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# Nuclear Translocation of FoxO3a Transcription Factor During Prelamin A Induced Cell Cycle Arrest in 3T3 Cells.

Jessica B. Keasler

*East Tennessee State University*

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NUCLEAR TRANSLOCATION OF FOXO3A TRANSCRIPTION FACTOR DURING  
PRELAMIN A INDUCED CELL CYCLE ARREST IN 3T3 CELLS

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Thesis submitted in partial fulfillment of Honors

By

Jessica B. Keasler

The Honors College

University Honors Scholars Program

East Tennessee State University

April 10, 2012

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Dr. Antonio Rusiñol, Faculty Mentor  
Department of Biochemistry  
Quillen College of Medicine

---

Dr. Katrina Heil, Faculty Reader  
Department of Literature and Language  
East Tennessee State University

---

Mrs. Jaime Parman-Ryans, Reader  
Department of Biochemistry  
Quillen College of Medicine

## Abstract

As the so-called “Mothership of the Human Genome,” the cell nucleus must keep all vital genetic information safe, but accessible, inside a strong protective envelope. The inner membrane of the nuclear envelope is lined by tough but adaptable proteins called lamins. While lamins polymerize into fibrous structures that hold up the “walls” of the nucleus, they also serve as an internal scaffold for the complex machinery involved in DNA replication and gene expression. It is in this later role that we have been looking for clues to premature and possibly to normal aging. One type of lamins, Lamin A is made through an unusual pathway involving a lipid dependent cleavage of a larger precursor called *prolamin A*<sup>1-3</sup>. The functional significance of this processing pathway is that prelamin A cannot assemble and is inhibitory of proper lamina formation. Pathological cases of immature lamin A accumulation include Hutchinson-Gilford progeria syndrome (HGPS) or Progeria characterized by premature aging and Restrictive Dermopathy (RD), a lethal prenatal disease. We have previously shown that accumulation of prelamin A leads to cell cycle arrest and drastic changes in expression of genes involved in cell cycle control, among those, several members of the FoxO family of transcription factors.

The goal of this study was to determine the mechanisms by which accumulation of uncleavable prelamin A activates FoxO-mediated cell cycle arrest. Cells expressing an uncleavable form of Lamin A in an inducible manner were used to determine subcellular distribution of FoxO3a upon accumulation of prelamin A. This was done by indirect immunofluorescence and Western blotting. The proliferation rate of these cells and controls expressing wild type Lamin A was also determined by measuring the incorporation of BrdU into DNA.

During these experiments, it was hypothesized and observed that overexpression of prelamin A leads to redistribution of FoxO3a from the cytoplasm of the cell to the nucleoplasm. Expression of FoxO3a target genes was accordingly increased, leading to a decrease in cell proliferation.

The information obtained from this study could not only be of interest in broadening our knowledge of the mechanisms of quiescence and aging in general, but also could inform the discussion of the use of several therapeutics for the treatment of Progeria and other diseases that result from the accumulation of prelamin A.

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## List of Abbreviations

HGPS	Hutchinson-Gilford Progeria Syndrome
RD	Restrictive Dermopathy
BrdU	Bromodeoxyuridine
FoxO	Forkhead box O
GADD45	Growth Arrest and DNA Damage genes
Pin1	Peptidylprolyl isomerase
HRP	Horseradish peroxidase
TMB	3,3',5,5'-tetramethylbenzidine
DMSO	Dimethylsulfoxide
PBS	Phosphate buffered saline

## Chapter 1. INTRODUCTION

The genetic diseases Hutchinson-Gilford Progeria Syndrome (HGPS) and Restrictive Dermopathy (RD) both arise from defects in the endoproteolytic processing pathway of prelamin A. The formation of lamin A, from its precursor prelamin A, is an unusual protein maturation pathway in that it requires two farnesylation-dependent endoproteolytic cleavages. The second cleavage in this pathway is unique to lamin A in higher vertebrates and carried out by the protease zmpste24.

While Restrictive Dermopathy is lethal, patients with HGPS live, but exhibit premature aging which leads to a life expectancy of ~13 years. In RD, the defect is a loss of function of zmpste24. In HGPS, there is a mutation in the prelamin A gene that deletes the second site where zmpste24 cuts prelamin A. Thus, in both diseases, immature prelamin A accumulates, inducing cell quiescence and senescence among other dramatic changes in the nuclear structure and function. In cell culture models, induction of cell quiescence is co-related with both zmpste24 down-regulation and prelamin A accumulation<sup>1</sup>.

In both diseases, similar forms of prelamin A accumulate. Remarkably, it has been shown that the form of farnesylated and carboxymethylated prelamin A that arises in HGPS, known as progerin or Lamin A  $\Delta 50$ , also occurs at low levels in normal human cells and that its expression produces aging-associated phenotypes. The current literature on prelamin A indicates a role for its accumulation in cellular senescence, as well as, quiescence.



The Forkhead box O (FoxO) transcription factors are emerging as an important family of proteins that modulate the expression of genes involved in apoptosis, the cell cycle, and other cellular functions<sup>4</sup>. Four isoforms of the FoxO transcription factors are found in mammals- FoxO1, FoxO3, FoxO4, and FoxO6. Akt-dependent phosphorylation critically regulates three of the four FoxO isoforms- FoxO1, FoxO3, and FoxO4. Furthermore, it has been shown that, in normal cells, Akt-dependent phosphorylation of FoxO factors promotes FoxO export from the nucleus to the cytoplasm, thereby repressing FoxO transcriptional function.

This family of transcription factors is characterized by a conserved DNA-binding domain (the 'Forkhead box' or FOX). They participate in diverse processes from apoptosis to cell-cycle progression to oxidative stress resistance.

As shown in Figure 1, FoxO factors can promote cell cycle arrest by upregulating the cell-cycle inhibitor p27<sup>Kip1</sup> to induce G1 arrest or GADD45 to induce G2 arrest<sup>5</sup>. FoxO proteins are negatively regulated by the P13K/Akt signaling pathway. Phosphorylation at three conserved residues by Akt results in the export of FoxO factors from the nucleus to the cytoplasm, thereby inhibiting FoxO dependent transcription. FoxO proteins are also negatively regulated by a peptidyl-prolyl isomerase called pin1<sup>6</sup>. This enzyme also prevents the nuclear accumulation of FoxO proteins.

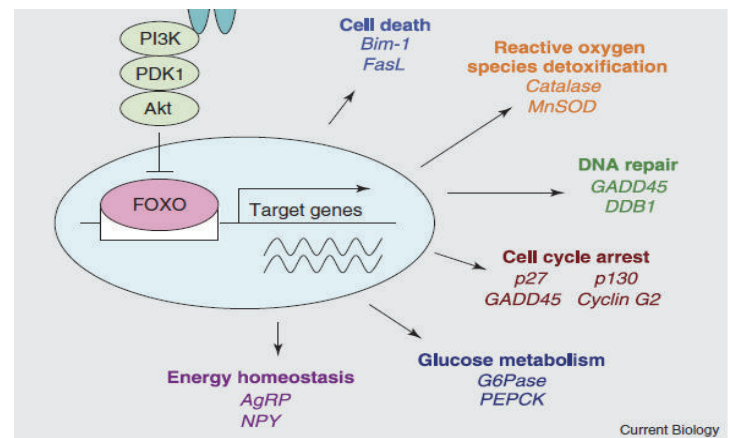


Figure 1. FoxO transcription factors. Adapted from Carter Me and Brunet A. (2007) **Current Biology** 7(4):R113-114.

In a study of *FoxO transcription factors in the maintenance of cellular homeostasis during aging*, Salih and Brunet have shown that the FoxO factors coordinate glucose homeostasis, angiogenesis, stem cell maintenance, immune, muscular, and neuronal functions, with implications for diabetes, cancer, autoimmune diseases and neurodegeneration <sup>7</sup>. FoxO factors have been shown to regulate other cellular responses in addition to those listed above. Elucidating how FoxO factors operate at the molecular level will provide important insights into their organismal function. For this reason, the focus of this study will lie in exploring the involvement of FoxO proteins in the regulation of cell cycle arrest induced by accumulation of prelamin A. It is hypothesized that when uncleavable prelamin A is expressed in 3T3 cells, FoxO proteins will migrate to the nucleus and induce cell cycle arrest through the upregulation of the p27<sup>Kip1</sup> cyclin-dependent kinase inhibitor.

## Chapter 2. METHODS AND MATERIALS

### Creation of uncleavable prelamin A expressing L647R cell line

In normal cells, Lamin A protein is processed by the post-translational pathway described in Figure 2.

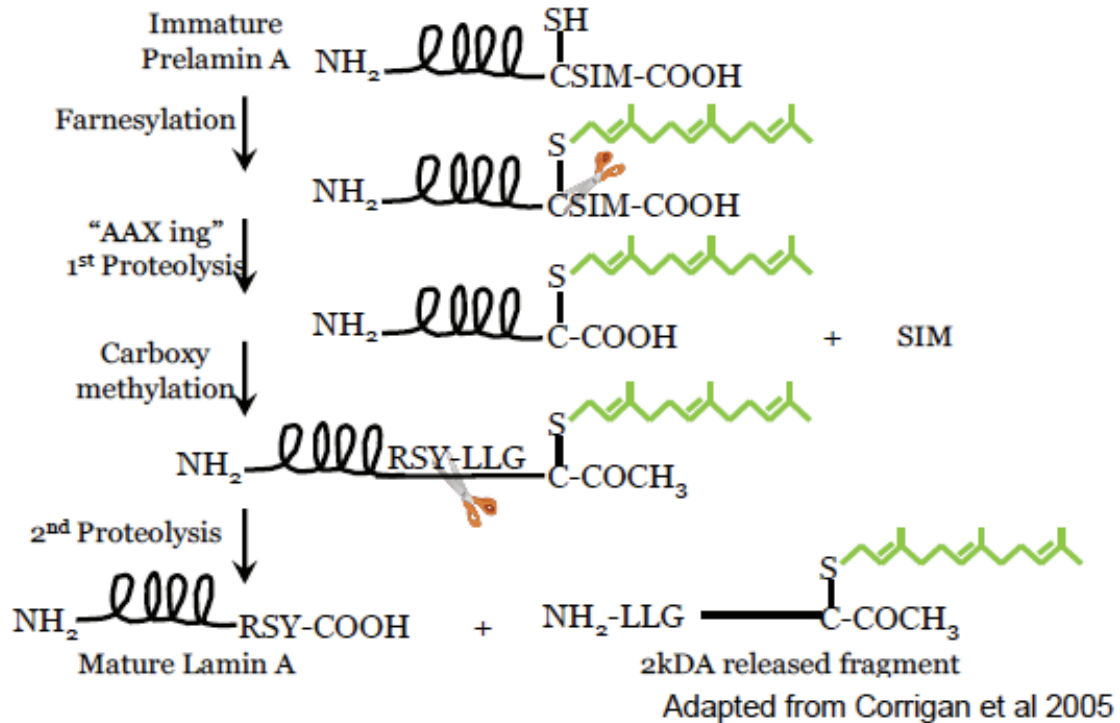


Figure 2. Maturation of Lamin A Protein by Proteolytic Post-Translational Processing. The precursor protein undergoes successive modifications: by the enzyme FT (which attaches a farnesyl moiety), followed by Zmpste24- or Rce1-mediated cleavage of the – SIM residues (AAXing), after which ICMT methylates the carboxyl terminus, signalling the 2nd proteolysis, by Zmpste24 (uniquely) between the tyrosine (Y) and leucine (L) residues of the RSYLLG Zmpste24 recognition site, releasing the 2kDa farnesylated c-terminal fragment to yield mature LA (72 kDa).

In order to develop cells which expressed an uncleavable prelamin A protein, site-directed mutagenesis was used to mutate the Zmpste24 cleavage site in the Lamin A cDNA sequence in a green fluorescent protein-tagged recombinant vector-based expression system, thus generating a full-length Prelamin A protein that was unable to undergo the final maturation cleavage to form mature Lamin A. The construct, pEGFP-C3-EGFP-LMNA-L647R, produces an EGFP-tagged protein termed “L647R Prelamin A,” in which the CaaX-box is intact, so the farnesyl modification, -aaX cleavage, and carboxyl methylation can occur as for Wild-Type Prelamin A<sup>8</sup>.

### **Measuring BrdU incorporation into DNA**

3T3 cells and L647R rheoswitch cells<sup>9</sup> (expressing an uncleavable prelamin A-GFP chimera) were seeded in triplicate at  $4 \times 10^3$  cells/well in a 96-well plate and incubated overnight. Cells were then treated with 0nM, 200nM, 400nM, 600nM, and 800nM concentrations of GenoStat inducer, or DMSO, as a control, for 48 hours. 10  $\mu$ M BrdU was then added to each well in the plate and cells were incubated for 4 hours. BrdU incorporated into DNA was detected by incubation with monoclonal anti-BrdU antibody linked to HRP. The HRP substrate TMB was used to develop the color. After 30 minutes of development, stop solution was added and absorbance at 450 nm was measured using a platereader. The quantification of absorbance represented the amount of BrdU incorporated into the cells, and indicated the level of proliferation occurring in the corresponding cells.

## **Indirect Immunofluorescence**

Indirect Immunofluorescence was used to examine intracellular protein localization. Before visualizing the proteins, growth medium was removed, the cells were washed with 1x PBS and pure DMEM medium was added to the cells for visualization. The cells were harvested by trypsinization before being transferred to coverslip chamber dishes and plated on glass coverslips, which were incubated overnight in regular growth medium. Cells received treatment and were then incubated for 48 hours. After incubation, the cells were rinsed with 1x PBS prior to fixing by incubation with 4% formaldehyde PBS for 15 minutes at room temperature. The cells underwent three PBS washes, and were then permeabilized with 0.2% Triton X-100 in PBS for five minutes on ice, washed, and blocked with 10% BSA in PBS for five minutes on ice. The cells were incubated with primary antibody for one hour at room temperature, washed and incubated with a secondary, fluorophore conjugated, antibody for one hour. Following the second incubation, cells were washed three times with PBS then treated with DAPI-containing anti-fade Mounting Medium to seal the stained cells between the coverslip and a microscope slide. Images were obtained using a Nikon Diaphot200 microscope with a Oimaging Retiga 2000 cooled CCD digital camera.

## **Determining the cellular location of FoxO3a protein in prelamin A induced and uninduced 3T3 cells**

Prelamin A expressing L647R Rheoswitch 3T3 cells and control 3T3 cells were plated on coverslips and incubated overnight in regular growth medium. Experimental cells were incubated for 48 hours in the presence of Genostat inducer (500 mM). Un-

induced control cells of both types were incubated for 48 hours in the presence of DMSO. Cells were then fixed in 4% formaldehyde and analyzed by indirect immunofluorescence with antibodies against FoxO3 over GFP. Secondary antibodies were Alexa 565 and 488 respectively.

### **Separation of Proteins by Electrophoresis**

All samples were solubilized by the addition of 1X NuPAGE LDS Buffer and 10% Bond Breaker (reducing agent) as well as heated in a sand bath for 5 minutes. The samples were then cooled back to room temperature before loading an aliquot of each sample onto a pre-cast one-dimensional denaturing NuPAGE Noxvex 4-12% Bis-Tris SDS PAGE gel. A constant 200 volts for approximately 1-1.5 hours were used to perform the electrophoresis. Protein standards were also run with the samples to enable the estimation of apparent molecular weights for each sample.

### **Protein Blotting and Immunodetection**

The protein gel transfer to a nitro-cellulose membrane was accomplished using the iBlot, a dry electroblotter. Manufacturer protocol was followed (Invitrogen/Life Technologies, USA). The transfer process lasted approximately 7 minutes. After transfer, the membranes were examined to verify protein transfer as evidenced by the visibility of standard bands. If transfer was confirmed, the membrane was then blocked in 1X TBST / 5% Nonfat milk for 1 hour at room temperature or overnight at 4°C. Following blocking, the membrane was washed for 10min, 3 x 5min, and 10min, respectively, in 1X TBST and then incubated with primary antibody diluted to the manufacturer suggested

concentration in 1X TBST / 5% Nonfat milk for 1 hour at room temperature or overnight at 4°C. The washings were repeated after primary incubation. The membrane was then incubated with a 1X TBST / 5% Nonfat milk diluted secondary antibody-HRP conjugate at room temperature for one hour. Washings were repeated to remove excess secondary antibody conjugate. Bathing the membrane in an ECL chemiluminescence reagent bath for 5 minutes at room temperature, then draining the excess reagent and wrapping the membrane in a plastic film allowed protein band detection. The film was imaged using a Chemiluminescence Imaging System with digital photo documentation capability.

### **Measuring changes in FoxO3a subcellular distribution in response to L647R prelamin A expression and Juglone**

L647R Rheoswitch 3T3 cells ( $2 \times 10^6$ ) were plated on 10 cm plates and incubated for 48 hours in the presence of GenoStat inducer (500 mM) or DMSO plus the addition of 0, 5, or 15 mM of Juglone, an inhibitor of pin1. Cells were washed with PBS and scraped in 1ML PBS plus 0.1 % NP40. Cells were pipetted up and down five times to break up the cells and then 300  $\mu$ L were taken from each tube and labeled as whole cell lysate (W). The remaining 700  $\mu$ L were centrifuged in an Eppendorf centrifuge for 10 seconds at maximum speed. The supernatant was separated from the precipitate and an aliquot of 300  $\mu$ L was taken (cytoplasm, C). The pellet was resuspended in PBS plus NP40 and centrifuged again for 10 seconds at maximum speed. The pellet was resuspended in 300 $\mu$ L of 1x PBS/NP40 (Nuclear, N). Aliquots containing DNA (W and N) were sonicated for 5 seconds to break up the DNA. Samples were analyzed by Western blotting and G-box image analysis.

**Measuring changes in expression and subcellular distribution of p27<sup>kip</sup> in response to L647R prelamin A expression and Juglone**

Experimental methods followed those of the above test, which measured changes in FoxO3a subcellular distribution in response to L647R prelamin A expression and Juglone.



## Chapter 3. RESULTS

### **Prelamin A induced cell cycle arrest**

The measurement of BrdU incorporation into cellular DNA was used to demonstrate the effects of prelamin A accumulation on cell cycle progression. In previous experiments we have shown that prelamin A accumulation is a cause, rather than a by product, of cell cycle arrest, and that, L647R prelamin A expressing cells exhibit decreased proliferation<sup>8</sup>.

To monitor cellular proliferation in control, 3T3 cells, and experimental, prelamin A expressing L647R cells, a bromodeoxyuridine (BrdU) uptake assay was performed. This assay was used to monitor cellular proliferation by labeling only newly synthesized DNA with BrdU. BrdU incorporation was measured by first treating the cells with an anti-BrdU antibody linked to HRP, and then measuring the absorbance of each cell group at 450nm to detect the presence of HRP (Figure 3). Control 3T3 cells that do not contain a lamin expression vector, and cells from the L647R prelamin A expressing cell line, were treated with various concentrations of GenoStat, an inducer, or DMSO, a control, for 48 hours before the addition of BrdU to the medium to label actively proliferating cells. Examination of Figure 3 reveals that BrdU incorporation and, thus, cell proliferation, drastically decreases with the GenoStat induction of prelamin A accumulation in the induced L647R cells. The possible concern that repression of proliferation in the L647R cells may result from the toxicity of increasing GenoStat dosage, is dismissed by the lack of inhibition of proliferation in the unmodified 3T3 cells which were also treated with GenoStat inducer. In addition, the lack of proliferation

inhibition seen in L647R cells treated with DMSO, provides evidence that the activation of prelamin A expression in GenoStat induced L647R cells results in a decrease in proliferation of these cells.

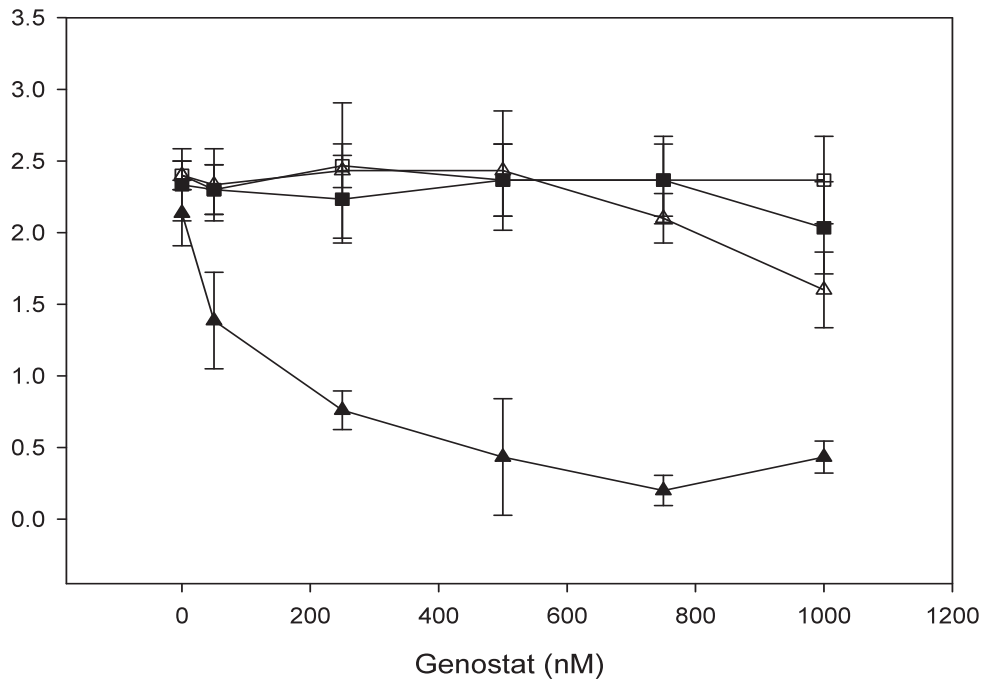


Figure 3. BrdU Incorporation into DNA. 3T3 cells (Squares) or L647R rheoswitch cells (Triangles) were seeded in triplicate at  $4 \times 10^3$  cells/well in a 96-well plate and incubated overnight. Cells were then treated with various concentrations of GenoStat inducer (filled symbols) or DMSO (empty symbols) for 48 hours. Finally,  $10 \mu\text{M}$  BrdU was added to the plate and cells were incubated for 4 hours. BrdU incorporated into DNA was detected by incubation with monoclonal anti-BrdU antibody linked to HRP. The HRP substrate TMB was used to develop the color and measure absorbance at 450 nm.

### **FoxO3a subcellular re-distribution upon prelamin A accumulation**

After showing that the accumulation of prelamin A induces cell cycle arrest, the second experiment was designed to investigate the impact of prelamin A accumulation on FoxO3a proteins within the cells. In this experiment, immunofluorescence imaging was used to determine if FoxO3a proteins were localized to the nucleus in L647R prelamin A expressing cells, as compared to uninduced control cells (Figure 4- top panel). Cells were treated with anti-FoxO3a antibody to reveal the diffuse expression of FoxO3a throughout the uninduced cells, and the significant increase in nuclear-concentrated FoxO3a expression in prelamin A induced cells.

Furthermore, treatment of the cells with an anti-GFP antibody (Figure 4-middle panel) revealed accumulation of GFP-tagged L647R prelamin A nucleoplasm and nuclear rim of the induced cells. This prelamin A induced fluorescence is absent from uninduced cells.

When the two immunofluorescent images are overlaid (Figure 4- bottom panel), the nuclear colocalization of FoxO3a and prelaminA becomes clear; however, neither nuclear localization nor prelamin A accumulation are present in the uninduced image overlay.

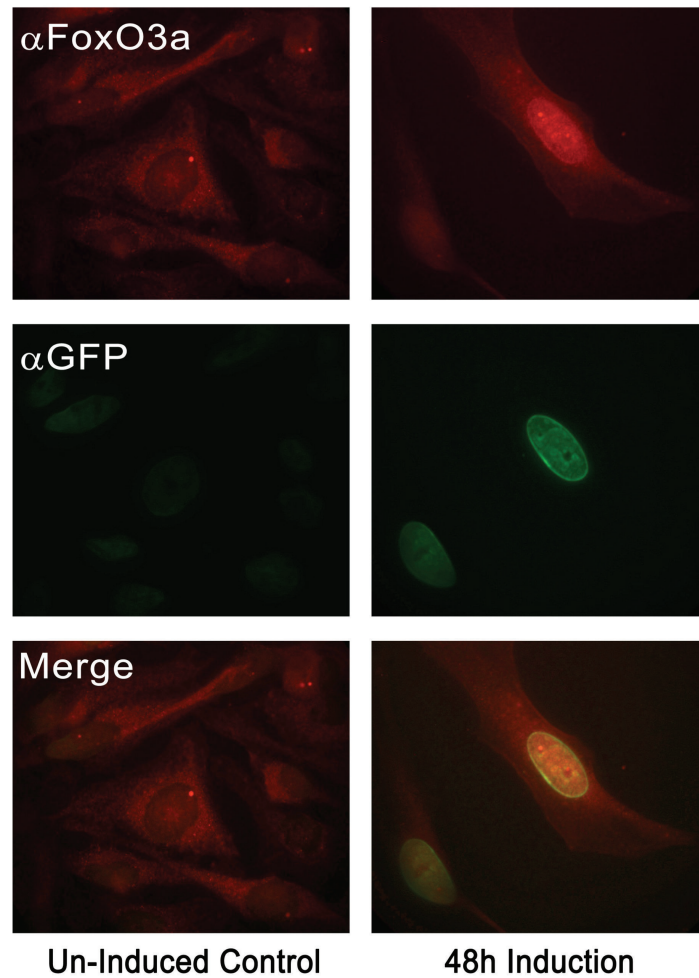


Figure 4. FoxO3a sub cellular re-distribution upon prelamin A accumulation in 3T3 cells. L647R Rheoswitch 3T3 cells were plated on coverslips and incubated overnight in regular growth medium. Cells were then incubated for 48 hours in the presence of GenoStat inducer (500 mM) or DMSO. Cells were then fixed in 4% formaldehyde and analyzed by indirect immunofluorescence with antibodies against FoxO3 over GFP. Secondary antibodies were Alexa 565 and 488 respectively.

### **Changes in FoxO3a subcellular distribution in response to Juglone**

The experiment “FoxO3a subcellular re-distribution upon prelamin A accumulation” in this study showed that L647R prelamin A expression increases expression of FoxO3a and the nuclear translocation of the protein. Because nuclear localization of FoxO3a is correlated with activation of the protein, these findings also indicate a higher level of FoxO3a activation in prelamin A induced cells. Furthermore, it was shown through additional experiments accompanying this study that, p27<sup>Kip1</sup>, a FoxO target for transcriptional activation, is also translocated to the cell nucleus, and, its expression increased upon prelamin A accumulation and FoxO3 nuclear translocation. This finding offers support for a prelamin A-induced effect on FoxO activation, and suggests a viable potential mechanism for prelamin A-mediated cell cycle arrest <sup>8</sup>.

Given that pin1 is known to suppress FoxO proteins function, it is expected that the increased presence of pin1 in the nucleus of prelamin A induced cells would result in decreased FoxO activity. However, when the pathway is examined as a whole, data suggests that pin1-mediated suppression of FoxO nuclear translocation is inhibited in prelamin A induced cells <sup>8</sup>. In order to test this hypothesis, the effects of treatment with Juglone, a pin1 inhibitor, on FoxO nuclear translocation were observed (Figures 4 and 5). This experiment was also used to biochemically confirm the translocation of FoxO3a to the nucleus of cells expressing uncleavable prelamin A. This was accomplished by performing a nuclear-cytoplasmic fractionation on the whole cell lysate of both control 3T3 cells and L647R cells expressing prelamin A. Nuclear, cytoplasmic, and whole cell samples of each cell type were electrophoresed using a 4-12% gradient SDS-PAGE gel. These gels were interpreted with western blotting techniques, knowing that the molecular

weight of FoxO3a is approximately 80Kd. The results of this experiment show that inhibition of pin1 by Juglone leads to the nuclear redistribution of FoxO3a in control, uninduced cells, and, that cells expressing uncleavable prelamin A show higher total levels of FoxO3a in the nuclear fractions at all concentrations of pin1 inhibitor administered.

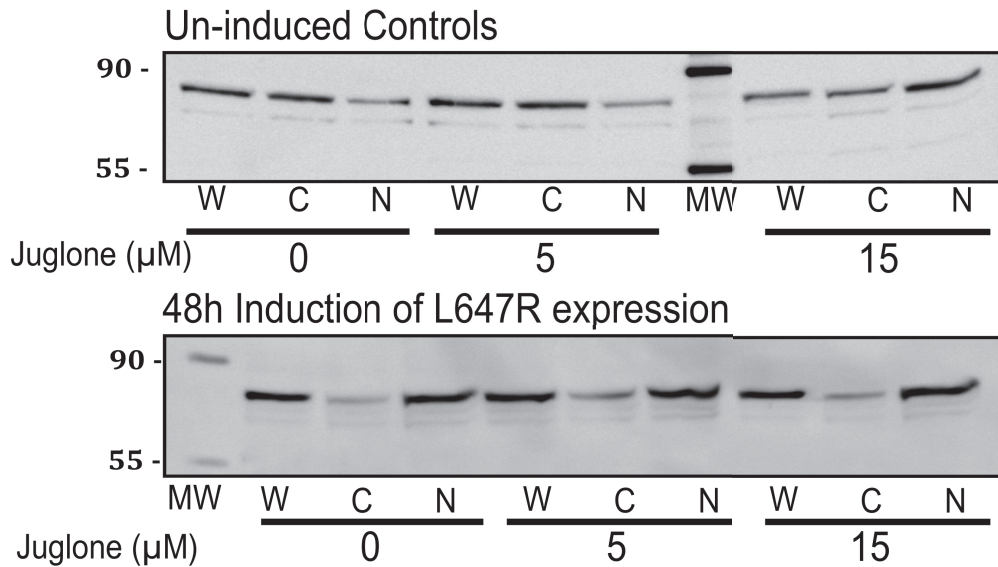


Figure 5. Western Blot Evidence for Changes in FoxO3a subcellular distribution in response to L647R prelamin A expression and pin1 inhibitor Juglone. L647R Rheoswitch 3T3 cells ( $2 \times 10^6$  were plated on 10 cm plates and incubated for 48 hours in the presence of GenoStat inducer (500 mM) or DMSO plus the addition of 0, 5, or 15 mM of Juglone, an inhibitor of pin1. Cells were washed with 1x PBS and scraped in 1ML 1x PBS plus 0.1 % NP40. Cells were pipetted up and down five times to break up the cells and then 300 μL were taken from each tube and labeled as whole cell lysate (W). The remaining 700 μL were centrifuged in an Eppendorf centrifuge for 10 seconds at maximum speed. The supernatant was separated from the precipitate and an aliquot of 300 μL was taken (cytoplasm, C).

The pellet was re-suspended in 1x PBS plus NP40 and centrifuged again for 10 seconds at maximum speed. The pellet was resuspended in 300  $\mu$ L of 1x PBS/ NP40 (Nuclear, N). Aliquots containing DNA (W and N) were sonicated for 5 seconds to break up the DNA. Samples were analyzed by Western blotting.

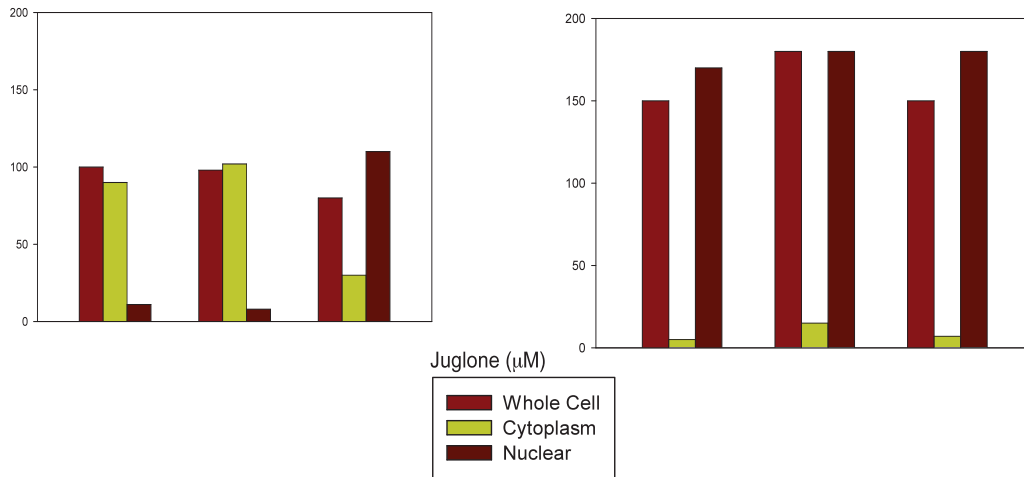


Figure 6. Graphical Analysis of Western Blot Evidence for Changes in FoxO3a subcellular distribution in response to L647R prelamina A expression and pin1 inhibitor Juglone. This bar graph shows a quantitation of the bands shown in Figure 5 using a G-Box image analysis instrument.

### Changes in p27<sup>Kip</sup> subcellular distribution in response to Juglone

In conjunction with the previous experiment, the effects of treatment with Juglone and prelamina A accumulation on p27<sup>Kip</sup> expression and localization were also observed. p27<sup>Kip1</sup> is a FoxO3a regulated molecule. The same membrane used in the previous experiment was stripped and re-probed. However, instead of probing for FoxO3a, the focus was p27<sup>Kip</sup>, which has a molecular weight of 27Kd. From this experiment, it can be seen that p27<sup>Kip</sup> is elevated in the induced uncleavable prelamina A cells as compared to the controls, and that the majority of the protein is localized in the nucleus. In addition,

the degree of p27<sup>Kip</sup> elevation increases as the Juglone treatment dosage is increased in both the control and induced cells. It is also important to note that the induced cells have higher initial levels of p27<sup>Kip</sup>, and that expression in these cells as compared to the uninduced cells without Juglone treatment, exhibits little significant change induced by pin1 inhibition.

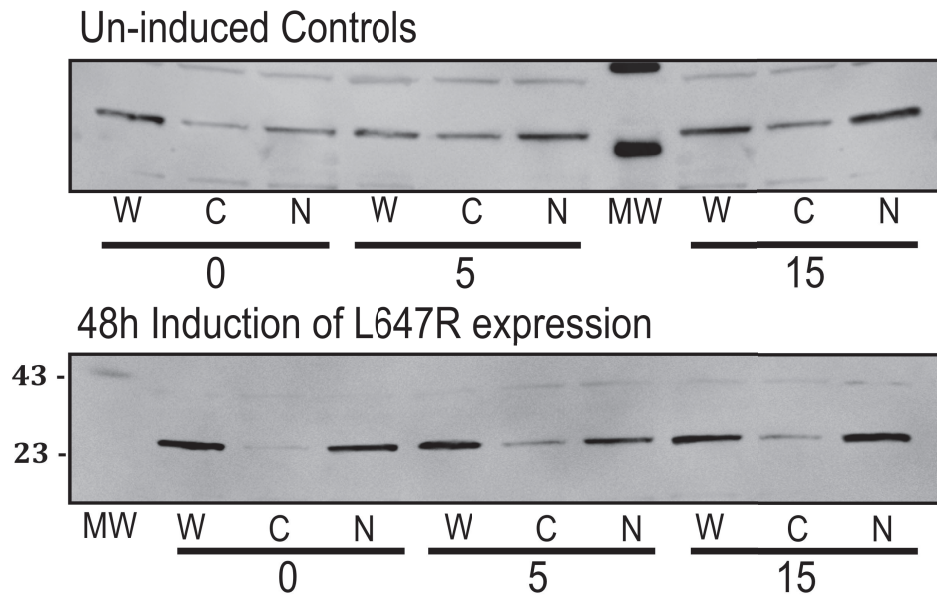


Figure 7. Western Blot Evidence for Changes in expression and subcellular distribution of p27<sup>Kip1</sup> (FoxO proteins regulated molecule) in response to L647R expression and pin1 inhibitor, Juglone. The membrane used in Figure 5 was stripped and reprobred using an anti-p27<sup>Kip</sup> antibody.



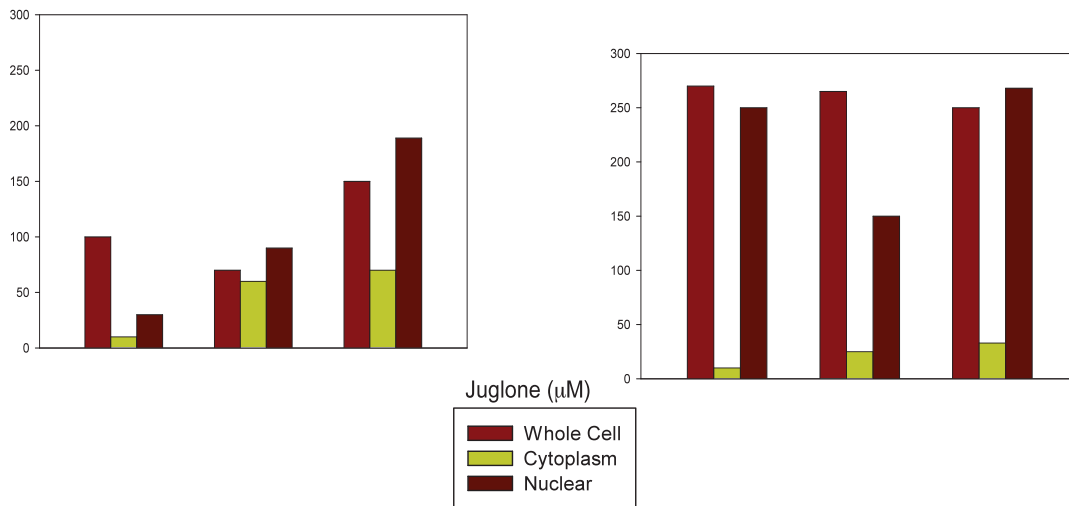


Figure 8. Graphical Analysis of Western Blot Evidence for Changes in expression and subcellular distribution of p27<sup>Kip1</sup> (FoxO proteins regulated molecule) in response to L647R expression and pin1 inhibitor Juglone. This bar graph shows a quantitation of the bands shown in Figure 7 using a G-Box image analysis instrument.

## Chapter 4. DISCUSSION AND CONCLUSION

Over the course of this study, techniques such as immunofluorescence and western blotting have been used to further study the mechanisms by which prelamin A activates FoxO-mediated cell cycle arrest. The mechanisms involved in this process were found to include: translocation of FoxO3a to the nucleus in the presence of uncleavable prelamin A, inhibition of pin1, and upregulation of p27<sup>Kip</sup>. It was hypothesized and observed that when uncleavable prelamin A is expressed in 3T3 cells, FoxO proteins migrate to the cell nucleus and induce cell cycle arrest through the upregulation of the p27 cyclin-dependent kinase inhibitor.

The first experiments of this study utilized BrdU incorporation into DNA to demonstrate how prelamin A accumulation induces cell cycle arrest, and indirect immunofluorescence to reveal the effects of this accumulation on sub cellular redistribution of FoxO3a. From the first experiment, it can be concluded that prelamin A accumulation is a cause, rather than an effect, of cell cycle arrest. In addition, the protein's accumulation during the cell cycle itself, as evidenced by prelamin A accumulation due to a lack of maturation processing, is a possible mechanism for proliferating cells to coordinate their exit from the cell cycle. The results of this experiment supported existing data concerning the role of prelamin A accumulation in the cell cycle, and prompted further exploration of this process with regards to the role of FoxO3a. The indirect immunofluorescence of both control and prelamin A induced cells shown in Figure 4 was used to provide visual evidence for the nuclear accumulation of prelamin A, as well as, the nuclear translocation of FoxO3a proteins in prelamin A expressing cells.

In studies by graduate student, Christina Bridges, Ph. D., it was shown that activity of the Akt phosphorylating pathway is downregulated when prelamin A is expressed<sup>8</sup>. Because Akt is the major inducing kinase of FoxO phosphorylation-dependent export from the nucleus, the observed downregulation of Akt is consistent with the nuclear localization and activated FoxO transcriptional regulation noted in this study. Additionally, pin1 has been shown to be a primary mediator of the Akt pathway, and inhibition of pin1 results in the destabilization and degradation of Akt. It is proposed that prelamin A binds and sequesters pin1, such that pin1 inhibition, and thus Akt downregulation can be mimicked by prelamin A accumulation. Therefore, if pin1 is inhibited by either Juglone or prelamin A accumulation, FoxO should be localized to the cell nucleus and actively transcribe target proteins such as p27<sup>Kip</sup>. These predictions are supported by Figures 4, 5, 6 and 7, in which Juglone was used to inhibit pin1 in control cells and prelamin A expressing cells. The results of both these studies support one another and the relationship between FoxO3a nuclear translocation, pin1 and p27<sup>Kip</sup> activity. Interestingly, while it was shown that treatment with pin1 inhibitor, Juglone, increased nuclear translocation of FoxO3a and p27<sup>Kip</sup> in uninduced, control cells, the prelamin A expressing, induced cells, which already exhibited an increased nuclear translocation of FoxO3a and p27<sup>Kip</sup> proteins, did not show a significant increase. These findings suggest that expression of prelamin A inhibits the negative effects of pin1 upon FoxO3a nuclear translocation, and the resultant p27<sup>Kip</sup> expression, as well as, that prelamin A acts through an undescribed mechanism to sequester pin1, limit its nuclear availability and binding to targets. Christina Bridges, Ph. D. remarks that this mechanism

of pin1 inhibition could have many consequences for cell cycle regulation due to the many targets of pin1 activity<sup>8</sup>.

In conclusion, these studies confirmed that expression of uncleavable prelamin A induces cell cycle arrest, and revealed that: the presence of uncleavable prelamin A is associated with the translocation of FoxO3a proteins to the nucleus; inhibition of pin1 leads to a redistribution of FoxO3a to the nucleus in uninduced cells; at all concentrations of pin1 inhibitor, cells expressing uncleavable prelamin A show higher total levels and increased amounts of FoxO3a in the nuclear fractions; inhibition of pin1 produces results similar to those seen with the accumulation of prelamin A in that FoxO3a translocates to the nucleus and upregulates p27<sup>Kip</sup>.

The goal of this study was to determine the mechanisms by which prelamin A accumulation activates FoxO-mediated cell cycle arrest. The mechanisms involved in this process were found to include: translocation of FoxO3a to the nucleus in the presence of uncleavable prelamin A, inhibition of pin1, and upregulation of p27<sup>Kip</sup>. Further experiments that can be done in order to gain a better understanding of these processes include: determining the effects of pin1 overexpression, inhibition of pin1 by means other than Juglone, exploration of the interactions between pin1 and prelamin A through site directed mutations and development of more effective ways to quantitatively explore the effects of pin1 and prelamin A accumulation in the translocation of FoxO3a to the cell nucleus. Hopefully, when the overexpression of pin1 is studied, a reversal of the effects of prelamin A accumulation will be seen, such that cell cycle arrest may be stopped. This project and the further studies associated with it have several implications ranging from improved treatment of rare disorders such as HGPS and RD, which involve abnormal

aging phenotypes, to implications for the more common age-dependent pathologies such as diabetes, cancer, autoimmune syndromes and neurodegeneration, and even the normal aging process.

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