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Proximity-Labeling of Near Neighbors of Lamin A and Lamin A-Δ50 (PROGERIN).

Mohammad Sabri
East Tennessee State University

PROXIMITY-LABELING OF NEAR NEIGHBORS OF LAMIN A AND LAMIN A-Δ50 (PROGERIN)

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By

Mohammad Sabri
The Honors College
University Honors Program
East Tennessee State University

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

Dr. Ismail Kady, Faculty Reader
Department of Chemistry
East Tennessee State University

Ms. Jaime Parman, Reader

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Abstract

In an attempt to isolate and identify proteins that differentially interact with or locate near lamin A and progerin, we used a previously described method named BioID (proximity-dependent biotin identification). This method is based on fusion of a promiscuous *E. coli* biotin-protein ligase (BL) to a targeting protein (in this study, lamin A-GFP and progerin-GFP). The biotin ligase biotinylates amino residues in proteins that are near-neighbors of the fusion protein. To create the fusion proteins, BL was sub-cloned from a pcDNA3.1 MCS-BirA(R118G)-HA plasmid donated by Kyle Roux from University of South Dakota. The BL fragment was ligated into a pNEBR-X1-lamin A-GFP and pNEBR-X1-progerin-GFP which inducibly express the fusion proteins in mammalian cells, under control of pNEBR-R1 Rheoswitch regulator plasmid.

Two stable cell lines expressing the GFP-BL-lamin A and GFP-BL-progerin chimeras were created. The expression of the chimeras was induced by incubation with 500nM of GenoStat for 24 hours in the presence of 50mM Biotin. Biotinylated proteins were isolated from cell lysates by incubation with streptavidin magnetic beads. Proteins were separated by SDS-PAGE and sent for identification by mass spectrometry. In conclusion, we isolated multiple proteins that differentially associate with and/or are proximate to lamin A and progerin *in vivo*. The identification of such proteins may shed light into the mechanism by which progerin causes its deleterious effects. The purpose of this research is to attempt to identify the neighboring proteins that differentially interact with or locate near lamin A and progerin.

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List of Abbreviations

HGPS	Hutchinson-Gilford Progeria Syndrome
BL	Biotin Ligase
LA	lamin A
LA-Δ50	lamin A-Δ50 (progerin)
GFP	Green Fluorescence Protein
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
MOPS	3-(N-morpholino) Propanesulfonic Acid
DMEM	Dulbecco's Modified Eagle's Medium
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
µL	Microliter
mL	Milliliter
nM	Nanomolar
mM	Micromolar

Chapter 1: INTRODUCTION

Proteins are vital and necessary for the continuation of human life, yet not enough is known about their interaction with other proteins or protein networks. It has gradually become more obvious that proteins can only be fully understood in the context of protein-protein interactions. These networks of interactions also are fundamental in understanding disease processes[1], such as Hutchinson-Gilford Progeria Syndrome. "BioID", a new approach to understand these interactions, was recently developed as an alternative to previous screening processes for protein-protein interactions[2]. Previous methods to identify protein-protein interactions have helped scientists understand more about these interactions, but there are significant drawbacks. The most common techniques for protein-protein interaction screening have been affinity capture screening and yeast two-hybrid systems; each has its positive and negative aspects.

Yeast two-hybrid systems are used to screen for protein-protein interactions[3] and protein-DNA interactions[4] by testing for physical interactions between two proteins or a protein and DNA molecule[3,4]. In most eukaryotic cell transcription factors, the activating and binding sites can function in close proximity with each other without physical binding[5]. In the yeast two-hybrid assay, a genetically modified yeast (lacking in certain vital nutrients) is infused with two separate bait and prey plasmids[6]. The bait protein's coding sequence is fused with the DNA binding domain (BD). The prey protein is fused into the transcription activation domain (AD). These vectors are then transfected into a yeast strain. If the "bait" and "prey" proteins interact, transcription of the gene LacZ occurs and a change in cell phenotype indicates success[3].

The yeast two-hybrid system is helpful and very effective when results are achieved, however, some studies have shown that this system can give up to 70% false

positives/negatives[7]. These false readings can be caused by many reasons. The most important reason is that all interactions take place *in vitro*[2]. While in vitro, the two-hybrid system only takes place in the nucleus; therefore, proteins that interact outside the nucleus (but not in the nucleus) may be read as negative[7]. Also, assay variants over-express fusion proteins which lead to false positives[7].

Another method called affinity capture screening may be used to purify an enzyme solution or identify compounds that bind to a particular substance[8]. It usually involves a cell lysate solution being passed through a gel to separate out molecules in the solution. The gel is commonly made of agarose. This method is effective when successful, but has disadvantages. High-throughput genetic approaches take place in vitro, usually not having the proper machinery for post-translational modifications to binding partners; this can lead to incomplete data[2].

A new approach was recently developed as an alternative to the previous stated screening processes for protein-protein interactions. Called "BioID", it is a groundbreaking method to screen for proteins in a natural cellular environment (*in vivo*)[2]. The main goal of the BioID method is to identify vicinal proteins by proximity-dependent biotinylation (marking of proteins with biotin)[2]. It was developed by Drs. Roux, Kim, Raida, and Burk at the University of South Dakota. In the development of the BioID method, these researchers used protein lamin A and "biotin ligase" BirA*.

Lamin A is an intermediate filament protein that is encoded by the LMNA gene[9]. This protein is found in the nuclear lamina. Lamin A is highly insoluble and has been proven difficult to define via conventional approaches[9]; therefore, is an ideal candidate for BioID[2]. BirA is a DNA-binding biotin:protein ligase in *Escherichia coli* that regulates the biotinylation of a subunit of acetyl-CoA carboxylase and represses transcription for the biotin biosynthetic

operon[10]. However, BirA is too selective to use in BioID[2].BirA*, also known as R118G, is a mutant of BirA. It biotinylates proteins more promiscuously than BirA[2].

In BioID, a biotin ligase is fused to a protein of interest; in Dr. Roux's experiment with his colleagues, they fused BirA* to lamin A[2]. The biotin ligase is then introduced into a live mammalian cell (Roux used HeLa cells) where it biotinylates (marks with biotin) vicinal proteins once the culture medium has biotin. These biotinylated proteins are then selectively isolated and identified[2]. Mass spectrometry is used to identify the biotinylated proteins[2]. BioID was used by Dr. Roux and his colleagues in this fashion to successfully biotinylate many known vicinal proteins in the nuclear envelope[2] of HeLa cells and identified a novel protein, SLAP75[2].

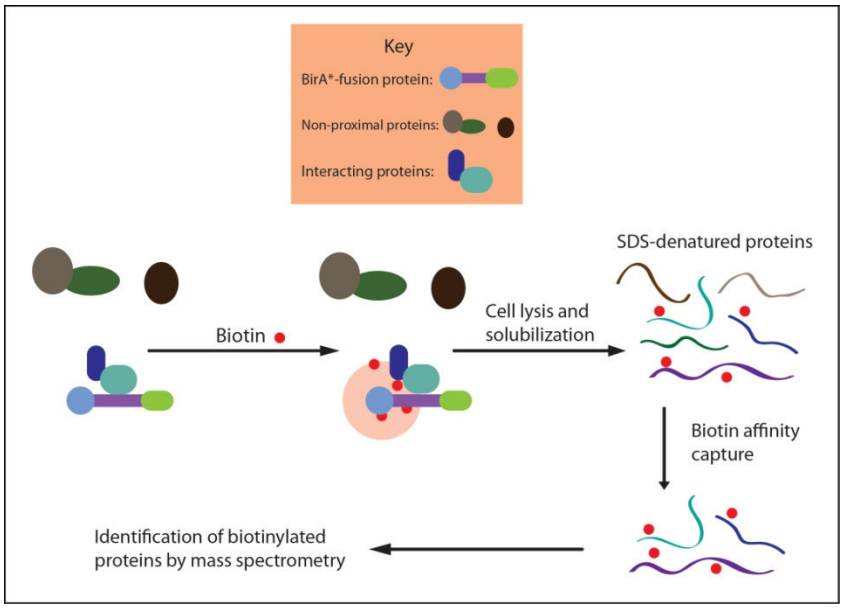


Figure 1. BioID Method. Adapted from Roux et al. (2012)

BioID was proven by Roux to be effective at biotinylating vicinal proteins. It has advantages over other common protein-protein interaction screening methods because it takes place *in vivo*. However, BioID has its weaknesses as well. It relies on the excess of biotin to be added to a culture in order to advance biotinylation. In theory, this can cause false positives or an inflated amount of biotinylated proteins[2]; however, that did not occur in Roux's experiment. Also, current studies have only been in mammalian cells so it cannot be said whether or not BioID is useful in all types of cells.

BioID is a new approach that is powerful and effective. It is broadly accessible and can be used by a wide range of researchers[2]. However, it should be used as a complement to the current conventional methods. The method is innovative and can be used to help elucidate the protein-protein interactions in laminopathies such as Hutchinson-Gilford Progeria Syndrome.

Mutations in the *LMNA* gene, which encodes the nuclear filament protein lamin A, cause the premature aging disease Hutchinson-Gilford Progeria Syndrome. One of these mutations results in the expression of a mutant lamin A, with a 50-aa in-frame deletion within its C terminus. Our laboratory previously demonstrated that this deletion leads to a stable farnesylation and carboxymethylation of the mutant lamin A. These modifications cause an abnormal association of progerin during mitosis, induce changes in the cell cycle progression, and may cause DNA damage (among many other effects).

We employed a recently developed technique, BioID, to identify vicinal proteins by proximity-dependent biotinylation. This *in vivo* technique has an advantage over previous screening methods such as affinity capture screening and yeast two-hybrid systems due to its ability to allow proteins to interact in a natural cellular environment. We use BioID to experiment with lamin AΔ50 (progerin) to understand its interactions in the hope of learning to regulate it and cure laminopathies such as Progeria.

Chapter 2. MATERIALS AND METHODS

Antibodies: Mouse anti-GFP was from ABCAM (Cambridge, MA) rabbit anti-biotin ligase was from Hoelzel-Biotech (Germany). Secondary antibodies conjugated to Alexa-488, Alexa-565, or Horse radish peroxidase were from Invitrogen. Dynabeads® MyOne™ Streptavidin C1 biotin binding beads were also from Invitrogen.

Plasmids: pcDNA3.1 MCS-BirA* plasmid encoding a bacterial biotin ligase (BL) was donated by Kyle Roux from University of South Dakota. pNEBR-X1-lamin A-GFP and pNEBR-X1-progerin-GFP were created by Christina Bridges in Dr. Rusiñol Lab.

Creation of biotin ligase fusion constructs: Biotin ligase/lamins fusion constructs were created by sub-cloning the biotin ligase open reading frame from pcDNA3.1 MCS-BirA* into either pNEBR-X1-lamin A-GFP and pNEBR-X1-progerin-GFP. Briefly, a biotin ligase fragment containing ectopic restriction enzymes sites for Not I and BamH I were generated by Polymerase Chain Reaction (PCR) using the following primers:

5'-GGAGAAATCTCCCTGAGAAGCGCAGAGAAGGGCGGCCGC-3'

5'-GGATCCCCCAAGCTGGCTAGCCACCATGGAACAAAAACTCATCTC-3'. For the restriction enzyme digestion reaction, a combination of 1µL 10x buffer, 6.5µL H20, 2µL DNA, and 0.5µL enzyme were combined in a microfuge tube in order. This mixture was incubated for 1 hour at 37 ˚C.

The biotin ligase fragment was ligated in frame after GFP into a pNEBR-X1-lamin A-GFP and pNEBR-X1-progerin-GFP. A Takara ligation kit was used and reaction was carried out for 12 h at 16˚C. E. coli cells were transformed with the products of the ligation reaction and plated on LB-agar plates containing 100µg/ml ampicillin. Colonies were picked after a 14 hour incubation at 30 ˚C. Cells from colonies were grown in 6 mL of LB media containing 100µg/mL

ampicillin for 12 hours at 30 ˚C. Plasmids were purified from these or larger (500 mL) cultures using Qiagen plasmid purification kits according to manufacturer protocol. Constructs were confirmed by DNA sequencing at the Quillen College of Medicine Core Facility.

Cell lines: To express the biotin ligase/lamin fusion proteins, we chose the Rheoswitch® inducible protein expression system from New England Biolabs. The RheoSwitch® Mammalian system allows for inducible gene expression achieved through the highly specific interaction of a synthetic inducer, RheoSwitch Ligand RSL1 (GenoStat, from Millipore), and a nuclear receptor/transcription factor. These factors are activated in the presence of GenoStat, and the level of gene transcription can be regulated by adjusting the concentration of GenoStat in the culture media.

Creation of stable Rheoswitch® cell lines expressing GFP-BL-lamin A and GFP-BL-progerin: pNEBR-X1-lamin A-BL-GFP and pNEBR-X1-progerin-BL-GFP plasmids were transfected into Rheoswitch T3T cells by using TransIT-3T3™ from Mirus Bio (Madison WI) or MegaTran 1.0™ from Origene (Rockville MD) in growth medium (DMEM, 10% heat inactivated bovine calf serum, Penicillin/Streptomycin) according to manufacturer protocol. After 24 hours, cells were trypsinized, diluted in a ration 1:10 and incubated in growth medium with 800ug/mL G418 and 200ug/mL Hygromycin for two weeks, changing medium every 3-4 days. Colonies of resistant cells were selected, isolated, expanded, induced with GenoStat and checked for expression by fluorescence microscopy. Clones with various levels of expression were obtained and stored on liquid nitrogen.

Inducible expression of biotin ligase-lamin fusion proteins: 10^7 cells were plated in 10 cm culture dishes and incubated in the presence of various concentrations of GenoStat for 48 hours. Cells were then washed twice with 5 mL of PBS and scraped in 1 mL of the ice-cold lysis buffer

(50 mM Tris, pH 7.4 250 mM NaCl 5 mM EDTA 50 mM NaF 1 mM Na3VO4 1% Nonidet P40 (NP40) 0.02% NaN3) containing Halt™ (protease and phosphatase inhibitors cocktail from Pierce). Cells lysates were kept on ice for 10 minutes and then frozen at -80 ˚C.

Immunoblotting: Lysates were next subjected to SDS-PAGE using the Novex electrophoresis system from Invitrogen according to manufacturer protocol. A constant 200 volts was used for approximately 45 minutes to perform the electrophoresis. Pre-stained molecular weight markers were ran alongside the samples. Proteins were then transferred to PVDF membranes using a semi-dry electro blotter, iBlot® from Invitrogen/Life Technologies. Manufacturer protocol was followed and the transfer time was set to seven minutes. Western Blotting with anti-GFP or anti-biotin ligase was performed following standard procedure using primary antibodies at a dilution of 1:10,000 and secondary antibodies at a dilution of 1:25,000.

Sub-cellular localization of biotin ligase-lamin fusion proteins by immunofluorescence

3T3 Rheoswitch cells were plated on coverslips and incubated with GenoStat for 24 hours. Next, cells were rinsed with 1x PBS prior to fixing by incubation with 4% formaldehyde PBS for 15 minutes at room temperature. 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. Cells were incubated with primary antibody for one hour at room temperature, washed and incubated with a secondary antibody for one hour. Dilution for mouse anti-GFP was 1:500 and for rabbit anti-biotin ligase 1:250 in blocking reagent. Dilution for the Alexa488 and Alexa565 secondary antibodies was 1:5000. 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 an EVOS-fl automatic microscope from AMG (Life Technologies). All image processing (contrast stretching and image overlays) was done by the microscope software.

BioID experimental protocol: The BioID protocol was followed as previously described by Roux[2] in order to identify proteins interacting with lamin A and progerin.

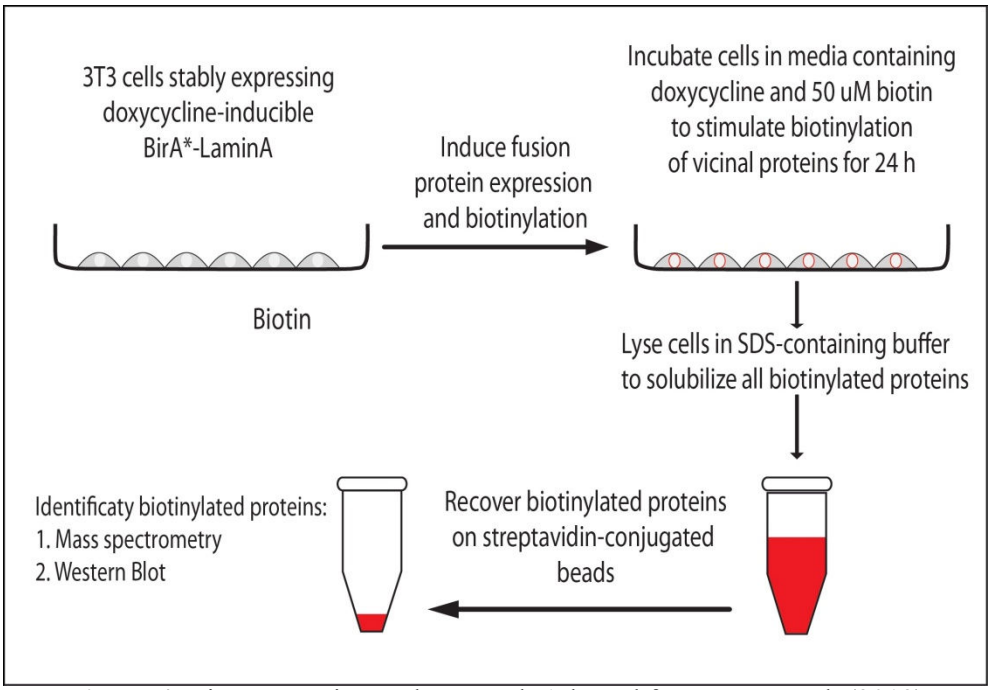


Figure 4. BioID experimental protocol. Adapted from Roux et al. (2012)

Fusion protein expression was induced by incubation with 500nM of Genostat for 24 hours in the presence of 50µM Biotin to stimulate biotinylation of vicinal proteins. The cells were lysed in SDS-containing buffer to solubilize all biotinylated proteins. The biotinylated proteins were then recovered with Dynabeads® MyOne™ Streptavidin C1 beads according to manufacturer protocol. Briefly, Lysates were incubated with the beads for 30 min at room temperature using gentle rotation. After separation with a magnet for 2–3 min, the coated beads were washed 4–5 times in PBS containing 0.1% BSA and resuspended in 50 to 100 µl. Immobilized biotinylated proteins were released by incubation for 5 min at 90°C in nonionic water or 0.1% SDS**.** Samples

were subjected to Sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE and protein containing lanes were cut into 5 mm pieces and send AB Sciex (Framingham, CT) for identification by MALDI-TOF mass spectrometry.

Chapter 3: RESULTS

Creation of biotin ligase-lamin fusion constructs

Biotin ligase-lamin constructs were successfully created.

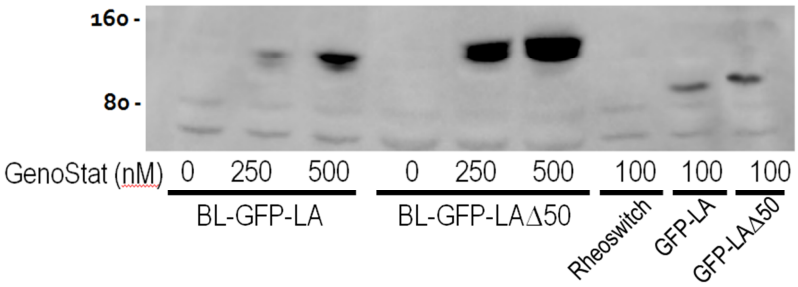
Creation of stable cell lines expressing biotin ligase-lamins fusion proteins

Two stable 3T3 Rheoswitch cell lines were created, one containing the GFP-BL-lamin A chimera and another cell line containing the GFP-BL-progerin chimera.

Inducible expression of biotin ligase-lamin fusion proteins

To ensure that the newly created cell lines contained the inducible protein chimeras, Western Blotting was used with various levels of GenoStat (0 nM, 250 nM, 500 nM). The experimental controls without the biotin ligase were in 100 nM of GenoStat. As expected, the fusion proteins were expressed in the cells with the biotin ligase BirA* proportionally with increasing levels of GenoStat. The fusion proteins were not expressed in the control cell groups.

Figure 5. Protein expression in Western Blot



Sub-cellular localization of biotin ligase-lamin fusion proteins

Immunofluorescence of 3T3 Rheoswitch BL-GFP-LA cells and 3T3 Rheoswitch BL-GFP-LAΔ50 cells showed the sub-cellular localization of biotin ligase-lamin fusion proteins. In the 3T3 Rheoswitch BL-GFP-

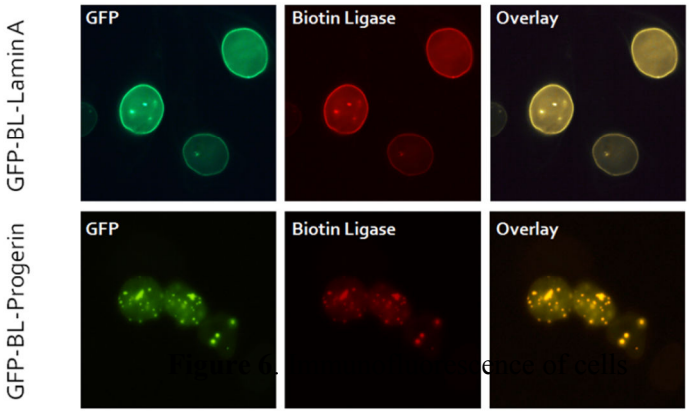


Figure C [illegible] ence of cells

LA, the fusion proteins were in the expected location of the nuclear lamina and nucleoplasma of the cell. In the 3T3 Rheoswitch BL-GFP-LAΔ50 cells, the biotin ligase-lamin fusion proteins were not in the nuclear lamina but rather in aggregates inside the nucleus and perhaps the cytoplasm or endoplasmic reticulum membranes.

BioID experimental protocol

Proteins biotinylated by biotin ligase in 3T3 Rheoswitch cells expressing BL-GFP-LA and BL-GFP-LAΔ50 were identified by mass spectrometry. Most of the identified proteins are known. There were proteins that were differentially biotinylated by BL-GFP-LA or BL-GFP-LAΔ50. Examples of these biotinylated proteins and their main functions are seen in Figure 7.

Labeled only by Lamin A-BL	Main Function
Zmpste24	Maturation of prelamin A
Ahctf1	Cell cycle, chromosome and centromere binding
Nup133	Constituent of nuclear pore
Xpo1	Exportin-1, participates in nuclear export
Tpr	Component of nuclear pore
Srebp	Transcription factor
Labeled only by Progerin-BL	**Main Function**
Rab2	Regulates vesicular trafficking
Pcna	Multiple functions in DNA replication and damage repair
H2Afz	Functions To Regulate Progression through the Cell Cycle
Mpp1	Interacts with the cytoskeleton and regulate cell proliferation
Sec61α1	Insertion of secretory and membrane proteins into the ER.
NapG	general elements of the cellular membrane transport apparatus

Figure 7. Examples of proteins differentially biotinylated by 3T3 RheoswitchBL-GFP-LA or 3T3 RheoswitchBL-GFP-LAΔ50

Chapter 4: DISCUSSION AND CONCLUSION

During this study, BioID and other techniques were used to identify neighboring proteins that differentially interact with or locate near lmin A and progerin. First, two new cell lines were successfully created with a biotin ligase BirA*: a 3T3 Rheoswitch BL-GFP-LA cell line and a 3T3 Rheoswitch BL-GFP-LAΔ50 cell line. This was done by transfecting lamin A-Biotin Ligase-Green Fluorescence protein constructs and Δ50Lamin A-Biotin Ligase-Green Fluorescence Protein constructs into 3T3 Rheoswitch cells. To ensure the inducible system functioned properly, the new cell lines were induced with GenoStat and the protein expression levels were compared to control cells. Immunofluorescence was also used to ensure proper location of the expressed proteins.

Once these cell lines were created, the BioID method was implemented to isolate vicinal proteins to lamin A and progerin. The BioID method consisted of the inducible expression of the biotin ligase fusion protein in 3T3 Rheoswitch cells, biotinylation of vicinal proteins, lysis of cells in SDS-containing buffer, recovery of biotinylated proteins on stravidin-conjugated beads, and identification via mass spectrometry. The technique was successful in isolating and identifying proteins known to interact *in vivo* with lamin A and progerin. It was seen that most of these interacting proteins are known. It was also determined that there are proteins that interact with either lamin A or progerin, but not both.

Certain interesting interactions were seen that may help understand laminopathies and similar disease processes. For example, progerin was found to interact with a protein of the endoplasmic reticulum, Sec61α1; this could mean that progerin leaves the nuclear lamina and interacts with the walls of the endoplasmic reticulum. Also, Zmpste24, a crucial protein involved in the maturation of lamin A, does not interact with progerin. It is important to continue studying

similar protein interactions unique to progerin and lamin A in order to learn why some protein dysfunctions occur. To shed more light on these processes, further studies can include synchronized cells in differing phases of the cell cycle such as G0, G1, S, etc. Also, further exploration of the protein interactions can be accomplished through fluorescence resonance energy transfer (FRET), co-immunoprecipitation (Co-IP), or site directed mutagenesis.

In conclusion, this study is a proof of concept for identifying the neighboring proteins that differentially interact with or locate near lamin A and progerin. This study's successful identification of near-neighbors of laminar proteins *in vivo* is a step on the path to completely understanding laminopathies and disease processes dealing with aging such as Hutchinson-Gilford Progeria Syndrome.

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