inner surface of the nuclear envelope to provide
attachment for various transmembrane proteins, organization of chromatin, transcription machinery, and distribution of nuclear pore complexes (Goldman et al. 2002; Dobrzynska et al. 2016).

A-type lamins are expressed mainly in highly differentiated cells and are soluble during the disassembly of the nuclear envelope that occurs during mitosis. Whereas, B-type lamins are expressed in all cell types and remain associated with nuclear membrane vesicles during lamina depolymerization and breakdown of the nucleus during mitosis (Goldman et al. 2002). Due to the various roles that A-type lamins play in the cellular functions, there is no wonder that they have been linked to various diseases referred to laminopathies; A-type lamins are also the main focus of this body of work. After the prelamin A has been fully processed into mature lamin A, multimeric polymerization with lamin B assists with the maintenance of the meshwork that lamin A forms on the inner layer of the nuclear membrane. Lamin A protein polymers also permeate the cell nucleoplasm contributing to nuclear integrity, shape, nuclear organization, and nuclear stability (Figure 3) (Goldman et al. 2002; Lammerding et al. 2004). Studies have shown that cells that are deficient in lamin A have a fragile nuclear membrane, therefore making them more subject to mechanical rupture or more likely to have nuclear membrane deformations such as blebbing and invaginations (Broers et al. 1997; Gruenbaum et al. 2000; Shumaker 2003; Bridger et al. 2007). In addition, there have been some studies that propose that mutations in lamin A have resulted in cell death due to the rupture of the fragile nucleus, because of either impaired access to membrane binding receptor proteins, abnormal placement or stability of nuclear pores, or altered membrane transport; any of which would
compromise the structure and integrity of the nuclear membrane (Hutchison 2002; Lammerding et al. 2006).

**Figure 3: Interactions of lamins with inner nuclear membrane proteins.** Lamin-binding proteins located in the INM, on chromatin, and in the nucleoplasm are thought to have mechanical and structural roles. The red lines are illustrations of lamins concentrated in the inner nuclear membrane and dispersed in the nucleoplasm (Adapted from Goldman et al. 2002).

Lamin A also plays a critical role in the functional control of many cellular processes including transcription. The spatiotemporal organization of lamin A allows them to participate
in maintaining cellular homeostasis. A-type lamins have been reported to associate retinoblastoma protein (Rb) (Ozaki et al. 1994), sterol response element binding protein 1 (SREPB1) (Lloyd et al. 2002), and zinc finger protein 239 (MOK2) (Dreuillet et al. 2002), thus affecting their expression levels. This occurs by preventing ubiquitination and subsequent proteasomal degradation of the bound proteins. It’s evident that if the structural integrity of the nucleus is compromised it could contribute to the dysfunction of these cellular processes; however, evidence has shown that these interactions are defective even in cases where the mutations in LMNA do not alter the structure of the nuclear envelope. Hence, disorders that have been related to lamin A dysfunction could not be attributed solely to nuclear integrity, but instead, to the control of the functions that depend on interactions with lamin A, independent of the structural maintenance of the nucleus (Gruenbaum et al. 2000; Hutchison 2002; Hutchison and Worman 2004; Lammerding et al. 2006; Bridger et al. 2007).

Several studies have shown that A-type lamins contribute to regulation of cell proliferation and tissue homeostasis (Chen et al. 2009; Naetar and Foisner 2009). A-type lamins are soluble during the disassembly of the nuclear envelope during mitosis (Gerace and Blobel 1980; Lutz et al. 1992; Goldman et al. 2002); however, unprocessed prelamin A cannot assemble into the lamina (Holtz et al. 1989; Lutz et al. 1992). These findings are consistent with the hypothesis that the carboxyl terminal tail of prelamin A is inhibitory for assembly of lamin A into the lamina and that the farnesylation-dependent cleavage of prelamin A allows assembly lamin A into the lamina to continue. Unpublished data from our lab showed that the 2KDa, 15aa peptide released in the processing of lamin A is rapidly degraded in cells. Microinjection of the same 2kDa prelamin A peptide (chemically synthesized) to cultured fibroblasts produced no
apparent effects. With these negative findings, there is a possibility that farnesylated and carboxymethylated prelamin A (FC-prelamin A) has a biological effect. Studies relating to the laminopathies have given some new insight into the possible function for FC-prelamin A (Davies et al. 2011).

**ZmpSte24 Crystal Structure**

ZmpSte24 is an inner nuclear membrane zinc metalloprotease that is essential to the maturation of lamin A. Zmpste24 is also known as farnesylated-protein converting enzyme-1 (FACE-1, Hs Ste24) and its crystal structure has recently been described by Quigley et al. 2013. The 3.4 angstrom (Å) resolution structure was determined from protein purified in two different detergent-lipid systems. This gave two independent crystal packing arrangements each with four copies of the structure in the asymmetric unit. The 3.4 Å structure was solved to help understand how zmpste24 cleaves prelamin A and how mutations in zmpste24 cause laminopathies (Quigley et al. 2013).

Zmpste24 has a seven transmembrane α-helical barrel structure that encloses a rather large chamber, and spans the membrane (Quigley et al. 2013)(Figure 4, A and B). The seven transmembrane helices (TMH) are packed in an antiparallel α-helical bundle, with the zinc metalloprotease fold inserted between TMH5 and TMH6. The nucleoplasmic side of the membrane is sealed by an M48 zinc metalloprotease domain, while the endoplasmic reticulum lumenal face of the membrane, and is sealed by three lumenal α-helices (LH), LH1, LH2, and TMH7A. There is access to the chamber from the nucleoplasm and the membrane by four fenestrations (Quigley et al. 2013)(Figure 4C). Simulations using molecular dynamics of zmpste24 in lipid bilayers, showed that the structure is stable when the chamber is filled with
water. The interior of the chamber has a mixed polar-nonpolar character with a prominent charged patch between TMH1 and TMH7 and three hydrophobic patches on the adjacent wall between TMH4 and TMH5, around the extensive S$_1$’ and S$_2$’ substrate specificity sites, and on the endoplasmic reticulum lumenal face of the chamber (Quigley et al. 2013)(Figure 4C). A synthetic peptide substrate that matches the C-terminus of prelamin A was used to show that purified zmpste24 has the expected proteolytic activity at the second cleavage site (Quigley et al. 2013)(Figure 4D). These findings were consistent in that zmpste24 has a longer substrate with the farnesyl group attached to allow for proper alignment of the peptide in the active site. To confirm this model they used shorter peptides, less than 15 residues, that were cleaved incorrectly at sites adjacent to the expected cleavage sites (Quigley et al. 2013)(Figure 4, D to F).

The catalytic zinc ion is found on the nucleoplasmic side of the membrane at the apex of the chamber (Quigley et al. 2013)(Figure 5A). The zinc ion is coordinated by His$_{335}$ and His$_{339}$ from the HEXXH zinc metalloprotease motif (H$_{335}$ ELGH in zmpste24) and Glu$_{415}$ from TMH7 (Quigley et al. 2013)(Figure 5A). Glu$_{336}$ from the HEXXH motif is predicted to be the catalytic residue, that activates a catalytic water molecule to attack the substrate peptide bond (Quigley et al. 2013). Structural alignments predict that Asn$_{265}$ and His$_{459}$ would stabilize the transition state during catalysis (Quigley et al. 2013).

A potential substrate-binding site was inferred from a 3.8 Å structure of a complex between zmpste24 and Cys-Ser-Ile-Met (CSIM) tetrapeptide from the C-terminus of prelamin A (Quigley et al. 2013)(Figure 5, B and C). The peptide-binding groove lies between the Zn$_{2+}$ ion and strand β3 of the metalloprotease β sheet and potentially interacts with highly conserved
residues, such as Asn^{265} on β3, His^{459} on a loop between metalloprotease helices (MHs) MH5 and MH6, and Arg^{465} on MH6 (Quigley et al. 2013). The deep pocket that encompasses both the S₁’ and S₂’ subsites (Figure 5B), is lined by highly conserved hydrophobic residues contributed by MH3, MH4, MH6 (Quigley et al. 2013). Such hydrophobic pockets could dictate the specificity of substrate binding for residues adjacent to the cleavage sites (Quigley et al. 2013).

Figure 4: Structure and activity of the zmpste24 zinc metalloprotease. (A) Cartoon representation of structure, viewed in the plane of the membrane. The structure is colored from blue to red from N to C-terminus. The catalytic zinc and its coordinating residues are highlighted in magenta. Horizontal lines indicate the approximate extent of the lipid bilayer as observed in MD simulations. The nucleoplasmic-cytoplasmic and ER lumen sides of the membrane are indicated. (B) Topology diagram of the arrangement of TMHs, colored as in (A). The metalloprotease fold is highlighted in pale blue. (C) Cut-away molecular surface representations highlighting the intramembrane chamber. The location of a
The fenestration between TMH5 and TMH6 in the chamber wall is marked with an asterisk. (D) ZmpSte24 is thought to cleave the C-terminus of prelamin A at the two sites indicated by black arrows. (E) Cleavage was assessed using synthetic peptides derived from the C-terminus of prelamin A with a mass spectrometric assay. Two peptides (26-amino acid peptide including fCys$_{661}$ and 11-amino acid peptide) probing the second cleavage site were cleaved between Tyr$_{646}$ and Leu$_{647}$, as expected. Additional cleavages were observed for the shorter peptide between Leu$_{647}$ and Leu$_{648}$ and Gly$_{649}$. (F) Two 9-amino acid peptides, with and without an fCys$_{661}$ were unexpectedly cleaved between Ser$_{662}$ and Ile$_{663}$. Green and blue triangles indicate expected and additional cleavages, with light colors for low-abundance reactions. Used with permission from Andrew Quigley et al. Science 2013; 339:1604-1607.

**Figure 5: Active site of zmpste24.** (A) Detailed view of the catalytic zinc-binding site. The active site is viewed looking from the ER lumen side of the membrane toward the nucleoplasmic end of the intramembrane chamber. Side chains are shown for highly conserved residues involved in zinc coordination, substrate binding, and catalysis. Molecular surface representation (B) and view of the active site (C) with a model of the prelamin A C-terminus bound in the active site, viewed from inside the chamber. Peptide carbons are colored green. (B) Is colored as for Fig. 4C and (C) as for (A). The fenestration, which could act as the entry and exit route for the prelamin A C-terminus, is marked with an asterisk, and the potential exit route for the SIM product is shown with a black triangle. The expected
The prelamin A substrate could enter the chamber via one of the four fenestrations in the chamber wall (Quigley et al. 2013)(Figure 4C). Since the chamber is too small to accommodate the whole 74-kD prelamin A, the unstructured C-terminus would insert into chamber, entering and exiting by the same route (Quigley et al. 2013). The most likely entry route is the fenestration between TMH5, TMH6, and the β hairpin connecting β3 and β4 (Quigley et al. 2013)(Figure 4C, 5B and 6A).

It connects the chamber with the nucleoplasm and the inner leaflet of the inner nuclear membrane, which are the locations of the protein and farnesyl moieties of the substrate (Quigley et al. 2013)(Figure 6, A and B). By using this entry route it would allow for correct alignment of the prelamin A C-terminus with the active site of zmpste24 for cleavage of the SIM sequence (Quigley et al. 2013). After cleavage, the tripeptide product would be able to exit via a fenestration between TMHs 1 and 2 (Quigley et al. 2013)(Figure 5B). The partially processed prelamin A C-terminus would then completely leave the chamber or project into the membrane through one of the fenestrations, which allows for carboxymethylation by ICMT, before prelamin A reenters the chamber (Quigley et al. 2013). The second cleavage site is 15 residues from the farnesylated Cys^{661} at the C terminus. There is sufficient space in the chamber for the additional residues and possibly a binding site for the farnesyl group at the endoplasmic reticulum lumen end of the chamber, which aligns the substrate for the second cleavage (Quigley et al. 2013)(Figure 6B). The C-terminus of lamin A would then leave the chamber as soluble nucleoplasmic lamin A (Quigley et al. 2013). Quigley et al. also showed the location of
the major laminopathy-causing mutations in zmpste24 as related to its structure (Figure 7) (Quigley et al. 2013). The crystal structures of zmpste24 provide a structural basis for protease-associated laminopathies; however, there is still much to be learned about this protein.

Figure 6: Proposed substrate entry route for the C-terminus of prelamin A and scheme for the reactions performed by zmpste24. (A) The fenestration between TMH5 and TMH6, below the β3-β4 hairpin is likely to be the entry route for the substrate. It provides a direct route into the active site, such that the C-terminus of prelamin A would be aligned with the peptide binding site identified in our protein-peptide complex. The 3D modelled peptide is shown with the carbon atoms in green. (B) Scheme for the reactions performed by zmpste24. The C-terminus of prelamin A is colored as in Fig. 3D, with the farnesyl group shown in pale green and the residues preceding the cleavage sites highlighted. Steps in the reaction scheme are numbered. Used with permission from Andrew Quigley et al. Science 2013; 339:1604-1607.
Figure 7: Laminopathy-causing mutations in zmpste24. Six mutations in zmpste24 lead to reduced enzyme activity and varying severity of disease. (A) Five of these mutations (N265S, L438F, L462R, W340R, and P248L, red spheres) lie in the zinc metalloprotease domain (gold) and one (L94P) is found in the ER lumen end of the chamber. The model for the bound CSIM tetrapeptide is shown in green. (B) Three mutations (N265S, L438F, and L462R) cluster around the substrate-binding or active site, and two MAD-B mutation sites (W340R and P248L) are located near the β3-β4 hairpin adjacent to the proposed substrate entry fenestration. (C) The L94P mutation lies at the elbow between TMH2 and LH1. These two helices, together with TMH3 pack together to form a fenestration, adjacent to Phe133, which links the chamber to the lipid bilayer. Mutated residues are shown as red sticks with semi-transparent molecular surfaces (B and C). Used with permission from Andrew Quigley et al. Science 2013; 339:1604-1607.

Laminopathies

Laminopathies are diseases that result from mutations in the lamins or the genes that encode the proteins that are responsible for lamin processing. The laminopathies are connected because they are all linked to very rare premature aging syndromes (Worman and Bonne 2007). Mutations in the LMNA gene are responsible for at least a dozen different
disorders, although there have also been a couple of disorders associated with the genes that encode for the B-type lamins (Worman et al. 2010). Laminopathies are classified into three different categories; those that affect striated muscle; adipose tissue; and diseases that involve peripheral neuropathy (Broers et al. 2004).

The first laminopathy identified was Emery-Dreifuss muscular dystrophy (EDMD), which occurs in two forms and is inherited in either an X-linked or autosomal dominant or recessive manner (Bione et al. 1994; Worman et al. 2009). The first manifestation of symptoms begins in the first decade of life with contractures of the elbows, ankles, and posterior neck. During the second decade of life, there is a slow progressive muscle weakening and wasting. The most significant of the EDMD symptoms is cardiomyopathy, which occurs in all cases and presents initially as a block in atrioventricular conduction followed by left ventricular dilation and congestive heart failure (Bione et al. 1994; Worman et al. 2009).

Dunnigan-type familial partial lipodystrophies (FPLD and mandibular acral dysplasia (MAD) are disorders that involve peripheral loss of adipose tissue, which leads to severe insulin resistance (Broers et al. 2004; Worman et al. 2009). At birth, patients have normal fat distribution, but at puberty they begin to lose subcutaneous adipose tissue in the extremities, which give them the appearance of a muscular physique; this is accompanied by an increase in adipose tissue in the face and neck (Broers et al. 2004; Worman et al. 2009). Additional phenotypes for MAD are typical of premature aging and include hypoplastic mandibles with severe dental crowding, short stature, and alopecia (Cao and Hegele 2000; Shackleton et al. 2000; Novelli et al. 2002).
Charcot Marie Tooth disorder type 2 (CMT2) is caused by a LMNA mutation that affects peripheral nerves. Patients affected by CMT2 have muscle weakness and wasting of the lower limb muscles (Worman et al. 2009). The velocity of motor nerve conduction is normal or slightly reduced due to axonal neuropathy (Worman et al. 2009). CMT2 patients will also sometimes exhibit muscular dystrophy and/or cardiomyopathy (Benedetti et al. 2005; Walter et al. 2005).

Hutchinson-Gilford progeria syndrome (HGPS) is a rare, autosomal dominant, fatal, progressive premature aging syndrome (Olive et al. 2010). Most HGPS cases are caused by a single de novo nucleotide substitution at position 1824 (C→T) in exon 11 of the LMNA gene. This mutation is also denoted as G608G because the encoded amino acid for codon 608 remains glycine (Eriksson et al. 2003; De Sandre-Giovannoli et al. 2003; Varga et al. 2006). This mutation creates a cryptic splice site, which deletes 50 amino acids (LAΔ50) (Eriksson et al. 2003; Corrigan et al. 2005; Shumaker et al. 2006; Dechat et al. 2010). In this deletion the second endoproteolytic cleavage site, in the prelamin A processing pathway, that is recognized by zmpste24 is deleted, thus preventing the release of mature lamin A. The resulting mRNA transcript codes for a mutant lamin A protein called progerin (Shumaker et al. 2006). Death of HGPS patients occurs at a mean age of 13 years because of myocardial infarction or stroke due to atherosclerosis (Olive et al. 2010). Currently, there is no cure for patients with HGPS.

A loss of zmpste24 leading to the accumulation of farnesylated prelamin A has been associated with the lethal disorder Restrictive Dermopathy (RD) (Worman et al. 2009). It was originally believed that RD was caused by a heterozygous mutation of zmpste24 in association with a mutation in another gene, but ultimately it has been shown that RD results from
homozygous loss of zmpste24 (Navarro et al. 2005). RD is characterized by tight translucent skin, slow growth during gestation, severe contractures of the joints, craniofacial abnormalities, epidermal hyperkeratosis, and death following birth due to pulmonary hypoplasia and restricted movement (Navarro et al. 2005). MAD has also been described as a milder form of RD, due to similarities associated with the accumulation of prelamin A (Agarwal et al. 2003).

Thus far, there have only been two disorders associated with the genes that encode for the B-type lamins. The first is an LMNB1 duplication with adult onset autosomal dominant leukodystrophy, which is a very slow and progressive fatal disorder characterized by pyramidal and cerebellar dysfunction along with symmetrical demyelination of the central nervous system (CNS) (Padiath et al. 2006; Worman and Bonne 2007). The other disease that has been linked to B-type lamins is a mostly sporadic acquired form of progressive lipodystrophy, called Barraquer-Simons syndrome (Worman and Bonne 2007). There is little data in how mutations in the genes encoding B-type lamins cause disease (Worman and Bonne 2007; Worman et al. 2009)
Aims of This Study

A long standing goal of our laboratory has been to understand the physiological significance of the prelamin A processing pathway. Studies on cultured fibroblasts from patients with HGPS and RD have provided clues as to the function of this processing pathway: expression of farnesylated and carboxymethylated prelamin A results in a gain of function and that function manifests itself in replicating cells in chromatin re-organization and a concomitant cellular senescence. Other literature on prelamin A and cell proliferation also indicates a role for its accumulation in quiescence. We propose that zmpste24 is down regulated in quiescent cells, resulting in the accumulation of FC-prelamin A and that down-regulation of zmpste24 serves to produce and/or maintain the quiescent state. We also propose that persistent accumulation of FC-prelamin A, in response to the down-regulation of zmpste24 or by other means, facilitates senescence by chromatin reorganization. We want to better understand the normal functions of FC-prelamin A, in particular how it relates to cellular quiescence and senescence. To accomplish this goal, we attempted to characterize the promoter and transcription factors regulating the expression of zmpste24 as well as identify and characterize specific FC-prelamin A interacting partners.
CHAPTER 2

MATERIALS AND METHODS

Mammalian cell culture

Various cell lines were used in these studies including: HeLa cells, BJ fibroblasts, WI-38 fibroblasts, HEK293T cells, LMNA cells, and L647R cells. All cell lines were maintained in a humidified incubator at 37°C with 5% or 10% CO₂, per cell requirements.

HeLa cells, BJ fibroblasts, WI-38 fibroblasts, and HEK293T cells were all cultured using standard Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% FBS and 1% Penicillin/Streptomycin antibiotics. Rheoswitch® cells expressing various lamin A protein chimeras were cultured in DMEM, supplemented with 10% heat inactivated FBS, 2.5 ml Hygromycin, 4 ml of G418 antibiotics, and 1% Penicillin/Streptomycin.

Transient Transfection

Two different methods were used to introduce plasmid DNA into mammalian cells. MegaTran 1.0 (Origene Technologies, Rockville, MD) is a non-lipid polymer-based reagent that provides high efficiency and low toxicity for transfected cells. Typically cells were plated in 35 mm or 60 mm plates for transfection and grown until cell density was approximately 60-70% confluent. Depending on the plate size used, 2-8 µg of DNA was transfected. Plasmids were diluted in 100 µl of DMEM without antibiotics and vortexed gently. MegaTran 1.0 reagent was added to the diluted DNA at a 3:1 ratio, then vortexed gently for 10 seconds and incubated at room temperature for ten minutes. Culture media was removed and the cells were washed with PBS, and refed with fresh culture media and the MegaTran 1.0/DNA mixture was added to
the culture plates. Generally the MegaTran 1.0/DNA mixture represented ~10% of the total volume of the culture media. The plates were gently rocked to ensure even distribution of the complexes and then incubated at 37°C for 24-48 hours. If cells were incubated for 48 hours, the media was replaced with fresh complete growth media 24 hours after the transfection.

Magnetofection™ Polymag reagent (OzBiosciences, France) was also used for some of the transient transfections. The Magnetofection™ Polymag reagent uses microscopic magnetic particles that adhere to the DNA. The DNA/magnetic particle suspension is added to adherent cells at 60-90% confluency at the time of transfection. The culture plates are then placed on a magnet for approximately 20 minutes to force the microscopic magnetic/DNA coated particles through the plasma membranes of the cells.

**DNA Digestion with Restriction Enzymes**

Plasmid DNA and insert DNA of interest were digested with corresponding restriction enzymes according to manufacturer’s protocol. Typically, 5 µg of DNA was digested using 5 units of restriction enzyme in 20-50 µl of 1X digestion buffer for one hour at 37°C.

**DNA Gel Electrophoresis**

The results of restriction digests were checked by DNA gel electrophoresis using 0.6% agarose gels made with 1X Tris-borate EDTA buffer (TBE) ran at 100V for 90 minutes. Ethidium bromide was added to the gel to a final concentration of 5 µg/ml for visualization of the DNA fragments. DNA fragments were observed using an ultraviolet (UV) light box.
**DNA Purification from Agarose**

Following DNA gel electrophoresis, regions of the gel containing fragments of interest were excised from the gel and weighed. The DNA was extracted from the gel using a QIAquick Gel Extraction kit according to the manufacturer’s instructions (Qiagen). Briefly, the gel slice was dissolved in solubilization buffer by incubating at 50°C for 10 minutes with vortexing every 2-3 minutes. Isopropanol was then added and the sample was transferred to a QIAquick spin column (Qiagen) and centrifuged for one minute. The column was washed twice using wash buffer and the DNA was eluted using 50 µl of elution buffer and centrifuging for one minute. The concentration of DNA was determined by spectral analysis using the NanoDrop™ 2000 (Thermo Fischer Scientific).

**DNA Ligation**

After restriction digest the plasmid vector of interest was treated with calf intestinal alkaline phosphatase (CIP) to dephosphorylate the 5’-ends of the DNA to maintain linearization. For the ligations, T4 DNA Ligase (Promega) was used according to the manufacturer’s protocol. Using a 3:1 molar ratio of vector: insert DNA all ligation reactions were carried out overnight at 4°C. After ligation the new constructs were transformed into bacterial cells using either DH5α™ competent cells (Invitrogen/Life Technologies) or SURE® competent cells (Stratagene/Agilent Technologies).

**DNA Transformation**

Transformation of plasmid DNA into cells was completed using one of two types of cells; DH5α™ Competent Cells (Invitrogen/Life Technologies) or SURE® Competent Cells (Stratagene/Agilent Technologies). For transformations using the DH5α™ Competent Cells,
after cells were thawed on ice; 5 ng of plasmid DNA was added to 50 µl aliquots and mixed gently, incubated on ice for 30 minutes, and then heat shocked for 20 seconds in a 42°C water bath without shaking. Cells were placed back on ice for two minutes and 950 µl of pre-warmed SOC media (Super optimal broth with catabolite repression) was added to each tube. Tubes were incubated at 37°C for one hour with shaking at 225 rpm to promote growth. After incubation 50-100 µl of each transformation was plated on selective plates containing 100 µg/ml of ampicillin and incubated overnight at 37°C. pUC19 control DNA was used as a positive control for the transformation, which was provided by the company.

For transformations using SURE® Competent Cells a 14 ml Falcon polypropylene tube was pre-chilled on ice and 100 µl of cells were added to each tube along with 1.7 µl of β-mercaptoethanol. The tubes were swirled gently to mix and incubated on ice for ten minutes with swirling every two minutes. Next, 20 ng of plasmid DNA was added the incubation continued for 30 minutes. The cells were then heat shocked in a 42°C water bath for 45 seconds, placed back on ice for two minutes before the addition of 0.9 ml of pre-warmed SOC media. The cells were then incubated at 37°C for one hour with shaking at 225-250 rpm. 100 µl of the transformation mixture was spread on selective LB agar plates containing 100 µg/ml of ampicillin and incubated overnight at 37°C. For all transformations, pUC18 plasmid provided with the competent cells, was used as a positive control.

**DNA Isolation**

For plasmid DNA purification, Wizard® Plus Midipreps DNA Purification System (Promega) was used. After transformation, isolated colonies were selected and added to 100 ml of LB broth containing 100 µg/ml of ampicillin and incubated overnight at 37°C with shaking...
at 225-250 rpm. Cultures were pelleted by centrifugation at 10,000 x g for ten minutes at 4°C. The supernatant was removed and excess liquid was allowed to drain off. Cell pellets were completely resuspended in 3 ml of cell resuspension solution and 3 ml of cell lysis solution was added and mixed by inversion. Neutralization solution (3ml) was added and mixed by inversion then centrifuged at 14,000 x g for 15 minutes at 4°C. The supernatant was carefully removed and transferred into a new centrifuge tube. Wizard® Midipreps DNA Purification Resin was added to the DNA solution and mixed. The DNA/resin mixture was transferred to Midicolumn and a vacuum was applied to pull the mixture into the Midicolumn. The column was washed with 40% isopropanol and column wash solution using a vacuum to pull the solution through the Midicolumn. The DNA was eluted using 300µl of nuclease free water that had been preheated to 70°C. DNA concentration was determined by spectral analysis using the NanoDrop™ 2000 (Thermo Fischer Scientific). DNA was stored at -20°C until use.

**Site-directed Mutagenesis**

The (His)₆-WT zmpste24 in pBacpak8 was used as the template for site-directed mutagenesis using the Stratagene QuickChange® Lightning Site Directed Mutagenesis Kit (Stratagene/Agilent Technologies). Six mutants were generated using this template: ΔHEXXH, H335A, Y379Stop, L290Stop, P230Stop, and C109Stop. According to the manufacturer’s instructions for the QuickChange PCR reaction, primers were used to insert the mutant sequences into the WT and are listed below; anti-sense primers were also designed for each mutant to correspond with the reverse complement for each one. The mutants were transformed into bacterial cells after PCR was complete, colonies selected, grown overnight and DNA was isolated as described previously.
Immunofluorescence

Cells were grown on glass coverslips overnight. After washing with PBS, cells were fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.3. Cells were permeabilized with 0.2% Triton X-100 in PBS for five minutes on ice, washed with PBS and blocked with 10% BSA in PBS on ice for 5 minutes. Coverslips were incubated for one hour at room temperature with primary antibody. Then cells were washed and incubated with the secondary antibody that was fluorophore conjugated at room temperature for one hour. Cells were washed three times with PBS and mounted to glass slides using an anti-fade mounting solution containing DAPI (Prolong). Images were acquired by digital deconvolution of 10-slice stacks using a Nikon Diaphot 200 microscope equipped with a Retiga2000 cooled CCD digital camera, Oncor Z-drive and Oncor Image Software.

Base Release Assay

Cells (1 x 10⁷) were labeled overnight with [methyl-³H] methionine (100 μCi/ml) (the ³H-methyl group will be incorporated into S-adenosyl methionine (The methyl donor on the
carboxymethylation step) and will also label all the remaining methionines in proteins. Prelamin A is isolated by immunoprecipitation with prelamin A specific antibody (α-PreA) followed by separation on SDS-PAGE. Lamin B is immunoprecipitated with commercially available antibody (Santa Cruz) as a control for a 100% carboxymethylation. Proteins were visualized by fluorography and the corresponding bands for proteins of interest are cut from the dried gel, placed into 0.5 ml microfuge tubes, and then rehydrated with 100 μl of distilled water. 200 μl of 1 M NaOH is added to the tubes and incubated at 37° for 24h in capped parafilm-sealed scintillation vials containing 2.5 ml of scintillation fluid. The base releasable counts (tritiated methanol) are trapped in the scintillation fluid. The gel slices are recovered and dissolved in Perchloric acid-H₂O₂ and counted. These counts represent the non-releasable counts. The ratio of counts released to counts retained are normalized relative to the number of methionines in the protein and expressed as a fraction of the normalized result for lamin B.

**ZmpSte24 Endoprotease Assay**

ZmpSte24 activity was assayed using nuclei from HeLa cells as the source of enzyme activity as described by Kilic et al. 1997, with the exception that we used the hexapeptide RSY*LLG, where Y* is a ¹⁴C-labeled tyrosine, as the substrate. Cells were collected by trypsinization and washed twice with ice-cold PBS. Cell pellets were resuspended to a final density of 4 x 10⁸ cells/ml in a lysis buffer (0.01 M Tris-HCl, pH 7.0, 0.01 M NaCl, 3 mM MgCl₂, 0.4% Nonidet P-40) (Kilic et al. 1997). Nuclei were washed two more times in the same buffer, isolated and then pelleted by centrifugation in a Beckman J-14 rotor for ten minutes at 365 x g. The nuclei were resuspended in the same buffer without the Nonidet P-40 and assayed. The Qubit® Protein Assay Kit was used to obtain the protein concentration. ¹⁴C-labeled peptide
(RSY*LLG final concentration of 5µM) was used to initiate the endoprotease reaction, leaving the nuclear preparation in a final volume of 200 µl in 10 mM MES, pH 6.0. The reaction was run for various periods of time at 37°C, then stopped by the addition of 5 µl of glacial acetic acid and chilled on ice for ten minutes. The reaction mix was cleared by centrifugation at 2,000 rpm for ten minutes using an Eppendorf centrifuge. The supernatant was collected and lyophilized, the RSY* residue was resuspended in 25 µl of water and isolated by reverse phase thin layer chromatography (TLC) (Analtech, Inc. Newark, DE). TLC plates were developed in 10% acetonitrile in water, and the spots were visualized by autoradiography. A synthetic, ^14C-RSY* peptide standard was run on each plate to aid in the identification of the expected product. The amount of labeled RSY formed in the assay was determined by scraping the appropriate spots into tubes and quantified by scintillation counting.

**Cell Proliferation Assay Using[^3H]-Thymidine**

DNA synthesis was examined by pulse labeling for 30min with[^3H]-Thymidine at various time points. Cells were washed two times with cold PBS, collected by scraping into 1 ml of PBS, homogenized by sonication, and a 0.2 ml aliquot was combined with 1 ml of cold trichloroacetic acid (TCA). The suspension was filtered to remove all insoluble material and washed with cold TCA. The amount of radioactivity that was incorporated was measured by scintillation counting.

**Cell Proliferation Assay Using BrdU**

Cells were seeded in triplicate at 4 x 10^3 cells/well in a 96-well plate or on glass coverslips and incubated overnight. Genostat inducer at concentrations of 0 nM, 200 nM, 400 nM, 600 nM, and 800 nM were used to treat cells for 48 hours, DMSO was used as a control.
Plates were incubated for four hours in the presence of 10 µM BrdU (5-Bromo-2’-deoxyuridine), then incubated for one hour with a monoclonal anti-BrdU antibody (1/100), and washed three times with TBST. Cells were then incubated with an anti-mouse IgG FITC conjugated secondary antibody for an hour. For the 96-well plates a plate reader was used to measure absorbance at 450 nm. Coverslips were mounted in an anti-fade mounting solution containing DAPI (Prolong). Samples were analyzed by fluorescence microscopy, and the number of BrdU positive cells were counted in twenty 40X fields per treatment/time point. The amount of BrdU that was incorporated into the cells was quantified, thus indicating the amount of cell proliferation taking place.

Induction of Quiescence

HeLa, WI-38 and BJ cells used in serum starvation experiments were maintained in normal growth medium containing 10% FBS until time for use, then washed with PBS. The medium was then replaced with medium containing 0.5% FBS. Cells were collected at the various time points as stated in the results after the serum removal. Contact inhibition studies used cells that were plated at 2.5 x 10^3 cells/cm² or at 1 x 10^5 cells/cm² and grown for the times indicated in the results.

Co-immunoprecipitation - Mass Spectrophotometry Analysis

Twenty-five micrograms of affinity-purified rabbit anti-prelamin A (Sinensky et al. 1994), monoclonal anti-Lamin A/C (clone 4C11, Sigma-Aldrich) or control rabbit antibodies were covalently linked to 50 µl of packed Affiprep protein A beads (Bio-Rad Laboratories), pre-eluted with glycine (Moritz et al. 1998), incubated with 700-1000 µL of fresh whole cell extract at 4°C for 1 h, washed five times with lysis buffer I (50 mM HEPES at pH 7.5, 100 mM KCl, 1 mM MgCl₂,
1 mM EGTA, 10% glycerol, 0.05% NP-40, 1 mM DTT, protease inhibitors) and once with lysis buffer without NP-40, and eluted by heating at 50°C in 50 μL of Novex® Tris-Glycine SDS Sample Buffer (Thermo Fisher).

SDS-PAGE was used to separate and purify proteins, which were stained using Colloidal Coomassie Brilliant Blue or by silver staining to visualize the proteins so that the appropriate bands could be excised. Isolated proteins were then treated with iodoacetamide and digested with trypsin. Ms. Gawinowic at the Protein Chemistry Core Facility at Columbia University performed the MADLI-TOF with PerSeptive Voyager DE-RP mass spectrometer.

**Preparation of Cell Lysates**

Transfected cells were washed twice with room temperature PBS, trypsinized, collected and centrifuged for 3 minutes at 1500 rpm. The cell pellet was washed twice with ice-cold PBS and centrifuged again to re-pellet the cells. The pellet was resuspended in 0.6 ml of 0.4% SDS RIPA buffer with 1X Halt Protease and Phosphatase Inhibitor Cocktail. Cells were transferred to a new tube and incubated for 30 minutes on ice and vortexed every 10 minutes for 30 seconds. Samples were centrifuged at 10,000 x g for ten minutes at 4°C, and the supernatant was transferred to a new tube. If a cell pellet was still visible, additional lysis buffer, sonication and centrifugation steps were performed until the pellet was completely solubilized. Lysates were stored at -20°C until use and protein concentrations were measured using the Qubit® Protein Assay.
Protein Assay

Protein assays were performed using the Qubit® Fluorometer (Invitrogen/Life Technologies). Thin walled, clear, 0.5 ml PCR tubes that were provided in the kit were used for preparation of the standards and samples. The Qubit® working solution was prepared by diluting the Qubit® Protein reagent 1:200 in Qubit® Protein buffer; 200µl was prepared for each sample including standards. Each standard included 190 µl of Qubit® working solution and 10 µl of standard, then mixed by vortexing for 2-3 seconds. Qubit® working solution was added to the samples so that the final volume was 200 µl, then mixed by vortexing for 2-3 seconds. All tubes were incubated at room temperature for 15 minutes. Standards and samples were read using the Qubit® 2.0 Fluorometer by inserting each tube individually into the sample chamber. Results were displayed on the instrument screen and recorded.

Protein Electrophoresis and Transfer

One dimensional SDS-PAGE was performed to separate the proteins under reducing conditions on a 4-12% gradient Bis-Tris NuPAGE precast slab gel from Invitrogen (Carlsbad, CA). Electrophoresis was performed at 200 volts for 45 minutes to an hour using the Novex Surelock System from Invitrogen. Broad range pre-stained protein markers from Invitrogen were used to estimate the relative molecular masses of the proteins. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a dry electroblotter called the iBlot (Invitrogen/Life Technologies, Carlsbad, CA). The transfer was approximately seven minutes and was performed according the manufacturer’s instructions.
Western Blot

Following transfer to PVDF, membranes were blocked for 1 hour at room temperature or overnight at 4°C in 5% nonfat milk in tris-buffered saline containing 0.1% Tween 20 (1X-TBST). Membranes were washed four times for five minutes each using 1X-TBST and probed with the indicated primary antibodies using the dilutions specified by the manufacturer. Antibodies were diluted in 1X-TBST containing 5% nonfat milk for 1 hour a room temperature or overnight at 4°C. Membranes were washed as previously described and incubated with the indicated secondary antibody conjugated to a horseradish peroxidase (HRP) (Santa Cruz) at room temperature for 1 hour. After incubation with the secondary antibody, membranes were washed and antibody-bound proteins were detected using enhanced chemiluminescence reagent (ECL) from Pierce (Rockford, IL). ECL was added to cover the membrane and incubated for five minutes at room temperature. Excess ECL was removed and membranes were covered with a plastic film. ECL signal was visualized using the Fuji Film Imager LAS-4000.

Dual-Luciferase® Reporter Assay

HEK293T cells were plated at 700,000 cells/60 mm culture dish, grown overnight, and transfected as previously described using the MegaTran 1.0 reagent. The vectors used for the transfections were pGL4 Luciferase Reporter Vectors (Promega, Madison, WI), pGL4.27 [luc2P/minP/Hygro] and pGL4.77 [hRlucP/Hygro]. Vector pGL4.27 [luc2P/minP/Hygro] contained the sequence of interest (e.g. ccgggcccgaactcg). For some experiments, co-transfections were also performed using an E2F1 expression vector under the control of a CMV promoter (Invitrogen). 48 hours after transfection growth medium was removed and transfected cells were washed with phosphate buffered saline (PBS). PBS was completely
removed and 500µl of 1X passive lysis buffer (PLB) was added to each well of the culture plate. The culture plate was placed on an orbital shaker with gentle shaking for 15 minutes at room temperature and cells were scraped into a microfuge tube. Luciferase Assay Reagent II (LARII) and Stop & Glo® were prepared according to manufacturer’s instructions (Promega, Madison, WI). 20µl of cell lysate suspended in PLB was dispensed into a 96 well plate, followed by sequential auto-injection of LARII and Stop & Glo® Reagents. Plates were read using a Molecular Devices L Max Microplate Reader, Luminometer, with Softmax Pro software. The luminometer was programmed to perform a 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay.

**Chromatin Immunoprecipitation (ChIP) qPCR or RT2 Endpoint PCR.**

ChIP was accomplished using the EpiTect® Chip qPCR assay for human zmpste24 (Qiagen). Cells expressing uncleavable prelamin A (L647R) and cells expressing mature lamin A were plated and grown overnight to 70-85% confluency in a 10 cm culture dish. Medium was removed from cells and 10 ml of fresh fixing buffer consisting of 1% formaldehyde in PBS was added and incubated at 37°C for 10 minutes, fixing the cells in formaldehyde cross-links the DNA sequences of interest to prelamin A. 1.1 ml of Stop buffer (Qiagen) was added to each plate and swirled to mix. Plates were incubated for 5 minutes at room temperature. The Stop buffer was removed and cells were washed with 9 ml of ice-cold 1X PBS two times.

**Cell Harvesting.** Cells were harvested, by scraping, in 1.5 ml of ice-cold harvesting buffer, containing 3 ml of PBS and 15 µl of protease inhibitor cocktail (PIC). The supernatant was collected in a new tube and placed on ice. Collected supernatant was centrifuged at 800 x g for 10 minutes at 4°C. The harvesting and centrifugation steps were repeated twice to ensure
that all cells were collected. Pelleted cells were resuspended in 100 µl of lysis buffer (Qiagen) containing PIC, then incubated on ice for 10-15 minutes with mixing every 5 minutes. Cell lysate was sonicated to shear the DNA. The probe was inserted to a 2-3 mm depth in the lysate. The sonicator was set on Power: 0.5 W; Time: 2 seconds ON/ 15 seconds OFF; Total time: 16 seconds (8 times per round); 3 rounds. Lysates were mixed by pipetting up-and-down after each round to prevent precipitation before the next round of sonication. The lysate was centrifuged at 14,000 x g for 10 minutes at 4°C to pellet the debris. Supernatant was carefully removed and transferred to a new tube and labeled as ChIP Ready Chromatin.

**Pre-Clear and Immunoprecipitation.** For each sample 900 µl of IP Buffer A (Qiagen) containing 7.5 µl of PIC was added to 100 µl of ChIP Ready Chromatin, mixed and placed on ice. 50 µl of Protein A beads (Qiagen) were added and incubated for 50 minutes at 4°C with rotation. Samples were centrifuged at 4000 x g for 1 minute at 4°C, then placed in ice for 1 minute to allow the Protein A beads to settle. After the beads settled, supernatant was carefully removed and placed in a new ice-cold 5 ml tube. A 1 ml aliquot of the supernatant was removed for a negative control, positive control, and the antibody fraction. The antibodies prelamin A, mature lamin and H2A.Z were used. Fractions were incubated with their respective antibodies overnight at 4°C with rotation. For the immunoprecipitation, 60 µl of Protein A beads (Qiagen) were added to each fraction and incubated at 4°C for one hour with rotation, then centrifuged at 4000 x g for one minute at 4°C. Tubes were placed on ice for one minute to allow the Protein A beads to settle, then supernatant was removed and discarded. The bead pellet, containing the Protein A-antibody/chromatin complex was resuspended and washed in 1 ml each of the four cold wash buffers, supplied in the kit according the manufacturer’s
instructions (Qiagen). After each wash, samples were centrifuged briefly for one minute and supernatant was carefully removed.

**DNA Isolation and Purification.** To elute the DNA, 30 µl of elution buffer (Qiagen) containing 2 µl of ChIP-Grade Proteinase K was added directly to each sample and incubated with shaking at 500 rpm at 45°C for 30 minutes. 100 µl of DNA extraction beads (Qiagen) was added to each sample and vortexed for 10 seconds, then incubated with shaking at 95°C for 10 minutes, and centrifuged at 14,000 x g for one minute at room temperature. Without disturbing the pellet, the supernatant was removed and placed in a new tube. Another 100 µl of elution buffer was added to each sample, vortexed for 20 seconds, and centrifuged as before. The supernatant was once again removed and added to the previous tube for a total of 200 µl.

DNA was purified using the DNA Spin Columns provided (Qiagen). To each 200 µl sample, 400 µl of column binding buffer (Qiagen) was added followed by mixing. The samples were transferred to individual DNA spin columns, in a 2 ml collection tube, and centrifuged at 11,000 x g for one minute at room temperature. The flow-through material was discarded, spin column was washed with 600 µl of Column wash buffer (Qiagen), then centrifuged again. The flow-through material was once again discarded, the DNA spin column was centrifuged at 11,000 x g for 2 minutes to remove any residual wash buffer. The DNA spin column was placed in a clean elution tube, and 100 µl of Elution Buffer (Qiagen) was added to the center of the column membrane and incubated for 1 minute at room temperature. Spin columns were centrifuged for 1 minute at 11,000 x g at room temperature. The elution step was repeated with another 100 µl of elution buffer, to yield a final volume of 200 µl for each ChIP sample.
After purification of DNA, the samples were analyzed by Endpoint PCR using RT² Endpoint PCR Kit (SABioiences) or taken to the Molecular Biology Core Facility at James H. Quillen College of Medicine for quantitative analysis by qPCR.

**Cell Lines for Proximity Labeling Experiments**

To create the Lamin-biotin ligase fusion proteins, biotin ligase was sub-cloned from a pcDNA3.1 MCS-BirA (R118G)-HA plasmid donated by Kyle Roux from University of South Dakota. The biotin ligase (BL) fragment was excised and ligated in frame into pNEBR-X1, pNEBR-X1-GFP-lamin A, and pNEBR-X1-GFP-L647R prelamin A (all created by Christina Bridges in our laboratory) to create BL-GFP-Lamin chimeras. The pNEBR-X1 plasmids are part of the Rheoswitch® inducible expression system from New England Biolabs. The BL plasmids were purified and constructs confirmed by DNA sequencing.

Stable Rheoswitch® cell lines expressing BL-GFP-Lamin A and BL-GFP-L674R prelamin A were created by transfecting Rheoswitch® 3T3 cells using Mirus TransIT 3T3 reagent. After 24 h, cells were incubated with 800ng/ml GenoStat and 50 μM biotin. For three PBS washes, 1-2 x 10⁷ cells were lysed by sonication in 0.4% SDS RIPA buffer containing complete protease/phosphatase inhibitor cocktail. After initial sonication, Triton X-100 was added to a final concentration of 2%
and centrifuged at 15,000 x g. Biotinylated proteins were isolated from the supernatant by incubation with Streptavidin-Dynabeads (Invitrogen) for 16 h, beads were collected using a magnetic tube rack and washed three times in 2% SDS for 5 min. Beads were washed once with wash buffer I (0.1% deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA, and 50 mM Hepes, pH 7.5), once with wash buffer II (250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, and 10 mM Tris, pH 8.1) and twice with wash buffer III (50 mM Tris, pH 7.4, and 50 mM NaCl). An aliquot of the sample (10%) was reserved for Western blot analysis. Bound proteins were eluted from the beads with Novex® Tris-Glycine SDS Sample Buffer (Thermo Fisher) saturated with biotin and incubated for 10 min at 90°C. Eluted proteins were reduced, alkylated and subjected to SDS-PAGE. Protein-containing lanes were cut into 5 mm pieces and sent to AB Sciex (Farmington, CT) or Columbia University core facility for tryptic in-gel digestion and identification by LC-MS/MS mass spectrometry. Raw data were converted into mzdata.xml using MassHunter Qualitative Analysis software (Agilent Technologies) and database search was performed using MASCOT 3.2 (MatrixScience) and human IPI database.
CHAPTER 3

RESULTS

Unsuccessful Attempts to Prove a Two-active Site Hypothesis for Zmpste24

Prior results from our laboratory had indicated that the prelamin A endoprotease responsible for the second endoproteolytic cleavage was a chymotrypsin like enzyme with a serine active site (Kilic et al. 1999); rather than a Zn$^{2+}$ dependent metalloprotease (Schmidt et al. 2000). This conclusion was based on the use of protease inhibitors to inhibit zmpste24 activity in both the “AAXing” and the second cleavage reactions in the conversion of prelamin A to lamin A. In more recent studies from our laboratory, site directed mutagenesis was used to delete the HEXXH motif in zmpste24 (Corrigan 2005). These results showed that the HEXXH motif deletion in zmpste24 abolishes its ability to perform the first cleavage (AAXing) activity but not second cleavage activity. Furthermore, zmpste24 was tagged by incubation with a fluorescent analogue of the suicide inhibitor TPCK, (5(6)-carboxyfluoresceinyl-L- phenylalanine chloromethylketone or FFCK), which specifically inhibits chymotrypsin-like serine proteases by the same mechanism as TPCK, namely by alkylation of the active-site histidine. Purification of fluorescently tagged fragments and MALDI-TOF analysis demonstrated that zmpste24 was indeed covalently modified by FFCK suggesting the presence of a chymotrypsin-like active site somewhere in zmpste24 structure (Corrigan 2005).

In order to narrow down a region containing the active site, or at least a residue associated with the active site, Corrigan used site-directed mutagenesis to introduce premature stop codons at predetermined locations in zmpste24. He then expressed and purified the
truncated zmpste24s in *E. coli*, and measured their activity in an AAXing, and second cleavage reactions in an assay using a prelamin A carboxy terminal fragment as a substrate (Corrigan et al. 2005; Corrigan 2005). His findings identified one truncation, Y379Stop, which abolishes the AAXing activity, while leaving the second cleavage activity intact (Corrigan 2005).

Patients with MAD or RD have a mutation 1085insT in exon 9 that causes a frameshift and a premature termination codon resulting in the truncated protein, Phe361fsX379. This is similar to our Y379Stop mutant (Agarwal et al. 2003; Navarro et al. 2004; Navarro et al. 2005). The loss of AAXing activity correlates with the results observed by Agarwal et al., which show that the Phe361fsX379 mutant form of zmpste24 could not complement a Ste24 deletion mutant of yeast that is defective in the processing of its prenylated yeast substrate, a-factor (Agarwal et al. 2003). The results from this endoproteolytic assay on prelamin A, show the MAD and RD phenotype observed in patients is more than likely a result of the inability of zmpste24 to properly AAX the prelamin substrate. Additionally, in this truncated form of zmpste24 the HEXXH motif, located at residues 335-339, is retained, thus suggesting the possibility that another sequence C-terminal to the HEXXH motif is also needed for AAXing to occur. Alternatively, it is possible that the residues located C-terminal to the Y379STOP truncation are needed for proper substrate recognition and/or proper folding of zmpste24.

Based on all of these results, we decided to test the ability of four truncated forms of zmpste24 to rescue the wild-type proliferation phenotype of fibroblasts from RD patients or Zmpste24-KO mice. These fibroblasts develop premature senescence in culture. To this end, premature stop codons were inserted into the predicted cytosolic or luminal loop regions located outside the boundaries of the transmembrane regions (Figure 8). This placement
allowed for proper transmembrane insertion of predicted transmembrane helices located N-terminal to the stop codon.

**Figure 8. Location of Stop codons in zmpste24 truncations.** Above is the predicted transmembrane topology map for zmpste24, as predicted by the TMHMM Server v. 2.0 server (http://cbs.dtu.dk/services/TMHMM/). Shown is the positioning of the four amino acids that were mutated into stop codons (C109, P230, L290, and Y379).

Truncated zmpste24 constructs were created as described in Materials and Methods, and expressed in fibroblasts from zmpste24 knock out mice donated by Dr. Carlos Lopez-Otín and Ignacio Varela from Oviedo University, Spain. While in the process of characterizing the
created cell lines, Quigley et al. solved the 3.4Å structure of human zmpste24 to understand how this enzyme cleaves prelamin A and how various mutations in zmpste24 cause laminopathies. They were able to confirm and show the seven transmembrane α-helical barrel structure of zmpste24 and that it is surrounding a large, water-filled, intramembrane chamber, and capped by a zinc metalloprotease domain with the catalytic site facing into the chamber (Quigley et al. 2013). They used a synthetic peptide substrate that matched the C-terminus of prelamin A (Ser\textsuperscript{636} to fCys\textsuperscript{661}), to show that purified zmpste24 did perform the proteolysis at the second cleavage site (Figure 4D). This group also showed that the catalytic site of zmpste24 is found at the apex of the chamber on the nucleoplasmic side of the membrane (Figure 4A)(Quigley et al. 2013). The zinc ion is coordinated by His\textsuperscript{335} and His\textsuperscript{339} from the HEXXH zinc metalloprotease motif (H\textsuperscript{335}ELGH in zmpste24) and Glu\textsuperscript{415} from TMH7 (Figure 5A) (Quigley et al. 2013). The observed second cleavage accomplished by zmpste24 between Tyr\textsuperscript{646} and Leu\textsuperscript{647}, was executed as expected and previously described (Barrowman et al. 2012; Quigley et al. 2013).

With the construction of this zmpste24 model there was no evidence of a second active site mediated by a serine protease type of activity. Therefore, disproving our “two site” hypothesis. The second endoproteolytic cleavage of prelamin A is indeed accomplished by the zinc metalloprotease zmpste24. Explanation for the results obtained with serine-protease suicide inhibitors followed by mass spectrometry was later provided by several groups (Grabarek et al. 2002)(Grabarek and Darzynkiewicz 2002).
Expression of Type A Lamins in Quiescence and Senescence

It has been extensively studied that actively proliferating cells including some cancer cell lines (Lin and Worman 1997), particularly embryonal carcinomas (Lebel et al. 1987; Stewart and Burke 1987; Mattia et al. 1992), and various hematopoietic stem cell types (Rober et al. 1990; Broers et al. 1997; Pierce et al. 1999), do not express lamin A/C. Additionally, several cell types that are terminally differentiated into a non-proliferative state (e.g. some classes of neurons) (Broers et al. 1997; Pierce et al. 1999) also lack lamin A/C expression. This provides a link between cells that can exhibit conditional proliferation and lamin A/C expression, which opens the possibility that a product of the LMNA gene expression may be involved in the maintenance of cells in a non-proliferative state like quiescence or senescence. Quiescence is defined as cells having the ability to re-enter the cell cycle; whereas, senescence is the loss of the ability to re-enter the cell cycle.

One of the goals of this study is to examine the mechanisms by which prelamin A accumulation affects cellular quiescence/senescence. Is prelamin A accumulation a byproduct or an mediator of quiescence? Does a persistent buildup of prelamin A lead to senescence? What are the molecular mechanisms?

Early work by Dr. Michael Sinensky and later by Christina Bridges in our laboratory showed that mevalonate and serum deprivation leads to accumulation of prelamin A and growth arrest in HeLa cells, F9 cells and human diploid fibroblasts (Sinensky et al. 1994; Bridges 2012). Here we show that incubation of NIH 3T3 cells in 0.5% FBS for 48hrs leads to accumulation of prelamin A in the nucleoplasm (Figure 9A). On the other hand, cells that were actively proliferating and
maintained in full serum and analyzed under the same conditions, did not show any detectable levels of prelamin A (Figure 9A lower panels).

![Figure 9: Accumulation of prelamin A upon serum starvation. A) Immunofluorescence using a prelamin A C-terminus specific antibody in proliferating or serum starved NIH 3T3 fibroblasts after 48 hours. Exposure for fluorescence photos was 5 seconds (Top) and 1 minute (Bottom). B) Western blot, using a lamin A/C antibody and lysates from NIH 3T3 cells grown in medium with 10% FBS, 0.5% FBS and 0.5% FBS for 48h and re-fed with 10% FBS for 24h. Lovastatin was used as a control to increase the levels of prelamin A. Upper band: Prelamin A; Middle band: Mature Lamin A; Lower band: Lamin C.](image)

Cell lysates were also collected after serum starvation and analyzed by Western blot (Figure 9B). The results show an increase in the accumulation of prelamin A in serum deprived cells. Also note the complete absence of detectable prelamin A when cells are grown in normal medium (10% FBS) and after serum deprived cells were re-fed with of 10% FBS containing medium for 24 h. Similar results were observed with serum-starved BJ fibroblasts, HeLa cells, and contact inhibited NIH 3T3 cells (data not shown).
Given the nature of the maturation pathway of lamin A, it was important to know whether the C-terminus of the prelamin A that accumulates in serum starved cells has been properly processed. Mammalian cells possess a second enzyme, Ras-converting enzyme 1 (Rce1) that is capable of performing the endopeptidase activity of step 1 in the prelamin A processing pathway (Winter-Vann and Casey 2005). The Rce1 enzyme has been demonstrated to be active in step 1 endoprotease processing of both Ras proteins (Winter-Vann and Casey 2005) and lamin B (Maske et al. 2003) raising the possibility that CAAX box processing, including carboxymethylation can occur even in the absence of zmpste24.

Figure 10: Prelamin A that accumulates in quiescent BJ cells is farnesylated and carboxymethylated. A) Mass spectral analysis of prelamin A from serum starved BJ cells. B) Carboxymethylation of prelamin A accumulating in serum starved BJ human diploid fibroblasts (incorporation of alkali-releasable counts from ([3H]-methyl) methionine). Lamin B was used as a positive control.
To assess degree of maturation of the prelamin A that accumulates in serum starved cells we used MALDI-TOF mass spectrometry to detect the signature mass peak for farnesylated and carboxymethylated prelamin A (Figure 10A). The C-terminal tryptic fragment at mass 996.23 was present in all samples from serum-starved BJ fibroblasts indicating that the prelamin A that accumulates has undergone the farnesylation, AAXing and carboxymethylation steps and is ready for the step 2 endoproteolysis by zmpste24. Since farnesylated peptides sometimes are under-represented on MALDI-TOF runs, a more quantitative measure of the accumulated farnesylated and carboxymethylated prelamin A can be obtained from the base-release assay (Figure 10B). In this assay, fully mature lamin A should not be labelled by ([3H]-methyl) from methionine since its C-terminal 2KDa peptide is lost after the step 2 endoproteolysis. On the other hand, lamin B should be fully labelled and can be used as a positive control. In the case of prelamin A, the base release assay provides a measure of the extent of accumulation of farnesylated and carboxymethylated protein. So, the vast majority of prelamin A that accumulates in quiescent cells is farnesylated and carboxymethylated (Figure 10B) as compared to the permanently farnesylated and carboxymethylated lamin B.

Since FC-prelamin A is a farnesylated protein, some general consideration of the properties of prenylated, particularly, farnesylated proteins is warranted. Prenyl modifications, including farnesylation, can be viewed as a mechanism for mediating protein-protein interactions, as opposed to simple binding to lipid bilayers. Recent reviews of this subject (Rusinol and Sinensky 2006; Davies et al. 2011) maintain the same perspective. Indeed, farnesylation and carboxymethylation are best viewed as weak membrane anchors which
provide a mechanism for membrane association that is reversible upon binding by other proteins that recognize these

![Northern blot of zmpste24](image)

Figure 11: Zmpste24 expression in serum-deprived WI38 cells. Northern blot of zmpste24. A $^{32}$P-labeled zmpste24 cDNA probe was hybridized to RNA collected from WI38 cells grown in 0.5% FBS medium. β–Actin was used as loading control.

structural elements and a second site on the farnesylated protein. In the case of FC-prelamin A, a specific heterodimeric partner was identified some years ago by the Worman lab (Barton and Worman 1999). We have now extended this number to nine proteins that interact with prelamin A but not lamin A; described later in this document (Table 1).

The nature of the prelamin A that accumulates in quiescence makes the second endoproteolytic step by zmpste24 the most desirable step to study. Previous data from our laboratory (Bridges, 2011) showed that zmpste24 protein levels and activity decrease when HeLa cells are serum deprived. Zmpste24 activity decreases in parallel to the rate of cellular proliferation, as shown by thymidine incorporation into DNA, thus indicating that prelamin A
accumulation in quiescence is due to zmpste24 inhibition or reduced expression. When serum was returned to the cultures, an increase in cell proliferation as well as zmpste24 activity was observed (Bridges, 2011).

Here we show the levels of zmpSte24 mRNA measured in actively proliferating WI38 cells in full serum and after serum starvation to induce quiescence. There was a drastic decrease in the level of mRNA transcript over a two-day period (Figure 11) suggesting a transcriptional regulation mechanism for intracellular levels of FC-prelamin A.

To determine whether the down-regulation of zmpste24 in quiescent cells has physiological significance, we examined, as a model, WI-38 fibroblasts rendered quiescent by growth to confluence. Cells were plated at low and high density so that the effect of expression of zmpste24 on quiescence produced by contact inhibition could be compared with DNA synthesis in proliferating and non-proliferating cells.
The results in Figure 12 show that overexpression of zmpste24 enhances the proliferation of confluent cells to essentially the same level as non-confluent cells.

Figure 12: Effect of zmpSte24 overexpression on proliferation of contact inhibited human diploid fibroblasts. WI-38 fibroblasts were plated at low (2500 cells/cm²) or high (25000 cells/cm²) density and transfected with an empty vector or a plasmid that expresses zmpSte24 under the control of a CMV promoter (Origene- pCMV6-XL5). Untransfected cells plated at low density (red circles), untransfected cells plated at high density (red triangles), zmpSte24 transfected cells plated at low density (blue circles), and zmpSte24 transfected cells plated at high density (blue triangles).
Characterization of Promoter and Transcription Factors That Regulate ZmpSte24 Expression

Computer analysis (Genomatrix or Promo) of the 5’ flanking sequence of the zmpSte24 gene (Accession # NM_005857) is consistent with several E2F1 binding sites with varied degrees of probability. This was accomplished by focusing on the sequence CCGGGCCCAGAACTCGG located about 100 bp (bp -124 to -108) upstream of the zmpste24 transcription start site, using a dual luciferase assay (Promega, Madison, WI) to determine if zmpSte24 is transcriptionally regulated by E2F1.

The putative E2F1 binding site sequence was ligated into minimal promoter pGL4 Luciferase Reporter Vector (Promega, Madison, WI), that were used expressed Firefly and Renilla luciferase (pGL 4.27 and pGL 4.77). These distinct luciferases have dissimilar enzyme structures and substrate requirements which make it possible to selectively discriminate between their respective bioluminescent reactions. This provides a convenient means of correcting for transfection efficiency by sequentially measuring both enzymes from a single sample and eliminating the need for using β-galactosidase for that purpose.

HEK293T cells were co-transfected with the luciferase reporter vectors that either contained the putative E2F1 binding site insert, a mutated version of it or empty vector. To directly explore regulation of zmpste24 expression by E2F1, cells were co-transfected with an E2F1 expression vector under the control of a CMV promoter (Invitrogen).
Figure 13: Reporter gene studies to test the putative E2F1 binding sites in the zmpste24 promoter. pGL4.27 [luc2P/minP] containing the wild type or mutated putative E2F1 response element and pGL4.77 [hRenilla lucP] (transfection efficiency control) were co-transfected into HEK293T cells. Where indicated, cells were also co-transfected with a CMV-driven E2F1 expression vector. Figure shows a representative experiment out of four repetitions. Data is expressed as the average of triplicate luciferase measurements, normalized for protein, hRenilla luciferase and compared to transfection of empty pGL4.27 [luc2P/minP] vector. ** p<0.003 comparing between ERE and ERE + E2F1.

Our results indicate that the promoter region in zmpSte24 contains a functioning E2F1 response element that leads to an increased transcription of the luciferase reporter gene in HEK293T cells (Figure 13). Of further interest, the putative E2F1 response element (ERE) was mutated, CCGGAAAAAGGAACCTCGG, this resulted in abolishment of expression of the reporter gene (Figure 13), including when cells were co-transfected with the E2F1 expression vector (Invitrogen). Although these experiments suggest that E2F1 may indeed upregulate zmpste24
activity in vivo, further experiments using E2F1 null cells or similar, may be necessary to establish whether E2F1 modulates zmpste24 expression.

Specific Prelamin A Interactions with Chromatin

A variety of studies have shown a direct and indirect interaction between lamin A, lamin C, and chromatin, suggesting the possibility of a differential gene expression program between cells that are expressing or not expressing FC-prelamin A (Gruenbaum et al. 2000; Maraldi and Lattanzi 2005; Maraldi et al. 2006; Rusinol and Sinensky 2006). The carboxyl terminal hydrophobic modifications of prelamin A localize it to the nuclear envelope (Hennekes and Nigg 1994). Lamin A has been shown to interact with various transcription factors as well as having more general binding interactions with chromatin (Goldman et al. 2005; Rzepecki et al. 2008; Piekarowicz et al. 2016). Consequently, as lamin A interacts with chromatin to promote or to silence gene expression, prelamin A might be expected to act in similar manner. However, the hydrophobic post-translational modification of prelamin A may also participate in bringing chromatin regions that interact with lamin A to the nuclear periphery where the new environment can affect gene expression. Supporting this possibility, expression of progerin was shown to produce a loss of heterochromatin (Lowndes and Toh 2005; Walter et al. 2006; Rzepecki et al. 2008; Piekarowicz et al. 2016). Additionally, a specific effect of prelamin A on heterochromatin sub-nuclear localization has been reported (Masny et al. 2004). The possibility that such differential expression occurs has been supported by the effects of progerin on gene expression in HGPS fibroblasts. A reported microarray analysis identified more than 60 differentially expressed genes in HGPS cells (Wang et al. 2006). For example, ankyrin 3 mRNA is expressed more than 100-fold higher in HGPS fibroblasts compared to
normal human diploid fibroblasts. However, the expression of ankyrin 3 at the protein level is similar between the HGPS and normal cells. Additionally, some isoforms of ankyrin 3 have been reported to diminish fibroblast proliferation in response to the mitogen, PDGF, through inhibition of the PDGF receptor (Ignatiuk et al. 2006).

Identification of prelamin A interacting proteins by proximity labeling

In an attempt to isolate and identify proteins that specifically interact with Prelamin A, Progerin or Lamin A, we decided to use the recently developed methodology called "BioID" as an alternative to more common screening techniques for protein-protein interactions (Roux et al. 2012). In BioID, a modified E. coli biotin ligase (BirA*) is fused to a protein of interest. The biotin ligase fusion protein is then introduced into a live mammalian cell where it biotinylates vicinal proteins. Biotinylated proteins are then selectively isolated by binding to streptavidin linked to paramagnetic beads and identified by mass spectrometry.

The main advantage of this method, is that the potential interacting partners are tagged in a natural cellular environment avoiding fixation, co-immunoprecipitation or other techniques that might lead to false positives. Furthermore, the nature of the tag (biotin) allows for extensive and convenient purification of the labeled proteins. Type A lamins, being members of the intermediate filament family, are relatively insoluble and therefore ideal candidates for BioID.

Four cell lines were created as described in Materials and Methods using NEB’s 3T3 Rheoswitch® cells as the background cell line. The cells expressed Biotin ligase (BL) or BL fusion proteins of GFP-Lamin A or GFP-L647R prelamin A, in a stable and inducible manner upon
incubation with GenoStat™ (Figure 14). The fusion proteins showed the appropriate molecular weight as determined by SDS-PAGE followed by Western blot with anti-GFP antibodies.

Expression and Sub-cellular Localization of Biotin Ligase-lamin Fusion Proteins

Immunofluorescence of 3T3 Rheoswitch® BL-GFP-LA cells and 3T3 Rheoswitch® BL-GFP-L647R-LA cells showed the sub-cellular localization of biotin ligase-lamin fusion proteins. In the 3T3 Rheoswitch® BL-GFP-LA, the fusion proteins were in the expected location of the nuclear envelope similar to both endogenous lamin A and lamin A with a GFP tag (Bridges, 2011). In the 3T3 Rheoswitch® BL-GFP-L647R-LA cells, the biotin ligase-lamin fusion protein localized to the nuclear lamina and also in nucleoplasmic aggregates (Figure 15).

![Figure 14: Expression of biotin ligase-lamins chimeras upon induction with GenoStat.](image)

3T3 Rheoswitch cells harboring biotin ligase constructs were incubated for 48h with various concentrations of GenoStat. Cells were then lysed and total cell extracts were analyzed by SDS-PAGE and western blotting using anti-GFP and Biotin Ligase antibodies. Rheo indicates untransfected cells.
**Proximity labeling experiment**

The stable cell lines were used in the proximity labeling experiment as follows: 1-2 X 10^7 cells were incubated for 24h in the presence of 500 µM GenoStat and 0 or 50µM biotin. Cells were then lysed under stringent conditions and biotinylated proteins were collected on streptavidin-conjugated beads (Dynabeads from Invitrogen) for subsequent analysis and identification by mass spectrometry. As a process intermediate control, aliquots of the eluted protein samples were subjected to SDS-PAGE and probed for biotinylated proteins by western blotting with streptavidin linked to horse radish peroxidase (Figure 16).

![Figure 15: Co-localization of GFP-L647R-lamin A (green) and biotin ligase (red) fluorescence.](image)

Immunofluorescence was carried out with anti-GFP and anti-biotin ligase antibodies as described in Materials and methods.

The following criteria was used for inclusion of the identified proteins as interacting partners: First, the average MASCOT (a software search engine that uses mass spectrometry
data to identify proteins from peptide sequence databases) score must be larger than 100.

Second, the proteins must not be present (or be present with a 5-fold lower MASCOT score) in the no-biotin background control samples. Third, mitochondrial proteins (based on Gene ontology (GO) annotations) and keratins were intentionally excluded to focus on nuclear proteins. In both cell lines the identified protein with the highest score was lamin A with an average MASCOT score of 3,123.

![Figure 16](image)

**Figure 16: Relative degree of biotinylation by BioID, BL-GFP-LA (LA) and BL-GFP-L647R-PreA (PreA).** Cells were treated with 0, or 50 µM biotin for 48h. After cell lysis and isolation with streptavidin-linked Dynabeads, biotinylated proteins were eluted in Novex Li-sample buffer and analyzed by SDS-PAGE and western blotting with streptavidin-HRP.
Interacting partners were categorized as interactors of lamin A, interactors of L647R-PreA or both. Many of the identified lamin A interactors were also detected in L647R-PreA samples and vice versa, requiring the use of additional criteria to identify differential interactors. Proteins were required to have a ratio of L647R-PreA over lamin A average MASCOT scores above 2.5 or below 0.25 in the samples from the respective cell lines. For example, Mascot scores were considered semi-quantitative only, and were used to distinguish proteins that might either have higher affinity for lamin A or L647R-PreA (or associated proteins) or were expressed more abundantly in BL-GFP-lamin A than in BL-GFP-L647R-cells. Whether these candidates bound lamins directly or indirectly cannot be revealed by this method.

Lamin A and prelamin A vicinal proteins identified by proximity labeling.

After applying the above described criteria to the data received from the proteomic core facilities, a total of 294 proteins were considered interactors of both L647R-PreA and lamin A (Table 1). Sixteen proteins were considered preferential interactors of either L647R-PreA or lamin A cells; seven with L647R-PreA interactors and seven with Lamin A. Two proteins, PCNA and SCYE1, were also labeled that did not meet the selection criteria for exclusive PreA interactor, but were more strongly represented in the prelamin A cells samples. Five out of the seven proteins in our screen that were biotinylated by BL-GFP-L647R-PreA were related to cell cycle control, in particular, regulation of processes involved in cell senescence. PIN1 and H2A.Z have been previously identified by our laboratory as prelamin A interactors.
Table 1: Differential interactors of BL-GFP-Lamin A or BL-GFP-L647RPreA.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2A.Z</td>
<td>H2A Histone Family Member Z</td>
</tr>
<tr>
<td>HIST1H2BP</td>
<td>Histone Cluster 1 H2B Member O</td>
</tr>
<tr>
<td>HIST1H2BA</td>
<td>Histone Cluster 1 H2B Member A</td>
</tr>
<tr>
<td>H2AFX</td>
<td>H2A Histone Family Member X</td>
</tr>
<tr>
<td>NAPG</td>
<td>NSF Attachment Protein g</td>
</tr>
<tr>
<td>TPM2</td>
<td>beta-tropomyosin</td>
</tr>
<tr>
<td>PIN1</td>
<td>Peptidylprolyl Isomerase</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>SCYE1</td>
<td>ATSCI Multifunctional Protein 1</td>
</tr>
<tr>
<td>Nup98</td>
<td>Nucleoporin 98</td>
</tr>
<tr>
<td>Tpr</td>
<td>Nuclear Basket Protein</td>
</tr>
<tr>
<td>Ahctf1</td>
<td>AT-Hook-Transcription Factor 1</td>
</tr>
<tr>
<td>Nup133</td>
<td>Nucleoporin 133</td>
</tr>
<tr>
<td>Tmem43</td>
<td>LUMA</td>
</tr>
<tr>
<td>ATL3</td>
<td>Atlastin GTPase 3</td>
</tr>
<tr>
<td>Rab5A</td>
<td>Rab 5, RAS Oncogene Family</td>
</tr>
</tbody>
</table>

Proteins were sorted according to MASCOT score. **Red**, proteins detected only in uncleavable PreA samples according to the inclusion criterion. **Orange**, proteins detected in both samples (meet the inclusion criterion but there is a big difference in the preA samples. **Green**, proteins detected only in the lamin A samples according to inclusion criterion.
Pin1 was found to be a potential interactor by an *in silico* approach that was confirmed by co-immunoprecipitation (Bridges, 2011). In that study, Bridges showed that Pin1 was a primary mediator in PreA regulation of the cell cycle. Pin1 was shown to have binding sites in the PreA C-terminus, to directly bind preA in a phosphorylation dependent manner, and to be sequestered in the nucleus upon prelamin A accumulation. Pin1 has been implicated in the control of a multitude of cell cycle regulators (Lin et al. 2015), so it was speculated that sequestration by interaction with prelamin A may affect that process.

**Figure 17:** Co-immunoprecipitation with lamin A or L647R prior to MALDI-TOF. Silver stained SDS-PAGE gel showing proteins co-immunoprecipitated with Lamin A or L647R-Prelamin A previous to band excision and analysis by MALDI-TOF. Proteins bands that were identified are labeled in the image.
Histone H2A.Z was more closely examined using an anti-prelamin A antibody developed by Sinensky et al. (Sinensky et al. 1994) and commercial anti-lamin A/C antibodies, which showed that H2A.Z is one of several proteins that co-immunoprecipitate with prelamin A. The experiment consisted of immunoprecipitation followed by MALDI-TOF identification as described in Materials and Methods (Figure 17).

H2A.Z is interesting because it is one of the specialized histone variants that is deposited in nucleosomes independent of replication and is constitutively expressed in many cell types. Many lines of evidence strongly suggest H2A.Z might have a role in gene transcription. The best described role for H2A.Z in aging and senescence was shown by Gevry et al. 2007 and Lee et al. 2012 They showed that depletion of H2A.Z by shRNA knockdown caused a p53-dependent increase in p21 expression resulting in premature senescence of WI-38 and IMR-90 human fibroblasts (Gevry et al. 2007) (Lee et al., 2012).

One of the most striking findings of zmpste24 K.O. mice is the upregulation of p53 and p53 clients like p21, and GADD45g in skin fibroblasts and other tissues (Varela et al. 2005). Using a focused RT-PCR array, increases in p21, p27, FOXO3 and other regulators of the cell cycle in response to L647R prelamin A accumulation in Rheoswitch 3T3 cells were found in our lab. (Bridges, 2011).

To determine whether the effect of uncleavable prelamin A on p21 expression levels might be mediated by H2A.Z a ChIP-PCR experiment was performed in which chromatin from Rheoswitch cells expressing L647R-PreA cells was isolated and immunoprecipitated with antibodies against H2A.Z. The purified genomic DNA was quantified by end-point PCR using
consensus primers specific for p21 (SABiosciences). Figure 18 shows that H2A.Z appears to be displaced from the p21 promoter region upon induction of L647R-PreA for 24h.

Figure 18: H2A.Z is displaced from p21 promoter region upon expression of L647R Prelamin A. Chromatin immunoprecipitation (ChIP) was performed with anti-H2A.Z on chromatin preparations harvested from L647R Rheoswitch cells incubated in the presence or absence of 800nM Genostat for 24h to induce the expression of uncleavable prelamin A. IgG represents non-specific background after immunoprecipitation with non-immune serum. The purified genomic DNA was quantified by end point PCR using consensus primers specific for p21 (SABiosciences). We used mouse GAPDN Internal Normalizer (SABiosciences) A) Representative separation of PCR products on agarose gel electrophoresis. B) Images were quantitated using G:Box image analysis system. Quantitation of Enrichment was determined as 100x (A\text{input} - A\text{enriched}) after adjusting the percentage of input DNA used. Shown are average ± s.d. of three biological replicates. * = p<0.05.

Next, changes in expression of p21 were measured in response to L647R-PreA accumulation. Transcription of the p21 gene was examined using a RT² Endpoint PCR system from SABiosciences on RNA isolated from L647R or control cells incubated with different GenoStat™ concentrations for 24 h. PCR products were separated on 2% agarose.
Figure 19 shows that p21 transcripts increase with the GenoStat concentration in cells expressing L647R-PreA but not in untransfected cells (Rheo).

![Image of gel electrophoresis](image)

**Figure 19: CDKN1a (p21) expression changes upon induction of L647R-PreA.** RNA was isolated from L647R-PreA Rheoswitch cells grown in the presence of various concentrations of GenoStat. First strand cDNA was synthesized and used in a RT2 endpoint PCR kit, using consensus primers for p21. PCR products were separated by electrophoresis in 2% agarose. We used mouse GAPDN Internal Normalizer (SABiosciences).

Lastly, the effect of L647R-PreA accumulation on p21 protein levels was determined. Rheoswitch cells, L647R PreA or wild type Lamin A, (not shown) were induced with increasing concentrations of Genostat for 48 hours.

Figure 20 shows that p21 levels parallel those of L647R-PreA upon induction with GenoStat.
Figure 20: p21 protein accumulation upon induction of L647R-PreA. 50 μg protein of total cell lysate per lane was separated by SDS PAGE on 4-12% gradient Bis Tris Gel and transferred to PVDF membrane, then the membrane was sequentially immunoblotted with anti-GFP antibody (Top Panel, equivalent to L647R-PreA), anti-p21 antibody and lamin B antibody (Bottom panel) after mild stripping of the membrane.
CHAPTER 4

DISCUSSION

Zmpste24 Structure/Function

Since the 3.4 Å structure of zmpste24 was solved by Quigley et al., we contemplated possible explanations for the inhibition and covalent modification of zmpste24 by fluorescent serine-protease suicide inhibitors as determined by mass spectrometry. In the absence of a crystal structure for zmpste24, these results (Corrigan 2005) formed the basis and rational of our initial approach (to rescue zmpste24 KO cells with truncated forms of the enzyme) to study the structure-function relationship of zmpste24 and to test the “two site” hypothesis. A possible explanation for the results that were observed is a non-specific inhibition (binding/covalent modification sterically alters the enzyme and affects the active site without actually binding to it) (Grabarek et al. 2002) and reviewed on (Grabarek and Darzynkiewicz 2002). Regardless, the truncated forms that we developed could still be used for further studies of zmpste24 function (e.g. protein-protein interaction, activity regulation, etc.).

Regulation of Zmpste24 Expression in Serum Starved Cells

The results described above provide a justification for the hypothesis that prelamin A may serve a function in quiescence in response to mitogen withdrawal. This function could be to support the quiescent state directly or to help block the other possible cell fates that can occur under these conditions: apoptosis or necrosis. We have also presented evidence that prelamin A accumulation during serum starvation is mediated by down-regulation of zmpste24 suggesting that the form of prelamin A that accumulates is the farnesylated and
carboxymethylated substrate of this enzyme. Consistent with this structural assignment, we utilized radiochemical methods to show that prelamin A that accumulates in serum starved BJ cells is prenylated (Figure 9), and carboxymethylated (Figure 9B). The relative carboxymethylation of prelamin A and lamin B, under these conditions is consistent with similar levels of CAAX-box processing. The predicted FC-prelamin A carboxyl terminal structure was confirmed by MALDI-TOF mass spectrometry (Figure 9A). Our promoter analysis experiments strongly suggest a mechanism of transcriptional regulation for zmpste24 levels in response to mitogen deprivation (Figure 21). Regulation seems to be mediated by the well-described Rb-E2F pathway. Under this condition, one mechanism may be that Rb is hypo-phosphorylated and binds to E2F1 transcription factor to inactivate it and in turn preventing upregulation zmpste24 expression (Figure 21).

Figure 21: Model of cell-cycle arrest by serum starvation. Our hypothesis is that zmpSte24 is regulated by E2F resulting in changes FC-prelamin A accumulation which facilitates or prevents entry into and/or maintenance of G0.
It is likely that transcriptional regulation is not the only mechanism for zmpste24 down-regulation since the zmpste24 mRNA levels decrease with slower kinetics (Figure 11) than the enzyme activity in response to serum deprivation (Bridges, 2011). In our hands a 50% decrease in zmpste24 enzyme activity occurs in ~10 hours whereas the mRNA levels decline to 50% after 16-32h. A potential explanation for this could be methodological or, more likely, a dual regulation where short-term enzyme inhibition can be accomplished by post-translational modification of zmpste24, perhaps by phosphorylation-de-phosphorylation and more persistent downregulation accomplished by modifying mRNA and/or protein turnover. In support of this hypothesis, zmpste24 has been shown to be phosphorylated by aurora and polo-like kinases in mitotic cells. (Kettenbach et al. 2011). These kinases orchestrate almost every step of cell division, from entry into mitosis to cytokinesis. In a recent proteome-wide survey, other post-translational modifications identified in zmpste24 were five ubiquitination sites (residues 46, 66, 251, 314 and 437) which were speculated to participate in modulation of cell cycle progression by zmpste24 (Wagner et al. 2011).

Using newer in silico detection algorithms (UCSC Genome Browser and the DECipherment Of DNA Elements database (DECODE), we have searched 20kb upstream and 10kb downstream of zmpste24 and detected several other potential transcription factor binding sites. Several members of the FOXO family have multiple putative binding sites in the 5’ flanking region of the zmpste24 gene. This is particularly interesting since we have shown that FOXO3 is involved in the up-regulation of p27, a mediator in the quiescence related cell
cycle arrest, by transient expression of uncleavable prelamin A (Manuscript in preparation and Bridges, 2011).

Another interesting result from our analysis of the zmpste24 gene promoter region is four binding sites for p53. Using commercially available primers for these sites in ChIP-PCR experiments using a ChIP certified p53 antibody (Qiagen), we have compared cells that are actively proliferating versus serum-starved quiescent WI38 cells. Preliminary evidence shows slightly increased occupancy of three out of the 4 sites in the 5’ untranslated region of zmpste24 in quiescent cells. The physiological consequences of these findings are hard to predict, because outcomes of increased or decreased p53-mediated gene expression have been described in the literature (Riley et al. 2008; Tang et al. 2012).

Identification of Prelamin A Interacting Proteins

The isolation and identification of lamin proteins interacting partners has been hindered by the insoluble nature of lamin forms. In this study we have applied a recently developed in situ and in vivo biotin-tagging and isolation approach to identify lamin A and prelamin A lamin interactors. The procedure used is based on creating fusion proteins with a promiscuous Biotin Ligase and the protein of interest. It was developed by Roux et al. 2012 and was focused on isolation of lamin A interacting proteins (Roux et al., 2012). This method is an improved variation of a similar technique that involves expression of lamin A and progerin modified with the high-affinity OneSTrEP-tag, precipitation of lamin-protein complexes after reversible protein cross-linking and subsequent protein identification by mass spectrometry (Kubben et al. 2010), a study focused on identification of differential lamin A and progerin interacting proteins. Here we focused on proteins that preferentially “interact” with prelamin A.
Analysis of Table 1 shows that the majority of these proteins are related to chromatin organization or cell cycle control. Of particular interest is H2A.Z. Under normal conditions H2A.Z seems to suppress the p53-dependent p21 transcription and senescence responses. H2A.Z is placed within the p21 promoter, by the p400 ATPase chromatin repressing p21 gene expression. Both, downregulation of p400 or siRNA knockdown of H2A.Z lead to p53/p21 senescence responses (Gévry et al. 2007; Lee et al. 2012). Our laboratory has previously shown that persistent expression of uncleavable prelamin A in 3T3 Rheoswitch cells led to a progressive accumulation of β-galactosidase in positive cells, a hallmark of the senescent state (Figure 22).

Figure 22: Cell senescence upon persistent L647RPreA expression. L647R PreA Rheoswitch cells, Induced for expression (500nM GenoStat, red bars) or Uninduced (blue bars) were split every other day in 1:3 ratio. At the indicated passage number, senescence was measured with a β-galactosidase staining kit. (Adapted from Bridges, 2011).
Therefore, a possible mechanism for the up-regulation of p21 seen in Figures 20 and 21 is by epigenetic displacement or sequestration of H2A.Z by prelamin A from promoter regions where H2A.Z exerts an inhibitory role on transcription. In conclusion, the association of H2A.Z with the accumulated L647R prelamin A could provide an epigenetic mechanism for the senescence phenotype observed in these cells.
REFERENCES


VITA

JAIME LYN PARMAN-RYANS

Education: South Greene High School, Greeneville, TN

A.S., General Studies, Walters State Community College, Morristown, TN

B.S., Health Sciences, East Tennessee State University, Johnson City, TN

M.S., Biomedical Sciences, East Tennessee State University, Johnson City, TN

Ph.D., Biomedical Sciences, East Tennessee State University, Johnson City, TN

Professional Experience: Graduate Assistant, East Tennessee State University, Johnson City, TN, June 2001 - December 2003

Adjunct Faculty, Walters State Community College, Morristown, TN, January 2004 - August 2008

Research Associate, East Tennessee State University, Johnson City, TN, January 2005 - August 2008

Graduate Assistant, East Tennessee State University, Johnson City, TN, August 2009 - December 2016

Associate Professor of Biology, Walters State Community College, Morristown, TN, August 2008 - Present

Publications:


Bridges, C.N., Parman-Ryans, J.L., Sinensky, M.S., Rusiñol, A.E. Regulation of prelamin A accumulation by the zmpste24 endoprotease during cellular quiescence.


Distinguished Adjunct Faculty Award, Walters State Community College, 2008
First Place, Student's Choice Award, Division II Research Forum, East Tennesse State University, 2002
Second Place, Division II-Research Forum, East Tennesse State University, 2002