The Ugly Sequestosome1: the role of p62/SQSTM1 in autophagy and multisystem proteinopathy

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LYSYL OXIDASE MUTATIONS IN THORACIC AORTIC ANEURYSM AND DISSECTION

by

Vivian Lee

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the requirements for the degree
of Doctor of Philosophy

May 2018
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Vivian Lee

Washington University in St. Louis

May 2018
Dedication

This dissertation is dedicated to the memory of my father, Haijune Lee PhD, whom I know
would be proud to know I followed in his footsteps.
ABSTRACT OF THE DISSERTATION

Lysyl Oxidase Mutations in Thoracic Aortic Aneurysm and Dissection

by

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Doctor of Philosophy in Biology and Biomedical Sciences
Developmental, Regenerative, and Stem Cell Biology
Washington University in St. Louis, 2018
Professor Robert P. Mecham, Chair

Aortic aneurysms are one of the leading causes of death in developed countries. While aneurysms can form anywhere along the arterial tree, thoracic aortic aneurysms and dissections (TAAD), in particular, are associated with a strong genetic component. Currently, mutations in 29 genes have been identified to be causative for TAADs. However, these mutations only account for 25% of known familial TAAD cases. Therefore, identification of more mutations in FTAAD is worth pursuing to expand the panel of screened mutations during clinical genetic testing. Moreover, elucidating the underlying mechanism for TAAD progression from these mutant proteins will be necessary to develop therapeutic agents for these individuals.

Our work revealed a lysyl oxidase (Lox) missense mutation in a family with an autosomal dominant form of TAAD. Introduction of this human mutation into the mouse genome demonstrated that animals homozygous for the mutation had ruptured TAADs at birth, leading to mortality. Conversely, mice heterozygous for the mutation developed into adulthood without aortic dilations, despite fragmented elastic fibers in the ascending aorta. Additionally, we
reported that the mutant Lox mRNA and protein were expressed but Lox activity in conditioned media from mutant cells was not detected.

Furthermore, we demonstrated that the mutant LOX is retained in the ER, through direct interactions with calnexin. However, this accumulation did not elicit an ER stress response and the ultimate fate of the retained mutant Lox remains to be determined. We also reported that the animals heterozygous for the mutation require a “second hit” to the cardiovascular system to develop aortic dilations. Together, our work and work by others that identified additional LOX mutations strongly suggest that families with idiopathic TAAD should be screened for missense variants in LOX. Further, future studies will be necessary to determine the catalytic potential for this mutant Lox enzyme; and if it is active, molecular chaperones for the mutant Lox will potentially be able to correct the impaired secretion.
Chapter 1:

Introduction
Introduction
Lysyl oxidase (Lox) is a key enzyme from a family of amine oxidases that utilizes copper and lysyl tyrosinquione (LTQ) cofactors to catalyze the synthesis of functional extracellular matrix (ECM) proteins in connective tissues. Specifically, Lox is responsible for the maturation of two key ECM proteins in the aorta: elastin and collagen. Elastin is responsible for arterial wall expansion and recoil during pulsatile blood flow in a closed circulatory system, and collagen ensures aortic wall tensile strength. *Lox*-deficient mice (Hornstra et al., 2003; Maki et al., 2002) and chicks fed a copper-deficient diet (Simpson et al., 1964) have demonstrated the critical role of Lox in vascular development. Without functional Lox, animals die from ruptured aortic aneurysms and pulmonary insufficiencies. Recently, multiple families with thoracic aortic aneurysms and dissections (TAAD) have been identified with missense variants in the human LOX gene (D. C. Guo et al., 2016; V. S. Lee et al., 2016).

Evolution of the closed circulatory system
A circulatory system is vital for transporting fluids in living organisms for the purpose of reducing the diffusion distance of nutrients, gases, and metabolic wastes (Monahan-Earley et al., 2013). Muscle contractions and one-way valves establish peristaltic waves to facilitate the movement of circulatory fluids through two different categories of circulatory systems: open and closed (Xavier-Neto et al., 2007). Most invertebrates have open circulatory systems in which blood or hemolymph directly bathes the surrounding tissues. The hemolymph is emptied from the heart and major vessels into the body cavity to immerse the various organ systems, thereby facilitating direct contact for diffusion. It is notable that upon the emptying into the body cavity, hemolymph becomes indistinguishable from the interstitial fluid. In contrast, all vertebrates and some invertebrates have a closed circulatory system. As organisms grew in complexity, the development of compartmentalized organs demanded a more sophisticated vascular network.
The closed circulatory system was evolutionarily selected to improve efficiency of the heart and the flow of fluids to vital organs (Reiber et al., 1997).

The emergence of the closed circulatory system necessitated the evolutionary adoption of elastin to allow vessel extensibility (Bussiere et al., 2006). The evolutionary emergence of elastin with the closed circulatory system can be observed in a study surveying the animal kingdom for elastin expression; elastin is present in all vertebrates, with the exception of jawless fish, but absent in all invertebrates (Sage, 1982). Furthermore, it was observed through electron microscopy that vertebrates with higher blood pressures, such as large mammals, have denser elastic fibers and increased number of elastin layers in the aorta.

**Aorta structure**

The aorta consists of three major layers- intima, media, and adventitia. The intima, the first layer closest to the lumen, acts as a barrier for blood vessels. The single layer of endothelial cells that make up the intima play a role in defining embryonic vascular pattern and recruiting vascular smooth muscle cells (vSMCs) to the artery (Carmeliet, 2000). The media is the second layer and is made of numerous concentric layers of lamellar units; a lamellar unit refers to a layer of vSMCs and a layer of ECM, which is mostly comprised of elastic fibers and collagen. The mouse aorta has 5~8 lamellar units, while the human aorta has 60~70 lamellar units. Collagen and elastin make up ~60% of aorta tissue dry-weight (Sauvage et al., 1999), marking their critical role in arterial integrity and physiologic properties. The most outer layer of the vessel is called the adventitia, which includes fibroblasts and collagen, and it defines the vessel’s outer surface.
Elastin

Elastin structure and assembly

Elastin is encoded by a single gene located on the long arm of the human chromosome 7 within the q11.1-21.1 region (Fazio et al., 1991). The gene has relatively short exons interspaced by longer introns. This particular organization allows for cassette-like splicing, which results in entire exon deletions. Recent studies have demonstrated that the proteins that arise from these known splice variants can alter elastin monomer assembly into networks, resulting in weakened tensile strength (Miao et al., 2017). It is also important to note that the hydrophobic (comprised of glycine, valine, and proline) and crosslinking (lysine-rich) domains of the proteins are encoded by separate exons. Therefore, the domain structure of the protein is a reflection of the exon organization in the gene (Burnett et al., 1982) (Figure 1.1).

The elastin gene is translated into the elastin monomer called tropoelastin and is secreted into the extracellular space, where it is deposited onto microfibrils that are predominantly composed of fibrillin-1 and fibrillin-2 (Sandberg et al., 1969). Tropoelastin and microfibrils form highly stable and insoluble polymer networks called elastic fibers (Rosenbloom et al., 1976), having a half-life of approximately 74 years (Campbell et al., 1991; Jackson et al., 1967).

Elastin expression

Elastin expression in the mouse aorta begins at embryonic day (E) 14 and continues to increase (with a dip at postnatal day (P) 0) until P14; at which point, its expression begins to decrease and remains depressed in adult tissue. Though translation is rapidly decreased after the neonatal period, the level of elastin transcription remains consistent for the life of the animal.
Studies have demonstrated that this regulatory mechanism is due to the binding of microRNAs to specific stability and instability elements within the mRNA sequence (P. Zhang et al., 2012).

**Elastin function**

Elastin’s primary function is to confer elasticity in connective tissues such as blood vessels, skin, lung, and bladder. The elasticity of a cross-inked elastin network is driven by both entropy and the hydrophobic effect (B. Li et al., 2001). Upon stretching, individual chains within the elastin polymer become highly ordered; however, entropy drives the polymer to recoil back to its favored, disordered state when the stretching force is removed. Concurrently, hydrophobic regions of elastin become exposed to water during stretching, which is also energetically unfavorable. Therefore, recoiling returns the hydrophobic regions of elastin back to their water-free environment.

Elastin-derived peptides (EDPs), peptide products of elastin degradation by elastases, have demonstrated bioactive properties. Matrix metalloproteinases (MMPs)-2, 7, 9, and 12 can degrade both soluble and insoluble elastin to generate EDPs (Heinz et al., 2012). In particular, the GxxPG consensus sequence in EDPs has been shown to be essential for the EDP’s bioactivity. EDP signaling is initiated by binding to their cell surface receptors—galectin-3 (Pocza et al., 2008), integrins αvβ3, αvβ5 (P. Lee et al., 2014; Rodgers et al., 2004), and the elastin receptor complex (ERC) (Hinek et al., 1988).

Among the cell surface receptors, ERC is the best studied. It is a heterotrimeric receptor composed of two membrane-associated subunits, neuraminidase-1 (Neu-1) and protective protein/cathepsin A (PPCA), and the peripheral protein, elastin binding protein (EBP) (Duca et al., 2007). Once EDP binds to the EBP, intracellular signaling is propagated through a second
messenger called lactosylceramide (LacCer) to prompt a wide range of biological effects on immune cells (Duca et al., 2002). Namely, an increase in inflammatory cell recruitment through chemotactic activity and an increase in elastase production (G. Guo et al., 2011; Hance et al., 2002; Houghton et al., 2006; Nowak et al., 1989) are initiated through ERC signaling.

The ERC has also been proposed to function as a mechanotransducer in vSMCs (Spofford et al., 2001, 2003). When cells were grown on a Flexercell Strain Unit coated with type I collagen matrix, the expression of c-fos, a gene shown to be upregulated in response to mechanotransduction pathway activation, was not altered upon stretching. Conversely, the expression of c-fos was significantly decreased with stretching when cells were grown on an elastin matrix. However, this change in c-fos expression was attenuated when the ERC was inhibited using a peptide inhibitor, suggesting that elastin-ERC mediated signaling can respond to stretch.

Together, these and other studies have demonstrated that elastin function is essential in elastic tissues for maintaining structural integrity and mediating physiologic demands to stretch and recoil. When the polymer is degraded into EDPs, it adopts bioactive properties that can propagate signaling through various cell surface receptors. This bioactivity has been implicated in chemotaxis, angiogenesis, and cell migration, adhesion, survival, and proliferation, making elastin receptors an attractive target for therapeutic agents in numerous diseases.

**Elastin knockout mouse**

Supravalvular aortic stenosis (SVAS), a narrowing of the aortic lumen, is associated with loss-of-function mutations in the elastin gene. To study this disease in mice, the elastin gene was inactivated using a standard gene targeting approach (D. Y. Li et al., 1997). The Eln−/− mice died
from complete arterial occlusion (D. Y. Li, Brooke, et al., 1998), while Eln\textsuperscript{+/−} mice developed arterial stenosis similar to SVAS seen in humans (D. Y. Li, Faury, et al., 1998; Wagenseil et al., 2010) (Figure 1.2). The Eln\textsuperscript{−/−} animals survived gestation but died around P4.5 from an obstructed aorta. The aortas from wild-type (WT) and Eln\textsuperscript{−/−} animals were indistinguishable until E17.5, at which point the smaller lumen and thicker medial layer became apparent in Eln\textsuperscript{−/−} aortas. Within days, the aortic lumen was completely obstructed by subendothelial proliferation of vSMCs. Thorough studies were conducted to eliminate the possibility of endothelial damage, inflammation, thrombosis, hemodynamic stress and fibrosis, for this arterial occlusion (Figure 1.3).

The Eln\textsuperscript{+/−} mice were similar in gross appearance, behavior, and life expectancy to those of the WT mice (D. Y. Li, Faury, et al., 1998). When compared to WT animals, Eln\textsuperscript{+/−} animals unsurprisingly had a ~47% reduction in elastin mRNA and 50% thinner aortic elastin lamellae at birth when observed by electron microscopy. The Eln\textsuperscript{+/−} animals also had hypertension (~160/100 mmHg compared to ~120/80 mmHg in WT) (Faury et al., 2003). However, despite the reduction of elastin in Eln\textsuperscript{+/−} aortas, extensibility at ~100 mmHg was the same as that of WT aortas. The pressure-diameter curve diverged only at pressures above normal physiologic pressures, which was measured at 125 mmHg and above. Visualization of the elastin in these arteries by Hart staining revealed that the Eln\textsuperscript{+/−} aortas maintained extensibility by increasing the number of elastin lamellae layers in the aorta wall. In mice, Eln\textsuperscript{+/−} animals had 35% and 25% more elastin lamellar units in the descending and ascending aorta, respectively, when compared to WT animals (Figure 1.4). Furthermore, individuals with SVAS had an aorta wall that was thicker and had a 2.5-fold increase in lamellar unit numbers when compared to control aortas.
In addition to highlighting the role of elastin in conferring elasticity to tissue, the above studies have demonstrated the regulatory role of elastin in SMC proliferation and organization. When elastin is completely absent in the aorta, subendothelial proliferation of SMCs ultimately leads to complete occlusion of the aorta. Furthermore, it was also demonstrated that elastin expression determines the number of elastin lamellar units. In mice hemizygous for elastin, the aorta maintained arterial wall extensibility by increasing the number of elastin lamellar units in both the ascending and descending aorta. This compensatory mechanism was also observed in humans with SVAS.

**Human elastin BAC mouse**

Although the *Eln-null* mouse has been instrumental in elucidating the role of elastin in development, it is important to consider the structural differences between the human elastin gene (*hELN*) and the murine ortholog (*mEln*). The *hELN* has 34 exons while *mEln* has 37 exons. Furthermore, there are six splice junctions that are not shared between the human and mouse elastin genes, resulting in different exon sizes (Szabo *et al.*, 1999). Mutations in *hELN* have been implicated in several human diseases, such as SVAS and autosomal dominant cutis laxa (ADCL); however, these human diseases cannot be modeled in mice due to the differences in the elastin gene structures. To address this issue, a humanized elastin mouse was established by expressing the human elastin gene as a bacterial artificial chromosome (BAC) in the *mEln-null* background (Hirano *et al.*, 2007).

The *hELN* transgene had temporal- and tissue-specific expression similar to that of the endogenous *mEln* and the protein was able to form proper cross-links when expressed in a mouse. Furthermore, when *hELN* was expressed in the *mEln*+/− background (*hELN-mHet*), it was
able to rescue the $mEln^{+/\cdot}$ phenotypes including hypertension, increased lamellar unit number, and decreased ascending aorta compliance. These results suggest that human elastin is able to functionally complement mouse elastin, despite the differences in gene structure. In addition, when $hELN$ was expressed in the $mEln^{+/\cdot}$ background ($hELN-mNULL$), the perinatal lethality phenotype was rescued.

The appropriate spatial and temporal expression of the $hELN$ in mice suggests that the regulatory elements responsible for spatiotemporal expression are conserved in humans and mice. However, a significant decrease in $hELN$ expression levels, compared to $mEln$ expression levels, suggests that the regulatory elements controlling this process are distinct. Further studies demonstrated that the specific transcription factors whose function is to bind to the elastin promoter in the developing human aorta were not expressed in the mouse. Moreover, quantification of the insoluble elastin in $hELN-mNULL$ animals revealed that the animals had only one-third of the normal amount. Overall, this mouse model has demonstrated that the phenotypes observed in individual with SVAS is a direct result of the decreased amount of elastin and these abnormalities can be rescued by increasing the amount of insoluble elastin.

**Lysyl oxidase**

**Lox biosynthesis**

The initiation of both collagen and elastin crosslinking begins with the conversion of a peptidyl lysine to $\alpha$-amino adipic-$\delta$-semialdehyde (allysine) (Miller et al., 1967). Lox was discovered as the enzyme responsible for the catalysis of this deamination process (Pinnell et al., 1968). It is synthesized as a pre-proenzyme with a signal peptide to facilitate secretion. In the ER and Golgi, Lox gains three N-linked glycosyl groups in its propeptide domain and incorporates its two cofactors, copper and lysyltryosine quinone (LTQ) in the catalytic domain. Once
secreted, the inhibitory propeptide is cleaved by cell-surface furin-like proteases and the catalytic domain assumes its oxidase activity. The Lox pre-proenzyme is synthesized as a 46 kDa protein that undergoes glycosylation to yield a ~50 kDa protein. The size of the catalytic domain following cleavage of the propeptide is ~32 kDa (Figure 1.5).

**Lox cofactors and mechanism of action**

Lox requires both copper and LTQ cofactors in the catalytic domain for enzymatic activity. Each mole of Lox requires one tightly bound copper (II) for maximum activity and the enzymatic activity is decreased when the copper is stripped from Lox. It has also been demonstrated that the Cu$^{2+}$ ion cannot be replaced by other divalent ions such as Ni, Cd, Zn, Co, Fe, Hg, Mg, or Cd (Gacheru et al., 1990). In addition, the LTQ cofactor, a covalently bound prosthetic group, is formed in the presence of copper by hydroxylation and oxidation of a tyrosine side chain (position 349 in bovine), which then reacts with the ε-amino group of lysine (position 314 in bovine) via Michael addition (Matsuzaki et al., 1994; Wang et al., 1996). The LTQ plays the critical role in the initial formation of the Schiff-base necessary for Lox-mediated catalysis reaction to proceed (Ling et al., 2001).

The catalytic mechanism of Lox has been described to be a ping-pong kinetic mechanism (Williamson et al., 1986). This means that the aldehyde group in the substrate is produced and released before molecular oxygen binds for the re-oxidation of Lox. At initiation of the reaction, the Schiff-base in the substrate formed via its interaction with the LTQ cofactor undergoes base-facilitated α-proton abstraction using nearby histidine side chains as the base (Gacheru et al., 1988). The electrons then move from the substrate carbanion to reduce the carbonyl cofactor, and the hydrolysis of the imine intermediate releases the aldehyde product. Upon release of the
substrate, molecular oxygen binds to Lox for reoxidation of the enzyme and release hydrogen peroxide and ammonia as reaction byproducts (Shah et al., 1993) (Figure 1.6).

The allysine that results from the amine oxidation reaction can then undergo a spontaneous reaction with other allysines via an aldol condensation reaction to form an aldol condensation product, a bifunctional cross-link (Davis et al., 1970). It can also react with the side chain amino group of lysines via the Schiff base reaction to produce the dehydrolysinonorleucine or lysinonorleucine, bifunctional cross-links (Davis et al., 1970). Some of these bifunctional cross-links can then react together to form the tetrafunctional cross-links called desmosine and isodesmosine (Figure 1.7).

**Proteolytic activation of Lox**

The enzymes that cleave the pro-peptide and thus activate Lox are members of the procollagen, C-proteinase peptidase family. The Bmp-1 gene encodes two such proteases: bone morphogenetic protein-1 (BMP-1) and mammalian Tolloid (mTLD) (Hartigan et al., 2003), which are distinguished as alternative splice variants. In addition, genetically distinct mammalian Tolloid-like (mTLL)-1 and -2 also belong to the BMP-1 related proteinase family (Scott et al., 1999). All four proteinases are capable of cleaving LOX at the correct site *in vitro*; however, BMP-1 is 3-, 15-, and 20-fold more efficient than mTLL-1, mTLL-2, and mTLD, respectively (Uzel et al., 2001). Sequencing studies have shown that BMP-1 cleaves the Arg-Arg sequence in pro-LOX at position 192-193 (Atsawasuwan et al., 2011). BMP-1 itself is also synthesized as a pro-enzyme that requires cleavage of the pro-peptide for activation. Studies show that pro-BMP-1 is cleaved in the trans-Golgi network by a furin-like pro-protein convertases (Leighton et al., 2003). These results indicate that BMP-1 is active intracellularly as well as at the plasma membrane, the major activity site for BMP-1.
**Lox inhibitors**

The specificity of Lox inhibitors remains critical from a therapeutic drug development standpoint. A naturally occurring Lox inhibitor, β-aminopropionitrile (BAPN), was first identified in grass peas that were fed to animals that developed a condition called lathyrism. Lathyritic animals developed pathologic changes in the bone and blood vessels with decreased collagen and elastin cross-links (Piez, 1968). Studies have demonstrated that BAPN is an irreversible inhibitor of all Lox isoforms (Pinnell et al., 1968). The inhibitor mechanism has been attributed to the inhibitor binding to Lox, causing derivatization of the enzyme. Other molecular candidates such as β-haloethylamines, β-nitroethylamines (Williamson et al., 1987), cis-1,2-diaminocyclohexane, and ethylenediamines (Gacheru et al., 1989) have also been identified as irreversible inhibitors of Lox.

The specificity for BAPN and another inhibitor, semicarbazide (SCZ), for Lox and semicarbazide-sensitive amine oxidase (SSAO) were compared in Brown Norway rats (Mercier et al., 2009). These studies demonstrated that both SCZ and BAPN are potent inhibitors of Lox activity. However, SCZ inhibited SSAO to a greater extent than BAPN, showing that BAPN can inhibit other amine oxidases in addition to Lox. Together the data suggests that the specificity for these Lox inhibitors must be further elucidated for the development of anti-Lox therapeutics.

**Lox and Lox isoform structures**

In 1991, the human gene for Lox was mapped to chromosome 5 (Hamalainen et al., 1991). The human *LOX* is about 15 kb in length with seven exons and six introns and its transcript size varies from 11-15 kb, depending on the size of the 3’ UTR. A year later, the mouse gene for Lox was mapped to chromosome 18 (Mock et al., 1992). The mouse *Lox*
consists of seven exons and six introns (Contente et al., 1993). It spans 14 kb in the mouse genome and transcribes two message products, 4.8 kb and 3.8 kb, with the difference in size also explained by a 3’ UTR length difference. There is no evidence of alternative splicing of the human or mouse Lox coding region. In subsequent years, several groups identified other members of the Lox gene family displaying sequence homology and assigned the nomenclature as lysyl oxidase-like 1-4 (Loxl1-4) (Csiszar, 2001).

At the protein level, all members of the Lox family have a conserved C-terminal catalytic region that contains a copper-binding site and a LTQ cofactor site. The N-terminus is less conserved between sub-families and distinguishes Lox and Loxl1 from Loxl2-4. Lox and Loxl1 have pro-peptides at the N-terminus while Loxl2-4 have multiple scavenger receptor cysteine-rich (SRCR) domains (Barker et al., 2012). The specific function for the SRCR domain has not been identified; however, it is predicted to have a role in protein-protein interactions (Figure 1.8).

While the crystal structure of Lox has not been obtained, a computational model of the enzyme has been refined using molecular modeling software. The overall Lox structure approximates a globular protein with 25% β-sheet, 20% α-helix, and 65% random coil (Ryvkin et al., 2004).

**Origin and evolution of Lox**

To study the evolutionary history of Lox, a wide selection of genomes were surveyed (Grau-Bove et al., 2015). This study found that Lox family of genes had a pre-metazoan origin and is not specific to just animals. The study found that Lox and its isoforms are expressed in bacteria and archaea as well. Furthermore, the analysis revealed that the Lox family expanded into the two subgroups during metazoan evolution, which differentiated, Lox and Loxl1 (and
Loxl5, specific to fish) from Loxl2-4. Together, these findings raise the possibility that Loxl2-4 may have been preferred for crosslinking collagen IV-based basement membranes, whereas Lox and Loxl1 contributed to chordate and vertebrate-specific ECM proteins such as, elastin.

**Lox knockout mouse**

While the *Eln-null* mice do not phenocopy BAPN-treated animals in developing aortic aneurysms, *Lox-null* animals do. Two independent groups generated *Lox<sup>+/−</sup>* mouse lines (Hornstra *et al.*, 2003; Maki *et al.*, 2002). Maki et al. generated a *Lox-null* allele by deleting exon1, while Hornstra et al. replaced the ATG start codon by inserting a NotI restriction enzyme site. Regardless of the differences in the targeting vector designs, both groups reported animals with similar phenotypes.

The *Lox<sup>+/−</sup>* animals developed to full-term but did not survive past a few hours after birth. Using timed-mating, *Lox<sup>+/−</sup>* pups were studied during late embryonic development. Starting at E14.5, *Lox<sup>+/−</sup>* aortas had thicker walls and smaller lumens compared to WT aortas. Histology showed that at E18.5, SMCs in the media were highly disorganized and elastic lamellae were very fragmented. These *Lox<sup>+/−</sup>* animals died within a few hours of birth due to ruptured aneurysms (Figure 1.9). Hornstra et al. performed desmosine/isodesmosine and hydroxyproline assays to assess Lox activity towards elastin and collagen, respectively. These studies revealed that elastin in the aorta were more severely affected by the absence of *Lox* than was collagen (*Lox<sup>+/−</sup>* aorta had 39% desmosine/isodesmosine compared to WT aorta, whereas *Lox<sup>+/−</sup>* aorta had 74% hydroxyproline compared to WT aorta). Maki et al. (Maki *et al.*, 2005) used histology to characterize the effect of *Lox* deletion on lung and skin of *Lox<sup>+/−</sup>* animals and found abnormal elastin and collagen fibers in both tissues. Together, these studies demonstrate that *Lox* deficiency most dramatically affects vascular development, as knockout animals are perinatal
lethal from ruptured aortas, but it also impacts other connective tissues such as lungs and skin, although not as severely.

**Lox11 knockout mouse**

All five members of the Lox family of enzymes share a highly conserved catalytic domain, but Lox and Lox11 are the only two isoforms within the family that are synthesized as proenzymes. Thus, to identify the physiological role of Lox11, *Lox11*−/− mice were developed (Liu *et al.*, 2004) by targeting exon 1 of *Lox11*. The *Lox11*−/− animals were viable and fertile, but *Lox11*−/− females developed pelvic organ prolapse post-partum resulting from changes in the elastic fibers in the uterine wall. The elastic fibers in the uterine wall before pregnancy appeared normal. However, after the first pregnancy, the uterine cervix remained stretched, indicating the loss of tissue resilience. Immunohistochemistry found the elastic fibers to be highly fragmented, which was consistent with the altered tissue mechanical properties. These data suggest that Lox11 plays a role in adult uterine tissue elastin maturation (Figure 1.10).

In both male and female *Lox11*−/− animals, elastic fibers were affected in various tissues. Knockout animals of both genders had rectal prolapse and intestinal diverticula. In addition, alveoli in the lung were enlarged and subdermal skin had fragmented elastic fibers. Elastin staining in the aorta of knockout mice was diffuse and weak, but the animals did not develop aneurysms or stenosis. Despite the complete absence of Lox11, morphology of collagen by histology or maturation measured by hydroxyproline assay was not altered in lung, skin, aorta, or uterine tissues.

Yeast two-hybrid assays identified Fibulin-5 (FBLN5) as a binding partner for LOXL1. This interaction is further supported by co-localization of the two proteins in cells, co-immunoprecipitation studies in tissue extract, and overlapping phenotypes between *Lox1-null*
and Fbln5-null animals. These studies demonstrate the non-redundant roles of Loxl1 and Lox in elastic fiber formation and homeostasis.

**Loxl2 knockout and overexpression mice**

Loxl2 knockout and overexpression mouse models were developed to study Loxl2 function (Martin et al., 2015). Loxl2-null mice were generated using a targeting vector to ablate exon 2 of Loxl2. Surviving Loxl2−/− animals were viable and fertile. However, at birth, the number of Loxl2−/− animals recovered was 2-fold less than the expected Mendelian ratio. Collection of embryos mid-gestation revealed that ~40% of Loxl2−/− animals died from disrupted ventricular septa formation and hepatic vessel dilation. Expression of other Lox isoforms was not significantly altered in the tissues examined except in the heart tissue, where Loxl3 protein levels were diminished. Furthermore, FAK2 and SRC protein levels were markedly decreased in Loxl2−/− heart tissue, which are components of the key signaling pathways in cardiac morphogenesis. These findings are in agreement with previous reports demonstrating that reduction of FAK and Src expression are associated with ventricular septal formation at late embryonic stages but with incomplete penetrance (Figure 1.11).

Martin et al. also developed a Loxl2 over-expressing mouse (R26L2/L2) using LOXL2-GFP expression under a constitutive ROSA26 promoter (Figure 1.12). Successful expression was confirmed by green fluorescence detection in the newborn pups. While R26L2/L2 animals did not have heart defects, 90% of the males were sterile due to epididymis hypertrophy and testicular degeneration that occurred as early as 3 months of age. Moreover, epididymis tubules were distended from acute inflammation and fibrosis, marked by increased fibronectin, elastin, and collagen, measured by immunostaining. Loxl2−/− and R26L2/L2 mice were further utilized to study the intracellular role of Loxl2. In brief, a 2-stage squamous cell carcinoma (SCC) model that
induces skin carcinogenesis was used to study the evolution of head and neck SCC. Results demonstrated that $R2\beta^{L2/L2}$ animals had an increase in tumor burden and malignant progression, whereas $Loxl2^{-/-}$ animals had the opposite phenotype.

These mouse models suggest that extracellular Loxl2 activity is specific to the heart and male reproductive organs. However, while loss of Loxl2 causes lethal cardiac defect in 40% of knockout animals, $R2\beta^{L2/L2}$ hearts were not affected. Similarly, 90% of $R2\beta^{L2/L2}$ males were sterile from epididymis hypertrophy and testicular degeneration, while $Loxl2^{-/-}$ male reproductive organs were not affected.

**Loxl3 knockout mouse**

Identification of a G2027A missense variant in $LOXL3$ human individuals with Stickler’s syndrome, commonly caused by mutations in various collagen genes leading to bone and cartilage deformities, shed light on $LOXL3$ as a candidate gene for human disease (Alzahrani et al., 2015). $Loxl3^{-/-}$ mice were developed (J. Zhang et al., 2015) using a targeting vector designed to remove $Loxl3$ exon 2. $Loxl3^{+/+}$ animals were viable and developed normally without any obvious abnormalities. For homozygous knockouts, however, only ~15% of $Loxl3^{-/-}$ animals survived to P0 and all were dead by P1. All $Loxl3^{-/-}$ animals developed cleft palates and ~90% of them also had severe spinal deformities. Analysis of these animals at embryonic stages determined that both of these phenotypes began as early as E13.5 and became more prominent by E15.5.

Analysis of immature and mature collagen cross-links identified a significant decrease in only dihydroxylysinoonorleucine (DHLNL). Furthermore, hydroxyproline and desmosine assays were performed to quantify mature collagen and elastin, respectively. Although hydroxyproline
levels were decreased in both \( \text{Loxl3}^{+/} \) and \( \text{Loxl3}^{-/-} \) embryos, there was no difference in the number of desmosine/isodesmosine cross-links. This suggests that Loxl3 prefers collagen as a substrate to elastin,

Individuals with Stickler’s syndrome often have non-progressive myopia associated with craniofacial deformities. However, despite the expression of Loxl3 in eyes, deletion of \( \text{Loxl3} \) did not cause myopia in mice. There were also no overt phenotypes in the heart, aorta, or trachea, of mutant mice assessed by histology.

Another tissue where changes were observed were the lungs, which had smaller alveoli than in WT animals (J. Zhang et al., 2016). By E14.5, abnormalities in thoracic architecture marked by deformities in the spine and ribs, reduced the thorax volume by \( \sim 30\% \). At E16.5, \( \text{Loxl3}^{-/-} \) terminal lung tubules did not expand as they did in WT littermates. By E18.5, attenuation of lung development in \( \text{Loxl3}^{-/-} \) animals became more obvious, as lung weight and lung weight to body weight ratios were significantly reduced. While there was no difference in the number of air saccules between WT and \( \text{Loxl3}^{-/-} \) lungs at this age, there was reduced saccular space volume and a \( \sim 40\% \) reduction in total lung volume based on body weight.

In addition to significant decrease in size of \( \text{Loxl3}^{-/-} \) lungs, there was a significant increase in lung elastin measured by resorcin-fuchsin staining and desmosine cross-link analysis. However, there was no difference in hydroxyproline levels between WT and \( \text{Loxl3}^{-/-} \) lungs, suggesting no difference in mature collagen levels. Interestingly, while \( \text{Lox} \) and \( \text{Loxl1} \) mRNA levels were unchanged in \( \text{Loxl3}^{-/-} \) lungs, \( \text{Loxl2} \) mRNA was reduced by 2-fold and \( \text{Loxl4} \) mRNA was increased by 2.5-fold. The increase in \( \text{Loxl4} \) expression was also confirmed by increased \( \text{Loxl4} \) protein detected by western blot and immunohistochemistry. To determine if increased
Loxl4 levels were responsible for the elevated elastin deposition in $Loxl3^{-/-}$ lungs, a pENTER-C-GFP-LOXL4 plasmid was transiently expressed in HEK-293 cells. This *in vitro* study demonstrated that Loxl4 expression was sufficient to induce increased elastin deposition from HEK-293 cells. This up-regulation of Loxl4 in $Loxl3^{-/-}$ lungs was associated with a modest increase in transforming growth factor β-1 (TGFβ-1), a growth factor previously demonstrated to induced Loxl4 expression.

The similarity in cell proliferation, apoptosis, and differentiation in $Loxl3^{-/-}$ lungs compared to WT lungs suggest that Loxl3 does not affect pulmonary cells directly. Instead, attenuation of lung size in $Loxl3^{-/-}$ animals is likely due to physical constraints imposed by the constrictive thoracic architecture alteration. Moreover, it would be of interest to pursue the molecular mechanisms behind how Loxl4 is up regulated in the absence of Loxl3. These studies will provide insight into interaction between various Lox isoforms and their tissue-specific functional overlap (Figure 1.13).

**Fibulin-4**

Fibulin-4 (Fbln4) is another ECM protein required for elastic fiber assembly. It belongs to a seven-member family (Fbln1-7), characterized by tandem repeats of calcium-binding epidermal growth factor like motifs and a C-terminal fibulin module (Kobayashi *et al.*, 2007). While the precise function of the fibulins has not been elucidated, mutations in the genes encoding for this family of proteins have been implicated in human diseases. Missense variants in *EFEMP1*, encoding FBLN3, have been shown to be responsible for inherited macular degenerative diseases, whereas mutation in *FBLN5* has been implicated in cutis laxa. Among the fibulin proteins, FBLN3, 4, and 5 have particularly high homology to one another and have been
shown to be indispensable in elastogenesis in different tissues. Thus, the three isoforms’
functions are not redundant and cannot compensate for one another.

Most interestingly, mice deficient in *Fbln4* (also known as *Efemp2*) die perinatally with
phenotypes similar to *Lox-null* animals (McLaughlin *et al.*, 2006). The *Fbln4* gene was
inactivated in mice using a target vector to disrupt exon 4. The *Fbln4*<sup>+/−</sup> animals were fertile and
lived normal lifespans, indistinguishable from their WT littermates. In contrast, all *Fbln4*<sup>−/−</sup>
animals die at birth and had torturous arteries with irregular narrowing and dilation, aneurysms,
and ruptures, which led to lethal hemorrhages. Histology of aortic cross-sections revealed that
*Fbln4*<sup>−/−</sup> animals did not have properly formed elastic fibers in the aortic wall. Instead of
continuous elastic fibers in concentric layers, the *Fbln4*<sup>−/−</sup> aortas had elastin aggregates dispersed
throughout the wall, similar to that seen in *Lox*<sup>−/−</sup> aortas. Furthermore, the amount of desmosine in
*Fbln4*<sup>−/−</sup> aorta is decreased by ~94% compared to WT, suggesting that the catalytic activity of
Lox is compromised in these animals (Figure 1.14).

There was significant decrease in desmosine cross-links in *Fbln4*<sup>−/−</sup> tissues despite
unaltered Lox mRNA and protein levels. To determine the relationship between FBLN4 and
LOX, *in vitro* studies were performed to assess their interaction (Horiguchi *et al.*, 2009). Tagged
protein studies demonstrated that the N-terminus of FBLN4 directly binds the pro-peptide of
LOX. Furthermore, this interaction was required for LOX to bind tropoelastin and all three of
these proteins co-localized in cultured human fibroblasts. Together, these studies suggests a
model for Fbln4 functioning as a molecule that tethers pro-Lox to tropoelastin to facilitate
activation of Lox only when it is positioned close to its substrate, tropoelastin. Further studies are
required to confirm their interaction *in vivo* to fully elucidate Fbln4 function.
Aortic Aneurysm and Dissection

Aortic aneurysms are defined as focal dilations that are at least 50% larger in diameter than normal (Goldfinger et al., 2014). Because the only treatment available for aneurysms is surgical repair after a rupture, identifying mutation in genes that predispose individuals to aneurysm formation and identifying mechanism behind aneurysm pathogenesis would greatly facilitate early detection and therapeutic intervention design.

Aortic aneurysms are categorized as abdominal aortic aneurysm (AAA) or thoracic aortic aneurysm (TAA) based on the localization below or above the diaphragm, respectively. AAAs are typically a degenerative condition, strongly linked to older age, cigarette smoking, hypertension, and hyperlipidemia. Conversely, TAAs are more strongly attributed to genetic perturbations (Lindsay et al., 2011; Rea et al., 2013). TAAs can be isolated or occur as a component of syndromic connective tissue disorders such as Marfan syndrome (Hayward et al., 1992), Loeys-Dietz syndrome (Loeys et al., 2005), and Ehlers-Danlos syndrome (Steinmann et al., 1980). Furthermore, familial TAA and dissection (FTAAD) can have faster growth rates compared to sporadic TAADs, leading to earlier presentation in life. In fact, 5.4% of sudden cardiac deaths in individuals at mean age 26.8 is from FTAADs (Puranik et al., 2005).

Currently, mutations in 29 genes have been identified to be responsible for FTAADs. These genes implicated in aortic aneurysms are classified into three categories based on molecular function: extracellular matrix proteins, TGFβ pathway components, and SMC contractile machinery (Campens et al., 2015; Pyeritz, 2014). Sequencing techniques continue to expand the panel of mutations that can be identified during clinical genetic testing. However, it is also critical to probe for molecular mechanisms that underlie TAAD pathogenesis for development of nonsurgical treatment.
Goals of the Thesis

Since its discovery, Lox has long been studied for its role in connective tissue maturation.

The genetic ablation of Lox in mouse models, as well as other animal models such as copper-deficiency and lathyism, established foundational insight into the role of Lox isoforms in development. The functional importance of Lox has recently expanded with direct genetic linkage of Lox isoforms to human diseases. A missense variant in LOXL3 was identified in a family with Stickler’s syndrome, marked by collagenopathy (Alzahrani et al., 2015). Simultaneously, multiple missense variants in LOX were also identified in families with TAAD, manifested by elastinopathy (D. C. Guo et al., 2016). This thesis uses CRISPR-Cas9 genome editing technology to introduce a LOX mutation our colleagues identified in a family with TAAD into the mouse genome with the intention to elucidate the molecular mechanism responsible for the aortopathy observed.

This thesis is based on the work of Dr. Nathan Stitziel at Washington University and his colleagues at Brigham Genomic Medicine, where they identified a family of patients with TAAD. The whole-genome sequencing data from the family identified the M298R missense variant in LOX, which was shared by the affected individuals in this family. The first goal of the work described in this thesis was to introduce this human missense variant into the corresponding locus in the mouse genome (M292R). The M292R Lox mice were then used to characterize in detail the effects of this mutation on connective tissue development and disease, with an emphasis on the aorta.

I have also used the M292R mouse model to show that this particular mutation in the catalytic domain of Lox causes TAAD. Lox is a complex enzyme that requires several post-translational modifications for it to ultimately reach the ECM for catalytic activity. I have
utilized primary MEFs isolated from the M292R Lox embryos, as well as the mammalian expression plasmids to express M292R Lox, and determined how the mutation affects Lox expression, secretion, and activity.
Elastin gene structure is organized by alternating hydrophobic and cross-linking exons, which are also reflected in the protein structure domains (Kanta, 2016).
Figure 1. 2: EM image of Eln KO aortas

At E18, smooth muscle cells (SMCs) in all genotypes are organized in mostly circumferential layers. Shown are representative electron microscopic (EM) images of the proximal ascending aorta with the lumen at the bottom of the image. Elastic laminae are thinner in Eln+/- aortas and absent in Eln-/- aortas, but the SMC arrangement looks similar to WT. Scale bars = 11 µm.

(Wagenseil et al., 2010)
Figure 1. 3: Subendothelial proliferation of vSMCs in Eln KO aorta

At P2.5, haematoxylin and eosin (H&E) staining of (A) Eln\(^{+/+}\) and (B) Eln\(^{-/-}\) aortas. (D. Y. Li, Brooke, et al., 1998)
Figure 1. 4: Increased number of lamellar units in Eln haploinsufficient aorta

Hart staining of aorta cross-sections. In Eln\(^{+/−}\) adult mice, both (B) ascending and (D) descending aortas had increased number of elastic lamellae compared to WT (A) ascending and (C) descending aortas. (D. Y. Li, Faury, et al., 1998)
Lox is synthesized as a proenzyme. The pro-peptide is cleaved in the ECM for activation of the catalytic domain. The catalytic domain of Lox is highly conserved among several species (V. S. Lee et al., 2016).
Figure 1.6: Mechanism of Lox action

Following initial Schiff base formation with LTQ (I-II), the bound substrate undergoes base-facilitated \( \alpha \)-proton abstraction. The electrons migrating from the substrate carbanion reduces the carbonyl cofactor (II-III). Aldehyde product is released from hydrolysis of the imine intermediate. The enzyme is re-oxidized using molecular oxygen (IV) and the byproducts hydrogen peroxide and ammonia are released (V). (Smith-Mungo et al., 1998)
Figure 1.7: Role of Lox in cross-link formation.

Lox catalyzes the amine oxidation reaction of lysine residues (Lys), resulting in an allysine (AAS). Subsequently, spontaneous condensation between Lys and AAS or ASS and another AAS results in bifunctional cross-links, dehydrolysinonorleucine (deLNL) or aldol condensation product (ACP) (Kagan et al., 1991). These bifunctional cross-links can further react together to form tetrafunctional cross-links, desmosine and isodesmosine (Luisetti et al., 2008).
Lox are a family of amine oxidase enzymes. Among the isoforms, only LOX and LOXL1 both have unique pro-peptides that are cleaved in the ECM for activation. Conversely, LOXL2, 3, and 4 isoforms have scavenger receptor cysteine-rich domains instead. The catalytic domain is highly conserved among all five isoforms and it includes the binding site for copper and residues responsible for LTQ formation.
Figure 1. 9: Lox KO mice

(A) Lox<sup>-/-</sup> animals are born in the correct Mendelian ratio and are similar in size compared to WT littermate controls. (B) Bulging aneurysms in the aorta of Lox<sup>-/-</sup> animals. (C) EM images of aorta cross-sections demonstrate the loss of proper elastin lamellae formation in Lox<sup>-/-</sup> aortas compared to WT controls. (Hornstra et al., 2003; Maki et al., 2002)
Gross dissections of the uterus and vagina of WT and Loxl1<sup>-/-</sup> mice, demonstrating dilation in both organs. (Liu et al., 2004)
Figure 1.11: Loxl2 KO mice

H&E sections of transversal sections from Loxl2\textsuperscript{−/−} hearts at different levels showing aberrant ventricular communication (left). Curved arrow points to disrupted ventricular septa. H&E sections of livers (right) from WT (top) and Loxl2\textsuperscript{−/−} (bottom) neonates. Arrowheads indicate regions of capillary distension. Scale bars: 1,500 µm (heart), 100 µm (liver). (Martin et al., 2015)
Figure 1.12: *Loxl2* overexpression mice

Macroscopic view of testis and epididymis from $R26^{+/+}$ and $R26^{L2/L2}$ mice (top) and H&E image of whole testis from both groups of animals showing testicular degeneration in the overexpressing mice (bottom). T: testis, E: epididymis. Scale bar in lower panels: 1,000 μm.

(Martin *et al.*, 2015)
Figure 1.3: Loxl3 KO mice

(A) Loxl3+/− mice had deformed spines (white arrow) as well as (B) craniofacial defects marked by cleft palate (black arrow) at P0. T= tongue. (C,D) Lateral view of Alizarin Red staining of mouse skeleton at E18.5. The side profiles of the LOXL3-deficient thoraxes also exhibited spine and rib deformities. Bar: 2 mm. (E) Decreased lung size in Loxl3+/− mice compared to WT controls. (J. Zhang et al., 2016; J. Zhang et al., 2015)
Figure 1.14: *Fbln4* KO mice

(A,B) At P1, *Fbln4*<sup>−/−</sup> animals have tortuous aortas with dilations. Elastic lamellae were largely absent in *Fbln4*<sup>−/−</sup> aortas. At E16.5, internal elastic lamina was identifiable in (C) WT aorta but not in (D) *Fbln4*<sup>−/−</sup> aorta. The aortic wall of (F) *Fbln4*<sup>−/−</sup> mice became progressively thicker over time compared to (E) WT controls and had irregular elastin aggregates. Arrow: internal elastic lamina. Arrowhead: Elastin aggregates. (McLaughlin *et al*., 2006)
References


Chapter 2:

Loss of function mutation in *LOX* causes thoracic aortic aneurysm and dissection in humans
This chapter was taken in full from the manuscript titled, “Loss of function mutation in LOX causes thoracic aortic aneurysm and dissection in humans” published in *Proceedings of National Academy of Science of the United States of America* on August 2, 2016.

Discovery of M298R mutation in LOX and human data were work by Dr. Nathan Stitizel and his colleagues. I carried out the development and characterization of M292R *Lox* mouse model in the lab of Dr. Robert Mecham.

Introduction

Thoracic aortic aneurysms and dissections (TAAD) comprise a large group of heterogeneous conditions with significant phenotypic diversity. Individuals presenting with an inherited form of TAAD may have evidence of a defined genetic syndrome (e.g. Marfan syndrome, Loeys-Dietz syndrome, Ehlers-Danlos syndrome type IV, or a TGF-β related vasculopathy) or simply a family history of TAAD (termed familial TAAD or FTAAD). Anatomically, FTAAD can affect various segments of the arterial system from the aortic root and ascending aorta to more distal arterial segments such as the hepatic or pulmonary arteries. In addition, FTAAD are characterized by significant locus heterogeneity with mutations in a diverse group of genes (TGFBRI, TGFBRII, TGFB2, ACTA2, SMAD3, COL3A1, FBN1, MYLK, MYH11, PRKG1 and SLC2A10) reported to date (Coucke et al., 2006; Guo et al., 2007; Guo et al., 2013; Lee et al., 1991; Loeys et al., 2005; Loeys et al., 2006; Tsipouras et al., 1986; van de Laar et al., 2011; Wang et al., 2010; Zhu et al., 2006). Despite significant progress in defining the genetic basis of FTAAD, the molecular etiology of many cases remains enigmatic.

Elastin and collagen are two of the major structural components that comprise the arterial wall. Lysyl oxidase (LOX) and its related gene family members are a group of copper-dependent oxido-deaminases that crosslink lysyl residues on these structural proteins in the process of forming proper elastic lamellae and collagen fibers (Smith-Mungo et al., 1998). The homozygous knockout of the murine Lox gene results in perinatal death from aortic aneurysm and spontaneous dissection and mutant Lox null mice exhibit highly abnormal aortic histology characterized by fragmented elastic fibers and aberrant smooth muscle cell layers (Hornstra et al., 2003; Maki et al., 2002). While these mouse model findings strongly support the functional significance of LOX in the maintenance of normal arterial wall integrity, until now LOX mutations have not been identified in human subjects (Lopez et al., 2011).
Here we report a LOX missense mutation discovered through whole genome sequencing in a family with autosomal dominant TAAD where prior clinical genetic testing was unrevealing. Using genome-engineering techniques, we created a mouse model of the specific human mutation to study its in vivo effects. In the heterozygous state, we found mice with the human mutation displayed abnormal aortas with disorganized assembly of elastic lamellae in the aortic wall, whereas mice homozygous for the human mutation died from peri-neonatal aortic aneurysm and spontaneous hemorrhage. Together, our data identify a novel genetic etiology for autosomal dominant TAAD with important implications for clinical patient care.
Results

Characterization of a family with FTAAD

The proband (Figure 1A, Individual III-1) presented to the Brigham Genomic Medicine Program (BGMP) as a 35-year-old Caucasian male for evaluation of his personal and family history of TAAD. His history included a surgical repair of pectus excavatum at the age of 2 and the diagnosis of a large ascending aortic aneurysm at age 19. The aneurysm, which did not involve the aortic root and extended to the brachiocephalic artery, measured 10.5 cm in diameter and was discovered on chest computed tomography imaging as part of an evaluation for complaints of chest pain and cough. The patient underwent a valve-sparing aortic root replacement. The histopathological analysis of his resected aortic tissue found a contained posterior rupture with evidence of cystic medial necrosis and fragmented external elastic lamella. On the basis of his medical history and suggestive physical features (which included tall stature, high arched palate, and dental crowding) he was given a clinical diagnosis of Marfan syndrome. However, genetic testing that included FBN1 gene sequencing and multiplex ligation-dependent probe amplification, TGFBR1 gene sequencing, and TGFBR2 gene sequencing was negative. His physical examination was notable for pectus excavatum, presence of venous varicosities in his right lower extremity, positive thumb but not wrist signs and skin striae on his flanks. A review of his family history suggested an autosomal dominant disorder (Figure 2.1A).

The mother of the proband (Figure 1A, Individual II-1) was also evaluated in our clinic because of her history of acute ascending aortic dissection with repair at the age of 52 (Figure 2.1B), abdominal hernia repair at the age of 34, and myopia. The histopathological analysis of her resected aortic tissue also demonstrated cystic medial necrosis with a disorganized appearance of collagen and elastic lamellae (Figure 2.1C). She underwent expanded genetic
testing which failed to reveal a causal mutation in \( TGFBR1, TGFBR2, ACTA2, COL3A1, MYH11, SLC2A10, SMAD3, \) or \( MYLK \). A variant of unknown significance c.703G>C (p.V235L) was found in the \( TGFBI \) gene. Clinical features of other family members are provided in Table 1.

**Identification of a missense mutation in \( LOX \) associated with FTAAD**

In November 2013, we performed whole genome sequencing in two first cousins with TAAD (Figure 1A, Individuals III-1 and III-3) to identify the causal gene and mutation underlying the disease in this family. We required putative causal mutations to be 1) shared in a heterozygous state between these two individuals; 2) rare with a minor allele frequency in all populations from the NHLBI Exome Sequencing Program and Exome Aggregation Consortium of 0.01% or less and not observed in a local database of individuals sequenced for other rare, non-vascular Mendelian disorders; and 3) exert a functional impact on the gene’s product, restricting our analysis to missense, nonsense, frameshift, or splice-site variants. This analysis resulted in a total of seven candidate mutations (Table 2); six of these were eliminated due to lack of co-segregation with disease (i.e. either not present in other affected individuals from the family or present in unaffected individuals in the family) or for other considerations (see Table 2 for details).

The remaining candidate mutation was a missense substitution (c.893T>G encoding p.Met298Arg, Figure 2.2) in lysyl oxidase (\( LOX \)) which was considered a strong candidate because of the gene’s known role in arterial wall biology (Hornstra et al., 2003; Maki et al., 2002). \( LOX \), an enzyme that requires copper for its activity, catalyzes the crosslinking of collagen and elastin by deaminating side chains of specific lysine and hydroxylysine residues
to the copper-binding domain of LOX (Figure 2.1D) suggesting this missense mutation might disrupt normal LOX function.

**Mice heterozygous for the Lox missense variant have longer ascending aortas with fragmented elastic fibers**

To explore the functional relevance of the LOX p.M298R mutation in terms of vascular disease and to determine how the mutation affects lysyl oxidase function, we used CRISPR/Cas9 genome editing to introduce the human mutation into the homologous site in the mouse genome which corresponds to amino acid 292 (Lox p.M292R) (Figure 2.3). Animals heterozygous for the mutation (Lox+/M292R, hereafter referred to as Lox+/Mut) appeared grossly normal and showed no increased mortality through six months of age. Aortic diameter in Lox+/Mut animals was normal, but ascending aortic length measured from the aortic root to the brachiocephalic artery was 10% longer in the Lox+/Mut animals (3.28 mm ± 0.05, n=9) compared to Lox+/+ littermate controls (2.94 mm ± 0.06, n=7) (Figure 2.4A). Compared to Lox+/+ littermate controls, the Lox+/Mut animals did not have significantly different systolic (110.71 mmHg ± 9.96 versus 118.22 mmHg ± 9.46 in Lox+/+ and Lox+/Mut animals, respectively; P=0.15) or diastolic (75.57 mmHg ± 8.30 versus 77.56 mmHg ± 4.19 in Lox+/+ and Lox+/Mut animals, respectively; P=0.87) blood pressure although we cannot exclude the possibility of a small difference (Figure 2.4B). Lox+/Mut animals had normal heart rate and did not develop cardiac hypertrophy (data not shown). Circumferential vessel wall stiffness, extrapolated from pressure/diameter measurements (Figure 2.4C and 2.4D), showed a stiffer carotid and ascending aorta at high pressures compared to Lox+/+ animals, but normal circumferential stiffness at lower pressures. These findings indicate
that the $Lox^{+/Mut}$ animals have altered vessel wall material properties but normal vessel wall mechanics at physiologic pressures.

Ultrastructural analysis of the unloaded $Lox^{+/Mut}$ aorta showed a thicker arterial wall with an appropriate number (7 to 8) of elastic lamellae and smooth muscle cell layers (Figure 2.5A). While there were regions of the wall that were morphologically normal, the majority of areas in the aortic tissue of $Lox^{+/Mut}$ mice had discontinuous elastic lamellae when compared with the normal lamellae seen in $Lox^{+/+}$ mice (Figure 2.5A). The abnormal lamellae observed in the aortic walls of $Lox^{+/Mut}$ mice were similar to $Lox^{+/+}$ mice. Autofluorescence of elastin in aortic tissue (Figure 2.5B) demonstrated these breaks were present at significantly higher density throughout the aorta of $Lox^{+/Mut}$ mice when compared to $Lox^{+/+}$ littermate controls (29.9 breaks/mm versus 11.9 breaks/mm, respectively; $P=0.0006$).

**Mice homozygous for the $Lox$ missense variant die shortly after birth due to ruptured aortic aneurysms**

Mice homozygous for the mutation ($Lox^{Mut/Mut}$) were born alive but did not survive more than a few hours. These animals were similar in size to their $Lox^{+/+}$ and $Lox^{+/Mut}$ littermates (Figure 2.6A), although cranial, thoracic, and abdominal hemorrhages associated with internal bleeding were frequently observed (Figure 2.6B). Some animals had severe kyphosis (Figure 2.6A) and ruptured diaphragms. All $Lox^{Mut/Mut}$ animals had highly tortuous vessels with aneurysms in the ascending aorta and/or aortic arch as well as frequent aneurysms in the descending abdominal aorta near the renal artery branches (Figure 2.6C).

**The $Lox$ missense mutation does not decrease mRNA expression or protein synthesis**
Gene array analysis showed that all lysyl oxidase family members (\textit{Lox}, \textit{Loxl1}, \textit{Loxl2}, \textit{Loxl3}, \textit{Loxl4}) are expressed in the developing aorta with \textit{Lox} having the highest expression level at every developmental time point (Figure 2.7). \textit{Loxl1} is the second most highly expressed member with an expression pattern similar to \textit{Lox} but at levels 2-4 fold less. Both \textit{Lox} and \textit{Loxl1} reach their highest expression levels during the late fetal period followed by relatively constant expression until decreasing rapidly around P30. Expression of \textit{Loxl3} in contrast, is highest postnatally (P0-P21). \textit{Loxl2} and \textit{Loxl4} have low but detectable levels that remain relatively unchanged from the embryonic period through adulthood.

To determine if the \textit{Lox} mutation alters expression of the mutant gene, quantitative real-time PCR was performed on mRNA from aortic tissue of newborn animals (P0). We found that \textit{Lox} and other lysyl oxidase isoforms were expressed in aortic tissue from \textit{Lox}^{+/Mut} and \textit{Lox}^{Mut/Mut} animals at the same levels as littermate controls (Figure 2.8A). In addition, the mutation did not block pro-\textit{Lox} protein synthesis (Figure 2.8A, inset).

**The \textit{Lox} missense mutation decreases enzymatic activity**

Given the evidence that the \textit{Lox} mutation did not decrease gene expression or protein synthesis, we next investigated the effect of the mutation on protein function. To determine the enzymatic activity level of the mutant \textit{Lox} protein, we cultured primary mouse embryonic fibroblasts (MEFs) from \textit{Lox}^{+/+} and \textit{Lox}^{Mut/Mut} animals. \textit{Lox} secreted into the culture medium was collected and enzyme activity was measured by the production of fluorescent resorufin using the Amplex Red assay at 0, 30, 60, 90, 120, and 150 minutes. \textit{Lox} activity in MEFs cultured from \textit{Lox}^{+/+} animals was significantly higher than those from \textit{Lox}^{Mut/Mut} animals beginning at 60
minutes (Figure 2.8B). There was no significant difference in Lox activity between conditioned media from $Lox^{\text{Mut/Mut}}$ MEFs and cell-free media samples.
Materials and Methods

Family recruitment

The family examined in this study was referred to our clinic because of the two-generational history of aortic dissection. Eight members of the family were recruited for the study (Figure 1A, Table 1). Informed consent was obtained from all study participants. Individuals I-2, II-1, II-2, II-3, III-1 and III-2 (Figure 1A, Table 1) were examined in our clinic. Individuals III-3 and III-4 were evaluated at another hospital and their records were subsequently reviewed.

Whole Genome Sequencing

Whole genome sequencing was performed by the Illumina Clinical Services Laboratory (Illumina, Inc). Briefly, genomic DNA was randomly fragmented and then sequenced using 100 base pair paired-end reads on an Illumina HiSeq 2000 Sequencer to an average depth of 30X across the genome. The resulting output was converted to FASTQ format. The paired-end FASTQ files were aligned to the human reference sequence (UCSC HG19 build) using the Burroughs-Wheeler Alignment too (Li et al., 2009) in paired-end mode followed by base quality recalibration and targeted local realignment focused around known short insertions and deletions (indels) using the Genome Analysis Toolkit (GATK) (DePristo et al., 2011). Duplicated reads from sequencing the same DNA fragment were discarded. Single nucleotide substitutions and indels were identified for both samples simultaneously using the Unified Genotyper tool from the GATK in multi sample calling mode. Variant quality score recalibration was performed using the GATK to identify a set of high-confidence variants. The functional consequence of the resulting set of variants was predicted using the Variant Effect Predictor from Ensemble (McLaren et al., 2010).
**Generation of animals harboring the Lox p.Met292Arg mutation**

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein-9 nuclease (Cas9) genome editing technology was used in the Washington University in St. Louis Hope Center Transgenic Vectors Core to generate the *Lox* p.M292R mouse. To design the CRISPR/Cas9 construct, *Lox* sgRNA-9 (5’-cattagacattaccacagca-3’) was cloned into BbsI digested plasmid pX330 (addgene # 42230). sgRNA activity was validated *in vitro* by transfection of NIH3T3 cells using ROCHE (San Francisco, CA) Xtremegen e HP, followed by T7E1 assay (New England Biolabs, Ipswich, MA). T7 sgRNA template was PCR amplified, gel purified, and *in vitro* transcribed with the MEGAscript T7 kit (Life Technologies, Carlsbad, CA). T7 Cas9 template was PCR amplified, gel purified, and *in vitro* transcribed with the T7 mMessage mMachine Ultra kit (Life technologies, Carlsbad, CA). After transcription, RNA was purified with Megaclear kit (Life Technologies, Carlsbad, CA). A 200 nt ssODN donor DNA with the mutation centered within the oligonucleotide was ordered from Integrated DNA Technologies (Coralville, IA) as an ultramer oligo. Founders were identified using Qiagen (Venlo, Netherlands) pyrosequencer and Pyromark Q96 2.5.7 software. B6CBA F1/J female mice (3-4 weeks old) (Jackson Laboratory, Bar Harbor, ME) were super ovulated and mated overnight with B6CBA F1/J male mice (>7 weeks old). Zygotes were harvested from the ampullae of super ovulated females and placed in KSOM medium (Millipore, MR-106D, Billerica, MA) before microinjection. Microinjection was performed in FHM medium (Millipore EmbryoMax, MR-024-D). The Cas9, sgRNA and single strand DNA template were co-injected into the pronucleus of 204 zygotes. The final concentration of the mixture was 50 ng/µl Cas9 WT RNA, 25 ng/µl of each sgRNA, and 20ng/µl of the ssODN DNA. After injection, zygotes were incubated at 5.5% CO₂ at 37°C for 2 h, and surviving embryos were transferred to ICR
recipients via oviduct transfer. Subsequently, we identified 4 founder animals that were mosaic for the c.875T>G (p.Met292Arg) missense variant. Further breeding of the founders gave rise to animals heterozygous \((Lox^{+/Mut})\) for the missense variant, which were bred for all the studies presented in this study. The missense variant was genotyped using Custom TaqMan® SNP Genotyping Assays from ThermoFisher Scientific (St. Louis, MO). The P0 and 3 mo old animals used in our studies were crossed to C57BL6/J (Jackson Laboratory, Bar harbor, ME), one generation. All surgical procedures in this study were performed according to protocols approved by the Animal Studies Committee of the Washington University School of Medicine.

**Blood pressure and ascending aortic length measurements**

\(Lox^{+/+}\) and \(Lox^{+/Mut}\) animals at 3 months were anesthetized with 1.5% inhaled isoflurane and placed on a 37°C-heated pad to maintain body temperature. A Millar pressure transducer (modelSPR-671; Houston, TX) was placed into the right common carotid artery and advanced to the ascending aorta. At the ascending aorta, systolic blood pressure and diastolic blood pressure were recorded via PowerLab data inquisition system (ADInstruments; Colorado Springs, CO). Blood pressure data were analyzed using LabChart7 for MAC software (ADInstruments). After blood pressure measurements, ascending aortic length was measured from the aortic root to the first branch point at the brachiocephalic artery using an electronic caliper.

**Arterial compliance measurements**

After blood pressure measurements, mice were sacrificed using isoflurane. The ascending aorta and left common carotid arteries were excised and placed in physiologic saline solution (PSS; 130 mM NaCl, 4.7 mM KCl, 1.6 mM CaCl₂, 1.18 mM MgSO₄·7H₂O, 1.17 mM KH₂PO₄, 14.8 mM NaHCO₃, 5.5 mM dextrose, 0.026 mM EDTA, pH 7.4) as previously described (Faury et al., 2003). The vessels were prepared by removing excess tissue and placed
on the pressure arteriograph (Danish Myotechnology, Aarhus, Denmark). In 37°C PSS, an inverted microscope was used to visualize the mounted vessels. The microscope is connected to a charged-coupled device camera and a computerized system. This system allowed measurement of the vessel’s outer diameter while gradually increasing the intravascular pressure by 25 mmHg increments from 0 to 125 mmHg.

Internal aortic latex casting

We collected post-natal day 0 (P0) pups from Lox+/Mut x Lox+/Mut breeders in order to visualize the vascular architecture of Lox+/+, Lox+/Mut, and LoxMut/Mut animals. The pups were anesthetized by placing them on ice for 5 minutes before opening of the thoracic cavity. Phosphate Buffered Saline (PBS) was flushed through the left ventricle of the heart followed by injection of 200 µl of yellow latex diluted in deionized water in a 1:1 ratio. The latex was allowed to polymerized in 4°C for 3 hours while moist before fixing the entire animals in 10% buffered formalin overnight in 4°C. The 10% buffered formalin was replaced with 70% ethanol 2 hours before dissection.

Quantitative real-time reverse transcription PCR

Aorta and lung tissue were collected from Lox+/+, Lox+/Mut, and LoxMut/Mut P0 animals and stored in RINalater solution (ThermoFisher Scientific, St. Louis, MO) at -20°C. Total RNA was collected using Trizol (ThermoFisher Scientific, St. Louis, MO) following manufacturer’s protocol. One microgram of RNA was reverse transcribed to cDNA using High-Capacity RNA-to-cDNA kit following manufacturer’s protocol. One microliter of cDNA was used for real-time PCR using Taqman Fast Universal PCR Master Mix and Taqman assays primer/probes (Life Technologies, Carlsbad, CA). 10 uL reactions were performed in duplicates using ViiA 7 real-time PCR system and Lox was normalized to Gapdh.
Light Electron Microscopy

For light electron microscopy, \( Lox^{+/+} \) and \( Lox^{+/Mut} \) animals were sacrificed at 3 months using \( \text{CO}_2 \). The animals were perfused with PBS through the left ventricle of the heart, then the ascending aortas were excised from the aortic root to the brachiocephalic artery. The tissues were fixed in 0.1 M sodium cacodylate/ 2.5% glutaraldehyde solution at 4°C over night. Then the tissues were sent for processing, staining with tannic acid, and thin sectioning by Washington University’s Electron Microscopy Core Facility.

MEF Culturing, Immunoblotting, and Lox Activity Assay

MEFs were collected from each embryo were cultured in a 10-cm dish using DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 1% nonessential amino acids. Five days after reaching visual confluency, the cell layers were extracted using buffered 8 M urea and prepared for immunoblotting as described (Thomassin et al., 2005). Protein concentration in each sample was assessed using Bio-Rad Reagent (Bio-Rad), and 25 µg MEF extract were separated by SDS 10% polyacrylamide gel and transferred to ProBlott Membrane (Applied Biosystems). The blot was incubated in 5% milk overnight at 4 °C to block non-specific binding sites followed by incubation with rabbit PP-Lox antibody targeting the propeptide portion of uncleaved pro-Lox (provided by Phillip Trackman, Boston University, Boston, MA) at 1:4,000 dilution for 1 h at room temperature (Hurtado et al., 2008). After washing, ECL Anti-Rabbit IgG HRP-Linked Secondary Anti-body (GE Healthcare) was added at 1:4,000 for 1 h at room temperature. The blots were then washed, and the immunoreactive bands were detected using Immobilon Western Chemilluminescent HRP Substrate (Millipore). The blot was then stripped of the PP-Lox antibody for immunodetection of β-actin (1:20,000; Sigma-Aldrich).
MEFs were derived as described above. After 5 d in culture, MEFs were passaged into a 15-cm dish and maintained as above until fully confluent. Twenty-four hours before collection, culture medium was replaced with DMEM lacking phenol red (ThermoFisher Scientific) supplemented with 50 µg/mL ascorbic acid and 0.1% BSA. Conditioned medium from each dish was collected and concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore) to a final volume of 1 mL. Lox activity was assessed in 50 µL concentrated culture medium and incubated with and without the Lox inhibitor β-aminopropionitrile (BAPN) using the Amplex Red assay as previously described (Palamakumbura et al., 2002). Resorufin fluorescence, the product of Amplex Red oxidation, was measured at excitation and emission wavelengths (540 and 600 nm, respectively) every 30 min for 150 min using a Biotek H4 Hybrid Reader. Lox activity was calculated as the difference between total activity and activity in the presence of BAPN. Differences in relative fluorescent units were tested using two-way ANOVA.


**Discussion**

Through whole genome sequencing in two affected individuals from a family exhibiting autosomal dominant TAAD we identified a missense mutation in the copper-binding region of *LOX* as the most likely causal variant. To further evaluate the significance and potential impact of this variant, we used genome-engineering techniques to insert the human mutation into the orthologous position in the mouse genome. Mice bred to homozygosity for the human mutation recapitulated the human phenotype, supporting the likely causal role of this mutation in the human disease. This conclusion is further bolstered by the recent identification of additional FTAAD probands harboring *LOX* mutations (Shendure *et al.*, 2008).

Several conclusions emerge from our results. First, we report another successful use of genome-scale sequencing in mapping a causal gene underlying human disease. With the advent of next-generation DNA sequencing technology (Bamshad *et al.*, 2011), the cost and time of genome-scale sequencing have both decreased substantially. In small kindreds such as the one presented in this study – in which the traditional mapping tools of linkage and directed sequencing are unlikely to provide a definitive result – genome-scale (either whole exome or whole genome) sequencing provides a rapid and cost-effective alternative means of identifying the causal variant and gene (MacRae, 2012).

Second, although the historically accepted criteria for considering a novel gene causal in Mendelian disease includes identifying additional kindreds with the same phenotype harboring independent variants in the same gene, this standard is likely to be increasingly difficult to meet in the future. After sequencing 410 unrelated FTAAD probands, Guo et al. (Guo *et al.*, 2016) identified five possibly causal mutations in *LOX*, suggesting this gene is probably responsible for $\leq 1\%$ of FTAAD. As gene-mapping studies continue to identify novel genes underlying FTAAD
and other Mendelian diseases, new gene discoveries will invariably represent increasingly smaller proportions of the inherited basis of disease. Thus, new techniques and criteria for proving causality in Mendelian disease should be considered. Genome-engineering techniques in animal models to study the in vivo effects of human alleles represent one path forward in the absence of identifying additional humans with disease.

Third, the generation of an animal model specific to this genetic defect provides insight into the potential mechanism underlying the disease. Lysyl oxidase is the major extracellular matrix crosslinking enzyme in blood vessels and loss of LOX activity, either through enzyme inhibition or gene inactivation, leads to vascular dilatation and rupture (Hornstra et al., 2003; Maki et al., 2002). Although mutations in LOX had not hitherto been associated with human disease, alterations in LOX expression levels and functional activity have been observed in diverse genetic disorders with some having vascular involvement. For example, reduced LOX activity has been reported in two X-linked recessively inherited disorders, Menkes disease and occipital horn syndrome (Andrews et al., 1975), which are caused by mutations in the ATP7A gene encoding a copper-transporting ATPase. Moreover, mottled blotchy mice, which carry mutations in Atp7a, also have high incidence of aortic aneurysms and exhibit disrupted elastic fibers (Norton et al., 1965). Functional inhibition of LOX by the toxin β-aminoproprionitrile, which is present at high levels in peas and lentils, has been shown to cause osteolathyrism (Horiguchi et al., 2009), a connective tissue disease characterized by skeletal abnormalities and aortic dissections. The vascular phenotype of the mutant mice, including fragmented elastic lamellae in Lox+/Mut animals and tortuous and aneurysmal vessels in animals homozygous for the mutation, resembles vascular changes seen in Lox knockout animals (Hornstra et al., 2003; Maki et al., 2002), implying that the arginine for methionine substitution leads to loss of Lox function.
In fact, we identified similar abnormalities of the aortic wall architecture in both $Lox^{+/Mut}$ and $Lox^{+/+}$ mice, suggesting the p.M298R missense change results in a functionally null enzyme. This was further corroborated by the observation that fibroblasts derived from $Lox^{Mut/Mut}$ animals did not secrete Lox with enzymatic activity that was detectable above baseline. This is in contrast to a recent study, which suggested FTAAD was associated with missense mutations in $LOX$ that only partially decreased protein function (Shendure et al., 2008) although methodological differences may account for this discrepancy. For example, our assay tested the function of Lox that was secreted from cells which were only producing mutant protein while the other study tested the function of LOX present in the cell lysates of cultured human cells (which express normal LOX) that were overexpressing the mutant protein.

There are several mechanisms by which the missense change we studied may result in loss of function. The introduction of an arginine residue within the copper-binding domain may reduce the ability of the enzyme to bind copper leading to loss of catalytic activity. Alternatively it might disrupt normal LOX protein processing or it could produce a dominant negative effect from decreased substrate binding. It is also possible that the mutant LOX protein is unable to interact with fibulin-4 (Atsawasuwan et al., 2008) or components of the TGF-beta signaling pathway (Liu et al., 2004) – both of which are known functions of LOX – thereby leading to aortic disease. It should be noted that even though $Loxl1$ is expressed at appreciably high levels in the aorta, it cannot compensate for loss of Lox activity and mice lacking $Loxl1$ do not have a vascular phenotype (Judge et al., 2004). Future studies will be needed to clarify the exact mechanism by which this mutation leads to loss of Lox function.

Similar to other genetically engineered mouse models of human aortic disease (Libby, 2015; Pereira et al., 1997), we did not observe any aneurysm or other arterial disease in $Lox^{+/Mut}$
mice. There are many reasons why the severity of phenotypes observed in murine models might differ from that seen in humans (Seidman et al., 2002), and for reasons that are not completely understood, mutations causing human disease in an autosomal dominant manner often reproduce disease in mouse models only when present in a homozygous state (Rucker et al., 1998). Despite the lack of overt disease in the heterozygous mouse, however, the increased ascending aortic length and fragmented elastic fibers in $Lox^{+/Mut}$ mice suggest that mutant animals may be predisposed to vascular diseases due to weakened vessel walls under stress conditions. This may explain why some individuals in the family (Individual II-3, for example) do not appear to develop aneurysm or dissection and are only affected with arterial tortuosity (Figure 2.9). Further studies in which heterozygous mice are subjected to hemodynamic stress conditions may provide further insight into this question.

Finally, our genome engineering approach has created a framework in which therapeutic hypotheses relevant to this genetic form of disease may be directly tested. For example, the observation that lysyl oxidase activity is directly correlated with dietary copper and increases even when dietary copper supplementation is above what is needed for normal growth and development (Busnadiego et al., 2015; Opsahl et al., 1982) promotes the hypothesis that copper supplementation in humans with TAAD due to $LOX$ mutations might augment the LOX enzymatic function of the normal allele and prevent vascular disease (although this is complicated by the fact that supplementation would need to be initiated early in embryonic development before a genetic diagnosis could easily be made). Regardless, hypotheses such as this are now directly testable using our mouse model of the human mutation. More generally, beyond individuals with TAAD due to $LOX$ mutations, there is evidence that LOX may also play a role in modifying other forms of TAAD (Choudhary et al., 2009; Jain et al., 2014; Oleggini et
al., 2007) and suggests that therapeutic manipulation of LOX activity may prove beneficial in other inherited aortopathies.

In summary, the discovery of LOX mutations underlying TAAD in this family and others (Shendure et al., 2008) suggests that this gene plays an important role in disease. Sequencing this gene may be useful in identifying the genetic basis for additional FTAAD probands and families. Future mouse model system studies of Lox mutations may provide further mechanistic insights into disease pathogenesis and potential therapeutic options specific to this genetic cause.
Figure 2.1: M298R LOX mutation in humans

(A) The family’s pedigree demonstrates autosomal dominant inheritance of FTAAD. Black symbols indicate affected individuals with arterial dissection or aneurysm. Grey symbols indicate individuals affected with arterial tortuosity. Open symbols indicate unaffected individuals. (-) symbol indicates presence of the mutation. (B) Contrast-enhanced axial CT image from Individual II-1 demonstrating ascending aortic aneurysm (arrow) with dissection and an intimal
flap separating the false and true lumen. (C) Histologic evaluation of aortic tissue resected from Individual II-1. Haematoxylin-eosin (H&E) staining shows abnormal architecture and a dissection tear. Masson's trichrome staining for collagen (blue) reveals disorganization of the collagen fibers and disruption of the medial architecture. Verhoeff-van Gieson staining for elastin (dark purple fibers) illustrates disarray and fragmentation of elastic fibers. (D) The location of the M298R missense mutation is depicted in relation to the domains of the lysyl oxidase protein. Methionine at position 298 is highly conserved as demonstrated by the homologous protein sequences from multiple organisms shown below. Histidine positions that are essential for copper binding and/or lysyl oxidase catalytic activity are highlighted in blue.
<table>
<thead>
<tr>
<th>Individual</th>
<th>Age, y</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>Died at 60</td>
<td>Height 183 cm; emphysema in the setting of tobacco use; pectus excavatum and scoliosis</td>
</tr>
<tr>
<td>I-2</td>
<td>Died at 92</td>
<td>Height 158 cm; normal screening echocardiogram; no history of aortic aneurysm or dissection</td>
</tr>
<tr>
<td>II-1</td>
<td>61</td>
<td>Height 179 cm; ascending aortic aneurysm and dissection at the age of 52 y old</td>
</tr>
<tr>
<td>II-2</td>
<td>76</td>
<td>Height 162 cm; normal echocardiogram; total body magnetic resonance angiography (MRA) shows no evidence of arterial tortuosity (Fig. 54) but reveals an incidental finding of a 2-mm brain aneurysm</td>
</tr>
<tr>
<td>II-3</td>
<td>73</td>
<td>Height 175 cm; normal serial echocardiograms; total body MRA shows arterial tortuosity (Fig. 54); however, this finding is also compatible with aging</td>
</tr>
<tr>
<td>III-1</td>
<td>39</td>
<td>Height 203 cm; 10.5-cm ascending aortic aneurysm with contained rupture diagnosed at the age of 19 y old requiring valve-sparing aortic root replacement; pectus excavatum, scoliosis, positive thumb sign, high arched palate, dental crowding, and skin striae</td>
</tr>
<tr>
<td>III-2</td>
<td>35</td>
<td>Height 173 cm; normal serial echocardiograms</td>
</tr>
<tr>
<td>III-3</td>
<td>46</td>
<td>Aortic arch dissection at the age of 41 y old; hepatic artery aneurysm</td>
</tr>
<tr>
<td>III-4</td>
<td>41</td>
<td>Height 196 cm; infrarenal abdominal aneurysm</td>
</tr>
</tbody>
</table>

Table 1: Clinical characteristics of family members from the study
Table 2: Candidate mutations identified from whole-genome sequencing

<table>
<thead>
<tr>
<th>Chr</th>
<th>Position</th>
<th>Ref/alt</th>
<th>Gene</th>
<th>Amino acid change</th>
<th>Affected without mutation</th>
<th>Unaffected with mutation</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51871629</td>
<td>T/C</td>
<td>EPST5</td>
<td>p.H542R</td>
<td>III-4</td>
<td>I-2, II-2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>215813765</td>
<td>C/T</td>
<td>ABCA12</td>
<td>p.G2321R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>121409850</td>
<td>A/C</td>
<td>LOX</td>
<td>p.M298R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>19156851</td>
<td>C/T</td>
<td>TWIST1</td>
<td>p.G325</td>
<td>III-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>65299132</td>
<td>T/C</td>
<td>SCYL1</td>
<td>p.M365T</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>12</td>
<td>32480750</td>
<td>A/G</td>
<td>BICD1</td>
<td>p.K454R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>9066474</td>
<td>G/A</td>
<td>MUC16</td>
<td>p.S6991F</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Affected indicates individuals from the pedigree with evidence of TAAD. Unaffected indicates individuals from the pedigree without evidence of TAAD. Alt, alternate allele; Chr, chromosome; Ref, reference allele.
Figure 2. 2: M298R LOX sequence chromatogram

Sequence chromatograms of genomic PCR products are shown. The sequence of the normal allele from the unaffected individual II-2 is shown in Upper. The sequence of the mutant c.893T>G (p.Met298Arg) LOX allele from the affected individual II-1 is shown in Lower.
Figure 2. 3: M292R Lox sequence chromatogram

Sequence chromatograms are shown for mice that are (A) $Lox^{+/+}$, (B) $Lox^{+/Mut}$, and (C) $Lox^{Mut/Mut}$. 
Figure 2.4: Lox\textsuperscript{+/M} mice have no overt phenotype

(A) The length of the ascending aorta from the heart to the brachiocephalic artery is significantly longer in 3 month-old Lox\textsuperscript{+/Mut} animals compared to Lox\textsuperscript{+/+} littermate controls. **** indicates P=0.0005. (B) Blood pressure measurements using an arterial catheter found no difference in systolic (P=0.15) or diastolic (P=0.87) blood pressure between Lox\textsuperscript{+/+} and Lox\textsuperscript{+/Mut} mice. n.s.=not significant. Pressure-diameter responses collected for the left common carotid artery (C) and ascending aorta (D) show that both vessels in Lox\textsuperscript{+/Mut} animals are slightly stiffer than controls at high pressure but are not different in the physiological pressure range. * indicates P=0.02; ** indicates P=0.001; *** indicates P=0.0002. Data are from n=9 Lox\textsuperscript{+/Mut} and n=7 Lox\textsuperscript{+/+} animals.
Figure 2.5: Fragmented elastic fibers in $\textit{Lox}^{+/M}$ aortas

(A) Electron microscopy of the ascending aorta from $\textit{Lox}^{+/+}$, $\textit{Lox}^{+/Mut}$, and $\textit{Lox}^{+/−}$ mice. The aortic wall of $\textit{Lox}^{+/+}$ animals demonstrated smooth and continuous elastic lamellae (white arrowhead) while aortas from both $\textit{Lox}^{+/Mut}$, and $\textit{Lox}^{+/−}$ mice were found to be thicker with fragmented (red arrowheads) and disorganized elastic lamellae. Scale bar = 10 µm. * indicates aortic lumen. (B) Autofluorescence of elastin in aortic tissue demonstrated this was not an isolated finding and found that $\textit{Lox}^{+/Mut}$ animals had a significantly higher density of elastic lamellae breaks (red arrowheads) compared to $\textit{Lox}^{+/+}$ mice ($P=0.0006$). Scale bar = 20µm. * indicates aortic lumen.
Figure 2.6: Ruptured aortic aneurysms in P0 $Lox^{M/M}$ mice

(A) $Lox^{Mut/Mut}$ animals were born in the expected Mendelian numbers but died within a few hours of birth. Body size of the mutant animals was comparable to wildtype and heterozygous littermates. Some $Lox^{Mut/Mut}$ animals had severe kyphosis (A, arrowhead), and cranial, thoracic, and abdominal hemorrhages (B, arrows) were common. Arterial architecture visualized by yellow latex injection into the left ventricle showed that $Lox^{Mut/Mut}$ animals have highly tortuous vessels together with ascending and abdominal aortic aneurysms (C, arrows). Vessel tortuosity or aneurysms were not observed in $Lox^{+/+}$ or $Lox^{+/Mut}$ littermates. Blood clots around blood vessels (C, arrowheads) indicated that aneurysmal rupture was a frequent occurrence in $Lox^{Mut/Mut}$ mice. Scale bar = 1 mm.
Figure 2. 7: Lox isoform expression in aortic development

Data from our previous microarray study were used to ascertain Lox family member expression levels in the developing mouse aorta. The data showed that Lox was the Lox gene family member with the highest expression levels at all developmental time points. E, embryonic day; P, postnatal day.
Figure 2.8: Lox expression and activity in M292R Lox mice

(A) qRT-PCR analysis of mRNA from the ascending aorta of $Lox^{+/+}$, $Lox^{+/Mut}$, and $Lox^{Mut/Mut}$ P0 animals showed normal expression levels of all Lox family members in all three genotypes (note that the $Lox$ primer/probe recognizes both $Lox^{+}$ and $Lox^{Mut}$ alleles). Two-way ANOVA $P=0.63$ for $Lox^{+/+}$ vs. $Lox^{+/Mut}$; $P=0.99$ for $Lox^{+/+}$ vs. $Lox^{Mut/Mut}$; and $P=0.63$ for $Lox^{+/Mut}$ vs. $Lox^{Mut/Mut}$. Lox protein immunoblotting confirmed that the mutant Lox protein is produced by $Lox^{Mut/Mut}$ cells at levels similar to the wild-type ($Lox^{+/+}$) protein (inset). The absence of a band in extracts of $Lox^{-/-}$ cells confirms the specificity of the Lox antibody.  

(B) Lox activity in the presence and
absence of β-aminopropionitrile (BAPN) was assayed in conditioned culture medium from 
$Lox^{+/+}$ and $Lox^{Mut/Mut}$ MEFs at 30 minute time intervals. Medium incubated without cells served 
as the baseline control. MEFs cultured from two $Lox^{+/+}$ and three $Lox^{Mut/Mut}$ embryos were tested 
in duplicates for n=4 and n=6, respectively and plotted according to genotype at each time point 
(mean +/- SEM). Lox activity, which is the difference between activity with and without BAPN, 
is expressed in relative fluorescent units (RFUs). **** indicates P<.0001.
Figure 2. 9: Arterial tortuosity in LOX^{+/M} individuals

(A) Individual II-3, who inherited the LOX p.M298R mutation, was found to have severely tortuous aortic branch and vessels, which included the common carotid artery (arrow). (B) Individual II-2, who did not inherit the LOX p.M298R mutation, was found to have normal carotid arteries without tortuosity.
References


Chapter 3:

Intracellular retention of mutant Lox leads to aortic dilations with a “second hit”
Introduction

There are 10.4 cases of thoracic aortic aneurysm and dissection (TAAD) per 100,000 people each year (Clouse et al., 1998). In contrast to abdominal aortic aneurysm (AAAs), TAAD has a strong genetic component (Lindsay et al., 2011; Rea et al., 2013). To date, there are mutations in 29 genes identified to be associated with TAAD. Identification of more mutations is worth pursuing, as these mutations still account for only 25% of familial TAAD cases. However, it is even more important to follow up on these known mutations to determine the mechanism of disease pathogenesis caused by the proteins encoded by these mutant genes.

Familial TAAD is classified into syndromic and non-syndromic categories. Marfan syndrome (MFS) is an example of syndromic TAAD, where a mutation in FBN1 causes detrimental changes in musculoskeletal and ocular systems in addition to having cardiovascular manifestations (Hayward et al., 1992). A mutation in the gene for myosin heavy chain (MYH11) is an example of non-syndromic TAAD, where individuals with this mutation are only affected by TAAD (Zhu et al., 2006). Regardless, all mutation associated with TAAD fall into one of three categories of gene function: ECM component, TGFβ pathway members, and smooth muscle cell contractility (Elefteriades et al., 2010; Goldfinger et al., 2014; Pyeritz, 2014).

In all cases of TAAD, a common histopathological feature is fragmented elastic lamellae in the aortic medial region (Saruk et al., 1977). Elastin is the most abundant ECM protein in the aorta, making up nearly 50% of its dry weight (Sauvage et al., 1999). It plays a critical role in the artery’s ability to expand and recoil during pulsatile blood flow and in maintaining arterial wall integrity. Elastin is synthesized as tropoelastin monomers, secreted by vSMCs and then assembled onto microfibril scaffolds between concentric layers of vSMCs. Subsequently, tropoelastin requires intra-and inter-molecular crosslinking, catalyzed by the enzyme Lox to form mature elastic fibers. In the absence of Lox in mice (Hornstra et al., 2003; Maki et al.,
2002), animals die within few hours of birth due to ruptured aortic aneurysms, emphasizing the critical role of functional elastin in aorta integrity and function.

Previously, we identified a *LOX* mutation in a family with a history of TAAD (Lee *et al.*, 2016). By introducing this human mutation into the mouse genome using CRISPR/Cas9 genome editing technology (c.857T>G encoding p.M292R), we confirmed that this specific mutation causes ruptured TAAD in mice in the homozygous state. However, in humans, one mutant allele of *LOX* is sufficient to cause TAAD. In this study, we elucidate the mechanism whereby the Lox mutation leads to protein LOF and show that a single mutant allele is sufficient to predispose animals to aneurysm formation when exposed to a “second hit.”
**Results**

**Active Lox is absent from Lox\(^{MM}\) MEF conditioned medium**

To further elucidate the mechanism of M292R LOF mutation, we measured LOX activity in both the cell layer and in conditioned medium from MEFS cultured from WT and mutant mice. Lox activity was absent in the cell layer of all MEF genotypes (\(Lox^{+/+}\), \(Lox^{+/M}\), \(Lox^{MM}\), and \(Lox^{-/-}\)) tested over 150 minutes (Figure 3.1A), which is consistent with Lox activation occurring extracellularly after secretion. In agreement with our previous studies, in the conditioned medium of \(Lox^{+/+}\) MEFs, there were discernable levels of Lox activity that increased linearly over the time of the assay. \(Lox^{+/M}\) and \(Lox^{+/+}\) MEF conditioned media had similar levels of activity whereas conditioned medium from \(Lox^{MM}\) MEFs had no detectable activity, similar to what was found for conditioned medium from \(Lox^{-/-}\) MEFs (Figure 3.1B).

**M292R LOX accumulates intracellularly**

Lox activity is dependent on proper delivery of the enzyme through the secretory pathway from the rough endoplasmic reticulum (ER) to the extracellular space (Rucker *et al.*, 1998). Once secreted, Lox is catalytically activated when the pro-peptide is cleaved to release the active domain of Lox. Because Lox requires proteolytic processing by BMP-1 from pro-Lox (50 kDa) to active Lox (30 kDa), only the full-length Pro-Lox was detected in the cell layer, whereas in the conditioned medium, only cleaved active Lox was detected. To determine whether the lack of Lox activity in M292R MEF conditioned medium is because the enzyme is catalytically inactive or the mutant protein is not being secreted into the conditioned medium, western blots with an anti-Lox antibody were performed on \(Lox^{+/+}\), \(Lox^{+/M}\), and \(Lox^{MM}\) MEF cell layer and conditioned media. Immunoblotting showed marked increased level of M292R Lox protein in cell extracts compared to WT Lox protein (Figure 3.2A). Conversely, in the conditioned medium, there was minimal M292R Lox, whereas WT Lox was abundant (Figure 3.2A). These
findings indicate that the reason Lox activity was not detected in the conditioned medium of $Lox^{M/M}$ MEFs is that the mutant protein is poorly secreted and retained intracellularly.

To determine if the mutant pro-Lox is capable of BMP-1-mediated cleavage, we treated $Lox^{+/+}$, $Lox^{+/M}$ and $Lox^{M/M}$ MEF lysates with recombinant BMP-1 (rBMP-1) and compared the amount of full-length pro-Lox to active Lox protein by western blot analysis. In all genotypes tested, pro-Lox was cleaved to the 30 kDa active form, in a rBMP-1 dose-dependent manner. These results indicate that the M292R mutation does not alter the BMP-1 proteolytic site and the ability of pro-Lox to be activated outside the cell (Figure 3.3).

**M292R LOX is retained in the ER, but does not elicit an ER stress response**

To determine where in the secretory pathway the M292R Lox protein is accumulating, we performed immunofluorescence imaging using an anti-Lox antibody. First, antibody staining for Lox in $Lox^{M/M}$ MEFs showed results consistent with the immunoblotting studies in that M292R LOX accumulates within the cells. Immunostaining of $Lox^{+/+}$ cells in contrast, showed minimal intracellular staining, confirming that wild-type LOX is properly secreted (Figure 3.2B). To determine in which compartment mutant LOX is accumulating within the cell, $Lox^{+/+}$, $Lox^{+/M}$, and $Lox^{M/M}$ MEFs were co-stained for LOX and calnexin, an ER resident protein. These results showed that M292R LOX co-localized with calnexin in the ER (Figure 3.4).

Accumulation of misfolded proteins in the ER leads to ER stress, which subsequently activates the unfolded protein response (UPR) (Hetz, 2012). UPR triggers three major stress sensors, PERK, IRE1, and ATF6 (Hampton, 2000). BIP is a molecular chaperone for unfolded proteins that can serve as ligands to activate all three of these stress sensors (Kozutsumi et al., 1989). Therefore, we assessed ER stress by measuring Bip transcript levels by qPCR. While there was a statistically significant increase in Bip mRNA in $Lox^{M/M}$ MEFs (Figure 3.5C)
compared to WT MEFs, the increase was modest compared to what was seen when ER stress was stimulated by treatment of MEFs with Lopinavir/Ritonavir (data not shown), which served as a positive control. We also measured mRNA levels of Atf4 and Chop, downstream transcriptional activators of the PERK pathway, and these transcript levels were also not elevated in \( Lox^{M/M} \) MEFs compared to \( Lox^{+/+} \) MEFs (Figure 3.5A,B). Together, these results suggest that ER stress is not elevated in the mutant cells.

The lack of ER stress response measured by downstream effectors was surprising given the significant intracellular accumulation of M292R LOX observed by both immunoblotting and immunofluorescence analysis. To determine if mutant LOX is being removed from the ER for degradation through the lysosomal pathway, cells were stained for LOX and the lysosomal marker, LAMP-2. No co-localization of the two proteins was observed, suggesting that the mutant protein was not trafficked through lysosomes (Figure 3.6).

**Calnexin directly interacts with M292R LOX**

Folding intermediates of most secreted proteins interact with the calnexin/calreticulin protein complex in the ER in an attempt to achieve the proper native conformation required for secretion (Zhang _et al._, 1997). The calnexin/calreticulin protein complex recognizes N-linked glycan structures (Rodan _et al._, 1996), which undergo cycles of sugar residue removal (trimming) and addition (processing), that act as quality control markers to determine the protein fate of either secretion or retrotranslocation into cytosol for ER-mediated degradation (ERAD) by proteasomes (Hiller _et al._, 1996). Lox has three N-linked glycosylation sites in the pro-peptide region (Trackman _et al._, 1992), suggesting that Lox folding intermediates interact with the calnexin/calreticulin complex. To determine if LOX directly interacts with calnexin, we performed co-immunoprecipitation studies using \( Lox^{+/+}, Lox^{+/M}, Lox^{M/M} \), and \( Lox^{-/-} \) MEF lysates.
The lysates were immunoprecipitated with an anti-calnexin antibody then immunoblotted using anti-Lox antibody. LOX was detected in cell lysates of all genotypes, suggesting in vivo interaction between LOX and calnexin. Interestingly, there were higher levels of LOX detected in $Lox^{M/M}$ MEF lysates immunoprecipitated with the anti-calnexin antibody, than in $Lox^{+/+}$ MEF lysates. This was consistent with M292R LOX remaining in an unfolded state and repeatedly interacting with calnexin for refolding (Figure 3.7A).

**Fibulin-4 interacts with LOX but is not a functional chaperone**

To test the possibility of a chaperone protein interaction necessary for proper LOX secretion, we tested several candidate proteins. Previous in vitro studies demonstrated that the pro-peptide domain of LOX directly interacts with the N-terminus of another extracellular protein, FBLN4 (Horiguchi et al., 2009). Interestingly, $Lox$-null and $Fbln4$-null animals have overlapping phenotypes, including TAAD formation at birth (Hornstra et al., 2003; Maki et al., 2002; McLaughlin et al., 2006). Expression of Fbln4 mRNA and protein in $Lox^{M/M}$ cells and aortic tissues, respectively, are not altered (data not shown). But to determine if these proteins interact in the secretory pathway and if this interaction is interrupted by the M292R mutation, we performed co-immunoprecipitation studies. $Lox^{+/+}$, $Lox^{+/M}$, $Lox^{M/M}$, and $Lox^{-/-}$ MEF lysates were precipitated with anti-Fbln4 antibody then immunoblotted using anti-Lox antibody. There was no difference in the amount of Lox protein associated with FBLN4 (Figure 3.7B). These results suggest that there is some interaction between LOX and FBLN4 inside the cells; however, this interaction is not perturbed by the M292R mutation and not necessary for proper secretion of LOX.

**WT Lox does not transport mutant Lox through the secretory pathway**
To determine if WT LOX is functioning as a secretory chaperone for M292R LOX and this serves as an explanation for why \( Lox^{+/M} \) animals do not have an overt disease phenotype, we transiently expressed a plasmid containing WT \( Lox \) or M292R \( Lox \) fused to the fluorescent protein, mApple, in \( Lox^{-/-} \) MEFs. Successful transfection was observed by mApple fluorescent protein expression using fluorescence imaging (data not shown). In the conditioned medium of untransfected \( Lox^{-/-} \) cells, which served as the negative control, no LOX was detected. In \( Lox^{-/-} \) MEFs transfected with 2.5 \( \mu g \) of WT \( Lox \)-mApple plasmid, a band at ~60 kDa was detected in the conditioned media, corresponding to the size of active LOX (30 kDa) fused to mApple (27 kDa). In \( Lox^{-/-} \) MEFs transfected with 2.5 \( \mu g \) of M292R \( Lox \)-mApple plasmid, no LOX was detected in conditioned medium, consistent with the inability of endogenous M292R LOX to be secreted. When \( Lox^{-/-} \) MEFs were transfected with 1.25 \( \mu g \) of WT \( Lox \)-mApple and 1.25 \( \mu g \) of M292R \( Lox \)-mApple, ~50% of LOX was detected compared to cells transfected with 2.5 \( \mu g \) of WT LOX-mApple construct alone. Together, these results suggest that only WT LOX was being secreted (Figure 3.7C). Therefore, WT LOX does not act as a transport protein for M292R LOX secretion.

**M292R mutation in \( Lox \) leads to an altered confirmation and changes in protease susceptibility**

The thymine to guanine change at the 893 position in human \( LOX \) leads to an amino acid change from a neutral methionine to a positively charged arginine. To explore whether the addition of the large, positively charged arginine side chain influences overall protein structure, we assessed protein mobility on native, non-reducing gels. When run in the absence of SDS and without disulfide bond reduction, LOX in cell lysates from \( Lox^{M/M} \) MEFs ran at a slightly higher
molecular weight than the WT protein (Figure 3.8A). Altered mobility of the mutant protein suggests it has a different overall shape than its WT counterpart.

We also explored possible structural changes by assessing differential sensitivity to proteolytic degradation. Changes in protein structure frequently expose or mask protease binding sites, leading to altered degradation products. Immunoblotting of cell lysate from $Lox^{+/+}$, $Lox^{+/M}$, and $Lox^{M/M}$ MEF cells incubated at 37°C for 6 hours using anti-Lox antibody revealed WT LOX degradation over time, while M292R LOX remained resistant to this degradation (Figure 3.8B). These results suggest that WT and M292R LOX have different susceptibility to degradation arising from endogenous proteases present in the whole cell lysate.

Degradation by purified proteases also suggested structural differences in WT and mutant protein. Treatment of cell lysates with trypsin resulted in extensive degradation of both proteins and was not informative (data not shown). However, porcine pancreatic elastase had a more restricted pattern of degradation and showed different degradation fragments among genotypes (it is important to note that porcine pancreatic elastase does not cleave after methionine or arginine, so the Met-to-Arg mutation does not create a new protease cleavage site). In $Lox^{+/+}$ cell lysate, there was a 25 kDa fragment evident with all doses of elastase, which was absent in $Lox^{+/M}$ and $Lox^{M/M}$ samples. When treated with the highest dose of elastase, pro-LOX from $Lox^{+/+}$ and $Lox^{+/M}$ samples degraded predominantly to a 30 kDa fragment. LOX from $Lox^{M/M}$ samples, in contrast, degraded further into mainly a 10 kDa fragment (Figure 3.8C). These studies suggest that M292R LOX has a different conformation than WT LOX, which exposes additional cleavage sites for elastase.
Lox^{+/M} adult animals are predisposed to aortic dilation following AngII administration

To test whether increased hemodynamic stress could induce aneurysm formation, angiotensin II (AngII) was subcutaneously delivered via Alzet osmotic pumps to increase blood pressure. Pumps containing saline served as the control. After 4 weeks of treatment, systolic and diastolic blood pressures were measured as described (Halabi et al., 2015) and the overall structure and compliance of the large vessels were determined using histology and pressure myography (Faury et al., 2003). Surprisingly, neither Lox^{+/+} nor Lox^{+/M} animals had increased blood pressure at the four-week time point. The same was true for animals treated with saline (Figure 3.9A). However, all AngII-treated animals had cardiac hypertrophy, indicating altered cardiac physiology as a result of AngII exposure (Figure 3.9B). Importantly, the ascending aorta of Lox^{+/M} mice exposed to AngII showed significant dilation compared to Lox^{+/+} mice exposed to AngII and to Lox^{+/M} mice exposed to saline alone (Figure 3.9C,D). This change translated to a 15-20% increase in wall diameter at both systolic and diastolic pressures. No dilation was evident in Lox^{+/+} animals treated with AngII or in any of the saline-treated animals. Interestingly, no dilation or change in vessel mechanics were observed in the left common carotid artery for either Lox^{+/+} or Lox^{+/M} animals with saline or AngII treatment (Figure 3.9E), nor were dilations observed in the aortic arch, descending aorta or abdominal aorta. This suggests that the ascending aorta is uniquely sensitive to injurious stimuli in Lox^{+/M} mice.

AngII treatment leads to increased cellularity and infiltration of immune cells in Lox^{+/M} aorta medial layer

We have previously reported that elastic lamellae are highly fragmented in the Lox^{+/M} aorta compared to Lox^{+/+} aorta. AngII treatment in both Lox^{+/+} and Lox^{+/M} animals did not alter elastic lamellar number or organization compared to saline-treated controls. There was,
however, an increase in the number of cells located between the elastic lamellae in AngII-treated $Lox^{+/M}$ animals, which is unusual since this area is normally occupied by one layer of vSMCs.

Another feature of these cells is that their nuclei were rounder compared to cells in $Lox^{+/+}$ vessels, suggesting a less polarized phenotype. There was also an increase in cell number in the adventia of both $Lox^{+/+}$ and $Lox^{+/M}$ aortas detected by DAPI stain.

A comparable number of cells in the adventitia were CD68+, indicating recruitment of monocytes in response to AngII administration (Figure 3.10). CD68+ cell infiltration into the medial layer was also observed in both $Lox^{+/+}$ and $Lox^{+/M}$ aortas after AngII treatment, but their localization within the wall was different. In AngII-treated $Lox^{+/+}$ vessels, CD68+ cells were located in the intima and within the first two elastic layers closest to the intima. CD68+ cells in vessels from $Lox^{+/M}$ animals treated with AngII, however, were in the outer layers of the media closest to the adventitia (Figure 3.10). These results suggest that the route of inflammatory cell infiltration, via lumen vs. adventitia, may be different between $Lox^{+/+}$ and $Lox^{+/M}$ aortas, respectively.
Materials and Methods

Generation of M292R *Lox* mice

As previously reported (Lee *et al.*, 2016), CRISPR/Cas9 genome editing technology was used in collaboration with Washington University School of Medicine Hope Center Transgenic Vectors Core to introduce the *LOX* mutation found in humans into the corresponding location in the mouse genome (c.857T>G encoding p.M292R). All animal studies were carried out following protocols approved by Animal Studies Committee of Washington University School of Medicine.

Primary mouse embryonic fibroblast culture

Primary MEFs were isolated following an established protocol (Jozefczuk *et al.*, 2012). In short, the uterine horn from a pregnant female, from a *Lox*+/M x *Lox*+/M cross, was dissected from staged E14.5. Individual embryo was decapitated and eviscerated before tissue homogenization in Dulbecco’s Modified Eagle Medium (DMEM). Homogenized tissue was digested in trypsin/EDTA at 37°C for 30 min, then supernatant was collected, and centrifuged. The remaining cell pellet was resuspended in complete medium (DMEM, 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin, and 1 mM L-glutamine). Cell pellet from each embryo was plated onto 10 cm tissue culture dish for the following experiments.

Lox activity assay

Lox activity assay was performed on *Lox*+/+, *Lox*+/M, *Lox*M/M, and *Lox*-/- MEF lysate and concentrated conditioned medium following a protocol, as previously described (Lee *et al.*, 2016; Palamakumbura *et al.*, 2002).

Immunofluorescence Imaging

Primary MEFs were seeded in 4-chamber glass slides at 3x10⁵ cells per/well. After 3 days in culture, cells were fixed with cold methanol for 5 seconds twice. Cells were then washed with
blocking solution 3 times before blocking the cells in the same solution for 30 min at room
temperature. After blocking, the cells were incubated with a primary antibody (1:200 rabbit anti-
Lox (#ab174316, Abcam), 1:200 mouse anti-Calnexin (#ab22595, Abcam), 1:500 rat anti-CD68
(#MCA1957, BioRad) or 1:500 rat anti-LAMP2 (#abl-93, DSHB)) overnight at 4°C. The next
day, cells were washed with blocking solution 3 times then incubated with a secondary antibody
(1:2000 goat anti-rabbit Fluor 488/Fluor 568 or 1:2000 goat anti-rat Fluor 488; Invitrogen, and
1:10,000 DAPI) for 30 min at room temperature. Cells were washed with blocking buffer 3 times
then mounted using ProLong Diamond Antifade Mountant (Invitrogen) and cover slipped.
Samples were imaged using a Zeiss Axioskop Fluorescence Microscope and QCapture Pro
software (Media Cybernetics, Inc.).

**Protein extraction from cell culture and aortic tissue**

For protein extraction from MEF cultures, after growing cells to full confluency in 10 cm
TC dishes, cells were incubated in 1 mL of cell lysis buffer containing protease inhibitors for 30
min at 4°C. The cells were then collected in 1 mL eppendorf tube then centrifuged at 12,500 rpm
for 5 min. Supernatant was collected and protein concentration was measured using
Bicinochoninic (BCA) assay (Thermofisher). Lamelli buffer with or without dithiol-threitol
(DTT) was added to samples for SDS-polyacrylamide or native gel electrophoresis, respectively,
and boiled at 100°C for 5 min.

For protein extraction from mouse aortic tissue, the aorta from the root to diaphragm,
skin from lower back, and whole lungs were collected and flash frozen before homogenization.
Tissues were homogenized using Tissue Lyser II (Qiagen) at 30 Hz for 5 min in 8 M urea/16
mM Na₂HPO₄ solution containing protease inhibitors. Samples were incubated overnight at 4°C
then diluted to 2 M urea using 16 mM Na₂HPO₄ solution. Protein concentration was then
measured using the BCA assay. Subsequently, the protein was precipitated using 10% trichloroacetic acid at 4°C for 1 hr, followed by 3 washes using ice-cold acetone. Protein pellet was then resuspended in Lamelli buffer containing DTT and boiled at 100°C for 5 min for SDS-PAGE.

**RNA extraction and quantitative real-time reverse transcription PCR**

MEFs from Lox+/+, Lox+/M, and Lox−/− animals were cultured until fully confluent, as described above, and RNA was extracted using TRIzol (Thermofisher Scientific) following manufacturer’s protocol. One microgram of RNA from cells was reverse transcribed to cDNA using High-Capacity RNA-to-cDNA Kit. Subsequently, one microliter cDNA was used for real-time PCR using Taqman Fast Universal PCR Master Mix and Taqman assay primer/probes (Life Technologies) for Bip (Mm00517691_m1), Atf4 (Mm00515325_g1), Chop (Mm01135937_g1), and Gapdh (Mm9999999915_g1). 10-µL reactions were performed in duplicate using ViiA 7 Real-Time PCR System. Bip, Atf4 and Chop mRNA levels were normalized to Gapdh.

**rBMP-1 Treatment**

Protein was extracted from Lox+/+, Lox+/M, and Lox−/− MEFs as described above. 50 µg of cell lysate and 30, 60, and 90 ng of rBMP-1 (NP_001190, R&D Systems) in reaction buffer (50 mM Tris, 150 mM NaCl, 5 mM CaCl2, pH 7.5) were incubated for 45 min at 37°C. Enzyme reaction was stopped by adding Lamelli buffer with DTT and boiled at 100°C for 3 min before immunoblotting.

**Western blotting**

50 µg of protein extract from cells or tissue were loaded in 10% Mini-PROTEAN TGX Stain-Free Protein Gel (Bio-RAD) and separated at 100 mV for one hour. The gel was then transferred to ProBlott Membrane (Applied Biosystems). The blot was incubated in 5% milk in
0.1% PBS-Tween for 1 h at room temperature, then incubated with a primary antibody (1:4,000 rabbit polyclonal Lox (#ab174316, Abcam); 1:10,000 rabbit polyclonal calnexin (#ab22595, Abcam), or 1:4000 rabbit poly clonal (Halabi et al., 2017) overnight at 4°C. The next day, the blots were washed with 0.1% PBS-Tween and incubated in anti-rabbit or anti-mouse ECL Anti-Rabbit IgG HRP-Linked Secondary Anti-body (GE Healthcare) for 1 h at room temperature. The blots were then washed and the immunoreactive bands were detected using Immobilon Western Cehimluminescent HRP Substrate (Millipore). The blots were then stripped for immunodetection of β-actin, for loading control (1:20,000; Sigma-Aldrich).

**Co-immunoprecipitation**

Lox+/+, Lox+/M, LoxM/M, and Lox⁻/⁻ MEFs were cultured for 5 days post-visual confluency then collected using cell lysis buffer containing protease inhibitor cocktail. Protein concentration was measured using the BCA assay (Thermofisher) and samples were diluted to 1 g/L. 50 uL of immobilized Protein A Anti-Rabbit IgG beads were pre-equilibrated by washing with lysis buffer then added to 250 uL of cell extract. After incubating on a rocking platform for 60 min at 4°C, the supernatant was collected and incubated with 8 ug of immunoprecipitation antibody for 1 h at 4°C. Then, 50 uL of precleared Protein A beads were added to the cells with immunoprecipitation antibody and incubated overnight at 4°C while rocking. Next day, immune complexes were collected by centrifugation and subsequently washed with the lysis buffer. Then 50 uL of 1x Lamelli buffer with DTT was added to the beads and boiled at 100°C for 10 min. Supernatant was then collected and separated by SDS-PAGE following the same immunoblotting protocol above.
Protein degradation analysis

$Lox^{+/+}$, $Lox^{+/M}$, and $Lox^{M/M}$ MEFs were cultured 5 days post-visual confluency. 4 h before collection with lysis buffer, Brefeldin A (B56542, Sigma) was added to the conditioned medium to inhibit ER to Golgi transport. This step ensures that WT LOX remains in the cells so that the starting amount of protein is the same across genotypes. For endogenous protease mediated degradation, cell extracts were incubated at 37°C and 50 uL samples were collected every hour. Lamelli buffer was added to these samples and boiled at 100°C for 5 min for SDS-PAGE analysis. For exogenous protease mediated degradation, porcine pancreas elastase in increasing concentrations (0.625, 1.25, 2.5, 5 ng/uL) was added to cell lysates at 1 mg/mL protein at 37°C for 30 min. After treatment, elastase activity was inhibited with protease inhibitor cocktail (P9599, Sigma) and Lamelli buffer. Samples were then boiled at 100°C for 5 min for SDS-PAGE analysis.

Angiotensin II treatment in mice

Angiotensin II was diluted in 0.9% saline solution for final concentration of 2 ug/kg/min. The micro-osmotic pumps (Alzet model 1004) were filled with the diluted AngII solution following manufacturer’s protocol. The osmotic pumps were subcutaneously implanted for 28 days in the interscapular region. Body weight was measured before and after AngII treatment.

Blood pressure and compliance measurement

After 28 days of AngII treatment, animals were anesthetized using 1.5 % inhaled isofluorane and placed on a 37°C heated pad to maintain body temperature. A milar pressure transducer was inserted into the right common carotid artery and advanced to the ascending aorta, where the blood pressure was recorded and analyzed as previously described (Faury et al., 2003). After the blood pressure measurements, the ascending aorta and left common carotid
artery were excised, and pressure-diameter curves were produced using a pressure arteriograph, which gradually increases the intravascular pressure by 25 mmHg increments from 0 to 175 mmHg while measuring the vessel’s outer diameter.
Discussion

Studies with cultured cells from $Lox^{MM}$ mice showed that mutant LOX is not secreted into the extracellular space. We demonstrated by immunofluorescence that M292R LOX accumulates in the ER and further confirmed the direct interaction between LOX and calnexin using co-immunoprecipitation studies. Secreted proteins pass through the calnexin/calreticulin checkpoint in the ER to ensure proper folding before secretion. If the calnexin-bound protein folds properly to its native state, it disassociates from calnexin and exits the ER for the Golgi. Proteins that do not fold properly associate with calnexin repeatedly and are not transported to the Golgi. M292R Lox appears to be one of these proteins. Immunolocalization studies showed a large proportion of M292R Lox co-localizing in the ER with calnexin whereas WT protein was efficiently secreted from cells. Co-immunoprecipitation studies established a direct interaction between calnexin and M292R LOX, confirming ER retention of the mutant variant. Neither decreased temperature nor chemical chaperones stimulated movement of M292R from the ER, and no appreciable degradation of the mutant protein was observed by immunoblot (data not shown).

The high levels of mutant Lox that accumulated within the ER suggested activation of the ER stress pathways, but expression levels of the ER stress-related genes $Atf4$ and $Chop$ were similar for both mutant and WT cells. Only $Bip$ expression in the mutant cells showed a statistically significant increase compared to control cells, but the overall change was minor (~20%), which makes its biological relevance questionable. There were also no morphological changes in ER volume, ER number, or cell proliferation rate that would indicate that mutant cells are experiencing ER stress (data not shown). The UPR is activated when misfolded proteins accumulate above a critical threshold (Hetz, 2012). The fact that no UPR is observed in Lox
mutant cells suggests that M292R Lox protein is removed from the ER before the UPR-activating threshold is attained. We explored whether removal was occurring via the lysosome pathway but did not find co-localization of mutant Lox with cytoplasmic lysosomes identified as Lamp-2-positive vesicles. While other clearance pathways are possible, it is likely that mutant Lox is being cleared by ER-associated degradation (ERAD), a ubiquitin-proteasome system that prevents the secretion and aggregation of misfolded proteins. Following ubiquitination, ERAD substrates are retrotranslocated across the ER membrane into the cytosol and degraded by 26S proteosomes (Berner et al., 2018).

The M292R mutation is located within the region of the catalytic domain that contains key histidine residues required for copper binding. The mutated methionine is five amino acids removed from the histidine that acts as the catalytic base for enzyme activity (Oldfield et al., 2017). Replacing the hydrophobic thioether side chain of methionine with the larger guanidino-containing side chain of arginine has implications for protein structure associated with both the size and charge of the side chain substitution. Our results show that M292R Lox is more sensitive to degradation by proteases than is the WT protein, implying a conformational alteration induced by the arginine substitution. This structural difference may explain why the ER folding machinery recognizes M292R Lox as misfolded and prevents its secretion. It should be noted, however, that cleavage of the mutant Lox pro-peptide by rBMP1 occurs normally, indicating that the structural changes associated with the M292R mutation largely affect the C-terminal catalytic domain.

An important question is whether the M292R mutant is capable of binding copper and becoming catalytically active if it could translocate from ER to Golgi, where copper loading occurs. Previous studies have shown that when a negatively charged residue is added to the
active site of LOX, the enzyme can no longer incorporate copper or form the LTQ cofactor (Oldfield et al., 2017). The same may be true for adding arginine to the active site. Inserting a large positively charged side chain within the negatively charged catalytic site could potentially disrupt enzyme activity by forming inappropriate salt bridges with neighboring acidic residues. The positively charge guanidino group of arginine could also inhibit lysine side chain binding through steric hindrance or charge repulsion.

The critical role played by Lox in vascular development and vessel function has been shown by numerous studies where animals do not survive if Lox is absent or its catalytic activity is inhibited (Hornstra et al., 2003; Maki et al., 2002; Tang et al., 1983). Mice homozygous for the M292R mutation die shortly after birth from vascular complications that include tortuous vessels and ruptured aneurysms. Humans heterozygous for M298R and other loss-of-function LOX mutations develop aneurysmal disease, usually as adults, although the penetrance of vessel disease in individuals with Lox mutations is unknown (Guo et al., 2016). Unlike the condition in humans, aneurysms were not found in mice with only one mutant M292R allele even when the mice were aged for 18 months. Lox⁺/M animals did, however, have alterations in vessel elastic fiber integrity that suggested that these mice might be more susceptible to vascular damage in response to an injurious stimulus.

This was indeed the case. Challenging these mice with AngII infusion resulted in cardiac hypertrophy in both genotypes and aortic dilation in Lox⁺/M but not in Lox⁺/+ animals. AngII induces hypertension by vasoconstriction and while cardiac hypertrophy and aneurysms formation are consistent with the treated animals experiencing increased hemodynamic stress in response to AngII infusion, blood pressure was essentially normal in Lox⁺/+ and Lox⁺/M animals at the four-week time point when blood pressure was measured. The absence of hypertension
after prolonged AngII infusion using Alzet osmotic pumps is not unprecedented, however. Other studies have demonstrated that AngII infusion leads to hypertension in the first week of treatment with blood pressure declining after 14 days and returning to baseline after 28 days (Daugherty et al., 1999; Kuroki et al., 2014). The cardiac hypertrophy and vascular changes that occurred in response to AngII treatment strongly suggest that both WT and $Lox^{+/M}$ mice experienced hypertension earlier in the study. Whatever the cause of the AngII-induced cardiovascular changes, the important point is that the aorta in $Lox^{+/M}$ animals is more susceptible than the WT vessel to damage when stressed. In terms of the human disease, these results suggest that individuals heterozygous for the M298R mutation and perhaps other Lox LOF mutations require a “second hit” for disease progression.
Figure 3.1: Lox activity in M292R Lox cell culture

Lox activity in the presence and absence of β-aminopropionitrile (BAPN) was assayed in (A) cell layer and (B) conditioned culture medium from $Lox^+/+$, $Lox^{+/M}$, $Lox^{Mut/Mut}$, and $Lox^{-/-}$ MEFs at 30 minute time intervals. Media incubated without cells served as the baseline control. Lox activity, which is the difference between activity with and without BAPN, is expressed in relative fluorescent units (RFUs). **** indicates $P<.0001$. 
Figure 3. 2: Intracellular retention of M292R Lox

Immunoblotting of cell lysate and conditioned medium from $Lox^{+/+}$, $Lox^{+/M}$, and $Lox^{M/M}$ MEFs using anti-Lox antibody that recognizes both 50 kDa pro-Lox and 30 kDa active Lox. In the cell layer, pro-Lox from $Lox^{M/M}$ accumulates, while most of it is secreted from WT cells. In the conditioned medias, there is abundant active Lox from WT cells, but almost no Lox protein secreted from $Lox^{M/M}$ cells.
Figure 3. 3: Proteolytic cleavage of intracellular Lox by rBMP-1

Cell extracts from $Lox^{+/+}$, $Lox^{+/M}$ and $Lox^{M/M}$ MEFs were isolated and incubated with increasing doses of rBMP-1 (30 60 and 90 ng) for 45 minute at 37°C. After incubation, cell lysates were separated on SDS-PAGE and immunoblotted with anti-Lox antibody.
Figure 3. 4: M292R Lox in the ER

Immunofluorescence imaging using anti-Lox and anti-calnexin antibodies. Almost no intracellular Lox in WT cells. In $Lox^{M/M}$ MEFs, Lox retained in the ER co-localized with calnexin. 100x images.
Figure 3. 5: Absence of ER stress response in M292R Lox cells

RNA from $Lox^{+/-}$, $Lox^{+/M}$, and $Lox^{M/M}$ MEFs were extracted and reverse transcribed to cDNA. qRT-PCR was then performed to measure transcript levels of ER stress markers, (A) $Atf4$, (B) $Chop$, and (C) $Bip$.
Figure 3.6: Absence of M292R Lox in lysosomal vesicles

Immunofluorescence imaging using anti-Lox and anti-Lamp-2 antibodies. The mutant LOX accumulating in $Lox^{+/M}$ MEFs do not co-localize with LAMP-2. 40X images.
Figure 3.7: Intracellular binding partners for Lox

Co-immunoprecipitation studies using cell extracts from \( \text{Lox}^{+/+} \), \( \text{Lox}^{+/M} \), and \( \text{Lox}^{M/M} \) MEFs immunoprecipitated with (A) anti-Calnexin, or (B) anti-Fbln4. The precipitated cell extracts were then separated by SDS-PAGE and immunoblotted with anti-Lox antibody. (C) \( \text{Lox}^{-/-} \) MEFs were transiently transfected with WT Lox-mApple, mutant Lox-mApple, or both plasmids. Conditioned medium from transfected cells were collected and separated by SDS-PAGE. Then, immunoblotting was performed using anti-Lox antibody.
Figure 3. 8: Structural changes in M292R Lox

(A) Only M292R Lox from mutant cells migrated slower on native PAGE. (B) Cell lysate from Lox<sup>+/+</sup> and Lox<sup>M/M</sup> MEFs were collected and incubated at 37°C for 6 h. Samples were collected every hour and separated by SDS-PAGE, then immublotted with anti-Lox antibody. (C) Cell lysate from Lox<sup>+/+</sup>, Lox<sup>+/M</sup> and Lox<sup>M/M</sup> MEFs were collected and treated with increasing concentration of porcine pancreatic elastase for 30 min at 37°C. After treatment, cell extracts were separated by SDS-PAGE and immunoblotted using anti-Lox antibody. Red arrowhead: ~25 kDa fragment unique to WT cells only. Red arrow: ~10 kDa fragment dominates mutant Lox extract incubated with highest dose of elastase.
Figure 3.9: AngII treatment in WT and Lox^{+/M} animals

(A) Despite AngII treatment, no animals displayed hypertension at the end of the 4 week treatment period. (B) Both WT and Lox^{+/M} animals with AngII did have significant cardiac hypertrophy. ****p<.00001 (C) Only AngII-treated Lox^{+/M} had visible dilation of the ascending aorta. (D) The AngII-treated Lox^{+/M} animals had significant increase in vessel extensibility at most pressures measured. # = AngII-treated WT vs. AngII-treated Lox^{+/M}. * = Saline-treated Lox^{+/M} vs. AngII-treated Lox^{+/M}. (E) No altered vessel mechanics in left common arteries.
Figure 3.10: Infiltration of macrophages in AngII-treated $Lox^{+/M}$ animals

Ascending aortas from saline- or AngII-treated $Lox^{+/+}$ and $Lox^{+/M}$ animals were excised and frozen in OCT. 3 µm sections were fixed in 4% PFA and immunofluorescence imaging was performed using anti-CD68 antibody. *= lumen.
References


Chapter 4:

Conclusions and future directions
Part I: Overall summary

Aortic aneurysms are the 18th most common cause of death in developed countries (Goldfinger et al., 2014; Lindsay et al., 2011). However, difficulty in diagnosis before a fatal rupture and the lack of preventative therapeutic agents persists. In particular, TAADs are strongly associated with genetic mutations; yet, mutations responsible for 75% of the FTAAD cases still remain unknown.

Using whole-genome sequencing, we identified a novel mutation in the catalytic domain of Lox that clearly segregated with the aortic aneurysm phenotype in a family with TAAD. To confirm the role of the M298R LOX mutation in the aortic disease, we introduced this mutation into the mouse genome in the corresponding position, M292R Lox, using CRISPR-Cas9 genome editing technology. The Lox\(^{M/M}\) animals were perinatal lethal due to ruptured aortic aneurysms, while the Lox\(^{+/-M}\) animals looked grossly normal and had comparable blood pressures and arterial tissue mechanics compared to the WT controls. However, the adult Lox\(^{+/-M}\) animals did have longer ascending aortas and severely fragmented elastin lamellae in the aortic wall, both of which are indicators of predisposition (Ardellier et al., 2017; Gillis et al., 2013) to TAA development (Lee et al., 2016). Furthermore, the role of Lox mutations in familial TAAD was further supported by the identification of additional Lox missense mutations in multiple families (Guo et al., 2016).

In order to determine if Lox\(^{+/-M}\) animals are indeed predisposed to TAA formation, we treated these animals with a vasoconstrictor, AngII, to increase blood pressure and hemodynamic stress on the arterial wall. While hypertension was not detected when measured at the end of AngII treatment period, both WT and Lox\(^{+/-M}\) animals developed significant cardiac hypertrophy. However, only Lox\(^{+/-M}\) ascending aortas had increased extensibility within the physiologic blood
pressure range, while AngII-treated WT and all saline-treated animals did not have altered arterial mechanics. Furthermore, only AngII-treated $Lox^{+/M}$ animals had increased cellularity in the media as well as infiltration of CD68+ macrophages in the medial layers closest to the adventitia.

To further elucidate the underlying mechanism of how this missense mutation in Lox leads to TAADs, MEFs isolated from the M292R Lox embryos were carefully studied. Our work demonstrated that M292R LOX has impaired secretion from cells, and therefore cannot reach the ECM for proteolytic activation. Thus, there was a significant decrease in Lox activity detected in the conditioned medium of $Lox^{+/M}$ MEFs compared to $Lox^{+/M}$ and WT conditioned media. Moreover, co-localization studies demonstrated that the intracellular M292R LOX is retained mostly in the ER, but did not elicit an ER stress response. To explain why M292R LOX is retained in the ER, we showed that the single amino acid change from a methionine to arginine causes a structural change of the enzyme. The altered structure was demonstrated by a slower migration on a native PAGE gel compared to WT and also by altered susceptibility to heat- and elastase-mediated degradation.

In summary, the work in this thesis confirmed the role of a $LOX$ mutation in familial TAAD development using a mouse model that recapitulated the human phenotypes. Furthermore, we demonstrated that the missense mutation caused retention of the mutant LOX in the ER, inhibiting its secretion to the ECM for activation and interaction with its substrates, elastin and collagen.
Part II: Miscellaneous observations

Musculoskeletal phenotypes in \( \textit{Lox}^{\text{M/M}} \) animals

Lox is responsible for the deamination of amine groups, the first step in cross-link formation, that is critical for both elastin and collagen maturation. Therefore, abnormalities in connective tissues with high collagen content are also expected with decreased Lox activity. In the proband individual with the \( M298R \textit{LOX} \) mutation, tall stature (height of 203 cm), pectus excavatum, scoliosis, a positive thumb sign, and high arched palate were observed during medical examination, suggesting abnormalities in collagen. We initially reported that some newborn \( \textit{Lox}^{\text{M/M}} \) animals had severe kyphosis, but a detailed analysis of the musculoskeletal system was not done (Lee et al., 2016).

Recently, we examined the musculoskeletal system in more detail and noticed that \( \textit{Lox}^{\text{M/M}} \) animals at P0 had bilateral forelimb contractures as well as craniosynostosis observed by skeletal staining (Figure 4.1). While these phenotypes were not previously reported in \( \textit{Lox-null} \) animals, we observed that \( \textit{Lox}^{\text{1/-}} \) animals in our colony also had forelimb contractures at birth (data not shown). However whether or not \( \textit{Lox}^{\text{1/-}} \) animals also have craniosynostosis still remains to be determined.

Individuals with ADCL1B associated with mutations in \( \textit{FBLN4} \) have connective tissue abnormalities including joint laxity and flexion contractures of the wrists. Similarly, \( \textit{Fbln4}^{\text{1/-}} \) mice have bilateral forelimb contractures (Markova et al., 2016), which are attributed to disrupted collagen fibrillogenesis. Further studies confirmed that Fbln4 is highly expressed in cartilage, bone, ligaments, and tendons, and it has been demonstrated that Lox expression is significantly reduced in \( \textit{Fbln4}^{\text{1/-}} \) long bones and calvaria (Sasaki, Stoop, et al., 2016).

Interestingly, although Lox mRNA was not reduced in \( \textit{Fbln4}^{\text{1/-}} \) tissues, studies demonstrated that
proteolytic activation of Lox is markedly reduced in Fbln4−/− cells, and this impairment was rescued when recombinant FBLN4 was added to the cell cultures.

Craniosynostosis is a condition where sutures in the infant skull are prematurely fused, which leads to facial deformities and delayed brain development. Although craniosynostosis has not been reported in either Lox−/− or Fbln4−/− mice, studies using osteoblasts isolated from Fbln4−/− calvaria demonstrated decreased active Lox expression (Sasaki, Stoop, et al., 2016). Furthermore, studies have shown increased BMP signaling, a pathway that may be altered in an aberrant ECM environment, led to premature suture fusion in mice (Komatsu et al., 2013). Thus, further studies will be necessary to identify the molecular mechanism underlying craniosynostosis observed in the LoxM/M animals.

Aortic root dilation, arterial tortuosity, and pulmonary artery dilation in the Lox−/M mice

Although the LoxM/M mice looked very similar to Lox−/− mice, suggesting that the M292R variant is a LOF mutation, we bred Lox+/M animals with Lox+/− animals early in our studies to assess the phenotype in Lox−/M animals. Out of four litters studied, Lox−/M animals were born in the correct Mendelian ratio. However, most Lox−/M animals died within a few hours of birth. During autopsy, we found that these animals had arterial tortuosity and elongated ascending aortas. However, ascending aortic aneurysms were absent in these animals and instead, there were pulmonary artery dilations. Furthermore, when one litter was left until weaning (3 weeks), there was one animal particularly smaller in size. An autopsy of this animal revealed extreme arterial tortuosity and a severe aortic root dilatation (Figure 4.2).

Pulmonary artery aneurysms are very rare, but can be found associated with congenital heart defects, pulmonary hypertension, pulmonary artery medial degeneration, or can be found as
idiopathic (Metras et al., 1987). Further analysis of the cardiovascular system will be necessary to determine the cause of pulmonary artery dilation in the $Lox^{+/M}$ animals at P0. Moreover, the surviving 3 week-old $Lox^{+/M}$ mouse suggests that the M292R LOX may retain a small level of catalytic activity.

The role of copper in Lox function

Copper ion is absolutely required for Lox activity. Without dietary copper, chicks died from ruptured aortas (Tang et al., 1983). In our initial studies, we hypothesized that the M292R missense mutation is affecting the ability for Lox to bind copper due the mutation’s close proximity to the copper-binding region. To determine if excess copper could rescue the aortopathy observed in $Lox^{M/M}$ animals, we subcutaneously delivered CuCl$_2$ to pregnant $Lox^{+/M}$ females. Resulting litters had no surviving $Lox^{M/M}$ animals at birth. The $Lox^{+/M}$ animals around 8 weeks of age lost most of their hair on the back and abdomen, a sign of copper toxicity (Pierard, 1979). We further analyzed the elastic lamellae of $Lox^{+/M}$ aortas from the copper-supplemented animals to determine if the fragmented elastic lamellae phenotype was rescued. While the elastin lamellae were still fragmented, there were areas within the medial layer with large elastin aggregates, observed by positive Alexa hydrazine 633 staining (Figure 4.3). These results suggest that increasing copper levels may suffice to increase Lox activity, thereby rescuing the Lox insufficiency associated with the $Lox^{+/M}$ genotype. A potential problem, however, is that newly cross-linked elastin may not be properly incorporated into the existing elastin network.

To determine if the M292R mutations alters Lox’s affinity for copper, we treated $Lox^{+/+}$ and $Lox^{M/M}$ MEFs with culture medium containing radiolabelled $^{64}$CuCl$_2$ to quantify the amount of copper bound to Lox. Post-radioactive $^{64}$CuCl$_2$ treatment, we separated protein from cell
extracts by SDS-PAGE. The gel transferred to PVDF membrane then exposed to x-ray film to obtain radiographs. The membranes were then immunoblotted with an anti-Lox antibody. We expected to be able to correlate the radioactive bands to the immunologically identified Lox. However, the radioactive bands were not at the proper molecular weight, giving rise to inconclusive results (Figure 4.4A,B).

In another set of experiments, we determined how copper chelation affects Lox expression and/or secretion. We treated WT MEFs with a copper-chelating agent, D-penicillamine and measured Lox protein the conditioned medium. Our data show that while the lack of copper did not affect Lox secretion, some pro-LOX from the penicillamine-treated cells remained in the inactive 50 kDa full-length form rather than all of LOX being cleaved to release the catalytically active 30 kDa LOX domain (Figure 4.4C).

Copper ion is incorporated into the catalytic domain of Lox in the Golgi apparatus. Thus, the above copper studies became irrelevant when we determined that the M292R LOX accumulates in the ER, which precedes the Gogi in the secretory pathway. Nevertheless, if we can identify chaperone proteins or chemicals that can transport M292R Lox from the ER to the Golgi, we will have to revisit these studies to determine the M292R Lox’s ability to bind copper for proper enzymatic activity.
Part III: Future directions

Ongoing work in the field of Lox biology has shown an expanded role for Lox in several diseases including fibrosis, cancer, and ocular disorders. In the work described in this thesis, I primarily focus on the role of LOX in arterial development and disease, particularly in ruptured aortic aneurysms where elastinopathy is the major cause of mortality in individuals lacking proper Lox function. While my work focuses on just one family with a specific missense variant in LOX, there are reports in the literature of additional LOX missense mutations (Guo et al., 2016), most of which are also in the catalytic domain, suggesting that these LOX mutants may all be behaving in a similar way. Together, these Lox missense mutations in families with TAAD call for understanding the underlying mechanism of disease that could aid in developing a therapeutic approach for the affected individuals.

While my work has provided new insights into Lox-related diseases, there are four areas where I would focus future research: 1) Why doesn’t the buildup of mutant LOX in the ER result in ER stress and the unfolded protein response? 2) Is mutant LOX capable of binding copper and acquiring catalytic activity if appropriately trafficked through the cell? 3) What role do macrophages play in the degradation of ECM and in the propagation of aneurysm formation? 4) What is the functional relationship between Fbln4 and Lox?

1: Mutant LOX in the ER

Analysis of 3-dimensional structure of LOX has been largely limited due to the absence of a Lox crystal structure (Ryvkin et al., 2004), owing to its low solubility and the failure to express the active enzyme in bacteria (Narayanan et al., 1974). Although we cannot model the effects of the missense mutation onto Lox overall structure, we hypothesized that the single amino acid change was sufficient to cause structural changes in Lox. If M292R LOX is
misfolded, we expected the cells to experience ER stress and in turn activate UPR. However, despite accumulation of M292R in the ER and its direct interaction with calnexin, a misfolded protein chaperone, UPR was not detected in $Lox^{M/M}$ cells.

An explanation for the absence of ER stress in $Lox^{M/M}$ cells could be that mutant Lox is being eliminated from the ER at a high rate, resulting in failure to induce ER stress. We already demonstrated that M292R LOX does not colocalize with lysosome marker, LAMP-2 (Figure 3.6). However, there are several other mechanisms of misfolded protein clearance from cells. First, it is possible that retrotranslocation of M292R LOX from the ER to the cytoplasm for ubiquitination results in degradation by proteasomes (Hiller et al., 1996). Second, when the capacity of the proteasome pathway is exceeded, aggregates of misfolded proteins are delivered to distinct pericentriolar structures termed, aggresomes for degradation (Johnston et al., 1998). Finally, Mutant LOX can be degraded in the ER by ER-localized ubiquitin ligases as well (Hara et al., 2014; Swanson et al., 2001). Future studies will determine if one of these mechanisms is employed to inhibit UPR in $Lox^{M/M}$ cells despite retention of mutant protein in the ER.

Alternatively, M292R LOX retention in the ER may not elicit a stress response because the mutant protein does not directly interact with UPR chaperone protein, Bip. Previous studies have been reported of a mutant peripheral myelin protein-22 (PMP-22) in Charcot-Marie-Tooth (CMT)-related neuropathies, where mutant PMP-22 is retained in the ER without activation of the UPR (Dickson et al., 2002). In this particular study, it was demonstrated that mutant PMP-22 binds to calnexin longer than WT PMP-22 but does not directly bind Bip. Therefore, without direct interaction with the UPR chaperon protein, ER stress was not activated in cells expressing the mutant protein.
The question of how M292R Lox is retained in the ER remains. In another study of CMT disease, it was demonstrated that some PMP-22 mutants lost the ability to bind a Golgi-sorting receptor, Rer1 (Hara et al., 2014). As a result, ER to Golgi transport was impeded in these mutants, leading to ER retention. Loss of interaction between M292R LOX and a Golgi-sorting receptor is a possible mechanism for its retention in the ER. Future studies will be needed to identify Lox protein binding partners. Identifying intracellular binding partners for LOX and determining whether or not that interaction is disrupted with the mutation will help elucidate the mechanism that must be targeted for proper trafficking of M292R LOX through the secretory pathway.

2: Catalytic activity of M292R LOX

Incorporation of copper into Lox is absolutely required for enzymatic activity. Because the M292R mutation site is close in proximity to the copper-binding region in the catalytic domain, the effect it may have on copper binding was a concern. More specifically, we hypothesized that the added positive charge from the arginine would lead to charge repulsion with Cu$^{2+}$, resulting in decreased affinity for copper in M292R LOX compared to WT LOX.

Although M292R LOX is retained in the ER, whether or not it can efficiently bind Cu$^{2+}$ for full enzymatic activity once it can be transported to the Golgi remains to be determined. The three histidine residues in the catalytic domain responsible for forming a stable complex with the Cu$^{2+}$ ion have been identified using short peptides containing the highly conserved copper-binding region of Lox and analyzed by NMR, electron paramagnetic resonance, and visible absorption and fluorescence (Lopez et al., 2011; Oldfield et al., 2017; Ryvkin et al., 2004). For future studies, similar short peptides with and without the mutation can be generated to apply
spectroscopic techniques to determine whether or not the mutation affects copper binding. An alternative approach will be to express WT and mutant Lox variants in bacterial or yeast expression systems. The success of this approach has been variable, with most laboratories not able to get catalytically active Lox using bacterial expression. However, several recent reports show promising results using new expression techniques in *E. Coli* that incorporate unique solubility tags (Smith et al., 2016).

3: Macrophages in aneurysms

A role of matrix metalloproteinases (MMPs) has been implicated in aortic aneurysm pathogenesis (Rabkin, 2017). In particular, MMP-2, -7, -9, and -12 have been identified as able to degrade elastin (Curci et al., 1998). In my studies, I developed a genetic model for TAAD where animals heterozygous for the *M292R Lox* mutation have fragmented elastic fiber in the ascending aorta wall, making these animals predisposed to aortic dilations when challenged with a vasoconstrictor, AngII. The abnormal elastic lamellae in *Lox*<sup>+/M</sup> aortas are present at P0 (Figure 4.5) and *MMP*-2, -7, -9, and -12 mRNA expression levels are not elevated in these tissues relative to WT controls (data not shown). Together, these data suggest that the fragmented elastic fibers in this model are a result of aberrant assembly, rather than elastase-mediated degradation.

While elastin degradation does not account for the fragmented elastic fibers in *Lox*<sup>+/M</sup> aortas, it does seem to play a role in the progression of arterial disease following a “second hit”. I have shown that treatment of *Lox*<sup>+/M</sup> animals with AngII resulted in the recruitment of macrophages to the adventitia and to the medial layers near the adventitia of aneurysmal vessels. Macrophages and neutrophils contain potent elastases and collagenases, and aortic dilation is generally associated with changes in the ECM in the adventitia as well as the media. The
presence of macrophages in this critical region following AngII treatment suggests that macrophage-derived proteolytic enzymes may play a role in weakening the vessel wall by degrading elastin and collagen. A question for future studies is what is the stimulus to recruit inflammatory cells when Lox-insufficient vessels experience a “second hit”? An intriguing possibility is that the matrix itself is playing and active role and that the under cross-linked elastic fibers in the $Lox^{+/M}$ aortas are serving as a source for bioactive peptides that participate in this alternative pathway for immune cell recruitment. Previous studies have demonstrated that the degradation of insoluble elastin releases EDPs that contain the bioactive GxxPG consensus motif (Heinz et al., 2012). These bioactive EDPs can then signal primarily through ERCs to elicit various biological responses including immune cell recruitment. The under cross-linked elastic fibers in $Lox^{+/M}$ may be more susceptible to proteolysis resulting in easier release of bioactive peptides that drive immune cell infiltration into the arterial wall. The Mecham lab has developed a function-blocking antibody to the bioactive sequence in elastin. It will be interesting to use this antibody to explore the role of EDPs in aneurysm progression. Also, understanding the role of the macrophages recruited to the AngII-treated $Lox^{+/M}$ arterial wall may shed light on a process that may be targeted for therapeutic development in individuals that develop TAADs from mutations in $LOX$.

4. Functional relationship between LOX and FBLN4

Since identifying the overlapping vascular phenotypes in $Lox$-$null$ (Hornstra et al., 2003; Maki et al., 2002) and $Fbln4$-$null$ mice (McLaughlin et al., 2006), efforts have been focused on determining the function of FBLN4 and its relationship to LOX. The currently accepted role of FBLN4, based on in vitro studies, is that it serves to target Lox to its substrate, elastin, for
elastogenesis (Horiguchi et al., 2009; Papke et al., 2014). However, this interaction between Fbln4 and Lox in vivo remains to be determined.

TAAs are observed not only in Lox KO and Fbln4 KO animals, but also in animal models with the M292R Lox mutation (Lee et al., 2016) and E57K Fbln4 mutation (Halabi et al., 2017; Igoucheva et al., 2015). This phenotypic overlap supports a similar functional role for both proteins. My work has shown that, in addition to the vascular abnormalities in animals with decreased Lox activity, these animals have musculoskeletal defects such as forelimb contractures and craniosynostosis. Interestingly, recent studies revealed forelimb contractures in Fbln4-null mice (Markova et al., 2016; Sasaki, Stoop, et al., 2016), suggesting that the interaction between Lox and Fbln4 in elastogenesis is also required for collagen fibrillogenesis in the musculoskeletal system.

In future research, M292R Lox mice can be used to help elucidate FBLN4 function and interactions between Fbln4 and Lox in vivo, as this has only been confirmed in in vitro studies (Sasaki, Hanisch, et al., 2016). Currently, unpublished work from Dr. Carmen Halabi has demonstrated that Fbln4<sup>E57K/E57K</sup>:Lox<sup>+/−</sup> animals have worsened vascular phenotypes in the aorta compared to Fbln4<sup>E57K/E57K</sup> or Lox<sup>+/−</sup> animals individually, suggesting that LOX and FBLN4 both play a critical role in the vasculature, but whether or not they directly interact still remains a question. Similarly, if the presence of Lox mutant allele in combination with Fbln4 mutant or null alleles gives rise to worsened cardiovascular and musculoskeletal phenotypes, we can further confirm that both protein work synergistically in elastogenesis and collagen fibrillogenesis. Primary cells from M292R Lox and E57K Fbln4 animals may be useful in identifying the in vivo interaction between Lox and Fbln4. Co-immunoprecipitation studies will
be instrumental in determining the affinity between the two proteins and whether this interaction is impaired by Lox mutations.
Figure 4.1: Musculoskeletal phenotypes in M292R Lox mice

A) $Lox^{+/+}$, $Lox^{+/-M}$ and $Lox^{M/M}$ animals were collected at birth and observed for any gross abnormalities. All $Lox^{M/M}$ animals had forelimb contractures (arrowhead) that were not observed in $Lox^{+/-M}$ or WT animals. (B) Alcian blue and Alizarin red staining was performed on P0 whole animals. Staining of skeletal sutures in red show (white arrows) that in WT animals, the sutures are still separated but they are prematurely fused in $Lox^{M/M}$ animals.
Figure 4.2: Pulmonary artery and aortic root dilation in Lox^−/M mice.

(A) The arterial tree in P0 animals was visualized using yellow latex injection casting. These animals had tortuous arteries but no ascending aorta aneurysm. Instead, they had dilated pulmonary arteries (arrowhead). (B) One Lox^−/M animal survived to 3 weeks but was markedly smaller in size compared to WT littermates. The yellow latex injection casting was performed in these animals to highlight the severely dilated ascending aorta root and extremely tortuous arteries.
Figure 4.3: in utero delivery of copper in M292R Lox mice

(A) The litter that received CuCl$_2$ in utero lost hair on their abdomen and back around 8 weeks after birth but the hair eventually grew back. (B) Ascending aortas from WT and Lox$^{+/M}$ at 3 months were cross-sectioned and stained with Alex hydrazide 633 for elastic fibers. The elastic fiber fragmentation in Lox$^{+/M}$ vessels was not reversed with copper treatment but both WT and Lox$^{+/M}$ vessels had increased accumulation of elastin aggregates in the media.
Figure 4.4: Radioactive copper and D-penicillamine treatment in M292R Lox cells

(A) Radioactivity detected on the PVDF membrane did not correspond to the correct molecular weight for pro-LOX, 50 kDa. (B) The same PVDF membrane was immunoblotted using anti-Lox antibody. The correct Lox bands were detected in all samples. (C) WT Lox MEFs treated with culture medium containing 5 mM D-Penicillamine demonstrated that without copper, proteolytic cleavage efficiency of the pro-peptide after secretion is decreased.
In $Lox^{+/+}$ aortas, vSMCs were well organized, comparable to WT vessels, but the elastic fiber thickness was irregular and was more fragmented compared to WT vessels. In $Lox^{M/M}$ aortas, vSMC organization was completely lost and the cells appeared more rounded, suggesting that they are less polarized. Instead of concentric layers of elastic lamellae, the elastin in $Lox^{M/M}$ aortas appeared as dispersed aggregates.
References


Curriculum Vitae

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EDUCATION
Doctor of Philosophy in Developmental, Regenerative, and Stem Cell Biology
Lucille P. Markey Special Emphasis Pathway in Human Pathobiology
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“Lysyl Oxidase Mutations in Thoracic Aortic Aneurysm and Dissection”
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Bachelor of Science in Biochemistry with minor in Chemistry
University of Washington, Seattle, WA
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RESEARCH EXPERIENCE
Research Scientist/Engineer I
Center for Lung Biology, Department of Pediatrics
William C. Parks PhD, John K. McGuire MD
“Pro-inflammatory Effects of Nanoparticles on Pulmonary Epithelial Cells and Macrophages”
University of Washington, Seattle, WA
March 2011-July 2013

Undergraduate Research Assistant
Center for Lung Biology- Division of Pulmonary and Critical Care Medicine
Anne Manicone MD
“Role of MMP-28 in ApoE Expression and Localization in Macrophages”
University of Washington, Seattle, WA
October 2009-March 2011

Undergraduate Research Assistant
Institute of Stem Cell and Regenerative Medicine-Department of Biochemistry
Hannele Ruohola-Baker PhD
“Identifying Regulators of Germline Stem Cell Differentiation/Maintenance in Drosophila Melanogaster”
University of Washington, Seattle, WA
December 2007-August 2009

PEER-REVIEWED MANUSCRIPTS
Lee VS, Halabi CM, Broekelmann TJ, Lin M, Stitziel NO, Mecham RP. Intracellular Retention of Mutant Lox Leads to Aortic Dilation with a “second hit.” [Manuscript in Preparation]


**AWARDS/HONORS**

**North American Vascular Biology Organization- Vascular Biology Meeting**  
Travel Award  
Monterey, CA  
*October 2017*

**Gordon Research Seminar- Elastin, Elastic Fibers, and Microfibrils**  
Best Poster Presentation Award  
Biddeford, ME  
*August 2017*

**National Institutes of Health- National Heart Lung and Blood Institute**  
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*March 2017- April 2018*

**American Society for Matrix Biology Biennial Meeting**  
Travel Award for Selected Talk  
St. Petersburg, FL  
*November 2016*

**European Elastin Meeting- Regenerative Medicine and Bioengineering**  
Young Scientist Award for Best Talk  
Stuttgart, Germany  
*June 2016*
Lucille P. Markey Special Emphasis Pathway in Human Pathobiology
Washington University School of Medicine in St. Louis
St. Louis, MO August 2014 – April 2016

Washington University in St. Louis- Annual Cardiovascular Research Day
2nd place in Pre-doctoral Poster Presentations
St. Louis, MO October 2015

University of Washington Alumnae Board- Full Tuition Scholarship Recipient

University of Washington Quarterly Dean’s List
Seattle, WA 2007-2011

INVITED PRESENTATIONS
Gordon Research Conference: Elastin, Elastic Fibers, and Microfibrils
Lee V, Mecham RP. Lysyl Oxidase in Vascular Disease.
Biddeford, ME August 2017

Washington University School of Medicine in St. Louis: Annual Cellular and Molecular Biology Symposium
Lee V, Mecham RP. Intracellular Accumulation of Mutant Lysyl Oxidase Leads to Familial Aortic Aneurysms.
St. Louis, MO November 2016

ABSTRACTS/PRESENTATIONS
Lee V, Mecham RP. Lysyl Oxidase in Vascular Disease.
Biddeford, ME August 2017

American Society for Matrix Biology Biennial Meeting-Oral Presentation
Lee V, Mecham RP. Lysyl Oxidase Mutation in Familial Aortic Aneurysm and Dissection.
St. Petersburg, FL November 2016

European Elastin Meeting-Oral Presentation
Lee V, Mecham RP. Lysyl Oxidase Mutation in Familial Aortic Aneurysm and Dissection.
Stuttgart, Germany June 2016

American Thoracic Society-Poster Presentation
Philadelphia, PA May 2013

Society of Toxicological Pathology Pacific Northwest Regional Meeting- Oral Presentation
Lee V. Effects of CdSe/ZnS quantum dots and silver nanoparticles on mouse lung epithelial cells and macrophages
Seattle, WA

National Institute of Environmental Health Sciences Centers for Nanotechnology Health Implications (NCNHIR) Consortium Meeting- Poster Presentation
Lee V, Hu X, Gao X, Faustman EM, Griffith WC, Kavanagh TJ, Eaton DL, Parks WC, McGuire JK. Effects and Health Assessment of Quantum Dot Exposure on Pro-inflammatory Responses by Lung Epithelial Cells and Macrophages
Portland, OR

American Thoracic Society- Poster Discussion Sessions
San Francisco, CA

Zhu X, Rims C, Lee V, McGuire JK. Deficiency of IL-10 Producing T cells in BAL and Lung Associated with Lack of CD103+Foxp3+ Tregs Lead to Delayed Recovery of ALI Induced by P. aeruginosa in CD103 Knockout Mouse
San Francisco, CA

National Institute of Environmental Health Sciences Centers for Nanotechnology Health Implications (NCNHIR) Consortium Meeting- Poster Presentation
Research Triangle Park, NC

University of Washington Undergraduate Research Symposium- Poster Presentation
Lee V, Lin M, Manicone AM. MMP28-Dependent Effects of APOE Expression and Localization in Macrophages
Seattle, WA

University of Washington Undergraduate Research Symposium- Poster Presentation
Lee V, Ward EJ, Ruohola-Baker H. Identifying Genes Required for Stem Cell Function
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BOOK CHAPTER PUBLICATION

PROFESSIONAL MEMBERSHIP
American Society of Matrix Biology
North American Vascular Biology Organization
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TEACHING EXPERIENCE
Washington University in St. Louis- Principles of Biology I
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St. Louis, MO
Spring Semesters 2015 & 2016 & 2017

RESEARCH MENTEES
University of Washington- Parks Lab
Maura Newell (University of Notre Dame)- Summer 2012 & Summer 2013

Washington University in St. Louis – Mecham Lab
Emily Braverman (Clayton High School)- Summer 2015
Erin Neely (Parkway South High School)- Summer 20

LEADERSHIP
Washington University School of Medicine in St. Louis- Young Scientist Program Summer Focus
Next Up Program Coordinator
Organize college preparatory class for high school scholars participating in summer research.
St. Louis, MO
January 2016- August 2017

University of Washington- Undergraduate Research Leader
Advocate undergraduate research program through outreach activities and annual undergraduate research symposium.
Seattle, WA
September 2009-May 2012

Global Medical Brigade- University of Washington Chapter Lead
Recruit students and medical professionals for summer service trips and direct operations on-site.
Seattle, WA; Honduras; Panama
January 2010-September 2011

Phi Lambda Upsilon: Honors Biochemistry/Chemistry Society-Vice President
Organize student memberships and career development panels for students in department
University of Washington, Seattle, WA
June 2008-September 2009
COMMUNITY SERVICE
KARE Program
St. Louis, MO  October 2015-December 2017
- Provide assistance and companionship for patients with Alzheimer’s disease during their visit to Washington University’s Kemper Art Museum

Young Scientist Program
St. Louis, MO  October 2013
- Chemistry Teaching Team: Introducing students to chemistry concepts as simple and everyday-related examples.