

2022

# Characterization of aortic carboxypeptidase-like protein mutations that cause Ehlers-Danlos syndrome

---

<https://hdl.handle.net/2144/45587>

*"Downloaded from OpenBU. Boston University's institutional repository."*

BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**CHARACTERIZATION OF AORTIC CARBOXYPEPTIDASE-LIKE PROTEIN  
MUTATIONS THAT CAUSE EHLERS-DANLOS SYNDROME**

by

**SIDRAH AHMED**

B.S., University of California, Los Angeles, 2019

Submitted in partial fulfillment of the  
requirements for the degree of  
Master of Science

2022

© 2022 by  
SIDRAH AHMED  
All rights reserved

Approved by

First Reader

---

Matthew D. Layne, Ph.D.  
Associate Professor of Biochemistry

Second Reader

---

Karen Symes, Ph.D.  
Associate Professor of Biochemistry

## **ACKNOWLEDGMENTS**

I would like to thank my PI, Dr. Matthew Layne, for all his support and guidance. I am incredibly appreciative of all the time he has taken to personally teach me. He has helped me grow into a better student, scientist, and researcher.

I would also like to thank my advisor, Dr. Karen Symes, who has been champion for me throughout this program. She has continually encouraged me and has given me many wonderful opportunities I am so grateful for.

I also thank Amber Liu for her collaboration and help with this research. Lastly I would like to thank my family and friends for keeping me motivated and always supporting me.

**CHARACTERIZATION OF AORTIC CARBOXYPEPTIDASE-LIKE PROTEIN  
MUTATIONS THAT CAUSE EHLERS-DANLOS SYNDROME**

**SIDRAH AHMED**

**ABSTRACT**

Ehlers-Danlos Syndrome (EDS) is a hereditary connective tissue disease that can cause a wide range of symptoms throughout the body. There are currently 14 different subtypes of EDS based on the molecular genetics of the disease with many subtypes due to mutations in collagen genes. Classical-like Type 2 EDS is caused by mutations in aortic carboxypeptidase-like protein (ACLP), gene name *AEBPI*. ACLP plays important roles in collagen polymerization, vascular smooth muscle differentiation, and myofibroblast differentiation. There have been multiple ACLP mutations that have been implicated in EDS. Previous studies have shown an ACLP mutation Asn490\_Met495delins, a mutation with intron inclusion in the protein, is intracellularly retained and causes endoplasmic reticulum (ER) stress. This study aims to characterize 5 additional human ACLP mutations that cause EDS including Leu642Pro, Asn236Ser, Trp356\*, Cys581\*, and Arg631\* to determine how these proteins disrupt the extracellular matrix (ECM). To achieve this goal, plasmid vectors harboring the desired mutation were generated. Immunofluorescent imaging revealed all mutated proteins remained in the secretory pathway and were properly translocated to the endoplasmic reticulum (ER). Western blot of cell lysate and media sample from cells transfected with the generated plasmid revealed that Leu642Pro was intracellularly retained while Asn236Ser, Trp356\*, Cys581\*, and Arg631\* were secreted

from the cell into the extracellular environment. Additionally, ACLP knockout mouse models were generated to further study the role of ACLP in aortic dysfunction observed in some ACLP null EDS patients. These studies provide further information about the molecular mechanisms in which ACLP causes EDS that could be possible targets for intervention in the future.

## TABLE OF CONTENTS

ACKNOWLEDGMENTS .....	iv
ABSTRACT.....	v
TABLE OF CONTENTS.....	vii
LIST OF TABLES .....	x
LIST OF FIGURES .....	xi
LIST OF ABBREVIATIONS.....	xii
CHAPTER I INTRODUCTION.....	1
EHLERS-DANLOS SYNDROME .....	1
EDS Classification .....	1
EDS Symptoms and Management .....	4
Treatment and Management .....	6
EXTRACELLULAR MATRIX.....	7
ECM functions .....	8
ECM Protein Trafficking .....	9
Endoplasmic Reticulum Stress Caused by Mutated ECM Proteins.....	10
AORTIC CARBOXYPEPTIDASE-LIKE PROTEIN.....	12
Structure and Function.....	12
Pathologies Associated with ACLP .....	15
Ehlers-Danlos Syndromes Caused by ACLP Mutations .....	16

GOALS AND OBJECTIVES .....	18
CHAPTER II METHODS .....	19
Generation of human ACLP mutants.....	19
Transformation.....	19
Miniprep and Maxiprep .....	20
Transient Transfections.....	21
Protein Extraction .....	22
Western Blot Analysis .....	22
Immunofluorescence.....	23
Mice Tissue Isolation.....	23
Mice Tissue Histology .....	24
CHAPTER III RESULTS .....	26
Generation of ACLP Mutants .....	26
Characterization of ACLP Mutants .....	29
Subcellular Localization of ACLP Mutants.....	30
Activation of ER Stress Pathways by Retained ACLP Mutants.....	31
Mouse Model ACLP loss of function .....	34
Summary of Results .....	35

CHAPTER IV DISCUSSION.....	37
ACLP-Leu642Pro is intracellularly retain .....	37
ACLP mutants Asn236Ser, Trp356*, Cys581*, and Arg631* are secreted.....	39
ACLP knockout Mouse Models.....	40
Additional ACLP mutants.....	42
ACLP (AEBP1) in cancer .....	43
Implications and future directions .....	44
REFERENCES .....	45
CURRICULUM VITAE.....	52

## LIST OF TABLES

<b>Table 1.</b> Clinical Classifications of EDS.....	2
--	---

## LIST OF FIGURES

<b>Figure 1.</b> IRE1 $\alpha$ Signaling in Response to ER Stress.....	11
<b>Figure 2.</b> ACLP structure and domains.....	12
<b>Figure 3.</b> Structure of metallocarboxypeptidase family members.....	13
<b>Figure 4.</b> Human mutations of ACLP causing EDS.....	17
<b>Figure 5.</b> Plasmid mutagenesis PCR cycling conditions and primers.....	20
<b>Figure 6.</b> Plasmid mutagenesis strategies.....	27
<b>Figure 7.</b> Sequencing verification of plasmids c.1925C>T (Leu642Pro) and c.707A>G (Asn236Ser).....	28
<b>Figure 8.</b> Cys581*, Arg631*, Trp356* and Asn236Ser are secreted from the cells while Leu642Pro is intracellularly retained.....	30
<b>Figure 9.</b> ACLP mutants exhibit perinuclear subcellular localization.....	32
<b>Figure 10.</b> ER Stress is increased in the intracellularly retained Ins40 mutant.....	33
<b>Figure 11.</b> ACLP expression is decreased in tamoxifen treated floxed ACLP Cre positive mice.....	35

## LIST OF ABBREVIATIONS

ACLP.....	aortic carboxypeptidase-like protein
AEBP1.....	adipocyte enhancer binding protein-1
ASK1.....	apoptosis signal-regulating kinase 1
DM.....	diabetes mellitus
ECM.....	extracellular matrix
EDS.....	Ehlers-Danlos Syndrome
ER.....	endoplasmic reticulum
FRD8.....	frizzled-8
GAG.....	glycosaminoglycans
IPF.....	idiopathic lung fibrosis
IRE1.....	inositol-requiring enzyme-1
LH1.....	lysyl hydroxylase-1
LRP6.....	lipoprotein receptor-related protein 6
MRTFA.....	myocardin-related transcription factor A
NASH.....	nonalcoholic steatohepatitis
PG.....	proteoglycan
SMA.....	smooth muscle actin
SRP.....	signal recognition particle
UPR.....	unfolded protein response
XBP1.....	X-Box binding protein 1

## CHAPTER I INTRODUCTION

### **EHLERS-DANLOS SYNDROME**

The Ehlers–Danlos Syndromes (EDS) are a group of heritable genetic connective tissue disorders with a variety of symptoms, manifestations, and genetic factors (Malfait et al., 2017). These disorders are characterized by tissue fragility, hypermobility in the joints, and skin hyperextensibility (Malfait et al., 2017). Due to the range of symptoms experienced and the abundance of connective tissues throughout the body, the severity of the disorders can vary from mild to more serious and debilitating (Syx et al., 2019). Disruptions to the connective tissue typically present itself as increased degree of motion in the joints, though some presentations may be more serious, such as fragility in tissue lining vascular walls (Syx et al., 2019).

#### ***EDS Classification***

Classification of EDS is dependent on the gene affected resulting in a variety of types of EDS that have a variable causes, symptoms, and treatment methods. There are currently fourteen subtypes of EDS and while some cases are inherited in either an autosomal dominant or autosomal recessive manner varying according to the subtype, other cases occur due to issues arising in early development (Malfait et al., 2017) (**Table 1**).

**Table 1. Clinical Classifications of EDS.** The 14 subtypes of EDS and their associated gene mutations. IP, inheritance pattern; AD, autosomal dominant; AR, autosomal recessive; NMD, nonsense-mediated mRNA decay. Adapted and modified from Malfait et al. 2017. Subtype 14 added from Vroman et al. 2021.

TABLE I. Clinical Classification of the Ehlers-Danlos Syndromes, Inheritance Pattern, and Genetic Basis					
Clinical EDS subtype	Abbreviation	IP	Genetic basis	Protein	
1 Classical EDS	cEDS	AD	Major: <i>COL5A1</i> , <i>COL5A1</i> Rare: <i>COL1A1</i> c.934C>T, p.(Arg312Cys)	Type V collagen Type I collagen	
2 Classical-like EDS	clEDS	AR	<i>TNXB</i>	Tenascin XB	
3 Cardiac-valvular	cvEDS	AR	<i>COL1A2</i> (biallelic mutations that lead to <i>COL1A2</i> NMD and absence of pro $\alpha 2(I)$ collagen chains)	Type I collagen	
4 Vascular EDS	vEDS	AD	Major: <i>COL3A1</i> Rare: <i>COL1A1</i> c.934C>T, p.(Arg312Cys) c.1720C>T, p.(Arg574Cys) c.3227C>T, p.(Arg1093Cys)	Type III collagen Type I collagen	
5 Hypermobile EDS	hEDS	AD	Unknown	Unknown	
6 Arthrochalasia EDS	aEDS	AD	<i>COL1A1</i> , <i>COL1A2</i>	Type I collagen	
7 Dermatosparaxis EDS	dEDS	AR	<i>ADAMTS2</i>	ADAMTS-2	
8 Kyphoscoliotic EDS	kEDS	AR	<i>PLOD1</i> <i>FKBP14</i>	LH1 FKBP22	
9 Brittle Cornea syndrome	BCS	AR	<i>ZNF469</i> <i>PRDM5</i>	ZNF469 PRDM5	
10 Spondylodysplastic EDS	spEDS	AR	<i>B4GALT7</i> <i>B3GALT6</i> <i>SLC39A13</i>	$\beta 4$ GalT7 $\beta 3$ GalT6 ZIP13	
11 Musculocontractural EDS	mcEDS	AR	<i>CHST14</i> <i>DSE</i>	D4ST1 DSE	
12 Myopathic EDS	mEDS	AD or AR	<i>COL12A1</i>	Type XII collagen	
13 Periodontal EDS	pEDS	AD	<i>C1R</i> <i>C1S</i>	C1r C1s	
14 Classical-like type 2 EDS	clEDS2	AR	<i>AEBP1</i>	ACLP	

EDS can be further categorized into groups based on the structures, mechanisms, or pathways disrupted or altered that give rise to the characterized symptoms (Malfait et al., 2017). These range from disorders affecting primary collagen structure and processing, collagen folding and crosslinking, the structure of myomatrix, glycosaminoglycan

biosynthesis, complement pathways, intracellular process, or disorders that are yet to be resolved in their cause (Malfait et al., 2017).

Each EDS subtype is caused specific genetic variations in processes and proteins within the extracellular matrix (ECM). Many subtypes originate from mutations in different types collagen including Classical, Cardiac-valvular, Vascular , Arthochalasia, and Myopathic EDS (Malfait et al., 2017). Vascular, Cardiac-valvular and Arthochlasia EDS are a result of mutations in the fibrillar type I collagen present in the ECM of most tissue (Ricard-Blum 2011). In rare cases type I collagen mutations in the alpha 1 chain can cause Classical EDS (Malfait et al., 2017). Classical EDS is mostly a result of mutations in type V collagen which is highly associated with type I collagen and is essential for fibril formation in the ECM (Birk 2001). Vascular EDS is additionally caused by mutation in the fibrillar type III collagen also known as reticular fibers that are also present throughout the ECM (Ushiki 2002).

Classical-like EDS has defects in the tenascin XB protein which interacts with collagen, fibril-associated collagen, and the proteoglycan (PG) decorin (Tokhmafshan et al., 2020). Dermatosparaxis EDS has defects in the ADAMTS-2 protein which is a procollagen N-proteinase, resulting in issues with the formation of collagen fibrils (Jiang et al., 2019). Kyphoscoliotic EDS has defects in both the lysyl hydroxylase 1 (LH1) and FK binding protein 22 (FKBP22) (Malfait et al., 2017). Defects in LH1 prevent lysine from being modified into hydroxylysine, resulting in a lack of cross-links (Hao et al., 2021). Defects

in chaperone protein FKBP22 lead to issues in the folding of procollagen in the ER (Tremmel et al., 2007). Brittle-cornea EDS has defects in the zinc finger transcription factor (ZNF469) which lead to issues with transcription and regulation of homeostasis of the proteins within the ECM (Wang & Chang et al., 2021). Spondylodysplastic EDS has defects in the  $\beta$ 4GalT7,  $\beta$ 3GalT6, and ZIP13 proteins (Mailfait et al., 2017). Defects in  $\beta$ 4GalT7 prevent the enzyme from catalyzing the synthesis of glycosaminoglycan protein linkage in proteoglycans, another structural component of the ECM (Mailfait et al., 2007). Defects in ZIP13 lead to a deficiency in zinc within the cell, as it regulates zinc homeostasis, and traps excess zinc within the vesicles rather than releasing it into the ECM (Jeong et al., 2012).

### ***EDS Symptoms and Management***

Symptoms manifest in a variety of tissues such as musculoskeletal, skin, or cardiovascular and have underlying abnormalities in the structure, production, or processing of collagen or other associated connective tissue proteins (Mailfait et al., 2007). The impacted connective tissue can present itself in a variety of types of tissues and it may have a greater effect on the patient's quality of life. Hypermobility EDS patients exhibit a higher degree of flexibility in the joint spaces, whereas a patient with vascular EDS might exhibit fragile tissue in a variety of organ systems, causing ruptures and bleeding (Byers et al., 2017). While phenotypic hallmarks, including joint hypermobility, skin hyperextensibility, and tissue fragility, are all accounted for when diagnosing EDS, definitive diagnosis relies on

molecular confirmation to identify causative variants and defective genes (Malfait et al., 2017).

Hypermobile EDS account for 80-90% of EDS cases (Tinkle et al., 2017). As a result, the hallmark symptom of joint hyperflexibility is significantly prevalent in EDS. Joint hypermobility can be screened for and assessed using The Beighton scoring system, however, the symptom may also present by altering biomechanics within the body, causing activity referred pain or promoting bodily pain in other affected areas (Tinkle & Levy, 2019). Joint hypermobility impacts young and developing children due to the inability to place weight-related stress on these joints, such as the knees or shoulders (Adib et al., 2005). As a result, the disorder can have lasting impacts on a patient's muscle strength and motor development, and impacts their psychological health due to the biomechanical defects (Engelbert et al., 2017).

Hyperextensibility and elasticity of the skin is another common symptom exhibited in a variety of EDS types, and a major criteria for diagnosis in specific subtypes, such as classical EDS (Remvig et al., 2010). The phenotypic defects in connective tissue present themselves in a several ways, such as velvety smooth skin, widened atrophic scarring, molluscoid pseudotumors, easy bruising, and manifestations of tissue fragility due to the extensibility of the skin (Bowen et al., 2017). Additionally, clinical practice regarding cases can be complicated due to the nature of the scarring exhibited by those with EDS, as it can be markedly similar to scarring and bruising exhibited by patients experiencing abuse (Morais et al., 2013).

More serious symptomatic expressions of EDS are less common and are often found in rarer subtypes, such as vascular EDS (Malfait et al., 2017). Defective tissue connectivity in this subtype can lead to increased bruising, arterial and bowel fragility, and uterine, cervical, and vaginal fragility, which serves to further complicate pregnancy and delivery (Byers et al., 2017). The weak tissue can also lead to ruptures in the blood vessels and inadequate blood flow due to stresses on the weakened cardiac muscles (Brady et al., 2017).

### ***Treatment and Management***

Due to the variety of genetic causes, affected systems, and spectrum of symptoms, there are no known cures for EDS and treatment is limited to supportive care. Additionally, treatment depends entirely on symptoms expressed and subtype, diagnosed through biomechanical defects (De Paepe & Malfait, 2012). In earlier developmental stages, physical therapy to support is effective in preserving muscle mass and encouraging proper developmental growth (Engelbert et al., 2017). Some treatments aim to target the biomechanics of subtypes of EDS by supplementing nutritional deficiencies caused by the defective enzymes or pathways (Mantle et al., 2005). To treat gastrointestinal pain, dietary restrictions and other nutritional supplements are often advised (Castori et al., 2015). For more serious subtypes and symptoms, treatments are aimed at minimizing the adverse events that might exasperate symptoms that would have greater risks to the patients' health.

With these subtypes, care teams comprised of specialists are necessary to meet the needs to the patients (Byers et al., 2017).

## **EXTRACELLULAR MATRIX**

The ECM is network of collagen, proteoglycans, elastin, laminin, fibronectin and glycoproteins that serves as a scaffold for all organs, tissues, and cells (Theocharis et al., 2015). The most abundant component is the fibrous collagen proteins, mostly types I, II, and III, which occupies over 30% of the ECM (Karamanos et al., 2021). There are approximately 28 forms of collagen with type I collagen being the predominant form found in the ECM (Theocharis et al., 2016). Fibrillar collagens consist of three polypeptides, also known as alpha chains, arranged as a triple helical rod. Collagen fibers form as procollagen fibers before they are secreted, processed, and stabilized by the covalent cross-linking constituent collagen molecules (Humphrey et al., 2014). This provides the collagen with the tensile strength necessary to support the surrounding tissues and cells (Humphrey et al., 2014). Elastic fibers additionally supply the ECM with the ability to provide recoil to tissues, which undergo repeating stretching forces. Elastic fibers present within tissues form as a large network composed of two components, elastin and microfibrils (Theocharis et al., 2016). Other important fibrous proteins found in the ECM are proteoglycans (PGs). PGs consist of a protein core with attached negatively charged glycosaminoglycans (GAGs) including heparan sulfate, heparin, chondroitin sulfate, keratan sulfate, and dermatan sulfate (Yanagishita 1993). PGs are responsible for the mechanical resistance to compression and hydration of the tissues and affect the activity of growth factors (GFs) in

the ECM (Theocharis et al., 2019). The ECM also contains proteases to maintain ECM homeostasis and allow for remodeling. These include metalloproteases and cathepsin proteases secreted by ECM stromal cells (Theocharis et al., 2015). The major stromal cells of the ECM are fibroblasts. Fibroblasts are cells that can secrete elastin, collagens, glycoproteins, and GAGs, differentiate into myofibroblast in response to tissue damage, act as signaling niche cells for tissue-resident stem cells and serve as progenitors for specialized differentiated mesenchymal cells (Plikus et al., 2021).

### ***ECM functions***

ECM function to provide tissues with structural integrity and mechanical properties essential for tissue functions or regulating cell phenotype (Theocharis et al., 2019). ECM provides the biochemical and biomechanical signals that influence a range of cell activities, including migration, adhesion, phenotypic modulation, and survival (Humphrey et al., 2014). The elastic fibers are deposited and organized prior to adulthood and provide a “mechanical memory” due to the fiber’s ability recoil when unloaded from their homeostatic state (Humphrey et al., 2014). The mechanical memory allows the ECM to use mechanosensing to alter cell signaling patterns that change the mechanical environment in the ECM. This is accomplished through a complex network of collagen, elastin, integrins, cytoskeleton, and focal adhesions (Humphrey et al., 2014). Not only do integrins support ECM modulation, they also further allow the ECM to alter cell signaling that can

affect cell behavior, migration, differentiation, proliferation, and survival (Humphrey et al., 2014).

### ***ECM Protein Trafficking***

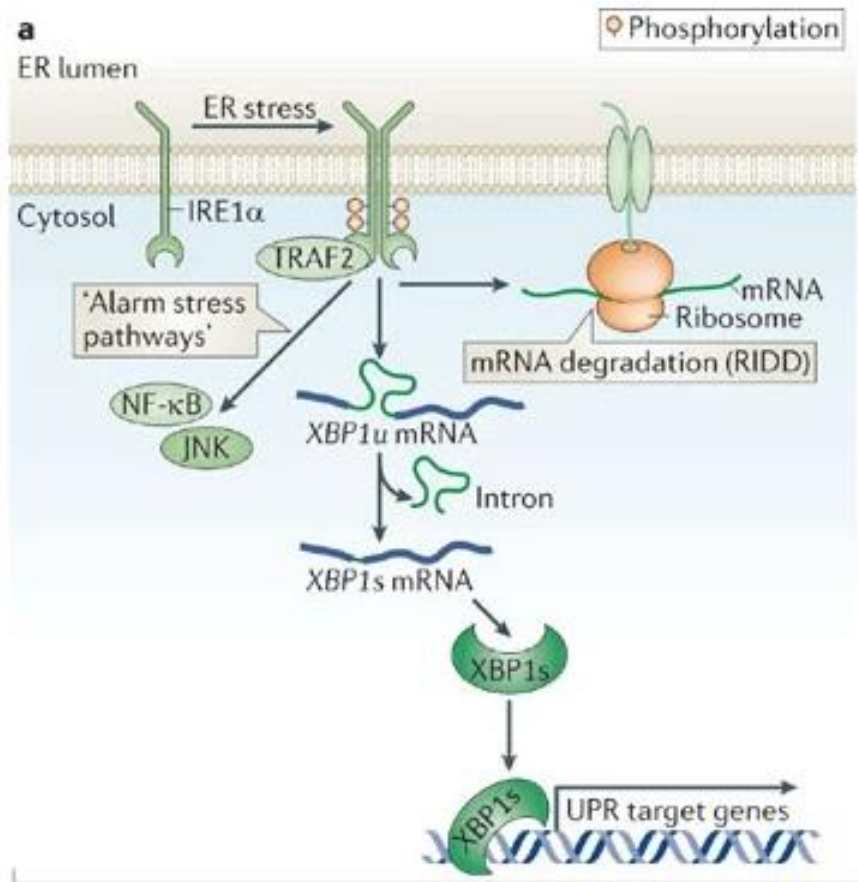
For ECM proteins to be trafficked outside the cell they must contain a signal sequence that is 20-30 amino acids long with a hydrophobic core followed by positively charged amino acids (Stroud and Walter 1999). As the protein is being translated by a cytosolic ribosome, a signal recognition particle (SRP) binds to the signal sequence halting translation until the SRP can bind to its endoplasmic reticulum (ER) receptor (Stroud and Walter 1999). Once SRP binds its receptor, the ribosome resumes translation through a transmembrane protein complex with an inner channel to the ER lumen.

The ER lumen contains chaperone proteins that aid the polypeptide to fold into its proper conformation (Lin et al. 2008). The signal peptide is usually cleaved from the fully translated polypeptide and protein often undergo N-linked glycosylation at specific asparagine residues (Stroud and Walter 1999). Glycosylated proteins that need to be secreted or inserted into the plasma membrane are transported in COPII vesicles from the ER to the Golgi (Kulkarni-Gosavi et al., 2019). Once in the Golgi, the proteins are further modified by enzymes that will trim N-linked glycans, add O-linked glycans, phosphorylate sugar residues or add sulfate to sugar chains (Kulkarni-Gosavi et al., 2019). The fully

processed proteins are packaged into vesicles to be secreted from the cell or incorporated into the plasma membrane.

### *Endoplasmic Reticulum Stress Caused by Mutated ECM Proteins*

The ER stress response is due to an accumulation of unfolded or misfolded proteins in the ER lumen. This can be caused by increased protein production or pathological stress caused by unfolded mutated proteins (Lin et al., 2014). In response to the stress, cells will activate the unfolded protein response (UPR) signaling cascades. The UPR is activated by several different ER resident transmembrane proteins including PERK, IRE1 $\alpha$ , ATF6, and IP<sub>3</sub>R (Hetz et al., 2020). These stress sensing proteins can activate signals cascades that will increase the ER ability to fold protein or promote apoptosis (Lin et al., 2014). In response to ER stress, inositol-requiring enzyme-1 (IRE1) will oligomerize which activates its kinase and endoribonuclease domains that are present in the cytosol (Lin et al., 2014). IRE1 endoribonuclease will then splice the X-Box binding protein 1 (XBP-1) mRNA resident in the cell cytosol. The spliced XBP-1 encodes for a basic-leucine zipper transcription factor that activates the transcription of proteins that enhance the ER protein folding capacity and induces degradation of misfolded protein (**Fig 1**).



**Figure 1. IRE1 $\alpha$  Signaling in Response to ER Stress.** IRE1 will dimerize and autophosphorylate in response to ER stress activating kinase and endoribonuclease domains present in the ER cytosol. IRE1 endoribonuclease splices the XBP-1 mRNA. The spliced XBP-1 encodes for a basic-leucine zipper transcription factor that will transcribes genes encoding proteins involved in ER protein folding. IRE1 additionally degrades certain mRNA through IRE1-dependent decay (RIDD). TRAF2 phosphorylation by IRE1 activates alarm stress pathways (JNK/NF $\kappa$ B). Adapted from Hetz et al., 2020.

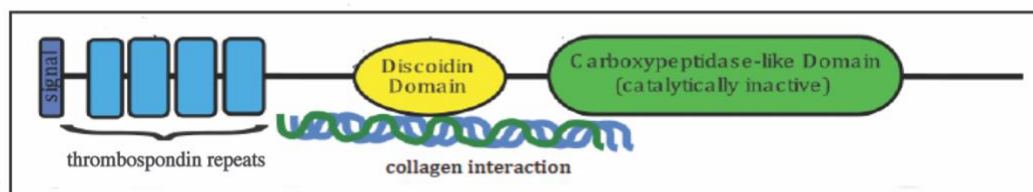
IRE1, through its kinase domain, can additionally bind and phosphorylate the TRAF2 adaptor molecule (Hetz et al., 2020). Phosphorylated TRAF2 can activate the apoptosis signal-regulating kinase 1 (ASK1) which phosphorylates and activates JNK (Hetz et al., 2020). The activation of JNK and its downstream targets leads to cell death.

ER stress serves as a driver for many different diseases including cancer, diabetes mellitus (DM), obesity, and neurodegeneration (Lin et al., 2014). The Wollocott-Rallison form of hereditary DM is caused by mutations in PERK that decrease the ability of  $\beta$ -cells to lower protein load in the ER (Lin et al., 2013). In cancer cells, UPR activation is upregulated and the oncogenic transformation of UPR signaling contributes to tumor growth, angiogenesis, and immune invasion (Hetz et al., 2020).

## AORTIC CARBOXYPEPTIDASE-LIKE PROTEIN

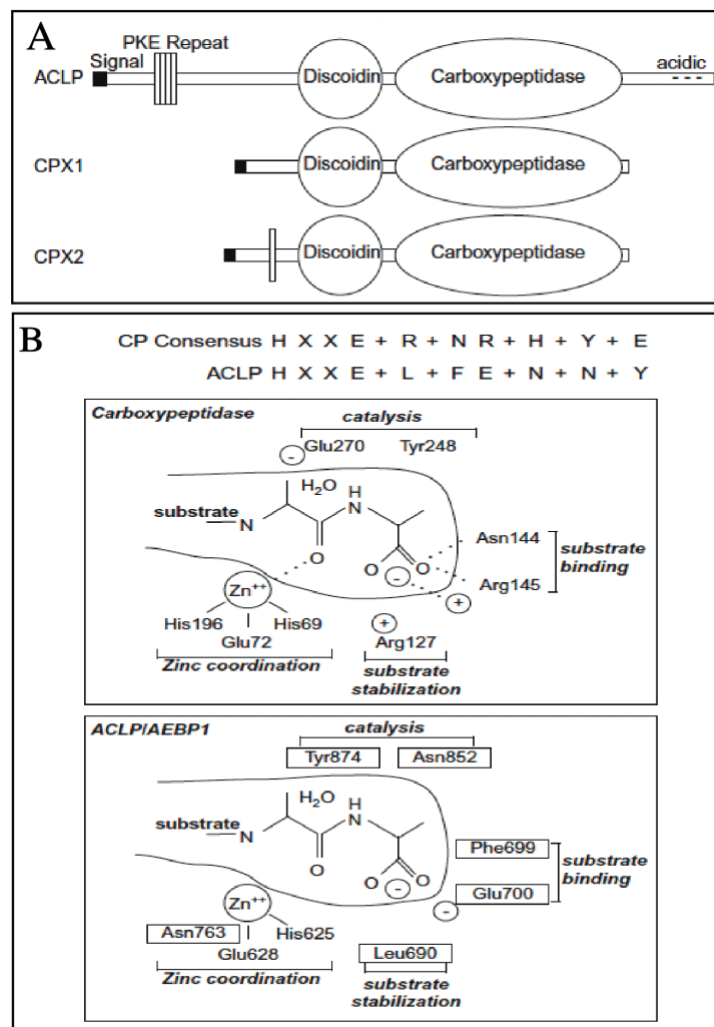
### *Structure and Function*

Aortic carboxypeptidase-like protein (ACLP) is a collagen binding ECM protein that is expressed in several tissues including in the bones, blood vessels, and skin (Layne et al., 2001). Human ACLP is 1158 amino acids in length and contains N-terminal thrombospondin repeats, collagen binding discoidin domain, and a catalytically inactive carboxypeptidase domain (Layne et al., 1998) (**Fig 2**).



**Figure 2. ACLP structure and domains.** N-terminal signal peptide is followed by thrombospondin repeats that interacts with TGF $\beta$ , a discoidin domain, and a catalytically inactive carboxypeptidase-like domain. Image by Dr. Matthew Layne.

ACLP belongs to a subfamily of metallo-carboxypeptidases although its sequence in the carboxypeptidase domain diverges from catalytically active members render it inactive toward usual substrates (Reznik and Fricker 2001). Other members of the ACLP metallo-carboxypeptidase subfamily CPX1 and CPX2 have similar discoidin domains followed by the inactive carboxypeptidase-like domain (Reznik and Fricker 2001) **Fig 3**).



**Figure 3. Structure of metallo-carboxypeptidase subfamily members.** (A) CPX1, CPX2, and ACLP have N-terminal signal peptides and discoidin domains followed by inactive carboxypeptidase domains. (B) The catalytic site of ACLP is mutated at residues critical for carboxypeptidase activity. Adapted from Layne and Tumelty 2013.

In mouse ACLP N-linked glycosylation occurs at positions Asn-471, Asn-519, Asn-913 and Asn-1030 with only Asn-471 and Asn-1030 required for secretion of the protein into the ECM (Vishwanath et al., 2020). Human ACLP has conserved glycosylation sites at positions Asn-480, Asn-528, Asn-922 and Asn-1039 (Vishwanath et al., 2020). Fibroblasts, myofibroblasts and smooth muscle cells produce and secrete ACLP (Wang et al., 2021).

ACLP has a variety of functions with many yet to be discovered. Expression of ACLP is primarily found in vascular and perivascular spaces where it is upregulated in response to vascular injury (Layne et al., 2002). Additionally, ACLP is expressed in lower level in the kidney and colon (Layne et al., 1998). Previous studies have determined ACLP upregulates smooth muscle actin (SMA) and collagen type I expression while it represses stem cell markers CD34 and KLF4 through myocardin-related transcription factor A (MRTFA) (Wang et al., 2021). MRTFA is an important regulator in myofibroblast differentiation and fibrosis (Small 2012). Myofibroblast differentiation is regulated by transformation growth factor  $\beta$  (TGF- $\beta$ ), collagen, and fibronectin (Tomasek et al., 2002). ACLP stimulates TGF- $\beta$  pathways which leads to myofibroblast differentiation (Tumelty et al., 2013). ACLP may also play a role in vascular smooth muscle cell differentiation due to the significant rise in ACLP levels as differentiation from neural crest cells (Layne et al., 1998). ACLP can repress adipocyte progenitor differentiation through TGF- $\beta$  pathways during white adipose tissue fibrosis (Jager et al., 2018). Studies have further show ACLP is highly expression

during mouse embryogenesis in the vasculature, dermis and developing skeleton (Ith et al., 2005).

### ***Pathologies Associated with ACLP***

In idiopathic lung fibrosis (IPF), there is increase ACLP expression in areas of dense collagen deposition (Schissel et al., 2009). Normal lung tissue exhibits limited ACLP expression in collagen rich areas such as blood vessels with an absence of ACLP expression in the lung parenchyma (Schissel et al., 2009). Mice injured with bleomycin has significant increase in ACLP levels (Schissel et al., 2009). ACLP knock out mice (ACLP<sup>-/-</sup>) are protected against bleomycin induced lung fibrosis due to a lower accumulation of myofibroblasts (Schissel et al., 2009). Bleomycin treated ACLP<sup>-/-</sup> mice had a 4.8 fold decrease in myofibroblast and a 28% decrease in collagen accumulation compared to wildtype bleomycin treated mice (Schissel et al., 2009). Fibroblasts with loss of ACLP exhibited reduced proliferation, cell spreading and contraction of collagen matrices, and the ACLP discoidin domain was sufficient for the restoration of these function in ACLP<sup>-/-</sup> fibroblasts (Schissel et al., 2009).

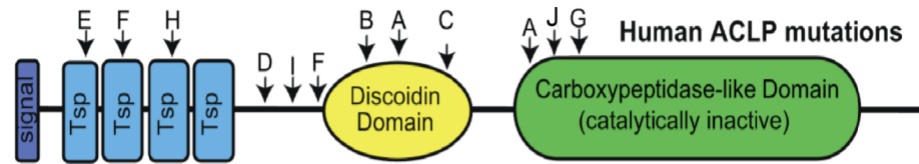
ACLP has been implicated in white adipose tissue fibrosis with mice fed chronic high fat diet significantly increasing expression of ACLP (Jager et al. 2018). In these mice, ACLP was co-expressed with collagens from the inflammatory cell depleted stromal-vascular fraction (SVF) and reduced adipogenesis and enhance myofibroblast differentiation

through TGF- $\beta$  signaling and potentiating fibrosis (Jager et al., 2018). Further studies have implicated ACLP in the progression of nonalcoholic steatohepatitis (NASH) (Teratani et al. 2018). Obesity related factors promote the production of ACLP in hepatic satellite cells (HSCs) which will activate the HSCs through the canonical WNT pathway (Teratani et al., 2018). WNT activation is achieved through the binding of ACLP to frizzled-8 (FRD8) and lipoprotein receptor-related protein 6 (LRP6) which forms a ternary complex that activates the pathway (Teratani et al., 2018). ACLP<sup>-/-</sup> mice exhibit lower levels of fibrosis in response to NASH (Teratani et al., 2018).

Additional ACLP<sup>-/-</sup> mouse studies have revealed a defective wound healing process in mice lacking ACLP after injury by dermal punch biopsy (Layne et al., 2001). ACLP<sup>-/-</sup> mice exhibited deficient dermal fibroblast proliferation (Layne et al., 2001). This study further revealed the importance of ACLP during mouse embryogenesis due to high expression and the development of fatal perinatal gastroschisis in ACLP<sup>-/-</sup> mice (Layne et al. 2001).

#### ***Ehlers-Danlos Syndromes Caused by ACLP Mutations***

Classical-like EDS type 2 has recently been identified in patients with mutation in adipocyte enhancer-binding protein 1 (*AEBP1*) which encodes for ACLP (Alazami et al., 2016; Blackburn et al., 2018; Syx et al., 2019). There have been multiple mutations in ACLP found in families with EDS (**Fig 4**).



Family	Trascript	Protein	Reference
A	c. 1470delC c. 1743C>A	p. Asn490_Met495delins (Ins40) p. Cys581*	Blackburn 2018
B	c. 1320_1326del	p. Arg440Serfs*3	Blackburn 2018
C	c. 1630+1 G>A	unknown/null	Alazami 2016 Blackburn 2018
D	c. 917dupA	p. Tyr306*	Hebebrand 2019
E	c. 362dupA	p. Glu122Glyfs*16	Syx 2019
F	c. 443dupA c. 1149_1150+2	p. Ala149Glyfs*57 in frame deletion 38 aa	Syx 2019
G	c. 1925T>C	p. Leu642Pro	Ritelli 2019
H	c. 707A>G	p. Asn236Ser	Alkuraya (personal comm.)
I	c. 1067G>A	p. Trp356*	Alkuraya (personal comm.)
J	c. 1894C>T	p. Arg631*	Sanai (personal comm.)

**Figure 4. Human mutations of ACLP causing EDS.** The location of known ACLP mutations causing EDS.

The clinical phenotypes of EDS patients with ACLP mutations includes severe joint and skin laxity, osteoporosis, osteoarthritis, soft acrogeria-like skin, delayed wound healing, and joint dislocations overlapping symptoms seen in classical, vascular, and arthrochalasia type EDS (Blackburn et al., 2018). EDS patients with ACLP mutations uniquely exhibited gastrointestinal dysfunction, mitral valve prolapse, aortic root dilation, and skeletal abnormalities (Blackburn et. Al 2018). The Ins40 mutation in ACLP is a result of a loss of the last amino acids encoded by exon 12 which leads to the inclusion of a 40 amino acid intron making this protein larger than wildtype (Blackburn et al., 2018). Cys581\* ACLP

mutant is caused by a nonsense mutation at position 1743 that leads to a premature stop codon and a truncated protein (Blackburn et al., 2018). Patients with Ins40 and Cys581\* ACLP mutations exhibited symptoms such as acrogeria-like skin, atrophic scars, cryptorchidism, and mitral valve prolapse (Blackburn et al., 2018). Recently 2 families with EDS have been identified in Saudi Arabia with mutations in ACLP, one mutation Trp356\* is another nonsense mutation resulting in a truncated protein and the other Asn236Ser is a missense mutation (Alkuraya, personal communication). A patient identified with the missense mutation Leu642Pro exhibited a range of symptoms including androgenetic alopecia, laxity of the digits, hyperextensible skin, easy bruising, and atrophic papraceous scars on knees (Ritelli et al., 2019). Another patient has been recently discovered in Japan with the mutation Arg631\* which is another nonsense mutation leading to a truncated protein (Sanai, personal communication).

## **GOALS AND OBJECTIVES**

EDS is a connective tissue disease that arises from a variety of different ECM protein mutations including mutations in ACLP. The goals of this research is to determine how Leu642Pro, Asn236Ser, Trp356\*, Cys581\*, and Arg631\* ACLP mutants alter the normal processing and function of ACLP which results in EDS. Previous studies have shown Ins40 ACLP mutations causes intracellular retention of the protein and induces ER stress, and our objective is to determine whether these mutants cause similar dysfunctions.

## CHAPTER II METHODS

### Generation of human ACLP mutants

Expression plasmids encoding the human ACLP mutations were generated from the expression vector pCMV6-hACLP (Origene SC309967). PCR was performed with Q5 polymerase (NEB M0491) and products were run on 1% Tris Acetate EDTA (TAE) agarose gels with 0.01% ethidium bromide at 100 V for approximately 30 minutes. Point mutants Leu642Pro and Asn236Ser vectors were generated using PCR and primers containing the mutant nucleotide. The truncation mutant Trp356\* vector was generated with loop primers designed with QuickChange Primer Design software. The remaining truncation mutant vectors, Cys581\* and Arg631\*, were generated with primers containing a XhoI site after the final nucleotide found in the truncation mutant. (Fig 5). Cys581\* and Arg631\* plasmids were then digested with XhoI, purified with the NEB gel extraction kit, and re-ligated to the pCMV6 backbone with NEB Quick Ligation Kit (NEB M2200S).

### Transformation

Competent DH10B bacteria (NEB C3019H) were transformed according to manufacturer's protocols. Briefly, 1  $\mu$ l of the ligation reaction was added to 50  $\mu$ l of bacteria and incubated on ice for 30 minutes. The tube was heat shocked at 47°C for 45 seconds and incubated on ice for 2 minutes. Super Optimal Broth (SOC) was added and bacteria were incubated at 37°C on a rotating wheel in for 1 hour. Ligation mixtures were plated on LB plates with kanamycin (concentration) and incubated overnight at 37°C. Colonies were inoculated in 3 ml LB broth with 50  $\mu$ g/ml kanamycin overnight.

<b>A Primers</b>		
	<b>Forward Primer</b>	<b>Reverse Primer</b>
<b>Asn236Ser</b>	5' CCACACTGGACTACAGTGACCAGATCG 3'	5' CGATCTGGTCACTGTAGTCCATGGTGG 3'
<b>Leu642Pro</b>	5' GCTCATGCAGTACCCGTGCCGAGAGTACC 3'	5' GGTACTCTCGGCACGGGTACTGCATGAGC 3'
<b>Trp356*</b>	5' GGAGACCGACAAGACGCGTACGCGGC 3'	5' GCCGCGTACGCGTCTTGTCGGTCTCC 3'
<b>Cys581*</b>	5' GACGAGATCGAGGAGTTC 3'	5' <b>CGCTCGAG</b> CTCCTCGTTCACCACCTT 3'
<b>Arg631*</b>	5' GACGAGATCGAGGAGTTC 3'	5' <b>CGCTCGAGG</b> CCCCAGCACCTCGTTGC 3'

<b>B Point Mutants</b>		
<b>Step</b>	<b>Temp</b>	<b>Time</b>
Initial Denaturation	98 °C	30 sec
25 Cycles	98 °C	10 sec
	55 °C	20 sec
	72 °C	5 min 40 sec
Final Extension	72 °C	11 min 20 sec
Hold	10 °C	—

<b>C Truncation Mutants</b>		
<b>Step</b>	<b>Temp</b>	<b>Time</b>
Initial Denaturation	98 °C	30 sec
25 Cycles	98 °C	10 sec
	55 °C	20 sec
	72 °C	60 sec
Final Extension	72 °C	2 min
Hold	10 °C	—

**Figure 5. Plasmid mutagenesis PCR cycling conditions and primers.** (A) Forward and reverse primers for each mutant generated. Cys581\* and Arg631\* XhoI site highlighted in green. (B) PCR cycling conditions for point mutants Asn236Ser and Leu642Pro. (C) PCR cycling conditions for truncation mutants Trp356\*, Cys581\*, and Arg631\*.

### Miniprep and Maxiprep

Plasmid DNA was extracted from the bacteria using NEB Miniprep Kit according to manufacturer's instructions (NEB T10105). Plasmids were characterized by restriction digestion with XhoI and SacI. Plasmids were then sequenced by Eton Bioscience with one of the following primers:

VP1-5: 5' GGACTTTCCAAAATGTCG 3'

XL39: 5' ATTAGGACAAGGCTGGTGGG 3'

After sequence verification, DNA for transfection was generated by growing bacteria in 500 ml of LB broth with kanamycin and grown overnight. Plasmid DNA was isolated using Invitrogen Maxiprep Kit (Invitrogen 0091853).

### **Transient Transfections**

AD-293 and 3T3 cells were cultured in DMEM (Corning 13821015) with 10% fetal bovine serum (FBS) (Hyclone) and penicillin, streptomycin, and glutamine (PSG). Cells were plated in 6 well plates at 200,000 cells per well or 4 well chamber slides at 25,000 cells per well. AD-293 cells were transfected with TransIT-293 transfection reagent (Mirus, MIR 2700) and 2.5 µg of DNA for 6 well plates or 0.5 µg of DNA for 4 well chamber slides in Opti-MEM media (Gibco 1676912). The Opti-MEM mixture was then added to cells in DMEM media. For secretion experiments, the media was replaced 24 hours post-transfection with DMEM/F12 (Gibco 2370939) and incubated for another 24 hours before extraction. 3T3 cells were transfected with TransIT-3T3 transfection reagent (Mirus MIR 2180) and 2.5 µg of DNA for 6 well plates or 0.5 µg of DNA for 4 well chamber slides in Opti-MEM media added to cells in DMEM media. The cells were then incubated at 37°C for 48 hours. For ER stress experiments, transfections with plasmid DNA and XBP1u-EGFP sensor (Vishwanath et al., 2020) were carried out 3:1 ratio respectively and incubated for approximately 18 hours before fixation and staining.

### **Protein Extraction**

Transfected cells were washed with phosphate-buffered saline (PBS) and extracted with a lysis buffer containing 25 mM Tris pH 7.4, 50 mM sodium chloride, 0.5% sodium deoxycholate, 2% NP-40, and 0.2% SDS + 1x Complete protease inhibitor (Roche, 04-693-123-001). Cells were scraped off plates and transferred to a chilled 1.5 ml microcentrifuge tubes and incubated for 15 minutes on ice. The tubes were then spun at 12,000 x g for 10 minutes at 4°C. Supernatants were transferred to a chilled 1.5 ml microfuge tubes and stored at -80°C. Media samples were collected and transferred to chilled 2 ml screw top tubes and clarified by spinning at 500 x g for 10 minutes. Supernatants were then transferred to 2 ml tube and stored at -70°C. Media samples were clarified, concentrated with a 10,000 molecular weight cut off concentrator by centrifuge at 3220 x g for 20 minutes at 4°C (Pierce WG330516).

### **Western Blot Analysis**

Protein extraction samples were aliquoted at equal volumes into 5x-SDS loading dye. Samples were heated at 99°C for 4 minutes in the thermocycler and loaded into a 4-20% Tris-glycine gel (Invitrogen 21081390) ran at 120 volts for approximately 1.5 hours. Gels were transferred to a nitrocellulose membrane (GE 10600002) with a transfer buffer containing 48 mM Tris pH 8.3, 39 mM glycine, 0.037% SDS, and 20% methanol and ran overnight at 22 volts at 4°C. Membranes were placed in a container on a shaker and equilibrated in 25 mM Tris pH 8.0, 125 mM sodium chloride, and 0.1% Tween (TBST) then blocked in 5% milk-TBST for 40 minutes at room temperature. Primary anti mouse-

FLAG (Sigma F1804) antibody was diluted 1:1000 in 2.5% milk-TBST and incubated with the membrane for 2 hours at room temperature. The membrane was then washed 4 times for 5 minutes in TBST. Secondary fluorescent anti-mouse antibodies (Invitrogen 680λ A21058) were diluted 1:10,000 in 2.5% milk-TBST and incubated with the membranes for 1 hour at room temperature. The membrane was washed 3 times with TBST for 5 minutes and one time in TBS for 10 minutes. Images were captured with LI-COR Odyssey CLX.

### **Immunofluorescence**

3T3 fibroblasts were grown and transfected on 4-well chamber slides with removable chambers (Lab-TEK 154917). Cells were washed twice with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-PBS (PBST). Cells were then blocked in 4% BSA-PBST for 1 hour. Anti-FLAG conjugated M2-Cy3 antibodies (Sigma A9594) were diluted 1:100 in 4% BSA-PBST. Cells were incubated with the conjugated antibodies for 1 hour at room temperature in a covered humid chamber. Slides were then washed twice with PBST and twice with PBS before mounting with ProLong Diamond Antifade Mountant with DAPI (ThermoFisher P36962). Slides were dried overnight and imaged using Zeiss fluorescent microscope.

### **Mice Tissue Isolation**

Floxed ACLP allele Cre mice were treated with 5 mg/ml tamoxifen injections for 5 days. The mice were allowed to recover for 2 week and were then euthanized with carbon dioxide. All mouse experiments were performed with the permission of BUSM IACUC

PROTO201800079. Mice were sprayed with 70% ethanol and sterilized blunt scissors and hemostats were used to open the body cavity. The right ventricle was injected with 1x DPBS to perfuse the lungs. The aorta was carefully removed with scissor and forceps and then washed to cold DPBS to remove excess blood. The lungs were then removed and washed in DPBS. Aorta and lung samples were then transferred to methyl Carnoy's fixative in 20 ml glass vials. Vials were incubated on a shaker at 4°C for 2 hours and 40 minutes. Samples were transferred to 70% ethanol and processed into paraffin blocks by standard techniques.

### **Mice Tissue Histology**

Processed slides were then de-waxed by heating the samples at 55°C in a hybridization oven followed by two 5 minute washes in xylenes, two 5 minutes washes in 100% ethanol, and three 1 minute washes in 95%, 85%, 75% ethanol respectively. Samples were then hydrated with a 1 minute wash in deionized water, three 5 minute washes in PBS, a 10 minute incubation in 0.1% triton X-100 PBS, and three final 5 minute washes in PBS. The slides were then placed in a humid chamber and blocked in 5% donkey serum in PBS for 1 hour at room temperature. Primary antibodies were added and incubated overnight in the humid chamber at 4°C. Anti-ACL<sub>P</sub> primary antibodies (Layne et al., 1998) were diluted 1:500 and anti-SMA antibodies (Sigma A5228) were diluted 1:300 in 5% donkey serum PBS. The following morning slides were washed three times in 0.1% Tween-PBST. Secondary antibodies were then added and incubated 1 hour at room temperature in the dark. Anti-rabbit Alexa 647 (Invitrogen A21245) diluted 1:300 and anti-mouse Alexa 568

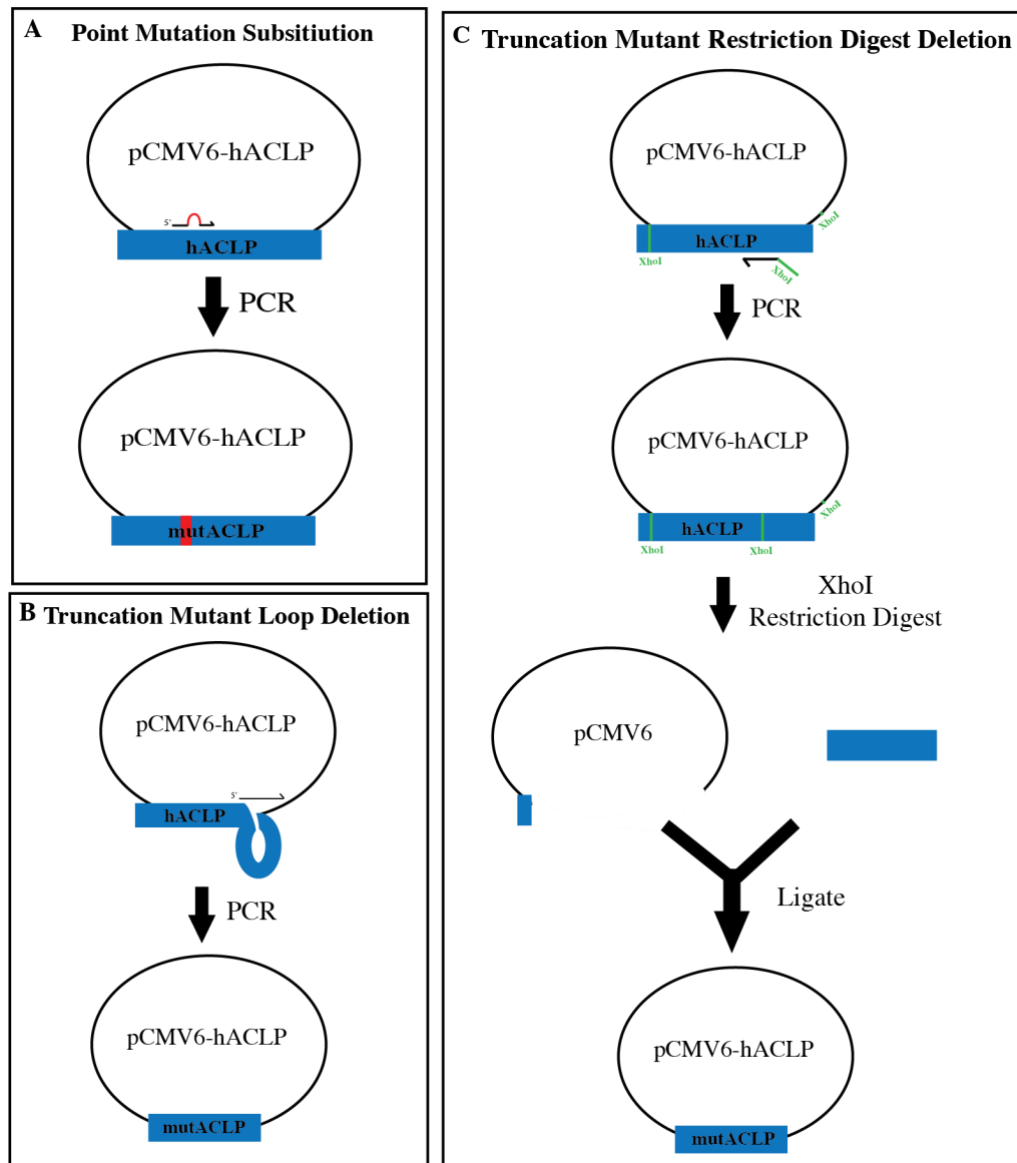
(Life Tech A11031) diluted 1:500 in 5% donkey serum PBS were used. Slides were washed again twice in 0.1% Tween-PBST for 5 minutes and twice in PBS for 5 minutes. The slides were placed in 50 ml tubes and dried by spinning at 100 x g for 3 minutes and mounted with ProLong Diamond Antifade Mountant with DAPI (ThermoFisher P36962). Slides were dried overnight and imaged using Zeiss fluorescent microscope.

## CHAPTER III RESULTS

EDS is a connective tissue disorder caused by mutations in ECM proteins including collagen and ACLP. The goal of this study was to characterize ACLP disease-causing mutations. ACLP is an important ECM protein that binds collagen, induces myofibroblast and vascular smooth muscle differentiation, and upregulates type I collagen production. (Tumelty et al., 2013; Layne et al., 1998., Wang et al., 2021). Previous studies have determined the ACLP mutation Ins40 leads to intracellular retention and ER stress (Vishwanath et al., 2020). Our objective were to create plasmid vectors harboring the mutations of interest to study their changes in expression and function.

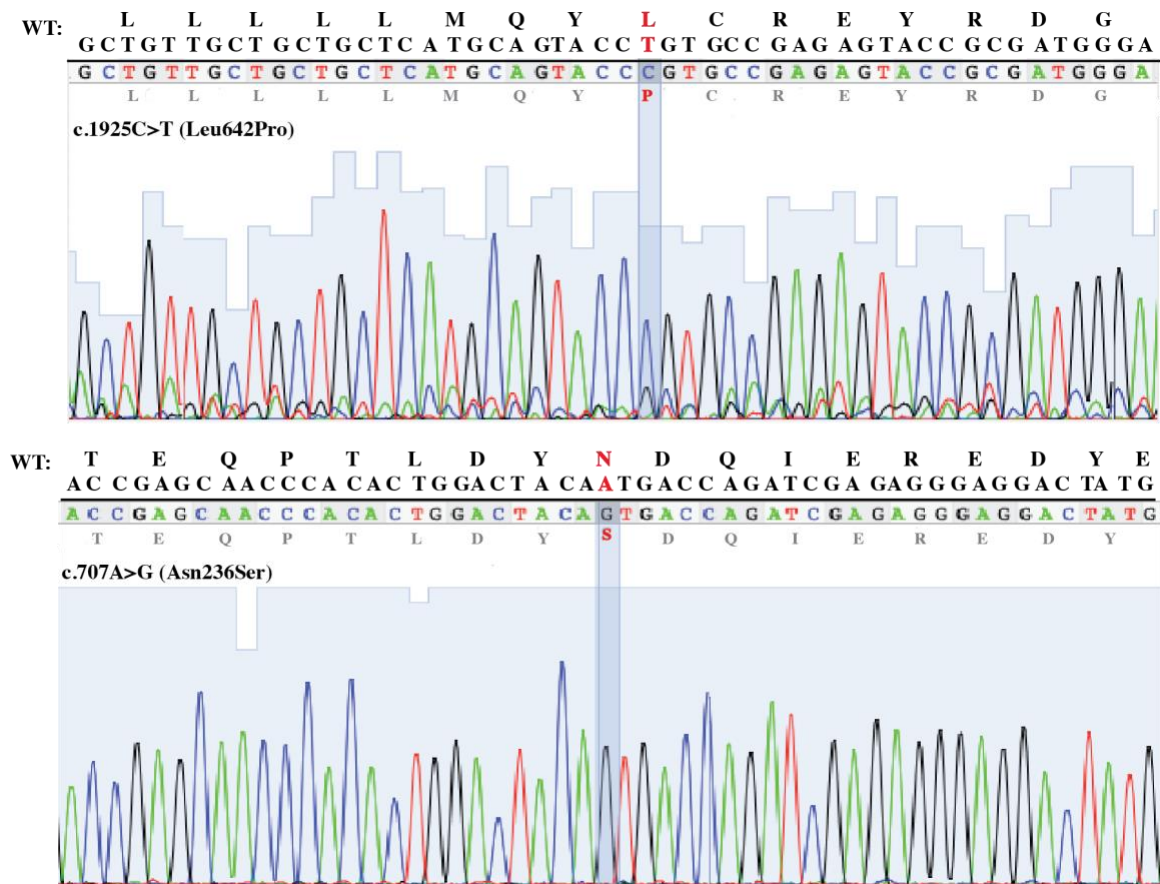
### **Generation of ACLP Mutants**

To generate plasmids with the desired mutations, an expression vector pCMV6 containing wild type ACLP was modified using PCR. Point mutants Leu642Pro and Asn236Ser vectors were generated with primers containing the mutant nucleotide. The Trp356\* vector was generated with loop primers designed to loop out the DNA between the end of the truncation and the beginning of the pCMV6 backbone. The remaining truncation mutant vectors, Cys581\* and Arg631\*, were generated with primers containing a XhoI site after the final nucleotide found in the truncation mutant. pCMV6-hACLP, Cys581\* and Arg631\* plasmids were then digested with XhoI. The pCMV6 backbone fragment and the mutant fragments were extracted from the agarose gel and re-ligated to generate the expression plasmid (**Fig 6**).



**Figure 6. Plasmid mutagenesis strategies.** (A) Point mutants Leu642Pro and Asn236Ser were generated with primers containing the mutant nucleotide. (B) Truncation mutant Trp356\* was generated with a primer that loops out the DNA from the end of the truncation to the pCMV6 backbone. (C) Truncation mutants Cys581\* and Arg631\* were generated with the insertion of a XhoI site at the last nucleotide found in the truncation by a primer. The plasmids were then digested with XhoI and re-ligated to remove the deleted region. Only one primer was shown for illustrative purposes.

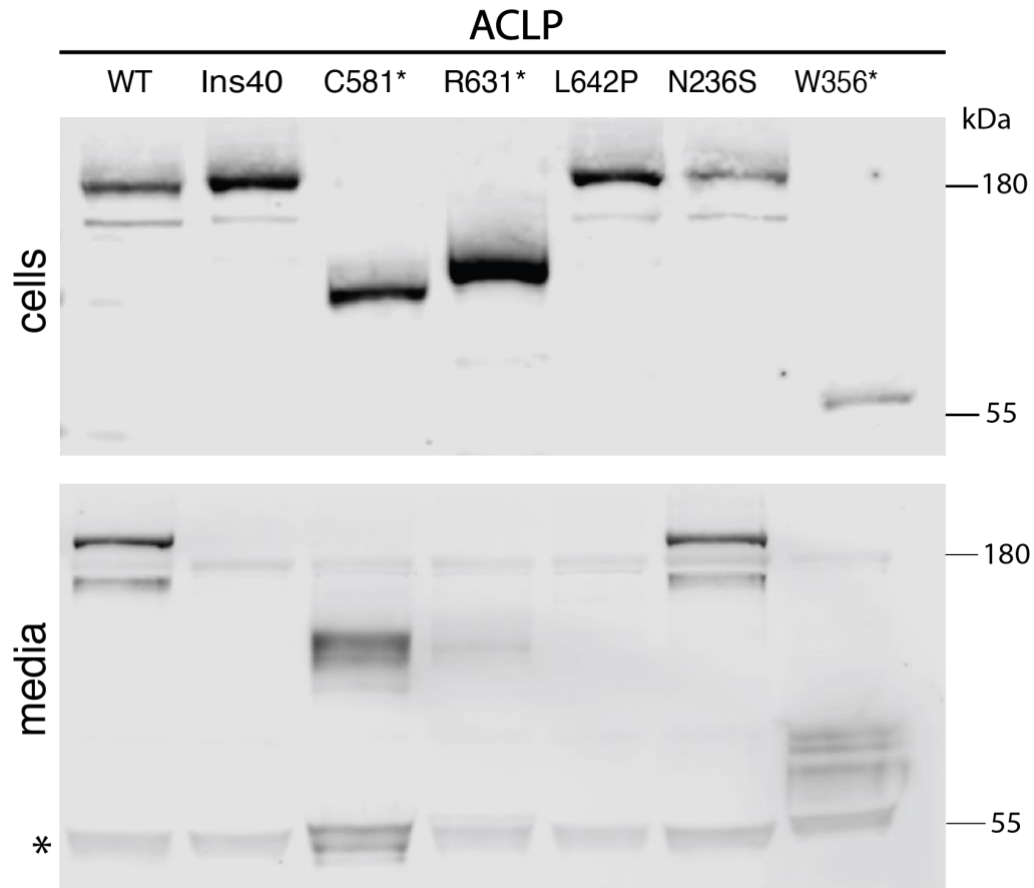
Leu642Pro, Asn236Ser, and Trp356\* plasmids were verified by restriction digest with XhoI. Cys581\* and Arg631\* plasmids were verified by restriction digest with both XhoI and SacI. All plasmids were verified by DNA sequencing (Fig 7).



**Figure 7. Sequencing verification of plasmids c.1925C>T (Leu642Pro) and c.707A>G (Asn236Ser).** Sequencing results confirm the desired mutations are found in the generated vector plasmids. All plasmids were confirmed with sequencing. Two plasmids are shown for illustrative purpose.

### **Characterization of ACLP Mutants**

ACLP is normally trafficked to the ER, Golgi, and then to the extracellular environment. (Vishwanath et al., 2020). Previous studies have identified an ACLP mutant, Ins40, that is intracellularly retained in the ER (Vishwanath et al., 2020). To determine whether the ACLP mutants being studied are excreted or retained intracellularly, expression plasmids were transfected into AD293 cells due to lack of endogenous ACLP expression. After 24 hours post transfection the serum media was replaced with DMEM/F12 and cells were incubated for another 24 hours to allow for ACLP secretion into the media. The media was then collected, clarified by centrifugation, and concentrated approximately 10-fold. The intracellular proteins were collected using lysis buffer containing protease inhibitors. Media and cell extracts were run on 4-20% Tris-glycine gel and transferred to a nitrocellulose member. The nitrocellulose membranes were blotted with anti-FLAG antibodies. Cell lysates were positive for mutant ACLP expression. Media samples showed secretion of WT, Cys581\*, Trp356\*, and Asn236Ser with weak secretion of Arg631\*. As expect there was no detectable Ins40 ACLP in the media. Leu642Pro was also not detected in the media indicating it is intracellularly retained (**Fig 8**).



**Figure 8. Cys581\*, Arg631\*, Trp356\*, and Asn236Ser are secreted from the cell while Leu642Pro is intracellularly retained.** AD293 cells were transfected with the generated mutant plasmids. Protein and media extracts were collected after 24 hours. Western blots were performed with anti-FLAG antibodies. All cell lysates detected the presence of the transfected ACLP. Media samples showed secretion of WT, Cys581\*, Trp356\*, and Asn236Ser with weak secretion of Arg631\*. Leu642Pro was absent in media samples. Asterix indicated background bands. Figure representative of 4 western blot experiments.

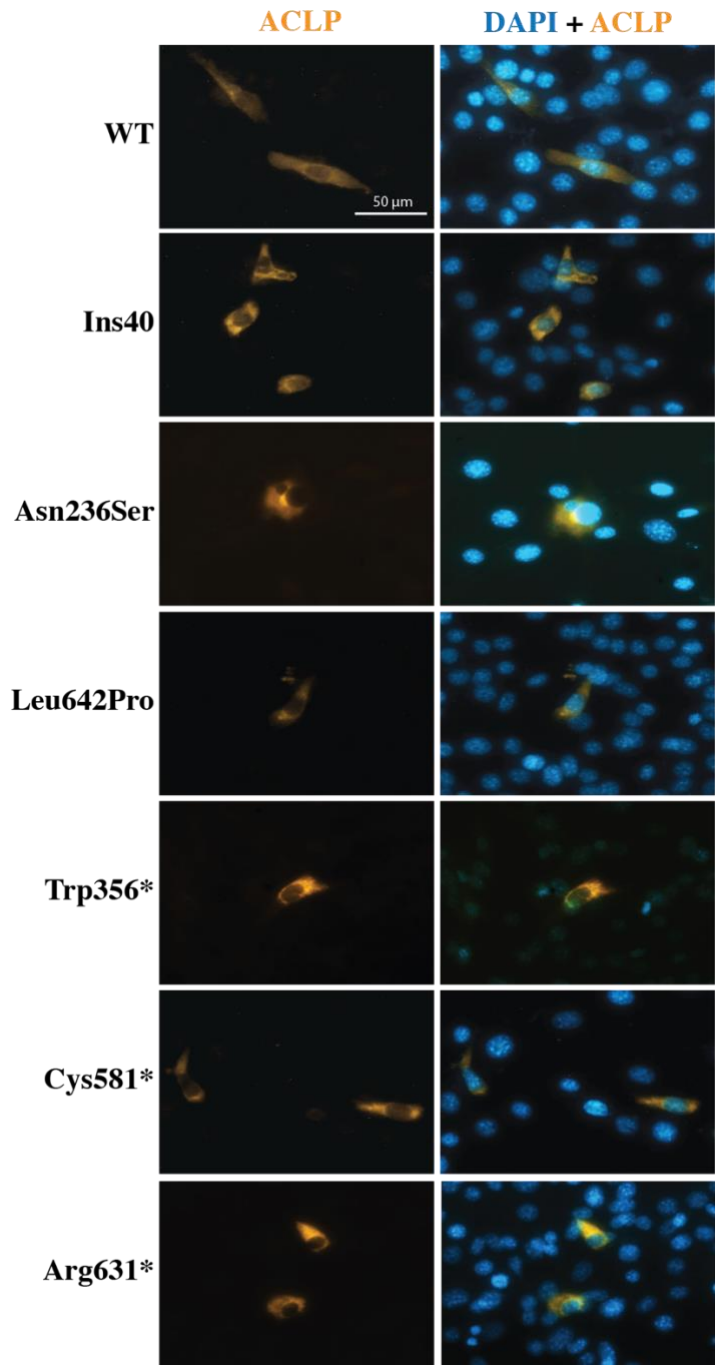
### **Subcellular Localization of ACLP Mutants**

To determine mutated proteins are being properly trafficked through the ER, immunofluorescent studies were performed. Fibroblasts, especially in settings of tissue injury produce ACLP and its secretion into the ECM, therefore 3T3 fibroblasts were chosen for these experiments. After 24 hours post-transfection, the cells were fixed, permeabilized

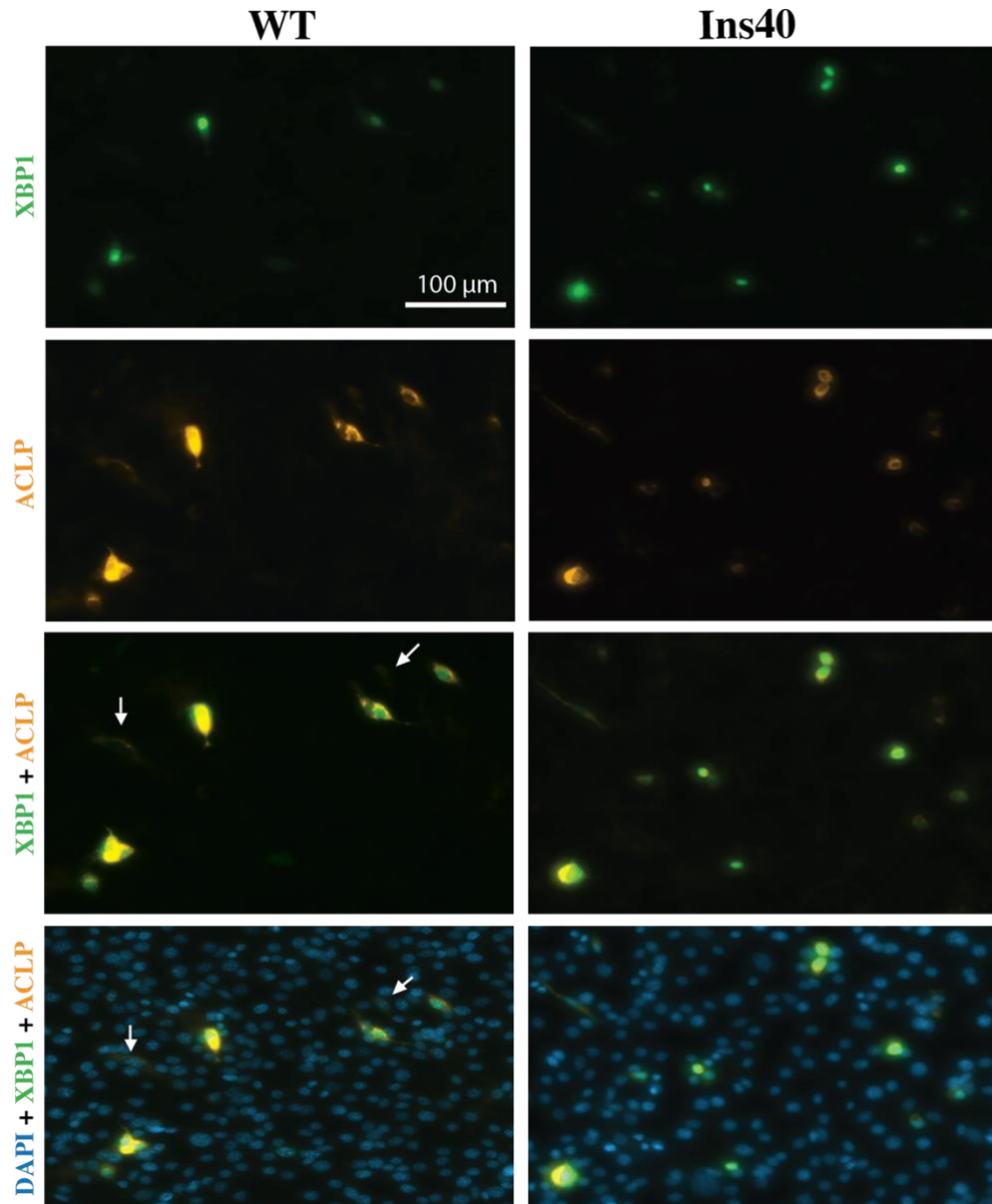
and stained with anti-FLAG antibodies conjugated to Cy3. Immunofluorescent imaging revealed all mutated proteins exhibited a perinuclear pattern indicating proper localization of the proteins to the ER (**Fig 9**).

### **Activation of ER Stress Pathways by Retained ACLP Mutants**

Previous studies have shown that the ACLP mutant Ins40 induces ER stress (Vishwanath et al. 2020). To determine whether the generated mutants induce ER stress, experiments were first performed with wildtype ACLP and Ins40 to confirm the efficacy XBP1-GFP reporter. The XBP1-GFP reporter expresses GFP when it is cleaved by IRE1 and transported to the nucleus in response to ER stress. 3T3 fibroblasts were co-transfected with the DNA plasmid and XBP1-GFP reporter and incubated for approximately 18 hours. Cells were then fixed and stained with anti-FLAG antibodies conjugated to Cy3. As anticipated, Ins40 had an increased expression of XBP1-GFP indicating Ins40 is causing increased ER stress (**Fig 10**). Future ER stress studies will be performed with the Arg631\* Cys581\*, Trp356\*, Leu642Pro and Asn236Ser mutants.



**Figure 9. ACLP mutants exhibit perinuclear subcellular localization.** 3T3 fibroblasts were transfected with DNA plasmids and stained with anti-FLAG antibodies conjugate to Cy3. Asn236, Leu642Pro, Trp356\*, Cys581\*, and Arg631\* mutants have perinuclear expression patterns indicating proper trafficking to the ER. Figure representative of 3 experiments. Collaboration with Amber Liu.

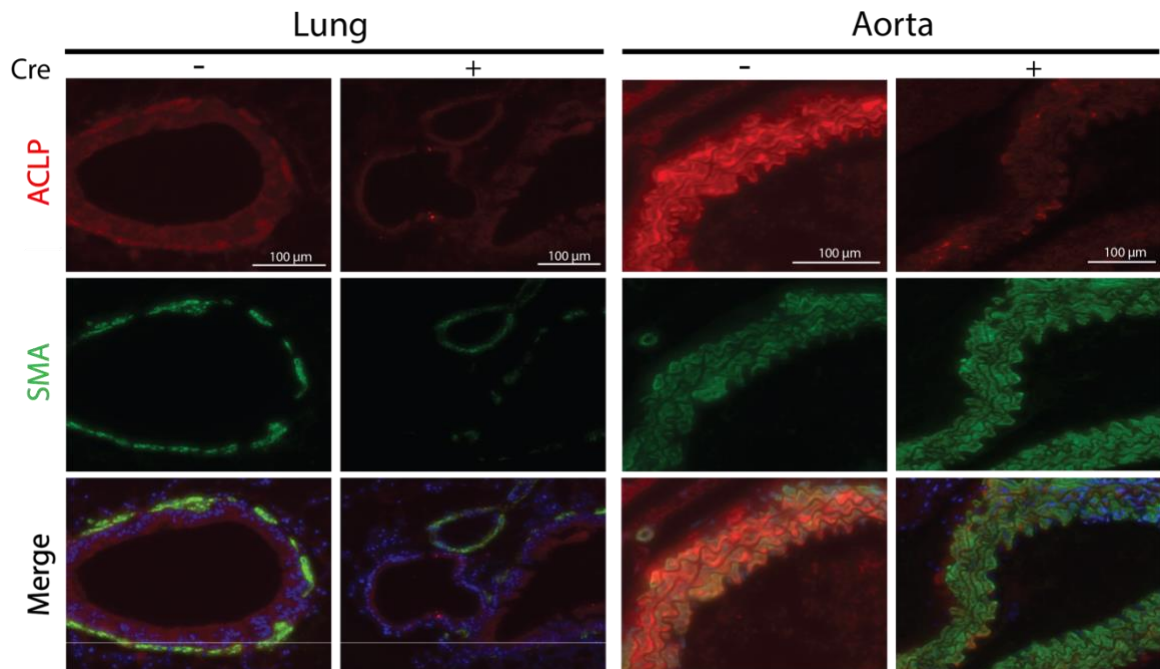


**Figure 10. ER Stress is increased in the intracellularly retained Ins40 mutant.** 3T3 fibroblasts were co-transfected with the DNA plasmids and XBPI-GFP. After 18 hours cells were fixed and stained. Ins40 transfected cells had greater expression of XBPI-GFP indicating an increase in ER stress. Arrows indicate cells without ER stress activation.

### **Mouse Model ACLP loss of function**

EDS patients with mutations in ACLP have exhibited delayed wound healing and aortic dysfunction similar to what has been observed in ACLP-null mice (Layne et al., 2001). To study the role of ACLP in connective tissue aortic dysfunction, ACLP<sup>-/-</sup> mice must be generated with confirmed ACLP knock out. This was accomplished with an inducible Cre lox mouse model with floxed ACLP. When mice are treated with tamoxifen the ACLP gene is deleted. Previous mouse models were made by injecting a targeting vector to disrupt the ACLP gene into mice blastocysts and crossing mice until homozygous ACLP<sup>-/-</sup> were generated (Layne et al., 2001). As previously mentioned ACLP is vital from mouse embryonic development therefore the majority ACLP<sup>-/-</sup> mice died prematurely from gastroschisis (Layne et al., 2010). The floxed ACLP Cre mouse model has been previously used in studies which found ACLP induces aortic contraction and adventitial fibrosis (Wang et al., 2021)

Floxed ACLP Cre negative and positive mice were injected with tamoxifen for 5 days and allowed 2 weeks for ACLP wash out. After 2 weeks the mice lung and aorta samples were isolated, fixed, sectioned, and stained with anti-SMA and anti-ACLP antibodies. There was significant reduction in ACLP expression in Cre positive mice in both the aorta and lungs (Fig 11).



**Figure 11. ACLP expression is decreased in tamoxifen treated floxed ACLP Cre positive mice.** Floxed ACLP Cre negative and positive mice were treated with tamoxifen to induce ACLP knock out. After a 2 week wash out period mice were euthanized and lung and aorta samples were removed, fixed, sectioned, and stained with SMA and ACLP antibodies. There is a significant reduction in ACLP in Cre positive mice in both the lung and aorta.

### Summary of Results

The goal of this research was to characterize ACLP mutants Leu642Pro, Asn236Ser, Trp356\*, Cys581\*, and Arg631\* that cause EDS. Plasmid vectors were generated with each mutation to study the mechanisms of the mutated proteins. Western blot studies showed there was intracellular retention Leu642Pro while all other mutants were secreted out of the cell. Imaging studies confirmed the mutants were properly trafficked to the ER as part of the secretory pathway. We have established the validity of a model to study the

activation of ER stress by ACLP mutants with an XBP1-GFP reporter. Additionally, we have generated a Cre lox mouse model to reduce ACLP expression.

## CHAPTER IV DISCUSSION

The goal of these studies was to further understand the mechanism in which ACLP mutations cause EDS. We have previously determined that the ACLP mutant Ins40 was intracellularly retained and activated ER stress pathways potentially contributing to EDS pathology (Vishwanath et al., 2020). To determine whether Leu642Pro, Asn236Ser, Trp356\*, Cys581\*, and Arg631\* ACLP mutants act in a similar manner, plasmid vectors were generated to study how the proteins are trafficked in cells and to determine whether they are secreted to the extracellular environment. We determined by western blot Leu642Pro was the only mutant studied that was intracellularly retained. Imaging studies revealed all mutants are properly trafficked to the ER following the secretory pathway. These studies revealed ACLP-Leu642Pro is a null mutation and Asn236Ser, Trp356\*, Cys581\*, and Arg631\* mutations result in ECM ACLP protein expression. We have additionally established an ACLP knock out mouse model that can be used to further study ACLP null mutations. The results of secretion or retention of these ACLP mutants and the generation of an ACLP null mouse model can give further insight to molecular mechanism behind ACLP associated EDS.

### **ACLP-Leu642Pro is intracellularly retain**

To understand the mechanisms by which the ACLP-Leu642Pro mutations cause EDS, we generated plasmid vectors harboring the Leu642Pro mutation. Transient transfections were performed in AD293 cells to determine whether Leu642Pro was able to be secreted into the extracellular environment. Multiple western blot experiments were performed on the

cell lysate and concentrated media samples with ACLP-Leu642Pro being completely absent from all media samples indicating Leu642Pro is intracellularly retained (**Fig 8**). It is possible secretion is inhibited by the disruption in protein folding caused by the substitution leucine with proline in the inactive carboxypeptidase-like domain. The structure of proline causes alpha-helix and beta-sheet breakage in globular proteins (Li et al., 1996). Classical-like EDS patients also exhibit truncation point mutations that result in null tenascin-X phenotypes (Schalkwijk et al., 2001). It is possible these mutations cause ER stress similar to ACLP-Ins40 or could possibly undergo nonsense mediated mRNA decay. Additional studies have seen collagen X mutations in metaphyseal chondrodysplasia type Schmid that similarly cause intracellular retention and ER Stress (Mullan et al., 2017). When mice were treated with carbamazepine, a drug aimed to decrease misfolded protein accumulation in the ER, there was a significant decrease in inhibition of hypertrophic chondrocyte differentiation indicating ER stress plays a major role in the disease phenotype (Mullan et al., 2017). Carbamazepine could possibly be used to treat EDS associated with intracellular ECM protein retention and ER stress.

To confirm proper translocation of the ACLP-Leu642Pro mutant to the ER immunofluorescent imaging studies were done in fibroblast. Antibodies against the FLAG epitope found in the plasmid vector were used to distinguish from endogenous ACLP. Imaging studies showed perinuclear ACLP-Leu642Pro expression indicating the protein is properly trafficked through the ER (**Fig 9**). We hypothesized that ACLP-Leu642Pro would similarly act as ACLP-Ins40 by activating ER stress pathways due to its lack of secretion

and accumulation in the ER. Future studies will be performed to determine if the ER stress pathway is activated by Leu642Pro expression.

ACL P is an important role in collagen polymerization and assembly through the fibrillar collagen binding discoidin domain (Blackburn et al., 2018). The discoidin domain found in ACL P preferentially binds collagen I, III, and V which are the same collagens that are mutated in Classical EDS (Blackburn et al., 2018). EDS patients with ACL P-Ins40 mutations have observed irregularity and disruption in collagen fibrils (Blackburn et al., 2018). These findings would likely be seen in ACL P-Leu642Pro patients given the lack of ACL P expression in the ECM. Additional studies have shown mice lacking ACL P exhibit delayed wound healing as have been observed in EDS patients with ACL P mutation (Layne et al., 2001; Blackburn et al., 2018).

#### **ACL P mutants Asn236Ser, Trp356\*, Cys581\*, and Arg631\* are secreted**

Additional plasmid vectors were generated with Asn236Ser, Trp356\*, Cys581\*, and Arg631\* ACL P mutants. Multiple western blot experiments were performed and showed strong secretion of Asn236Ser, Cys581\*, and Arg631\* and weaker secretion of Trp356\* (**Fig 8**). The ACL P-Asn236Ser mutation is in the N-terminal region of thrombospondin repeats (**Fig 3**). Interestingly, thrombospondin type 1 repeats are also found in ADAMTS-2 which is mutated in Dermatosparaxis EDS (Mead and Apte 2018; Malfait et al., 2017). Although the role of the thrombospondin repeats in ACL P have yet to be discovered, thrombospondin repeats have been shown to play a role in binding other ECM proteins and

may have a role in ECM remodeling during inflammatory response (Adams and Tucker 2000). It is possible through this mechanism ACLP-Asn236Ser mutations alter the stability of the ECM leading to the EDS phenotype. The ACLP-Trp356\* mutation results in an ACLP protein without its discoidin and carboxypeptidase-like domain (**Fig 3**). As previously mentioned, the discoidin domain plays a vital role in collagen polymerization and assembly (Blackburn et al., 2018). The lack of the discoidin domain and the weak secretion of the protein could likely give ACLP-Trp356\* similar disruptions in collagen fibrils as seen in ACLP-Ins40 EDS patients (Blackburn et al., 2018). The ACLP-Arg631\* mutation results in a truncation within the carboxypeptidase-like domain leaving the discoidin domain and thrombospondin repeats intact (**Fig 3**). EDS phenotypes in these patients could possibly indicate the catalytically inactive carboxypeptidase-like domain or C-terminus has some function in the maintenance of the ECM.

All mutants exhibited perinuclear patterns when imaged with immunofluorescence against the FLAG epitope. Future studies will be done to determine if any of the secreted mutants additionally activate ER stress pathways. ER stress activation could play an important role in EDS as it have been shown to be a driver in many diseases such as cancer, diabetes, chronic inflammatory diseases, and neurodegeneration (Lin et al., 2013).

### **ACLP knockout Mouse Models**

Mouse model studies can be used to further understand the mechanisms of EDS by induction of ACLP knockout. Mouse models have been widely used to study other

subtypes of EDS mimicking several hallmarks of EDS including dermatological, cardiovascular, musculoskeletal, and biomechanical symptoms seen in human patients (Vroman et al., 2021). In our study, floxed ACLP allele Cre mice were treated with tamoxifen to induce ACLP knockout. The mice were treated with tamoxifen injections for 5 days and allowed 2 weeks for ACLP wash out although the half-life of ACLP is yet to be determined. Samples were taken from the mice for genotyping and recombination PCR and for tissue histology to confirm knockout. Histology samples were stained with anti-ACLP and anti-SMA antibodies and imaged with immunofluorescence. Imaging confirmed significant ACLP reduction in the lungs and aorta although small amounts of ACLP were still present (**Fig 11**). Confirmation of ACLP knockout with qRT-PCR are still needed.

EDS patients with ACLP mutations have delayed dermal wound healing after injury similar to what has been shown in previous studies with ACLP<sup>-/-</sup> mice (Layne et al., 2001, Blackburn et al., 2018). In a bleomycin induced IPF model, ACLP<sup>-/-</sup> mice have additionally shown decreased fibrosis with less collagen deposition and myofibroblast accumulation (Schissel et al., 2009). Similarly in EDS patients with ACLP mutation there is observed decreased dermal collagen compared to carriers and control subjects (Blackburn et al., 2018). Some patients with EDS caused by ACLP mutations exhibit vascular abnormalities such as mitral valve prolapse, aortic dilation, and vascular stenosis (Blackburn et al., 2018).

It is our goal to use ACLP<sup>-/-</sup> mice to study the molecular mechanisms of vascular dysfunction. Mouse models of Vascular EDS with mutations in collagen type III have shown abnormalities in the phospholipase C/inositol triphosphate/protein kinase C pathway that mediated vascular defects (Bowen et al., 2020). Treatment of inhibitors of this pathway were shown to prevent death due to spontaneous aortic rupture (Bowen et al., 2020). ACLP has been shown to preferentially bind type III collagen so it is possible the collagen dysfunction could additionally cause vascular defects through this signaling pathway (Blackburn et al., 2018). Previous studies have determined ACLP plays an important role in vascular smooth muscle differentiation (Layne et al., 1998). Additional studies have shown ACLP is upregulated in differentiated neointimal vascular smooth muscle cells in the carotid artery in response to injury (Layne et al., 2002). Future studies will be performed with ACLP<sup>-/-</sup> mice to study aortic dysfunction and the role of ACLP in aortic aneurysm formation using angiotensin-II mediated aneurysm models.

### **Additional ACLP mutants**

As of March 2022, the Genome Aggregation Database (gnomAD) has 1808 mutations in the *AEBPI* gene that encodes ACLP with 12 of those mutations predicted to being pathogenic. Interestingly, 5 of the pathological mutations appear to occur in the catalytically inactive carboxypeptidase-like protein indicating the domain could possibly be playing role in ECM maintenance and assembly or mutations could be leading to intracellular retention of ACLP similar to Leu642Pro. The remaining mutations are located in the discoidin domain which is essential for collagen polymerization and ECM stability

or the N-terminal region which is hypothesized to play a role in signaling including TGF- $\beta$  signaling (Blackburn et al., 2018).

According to the gnomAD database all pathological ACLP mutations have very low allele frequencies with the highest frequency of  $3.61e5$  and some pathogenic mutations are limited to one family. The studies performed on Leu642Pro, Asn236Ser, Trp356\*, Cys581\*, and Arg631\* could be replicated with the other identified pathogenic mutants to determine if these proteins are able to be secreted into the ECM and could have partial function.

### **ACLP (*AEBPI*) in cancer**

In addition to fibrosis and EDS, other studies have identified the gene encoding ACLP, *AEBPI*, as a possible oncogene that has been shown to be upregulated in breast, bladder, and ovarian cancers (Liu et al., 2018). In gastric cancer, both mRNA and protein levels of *AEBPI* are increased in cancer cells and when *AEBPI* is silenced there is significant repression of proliferation, migration, metastasis, and epithelial-mesenchymal transition (Lui et al., 2018). In colon adenocarcinoma, *AEBPI* expression levels correlated to tumor size, level of histological differentiation and poor prognosis (Xing et al., 2019). This study further showed *AEBPI* promotes proliferation, migration and invasion through upregulating matrix metalloproteinases and vimentin (Xing et al., 2019). *AEBPI* has been further linked to acquired resistance to BRAF inhibition in melanoma (Hu et al., 2013). The upregulation of *AEBPI* in melanoma occurs through hyperactivation of the PI3K/Akt-

CREB pathway (Hu et al., 2013). These studies have identified *AEBPI* as a possible target for cancer treatment.

### **Implications and future directions**

Our studies have shown two different molecular mechanisms of ACLP mutants causing EDS. ACLP-Leu642Pro mutations result in intracellular retention of ACLP with complete lack of secretion to the extracellular environment while Asn236Ser, Trp356\*, Cys581\*, and Arg631\* ACLP mutants are secreted to the extracellular environment where they possibly partly function. As mentioned previously additional experiments will be performed to determine whether any of these mutants activate the ER stress pathway contributing to EDS symptoms. Additionally, collagen binding studies on the secreted mutants with intact or partial disoidin domains could be done to determine if there is any change in the disoidin domain function which is important for collagen polymerization and assembly. Studies should also be done to elucidate the function of the carboxypeptidase-like domain in the ECM since mutations involving this domain result in pathogenic phenotypes. Further development of the molecular model in which ACLP mutations cause EDS can lead to the discovery of new treatments that can target possible signaling pathways or underlying protein dysfunction to alleviate EDS symptoms.

## REFERENCES

- Adib, N., Davies, K., Grahame, R., Woo, P., & Murray, K. J. (2005). Joint hypermobility syndrome in childhood. A not so benign multisystem disorder? *Rheumatology*, *44*(6), 744–750.
- Alazami, A. M., Al-Qattan, S. M., Faqeih, E., Alhashem, A., Alshammari, M., Alzahrani, F., Al-Dosari, M. S., Patel, N., Alsagheir, A., Binabbas, B., Alzaidan, H., Alsiddiky, A., Alharbi, N., Alfadhel, M., Kentab, A., Daza, R. M., Kircher, M., Shendure, J., Hashem, M., ... Alkuraya, F. S. (2016). Expanding the clinical and genetic heterogeneity of hereditary disorders of connective tissue. *Human Genetics*, *135*(5), 525–540.
- Birk, D. E. (2001). Type V collagen: Heterotypic type I/V collagen interactions in the regulation of fibril assembly. *Micron*, *32*(3), 223–237.
- Blackburn, P. R., Xu, Z., Tumelty, K. E., Zhao, R. W., Monis, W. J., Harris, K. G., Gass, J. M., Cousin, M. A., Boczek, N. J., Mitkov, M. V., Cappel, M. A., Francomano, C. A., Parisi, J. E., Klee, E. W., Faqeih, E., Alkuraya, F. S., Layne, M. D., McDonnell, N. B., & Atwal, P. S. (2018). Bi-allelic Alterations in AEBP1 Lead to Defective Collagen Assembly and Connective Tissue Structure Resulting in a Variant of Ehlers-Danlos Syndrome. *The American Journal of Human Genetics*, *102*(4), 696–705.
- Bowen, C. J., Calderón Giadrosic, J. F., Burger, Z., Rykiel, G., Davis, E. C., Helmers, M. R., Benke, K., Gallo MacFarlane, E., & Dietz, H. C. (2019). Targetable cellular signaling events mediate vascular pathology in vascular Ehlers-Danlos syndrome. *Journal of Clinical Investigation*, *130*(2), 686–698.
- Bowen, J. M., Sobey, G. J., Burrows, N. P., Colombi, M., Lavalley, M. E., Malfait, F., & Francomano, C. A. (2017). Ehlers-Danlos syndrome, classical type. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, *175*(1), 27–39.
- Brady, A. F., Demirdas, S., Fournel-Gigleux, S., Ghali, N., Giunta, C., Kapferer-Seebacher, I., Kosho, T., Mendoza-Londono, R., Pope, M. F., Rohrbach, M., Van Damme, T., Vandersteen, A., van Mourik, C., Voermans, N., Zschocke, J., & Malfait, F. (2017). The Ehlers-Danlos syndromes, rare types. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, *175*(1), 70–115.

- Byers, P. H., Belmont, J., Black, J., De Backer, J., Frank, M., Jeunemaitre, X., Johnson, D., Pepin, M., Robert, L., Sanders, L., & Wheeldon, N. (2017). Diagnosis, natural history, and management in vascular Ehlers-Danlos syndrome. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, 175(1), 40–47.
- Castori, M. (2020). Joint hypermobility in children: A neglected sign needing more attention. *Minerva Pediatrica*, 72(2), 123-133.
- Castori, M., Morlino, S., Pascolini, G., Blundo, C., & Grammatico, P. (2015). Gastrointestinal and nutritional issues in joint hypermobility syndrome/ehlers-danlos syndrome, hypermobility type. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, 169(1), 54–75.
- Danzer, E., Layne, M. D., Auber, F., Shegu, S., Kreiger, P., Radu, A., Volpe, M., Adzick, N. S., & Flake, A. W. (2010). Gastroschisis in Mice Lacking Aortic Carboxypeptidase-Like Protein Is Associated With a Defect in Neuromuscular Development of the Eviscerated Intestine. *Pediatric Research*, 68(1), 23–28.
- De Paepe, A., & Malfait, F. (2012). The Ehlers-Danlos syndrome, a disorder with many faces. *Clinical Genetics*, 82(1), 1–11.
- Engelbert, R. H. H., Juul-Kristensen, B., Pacey, V., de Wandele, I., Smeenk, S., Woinarosky, N., Sabo, S., Scheper, M. C., Russek, L., & Simmonds, J. V. (2017). The evidence-based rationale for physical therapy treatment of children, adolescents, and adults diagnosed with joint hypermobility syndrome/hypermobile Ehlers Danlos syndrome. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, 175(1), 158–167.
- Hebebrand, M., Vasileiou, G., Krumbiegel, M., Kraus, C., Uebe, S., Ekici, A. B., Thiel, C. T., Reis, A., & Popp, B. (2018). A biallelic truncating AEBP1 variant causes connective tissue disorder in two siblings. *American Journal of Medical Genetics Part A*, 179(1), 50-56.
- Hetz, C., Zhang, K., & Kaufman, R. J. (2020). Mechanisms, regulation and functions of the unfolded protein response. *Nature Reviews Molecular Cell Biology*, 21(8), 421–438.
- Ith, B., Wei, J., Yet, S.-F., Perrella, M. A., & Layne, M. D. (2005). Aortic carboxypeptidase-like protein is expressed in collagen-rich tissues during mouse embryonic development. *Gene Expression Patterns*, 5(4), 533–537.

- Jager, M., Lee, M.-J., Li, C., Farmer, S. R., Fried, S. K., & Layne, M. D. (2018). Aortic carboxypeptidase-like protein enhances adipose tissue stromal progenitor differentiation into myofibroblasts and is upregulated in fibrotic white adipose tissue. *PLOS ONE*, *13*(5), e0197777.
- Jeong, J., Walker, J. M., Wang, F., Park, J. G., Palmer, A. E., Giunta, C., Rohrbach, M., Steinmann, B., & Eide, D. J. (2012). Promotion of vesicular zinc efflux by ZIP13 and its implications for spondylocheiro dysplastic Ehlers–Danlos syndrome. *Proceedings of the National Academy of Sciences*, *109*(51), 3530–3538.
- Jiang, C., Zhou, Y., Huang, Y., Wang, Y., Wang, W., & Kuai, X. (2019). Overexpression of ADAMTS-2 in tumor cells and stroma is predictive of poor clinical prognosis in gastric cancer. *Human Pathology*, *84*, 44–51.
- Karamanos, N. K., Theocharis, A. D., Piperigkou, Z., Manou, D., Passi, A., Skandalis, S. S., Vynios, D. H., Orian-Rousseau, V., Ricard-Blum, S., Schmelzer, C. E. H., Duca, L., Durbeej, M., Afratis, N. A., Troeberg, L., Franchi, M., Masola, V., & Onisto, M. (2021). A guide to the composition and functions of the extracellular matrix. *The FEBS Journal*, *288*(24), 6850–6912.
- Kulkarni-Gosavi, P., Makhoul, C., & Gleeson, P. A. (2019). Form and function of the Golgi apparatus: Scaffolds, cytoskeleton and signalling. *FEBS Letters*, *593*(17), 2289–2305.
- Layne, M. D., Endege, W. O., Jain, M. K., Yet, S.-F., Hsieh, C.-M., Chin, M. T., Perrella, M. A., Blonar, M. A., Haber, E., & Lee, M.-E. (1998). Aortic Carboxypeptidase-like Protein, a Novel Protein with Discoidin and Carboxypeptidase-like Domains, Is Up-regulated during Vascular Smooth Muscle Cell Differentiation. *Journal of Biological Chemistry*, *273*(25), 15654–15660.
- Layne, M. D., Yet, S.-F., Maemura, K., Hsieh, C.-M., Bernfield, M., Perrella, M. A., & Lee, M.-E. (2001). Impaired Abdominal Wall Development and Deficient Wound Healing in Mice Lacking Aortic Carboxypeptidase-Like Protein. *Molecular and Cellular Biology*, *21*(15), 5256–5261.
- Layne, M. D., Yet, S.-F., Maemura, K., Hsieh, C.-M., Liu, X., Ith, B., Lee, M.-E., & Perrella, M. A. (2002). Characterization of the Mouse Aortic Carboxypeptidase-Like Protein Promoter Reveals Activity in Differentiated and Dedifferentiated Vascular Smooth Muscle Cells. *Circulation Research*, *90*(6), 728–736.

- Li, H., Xu, H., Wen, H., Wang, H., Zhao, R., Sun, Y., Bai, C., Ping, J., Song, L., Luo, M., & Chen, J. (2021). Lysyl hydroxylase 1 (LH1) deficiency promotes angiotensin II (Ang II)-induced dissecting abdominal aortic aneurysm. *Theranostics*, *11*(19), 9587–9604.
- Li, S.-C. (1996).  $\alpha$ -Helical, but not  $\beta$ -sheet, propensity of proline is determined by peptide environment. *Proceedings of the National Academy of Science USA*, *93*(1), 6676–6681.
- Lin, J. H., Walter, P., & Yen, T. S. B. (2008). Endoplasmic Reticulum Stress in Disease Pathogenesis. *Annual Review of Pathology: Mechanisms of Disease*, *3*(1), 399–425.
- Liu, J.-Y., Jiang, L., Liu, J.-J., He, T., Cui, Y.-H., Qian, F., & Yu, P.-W. (2018). AEBP1 promotes epithelial-mesenchymal transition of gastric cancer cells by activating the NF- $\kappa$ B pathway and predicts poor outcome of the patients. *Scientific Reports*, *8*(1), 11955.
- Malfait, F. (2018). Vascular aspects of the Ehlers-Danlos Syndromes. *Matrix Biology*, *71–72*, 380–395.
- Malfait, F., Francomano, C., Byers, P., Belmont, J., Berglund, B., Black, J., Bloom, L., Bowen, J. M., Brady, A. F., Burrows, N. P., Castori, M., Cohen, H., Colombi, M., Demirdas, S., De Backer, J., De Paepe, A., Fournel-Gigleux, S., Frank, M., Ghali, N., Tinkle, B. (2017). The 2017 international classification of the Ehlers-Danlos syndromes. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, *175*(1), 8–26.
- Mantle, D., Wilkins, R. M., & Preedy, V. (2005). A novel therapeutic strategy for Ehlers-Danlos syndrome based on nutritional supplements. *Medical Hypotheses*, *64*(2), 279–283.
- Mead, T. J., & Apte, S. S. (2018). ADAMTS proteins in human disorders. *Matrix Biology*, *71–72*, 225–239.
- Morais, P., Ferreira, O., Magina, S., Silva, C., Leão, M., Maia, A., & Azevedo, F. (2013). Classic Ehlers-Danlos syndrome: Case report and brief review of literature. *Acta Dermatovenerologica Croatica: ADC*, *21*(2), 118–122.
- Mullan, L. A., Mularczyk, E. J., Kung, L. H., Forouhan, M., Wragg, J. M. A., Goodacre, R., Bateman, J. F., Swanton, E., Briggs, M. D., & Boot-Handford, R. P. (2017). Increased intracellular proteolysis reduces disease severity in an ER stress-associated dwarfism. *Journal of Clinical Investigation*, *127*(10), 3861–3865.

- Plikus, M. V., Wang, X., Sinha, S., Forte, E., Thompson, S. M., Herzog, E. L., Driskell, R. R., Rosenthal, N., Biernaskie, J., & Horsley, V. (2021). Fibroblasts: Origins, definitions, and functions in health and disease. *Cell*, *184*(15), 3852–3872.
- Remvig, L., Duhn, P., Ullman, S., Arokoski, J., Jurvelin, J., Safi, A., Jensen, F., Farholt, S., Hove, H., & Juul-Kristensen, B. (2010). Skin signs in Ehlers–Danlos syndrome: Clinical tests and para-clinical methods. *Scandinavian Journal of Rheumatology*, *39*(6), 511–517.
- Reznik, S. E., & Fricker, L. D. (2001). Carboxypeptidases from A to Z: Implications in embryonic development and Wnt binding. *Cellular and Molecular Life Sciences*, *58*(12), 1790–1804.
- Ricard-Blum, S. (2011). The Collagen Family. *Cold Spring Harbor Perspectives in Biology*, *3*(1), a004978.
- Ritelli, M., Cinquina, V., Venturini, M., Pezzaioli, L., Formenti, A., Chiarelli, N., & Colombi, M. (2019). Expanding the Clinical and Mutational Spectrum of Recessive AEBP1-Related Classical-Like Ehlers-Danlos Syndrome. *Genes*, *10*(2), 135.
- Schissel, S. L., Dunsmore, S. E., Liu, X., Shine, R. W., Perrella, M. A., & Layne, M. D. (2009). Aortic Carboxypeptidase-Like Protein Is Expressed in Fibrotic Human Lung and its Absence Protects against Bleomycin-Induced Lung Fibrosis. *The American Journal of Pathology*, *174*(3), 818–828.
- Small, E. M. (2012). The Actin–MRTF–SRF Gene Regulatory Axis and Myofibroblast Differentiation. *Journal of Cardiovascular Translational Research*, *5*(6), 794–804.
- Stroud, R. M., & Walter, P. (1999). Signal sequence recognition and protein targeting. *Current Opinion in Structural Biology*, *9*(6), 754–759.
- Syx, D., De Wandele, I., Symoens, S., De Rycke, R., Hougrand, O., Voermans, N., De Paepe, A., & Malfait, F. (2019). Bi-allelic AEBP1 mutations in two patients with Ehlers–Danlos syndrome. *Human Molecular Genetics*, *28*(11), 1853–1864.
- Teratani, T., Tomita, K., Suzuki, T., Furuhashi, H., Irie, R., Nishikawa, M., Yamamoto, J., Hibi, T., Miura, S., Minamino, T., Oike, Y., Hokari, R., & Kanai, T. (2018). Aortic carboxypeptidase-like protein, a WNT ligand, exacerbates nonalcoholic steatohepatitis. *Journal of Clinical Investigation*, *128*(4), 1581–1596.

- Theocharis, A. D., Manou, D., & Karamanos, N. K. (2019). The extracellular matrix as a multitasking player in disease. *The FEBS Journal*, 286(15), 2830–2869.
- Theocharis, A. D., Skandalis, S. S., Gialeli, C., & Karamanos, N. K. (2016). Extracellular and Ehlers-Danlos syndrome hypermobility type): Clinical description and natural history. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, 175(1), 48–69.
- Tinkle, B., Castori, M., Berglund, B., Cohen, H., Grahame, R., Kazkaz, H., & Levy, H. (2017). Hypermobility Ehlers-Danlos syndrome (a.k.a. Ehlers-Danlos syndrome Type III and Ehlers-Danlos syndrome hypermobility type): Clinical description and natural history. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, 175(1), 48–69.
- Tinkle, B. T., & Levy, H. P. (2019). Symptomatic Joint Hypermobility. *Medical Clinics of North America*, 103(6), 1021–1033.
- Tokhmafshan, F., El Andaloussi, J., Murugapoopathy, V., Fillion, M.-L., Campillo, S., Capolicchio, J.-P., Jednak, R., El Sherbiny, M., Turpin, S., Schalkwijk, J., Matsumoto, K.-I., Brophy, P. D., Gbadegesin, R. A., & Gupta, I. R. (2019). Children with vesico-ureteric reflux have joint hypermobility and occasional TNXB sequence variants. *Canadian Urological Association Journal*, 14(4), 128–136.
- Tremmel, D., Duarte, M., Videira, A., & Tropschug, M. (2007). FKBP22 is part of chaperone/folding catalyst complexes in the endoplasmic reticulum of *Neurospora crassa*. *FEBS Letters*, 581(10), 2036–2040.
- Tumelty, K. E., & Layne, M. D. (2013). Adipocyte Enhancer Binding Protein 1 and Aortic Carboxypeptidase-Like Protein. In *Handbook of Proteolytic Enzymes* (pp. 1348–1353). Elsevier.
- Tumelty, K. E., Smith, B. D., Nugent, M. A., & Layne, M. D. (2014). Aortic Carboxypeptidase-like Protein (ACLCP) Enhances Lung Myofibroblast Differentiation through Transforming Growth Factor  $\beta$  Receptor-dependent and -independent Pathways. *Journal of Biological Chemistry*, 289(5), 2526–2536.
- Ushiki, T. (2002). Collagen Fibers, Reticular Fibers and Elastic Fibers. A Comprehensive Understanding from a Morphological Viewpoint. *Archives of Histology and Cytology*, 65(2), 109–126.

- Vishwanath, N., Monis, W. J., Hoffmann, G. A., Ramachandran, B., DiGiacomo, V., Wong, J. Y., Smith, M. L., & Layne, M. D. (2020). Mechanisms of aortic carboxypeptidase-like protein secretion and identification of an intracellularly retained variant associated with Ehlers–Danlos syndrome. *Journal of Biological Chemistry*, 295(28), 9725–9735.
- Vroman, R., Malfait, A.-M., Miller, R. E., Malfait, F., & Syx, D. (2021). Animal Models of Ehlers–Danlos Syndromes: Phenotype, Pathogenesis, and Translational Potential. *Frontiers in Genetics*, 12(1), 726474.
- Wang, D., Rabhi, N., Yet, S.-F., Farmer, S. R., & Layne, M. D. (2021). Aortic carboxypeptidase-like protein regulates vascular adventitial progenitor and fibroblast differentiation through myocardin related transcription factor A. *Scientific Reports*, 11(1), 3948.
- Wang, X., Chang, H., Gao, G., Su, B., Deng, Q., Zhou, H., Wang, Q., Lin, Y., & Ding, Y. (2021). Silencing of PRDM5 increases cell proliferation and inhibits cell apoptosis in glioma. *International Journal of Neuroscience*, 131(2), 144–153.

## CURRICULUM VITAE

