

## **LOX Mutations Predispose to Thoracic Aortic Aneurysms and Dissections**

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**Running title:** LOX Mutations Predispose to TAAD



# Circulation Research

ONLINE FIRST

### **Subject Terms:**

Aneurysm  
Aortic Dissection  
Genetics  
Vascular Biology

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**In December 2015, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13.05 days.**

## ABSTRACT

**Rationale:** Mutations in several genes have been identified that are responsible for approximately 25% of families with familial thoracic aortic aneurysms and dissections (TAAD). However, the causative gene remains unknown in 75% of families.

**Objectives:** To identify the causative mutation in families with autosomal dominant inheritance of TAAD.

**Methods and Results:** Exome sequencing was used to identify the mutation responsible for a large family with TAAD. A heterozygous rare variant, c.839G>T (p.Ser280Arg), was identified in *LOX*, encoding a lysyl oxidase, that segregated with disease in the family. Sanger and exome sequencing was performed to investigate mutations in candidate genes in an additional 410 probands from unrelated families. Additional *LOX* rare variants that segregated with disease in families were identified, including c.125G>A (p.Trp42\*), c.604G>T (p.Gly202\*), c.743C>T (p.Thr248Ile), c.800A>C (p.Gln267Pro), and c.1044T>A (p.Ser348Arg). The altered amino acids cause haploinsufficiency for LOX or are located at a highly conserved LOX catalytic domain, which is relatively invariant in the population. Expression of the LOX variants p.Ser280Arg and p.Ser348Arg had significantly lower lysyl oxidase activity when compared with the wild type protein. Individuals with LOX variants had fusiform enlargement of the root and ascending thoracic aorta, leading to ascending aortic dissections.

**Conclusions:** These data, along with previous studies showing the deficiency of *LOX* in mice or inhibition of lysyl oxidases in turkeys and rats causes aortic dissections, support the conclusion that rare genetic variants in *LOX* predispose to thoracic aortic disease.

### **Keywords:**

Aortic aneurysm, aortic dissection, lysyl oxidase, gene mutation, aortic disease, exome sequencing.

### **Nonstandard Abbreviations and Acronyms:**

BAPN	β-aminopropionitrile
ECM	extracellular matrix
ESP	the NHLBI Exome Sequencing Project
ExAC	Exome Aggregation Consortium
FTAAD	familial thoracic aortic aneurysms and dissections
HA	hemagglutinin
MAF	minor allele frequency
OHS	occipital horn syndrome
SMC	smooth muscle cell
TAAD	thoracic aortic aneurysms and dissections
WT	wild type

## INTRODUCTION

Thoracic aortic aneurysms, involving the aortic root, the ascending aorta, or fusiform dilatation of both segments, are typically asymptomatic as they progressively enlarge. If undiagnosed, these aneurysms can cause an acute dissection of the ascending aorta. Family studies indicate that up to 20% of individuals with thoracic aortic aneurysms leading to acute dissections (TAAD) who do not have a known genetic syndrome (e.g., Marfan syndrome [MIM: 154700]) have a family history of TAAD, termed familial TAAD (FTAAD).<sup>1,2</sup> The 11 genes identified to date for FTAAD are only responsible for disease in 25% of FTAAD families, thus demonstrating further genetic heterogeneity for this disorder. The identified genes predisposing to TAAD encode proteins involved in the following: the extracellular matrix (ECM) of the aortic wall (*FBN1*, *MFAP5*), smooth muscle cell (SMC) contraction or metabolism (*ACTA2*, *MYH11*, *MYLK*, *PRKG1*, *MAT2A*) and canonical TGF- $\beta$  signaling (*TGFBR1*, *TGFBR2*, *TGFB2*, *SMAD3*).<sup>3-10</sup>

The pathologic changes associated with TAAD primarily involve the thick medial layer of the aortic wall, which is composed of alternating layers of SMCs and elastic lamellae. With thoracic aortic disease, there is fragmentation and loss of the elastic lamellae, focal areas lacking SMCs, and accumulation of proteoglycans. The layers of elastin in the aorta are laid down during development and have an estimated half-life of approximately 40 years.<sup>11</sup> Additionally, there is a collagen deposition between the SMCs and the elastin lamellae. The proper construction of the elastic lamellae and collagen fibers is dependent on lysyl oxidases, which are extracellular copper enzymes that catalyze the formation of lysine-derived crosslinks in elastin and hydroxylysine-derived crosslinks in collagens. Mammalian genomes have 5 lysyl oxidase gene family members, including the prototypic LOX and LOX-like proteins 1 through 4, all of which have highly similar catalytic domains. We describe here variants in one of the lysyl oxidase genes, *LOX*, that disrupt enzyme function and predispose to TAAD.

## METHODS

Exome sequencing was used to identify the mutation responsible for a large family with TAAD. Sanger and exome sequencing was performed to investigate rare variants in candidate genes in an additional 410 probands from unrelated families and tested the segregation of these variant with aortic diseases in families. Expression of the LOX variants and lysyl oxidase activities were measured to investigate biological function of rare LOX variants. An expanded Methods section is available in the Online Data Supplement at <http://circres.ahajournals.org>.

### ***Family recruitment and characterization.***

Blood or saliva samples were collected from affected individuals and family members after obtaining informed consent and approval from all participating institutions, including the University of Texas Health Science Center at Houston, Cleveland Clinic, and the Centre de Reference pour les Syndromes de Marfan et Apparente's in France. Medical records, including imaging studies of the aorta, surgical reports, hospital records, death certificates and physicians' notes were reviewed.

## RESULTS

### *Identification of LOX rare variants associated with familial thoracic aortic aneurysms and dissections.*

To identify additional novel genes that cause an inherited predisposition to TAAD, exome sequencing was pursued using DNA from three affected individuals from family TAA602 with autosomal dominant inheritance of thoracic aortic disease but no mutations in the known TAAD genes (Figure 1A).<sup>8</sup> Gene variants identified by whole exome sequencing were filtered based on the following: 1) variants that altered the amino acid sequence of proteins, including nonsynonymous, stop-lost, stop-gain, coding indels, frameshift, or splice site variants; 2) variants that had minor allele frequency (MAF) of less than 0.05% in the NHLBI Exome Sequencing Project (ESP) database; and 3) variants shared between the three affected relatives.<sup>9</sup> The variants that met these criteria were assessed for segregation with aortic disease in TAA602, decreasing the number of genes with candidate rare variants down to 10 (Supplemental Table I). Among these genes, *LOX* (NM\_002317.5) was the best candidate gene for causing aortic disease because of the established role of lysyl oxidases in connective tissue mechanical stability, the relatively high expression in SMCs, and the fact that *Lox*<sup>-/-</sup> mice die of thoracic aortic rupture shortly after birth.<sup>12</sup> The *LOX* variant identified in TAA602, p.Ser280Arg, falls in the catalytic domain of the enzyme and is predicted to be damaging by five of seven functional prediction programs. The C score of combined annotation dependent depletion prediction (<http://cadd.gs.washington.edu/score>) is 17.36. This variant is absent in 13,000 chromosomes in the ESP database and only present in two out of 121,214 chromosomes, i.e., MAF  $1.65 \times 10^{-5}$  in the ExAC (Exome Aggregation Consortium) database. Additionally, serine 280 and its flanking amino acids are highly conserved from human through zebrafish (Figure 1B).

Exome and Sanger sequencing of an additional 410 unrelated FTAAD probands identified five additional *LOX* rare variants that disrupted the protein sequence: c.125G>A (p.Trp42\*), c.604G>T (p.Gly202\*), c.743C>T (p.Thr248Ile), c.800A>C (p.Gln267Pro), c.1044T>A (p.Ser348Arg), c.235G>A (p.Ala79Thr), and c.460C>T (p.Leu154Phe). The nonsense variant p.Trp42\* identified in TAA-92291 is predicted to lead to nonsense mediated decay and co-segregated with thoracic aortic aneurysms in the family. The p.Gly202\* variant identified in TAA271 is predicted to lead to nonsense mediated decay. This *LOX* variant is not present in the proband's unaffected mother; however, samples from the affected father and uncle were not available. Notably, no *LOX* nonsense or indel variants is found in the ESP database and only one frameshift variant is found in one of 121,182 chromosomes in the ExAC database. Variants, p.Thr248Ile, p.Gln267Pro, and p.Ser348Arg, are absent in the ESP and ExAC databases, are predicted to be damaging or deleterious by more than four of seven programs, have C scores of 11.7, 28.0 and 24.7, respectively, and are located in the highly conserved catalytic domain of the protein. Glutamine 267, serine 280, serine 348 and are conserved from human through zebrafish, and the amino acid at position 248 is threonine or serine among these species (Figure 1B and 1C). The ESP database has only one non-synonymous rare variant in European Americans, p.Lys320Glu, in the *LOX* catalytic domain. Additional samples were not available from other affected members of TAA111 and TAA703. The last two rare variants identified disrupt Ala79 and Leu154, both of which are located in the propeptide region of *LOX* and this region is removed after post-translational procedure. This region of *LOX* contains multiple rare variants in the general population and *LOX* p.Ala79Thr and p.Leu154Phe are predicted to be benign or neutral by six of seven programs and are not conserved (Figure 1C and Table 1). Thus, these *LOX* rare variants are likely to be benign. Additionally, *LOX* p.Ala79Thr was identified in a family with a disease-causing *ACTA2* mutation. Therefore, we determined that possible disease-causing *LOX* variants are responsible for approximately 1% of familial thoracic aortic disease and associated with decreased penetrance.

### *Clinical characterization of affected individuals with LOX rare variants.*

The proband of family TAA602 is a 37 year old European American female with fusiform dilatation of the aortic root and ascending aorta, measuring 3.8 cm and 4.1 cm, respectively. Mild mitral regurgitation was also noted, and her physical exam was negative for syndromic features. Her father was 62 years old when he was diagnosed with a 5.2 cm aortic root aneurysm that involved the ascending aorta (4.2 cm) and aortic arch (4.0 cm). He also has a bicuspid aortic valve, a three-vessel coronary artery disease and severe left ventricular hypertrophy. He underwent an aortic valve replacement and root and ascending aorta replacement, along with a coronary artery bypass graft. The proband's paternal uncle had an ascending aortic dissection at 60 years of age. A paternal aunt was diagnosed with a 3.9 cm aortic root aneurysm and an ectatic ascending aorta at the age of 54 years.

The proband of family TAA111 is a European American male who had an incidental finding of a 12.5 cm ascending aortic aneurysm and underwent an aortic valve replacement and supracoronary ascending aortic replacement at the age of 41 years and aortic root aneurysm repair at the age of 51 years. His father died suddenly at the age of 56 years. The proband of family TAA271 is a 36 year old European American male with an unremarkable physical exam, who was diagnosed with a 4.2 cm aortic root with fusiform enlargement of the ascending aorta. Due to the dissection deaths of his father and paternal uncle, he underwent an elective aortic root and ascending aorta replacement and aortic valve repair. The proband of TAA703 is a European American female, who was diagnosed with a 5.2 cm ascending aortic aneurysm and transverse aortic arch aneurysm and bicuspid aortic valve at the age of 40 years. She has no features of a genetic syndrome. Her father died at the age of 53 years due to a ruptured ascending aorta. The proband of TAA-92291 underwent surgery for a thoracic aortic aneurysm at 11 years of age and has additional Marfan-like features, including dolichostenomelia, scoliosis and pectus excavatum. Three of the proband's relatives with the *LOX* variant (II:3, II:4, III:1) underwent thoracic aortic aneurysm repair in their 50's. Individual II:1 died at 70 years of age after thoracic aortic surgery and individual III:2 has a thoracic aortic aneurysm requiring surgery; DNA samples from these individuals were not available. Limited clinical information was available for this family. The proband of TAA-9544 had a bicuspid aortic valve and thoracic aortic aneurysm, and underwent a Bentall procedure at 16 years of age. He also has features of Marfan syndrome, including a highly arched palate, joint laxity and skin striae. A maternal uncle (II:1), who also underwent a Bentall procedure at the age of 51 years, and a first cousin (III:3) and first cousin once removed (IV:1) with aortic root enlargement carry the *LOX* variant. DNA samples were not available from the proband's maternal grandparents, but the grandfather had a thoracic aortic surgery at the age of 54 years. Dural ectasia, pectus deformity, joint hypermobility and skin striae were variably present in the family members.

The aortic pathology in two patients, the proband's father in TAA602 (II:5) with p.Ser280Arg and the proband in TAA271 (III:4) with p.Gly202\*, both of whom underwent prophylactic repair of thoracic aortic aneurysms, was assessed. The control aorta shows the orderly structure of the alternating layers of wavy elastic fibers (black) and SMCs (red; Figure 2). The aortas from both patients with *LOX* variants showed mild medial degeneration characterized by focal loss of elastin fibers and smooth muscle cells, but limited deposition of proteoglycans (top panels, Figure 2). Perhaps more significant in the patients' aortic pathology is the disorganization of the elastic fiber deposition when compared to the control aorta (middle panels, Figure 2). In addition, collagen deposition is increased in the patients' aortas (lower panel, Figure 2).



### *LOX missense variants in the enzymatic domain decrease LOX activity.*

The protein product of *LOX* (referred to as LOX) is synthesized as a 50-kDa inactive proenzyme (pro-LOX) that subsequently undergoes N- and O-glycosylation at sites in the propeptide domain, and then is secreted from the cells and cleaved extracellularly to form the functional C-terminal 30-kDa enzyme and 18-kDa N-terminal propeptide fragment (Figure 1C). Recombinant plasmids were constructed to express either the wild type (WT) LOX or each of the mutant LOX proteins (Thr248Ile, Ser280Arg and Ser348Arg) with either a human influenza hemagglutinin (HA) or FLAG tag at the C-terminus. The plasmids were transfected individually into HeLa cells and immunoblot analyses using antibodies against either LOX or the protein tags showed similar levels of the pro-LOX protein for all four constructs (Figure 3A). At the same time, immunoblot analysis using the LOX antibody showed that the levels of active mutant LOX (molecular weight 34 kDa) was lower than the WT, with the Thr248Ile mutation 4% lower, the Ser280Arg 27% lower and the Ser348Arg 14% lower (Figure 3A). Surprisingly, the antibodies directed against either the FLAG or HA tag failed to recognize the active protein. Lysyl oxidase activity was assayed at five time points by spectrophotometric detection of the byproduct of LOX enzymatic activity, hydrogen peroxide, and the enzymatic activity corrected for the decreased protein levels of the active LOX mutants relative to the WT level. The mutant LOX constructs, Thr248Ile, Ser280Arg and Ser348Arg, had lysyl oxidase activity levels that were 92%, 50%, and 79% that of WT LOX, respectively (p values 0.03 to  $5.2 \times 10^{-8}$ ; (Figure 3B).

## DISCUSSION

The data presented here support the conclusion that heterozygous loss-of-function mutations in *LOX*, specifically variants that disrupt the catalytic activity or lead to haploinsufficiency, predispose to thoracic aortic aneurysms and acute aortic dissections. Overlapping syndromic features of Marfan syndrome, such as pectus deformities and striae, were reported in family members with *LOX* variants but these features were not sufficient to meet diagnostic criteria for Marfan syndrome.<sup>13</sup> Thoracic aortic aneurysms in these individuals are either aortic root aneurysms or fusiform aneurysms, involving both the aortic root and ascending aorta. Although mutation carriers died of ascending aortic dissections, there were no reports of aortic dissections with minimal enlargement of the ascending aorta. None of the affected individuals presented with descending thoracic aortic aneurysms or dissections. Interestingly, a bicuspid aortic valve was identified in three out of 18 individuals with *LOX* mutations. It is also notable that probands from two families, TAA-92291 and TAA-9544, underwent thoracic aortic aneurysm repair at 11 and 16 years of age, respectively, which is younger than their affected family members and other *LOX* mutation carriers. This young age of onset could be due to environmental modifiers of the phenotype but is perhaps more likely due to a second genetic alteration leading to an early onset of the disease.

Lysyl oxidases are extracellular copper enzymes that initiate the formation of lysine-derived crosslinks in elastin and collagen. There are five lysyl oxidase gene family members with highly similar catalytic domains. The specific function of some of these genes has been determined through deletion of these genes in mice. *Lox*<sup>-/-</sup> mice die at the end of gestation or soon after parturition due to aortic rupture with evidence of aortic aneurysms and aortic tortuosity.<sup>11,12</sup> Importantly, 80% of lysyl oxidase activity is lost with deletion of *Lox*, suggesting *LOX* encodes the majority of the lysyl oxidase in the aorta.<sup>14</sup> Electron microscopic examination of *Lox*<sup>-/-</sup> mouse aortas shows decreased amount and fragmentation of the elastic laminae, along with marked disorganization of the elastin fibers, similar to what was observed in the aortic pathology associated with *LOX* mutations (Figure 2). Thus, other lysyl oxidase genes cannot compensate for the loss of *Lox* in aortas of mice to assure proper development of the aorta. In contrast, deletion of *Loxl* does not disrupt aortic development in mice, but instead leads to uterine and bowel prolapse and enlarged airspaces in the lung.<sup>15</sup>

Loss of lysyl oxidase activity due to the *LOX* mutations may predispose to thoracic aortic disease through aberrant development of the thoracic aorta due to fewer elastin cross links, leading to decreased and fragmented elastic fiber as observed in the *Lox*<sup>-/-</sup> mice. It is also established that irreversible inhibition of lysyl oxidase activity by administration of  $\beta$ -aminopropionitrile ( $\beta$ APN) induces thoracic aortic aneurysms and dissections in both young turkeys and rats.<sup>16-21</sup> The aortic pathology in BAPN-treated young rats shows features of medial degeneration, including elastin degradation, thickening of the medial layer, and SMC apoptosis.<sup>22</sup> Thus, inhibition of lysyl oxidase activity postnatally also induces thoracic aortic disease. Since elastin layers are laid down during development, it raises the possibility that the stability of the aorta provided by lysyl oxidase activity may result from the cross linking of collagen, which has a relatively short half-life in the aorta in comparison to elastin.

Although *LOX* mutations have not been described in humans until this report, secondary lysyl oxidase deficiency can occur in individuals with Menkes syndrome and the less severe occipital horn syndrome (OHS). Mutations in *ATP7A*, a copper transporter necessary for copper absorption, cause both Menkes and OHS and loss of copper leads to reduced activities of copper-dependent enzymes, including all lysyl oxidases.<sup>23</sup> Vascular abnormalities such as vascular tortuosity and arterial aneurysms have been observed in individuals with Menkes syndrome and OHS and are associated with fragmentation and disruption of the elastic lamellae. Furthermore, a mouse model of Menkes syndrome develops aneurysms in the ascending, descending thoracic, and abdominal aortas within six months of age.<sup>24</sup> The overlap of the aortic disease in Menkes with the phenotype of FTAAD due to *LOX* mutations provides further evidence that lysyl oxidase activity is important for maintaining structural integrity of the aorta throughout a life time.

The rare variants in FTAAD that were classified as disease-causing fall in the catalytic domain, which is relatively invariant in the general population. Lysyl oxidase activity is predicted to be decreased by 50% with the decrease by p.Trp42\* and p.Gly202\* and we found the other rare variants, p.Thr248Ile, p.Ser280Arg and p.Ser348Arg, decrease activity from 50 – 92% of that of the wild type allele. Thus, with the addition of the wildtype allele, total *LOX* activity is predicted to be 75% to 96% that of normal, raising the question why the relatively modest decrease in enzymatic activity would cause thoracic aortic disease. One possibility is that the active mutant protein is less stable than the active wild type protein. We found that all the mutations lead to decrease active *LOX* protein levels when the proteins were overexpressed in HeLa cells, supporting this possibility. Additionally, although we have focused on disruption of the catalytic activity by the rare variants associated with TAA, it is important to note that *LOX* has other functions in the cell. The *LOX* propeptide region targets *LOX* to elastin fibers in the extracellular matrix by mediating its interactions with tropoelastin.<sup>25</sup> *LOX* is also reported to localize the nucleus in SMCs, where data indicate that it has a role in chromatin structure regulation and gene transcription.<sup>26, 27</sup> Thus, the *LOX* rare variants identified associated with FTAAD may disrupt other functions of the protein than just the catalytic activity. Finally, it is important to note that we were not able to confirm that the *LOX* variant that disrupted protein stability and activity minimally, p.Thr248Ile, segregated with disease in the family. Therefore, this variant may not be disease-causing.

In summary, accumulating evidence indicates that loss of lysyl oxidase activity due to mutations in *LOX* predisposes individuals to fusiform thoracic aortic aneurysms and ascending aortic dissections. The presence of bicuspid aortic valve in three of the 20 carriers of *LOX* mutations. Normal heart valves develop from cardiac cushions, which undergo extensive remodeling that involves cell differentiation, apoptosis, and remodeling of extracellular matrix. Thus, defects in *LOX* function has the potential to contribute to this congenital valve abnormality and thoracic aortic disease. Additional studies are needed to define the spectrum of mutations and further delineate the clinical phenotype.

## ACKNOWLEDGEMENTS

The authors are extremely grateful to the individuals and families who participated in this study and the physicians and genetic counselors who aided in the collection of clinical data from the families.

## SOURCES OF FUNDING

The following sources provided funding for these studies: RO1 HL62594, P01HL110869-01, UL1 RR024148, John Ritter Foundation, Vivian L. Smith Foundation, and Richard T. Pisani Funds (all to D.M.M.) and GIS-Maladies rares (C.B.), PHRC AOM09093 (G.J.), and ANR 2010 BLAN 1129 from the French National Research Agency (G.J.). Sequencing was provided by the University of Washington Center for Mendelian Genomics (UW CMG) and was funded by the National Human Genome Research Institute and the National Heart, Lung and Blood Institute grant 1U54HG006493 to D.A.N, J.S. and M.J.B.

## DISCLOSURES

None.

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## FIGURE LEGENDS

**Figure 1. *LOX* rare variants identified in FTAAD families.** **A.** Pedigrees of families with *LOX* rare variants. The legend indicates the disease and mutation status of the family members. The age at diagnosis of aortic aneurysm or dissection (“dx”) and age at death (“d”) are shown in years. An asterisk indicates individuals with a bicuspid aortic valve. A dashed circle around a symbol indicates individuals whose DNA was used for exome sequencing. **B.** Orthologue conservation of *LOX* variants identified in individuals with TAAD and surrounding amino acids. **C.** Schematic representation of lysyl oxidase encoded by *LOX* with the domains of the proteins indicated. The *LOX* rare variants identified in this study are on the top of the protein diagram and the rare variants identified in European Americans in the NHLBI ESP database are indicated below. Red or blue font designates variants predicted to be possibly or probably damaging, respectively, and black font indicates variants predicted to be benign by PolyPhen-2 analysis.

**Figure 2. Aortic pathology associated with aneurysms in individuals with *LOX* variants.** Movat pentachrome and picro sirius red staining was done on control aortic tissue and aortic tissue removed at the time of prophylactic repair of a thoracic aortic aneurysms from patients with the p.Gly202\* and p.Ser280Arg mutations. Evidence of medial degeneration is evident by Movat staining in the patients’ aortas as indicated by focal fragmentation of elastic fibers (black) and a decreased number of SMCs (red) when compared to the control aorta. Movat staining also shows the disorganization of elastin deposition in the patients’ aortas compared with control aortas. Picro sirius red staining of collagen fibers shows increased collagen deposition in the patients’ aortas compared with the control aorta.

**Figure 3. Missense variants identified in FTAAD patients decrease the enzymatic activity of *LOX*.** **A.** Immunoblot analyses of HeLa lysates transfected with plasmid containing WT *LOX* or the individual *LOX* variants using an antibody directed against HA tag (top) and the *LOX* protein (middle) show equal expression of a pro-*LOX*-HA with all plasmids. **B.** Lysyl oxidase enzymatic activity assays show that *LOX* activities were significantly decreased when compared with WT for Ser280Arg and Ser348Arg *LOX* variants, but not for Thr248Ile. All *LOX* activity data are resulted from two separate transfections, and triplicate analysis of the lysates from each transfection. Lysyl oxidase activities were measured at 30, 60, 90, 120, and 150 minutes, respectively. Data are normalized to activity of the WT *LOX* with the non-transfected cells activity subtracted. Activity is presented in relative fluorescent units with each data point, error bars indicate standard deviation. \* $p < 0.05$  for the comparison of mutant versus WT *LOX* activity levels.

## Novelty and Significance

### *What Is Known?*

- Approximately 25% of patients with thoracic aortic aneurysms that can lead to acute aortic dissections have an inherited predisposition for the condition and there is significant genetic heterogeneity in this inherited predisposition, with 11 genes identified to date.
- The stability and integrity of elastin and collagen, two major matrix components of the aortic wall, are dependent on lysine-derived crosslinks formed by lysyl oxidases.
- Mammalian genomes have 5 lysyl oxidase genes, including the prototypic *LOX* gene and *LOX*-like genes 1 through 4, but no genetic mutations predisposing to thoracic aortic disease have been identified to date.

### *What New Information Does This Article Contribute?*

- Mutations in *LOX* were identified in families with an inherited predisposition for thoracic aortic disease.
- These *LOX* variants are loss-of-function mutations, leading either to haploinsufficiency or disruption of the catalytic domain and decreased enzymatic activity.
- Individuals with *LOX* mutations had fusiform enlargement of the root and ascending thoracic aorta, and in the absence of surgical repair of the aneurysms, this leads to acute type A (ascending) aortic dissections.

Identification of the genes predisposing to thoracic aortic aneurysms and acute aortic dissections is important to identify at-risk individuals so that medical and surgical management can be initiated to prevent premature death. However, no mutations causing thoracic aortic disease have been identified in any of the five genes for human lysyl oxidases. Here we report loss-of-function mutations in *LOX* that predispose to thoracic aortic disease. This finding adds to the list of genes predisposing to thoracic aortic disease and confirms the role of lysyl oxidase in regulating the stability and integrity of elastin and collagen in the aortic wall.

**TABLE 1.** Computational and predicted effect on function of rare variants in *LOX*

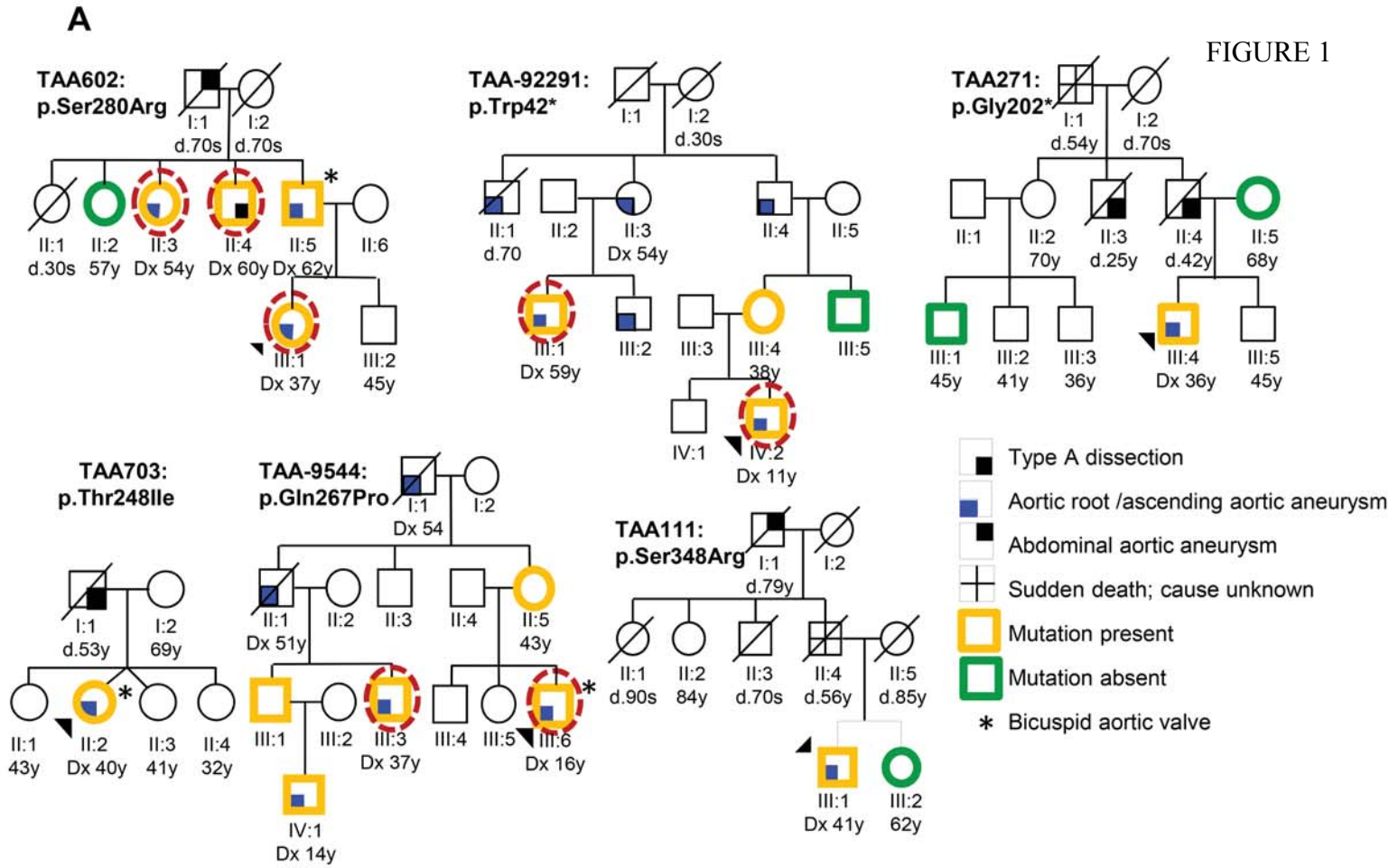
Amino-acid substitution	Family ID	Conservation <sup>a</sup>	Cscore	Predicted effect on function <sup>b</sup>	MAF in ExAC
p.Ser280Arg	TAA602	1	17.7	5/7	1.65E-05
p.Trp42*	TAA-92291	1	22.2	1/1	0
p.Gly202*	TAA271	1	22.5	1/1	0
p.Thr248Ile	TAA703	1	24.6	4/7	0
p.Gln267Pro	TAA-9544	1	28.0	7/7	0
p.Ser348Are	TAA111	1	11.7	7/7	0
p.Leu154Phe	TAA289	0.15	15.9	1/7	1.7E-05
p.Ala79Thr	TAA296/TAA806	0.011	0.53	0/7	3.8E-04

<sup>a</sup> PhastCons conservation score<sup>b</sup> LRT, MutationTaster, Polyphen2 HDIV, Polyphen2 HVAR, SIFT, PROVEAN, and MutationAssessor.

# Circulation Research

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FIGURE 1



**B**

	T248	Q267	S280	S348
Human	ENCLAS <b>T</b> AYRADV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Pig	ENCLAS <b>S</b> AYRADV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Dog	ENCLAS <b>S</b> AYRADV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Cow	ENCLAS <b>S</b> AYRGDV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Microbat	ENCLAS <b>S</b> AYRSDV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Hedgehog	ENCLAS <b>S</b> AYRADV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Rabbit	ENCLAS <b>S</b> AYRADV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Mouse	ENCLAS <b>S</b> AYRADV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Zebra_Finch	ENCLAS <b>S</b> AYRADV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Chicken	ENCLAS <b>S</b> AYRADV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Xenopus	ENCLAS <b>S</b> AYRSDI	LRFPQ <b>R</b> VKNQGTADFLP <b>S</b> RPRYT <b>W</b>	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
Coelacanth	ENCLAS <b>S</b> AYRSDV	LRFPQ <b>R</b> VKNQGTADFLP <b>S</b> RPRYSW	AHTQGL <b>S</b> PGCYDT	AHTQGL <b>S</b> PGCYDT
zebrafish	ENCLAS <b>S</b> AYRSSV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYT <b>W</b>	SHTQGL <b>S</b> PGCYDT	SHTQGL <b>S</b> PGCYDT
Spotted_gar	ENCLAS <b>S</b> AYRSEV	LRFPQ <b>R</b> VKNQGTSDFLP <b>S</b> RPRYSW	SHTQGL <b>S</b> PGCYDT	SHTQGL <b>S</b> PGCYDT
	*:****:***:..	*****:*****:*	:*****	:*****

**C**

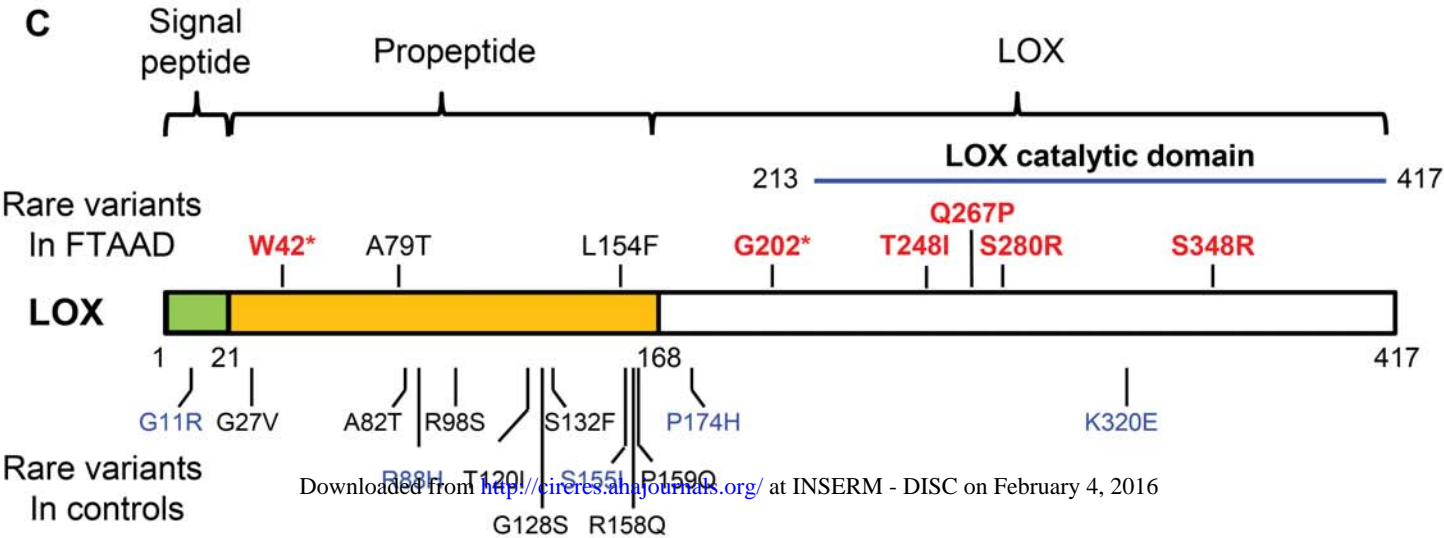




FIGURE 2

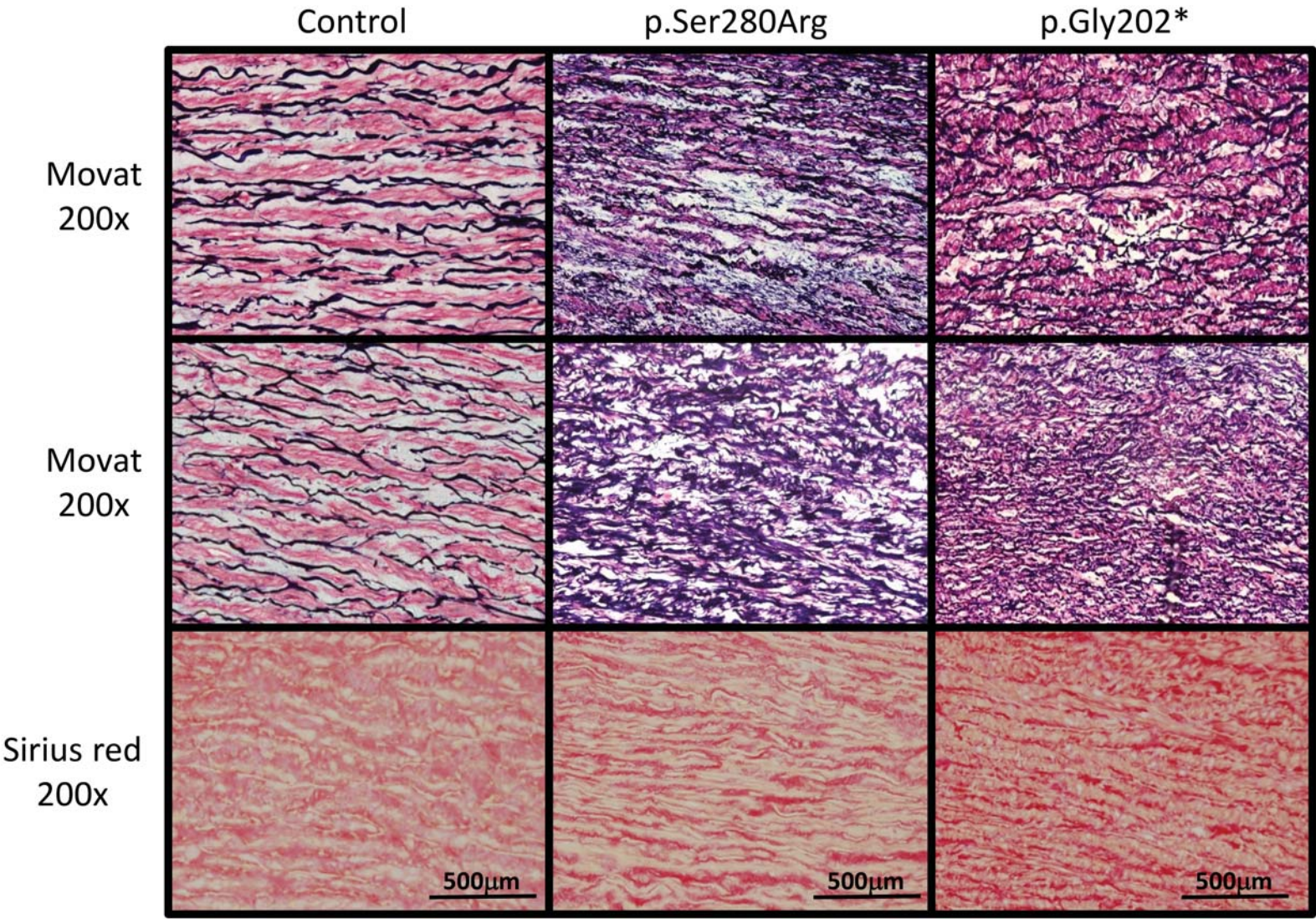
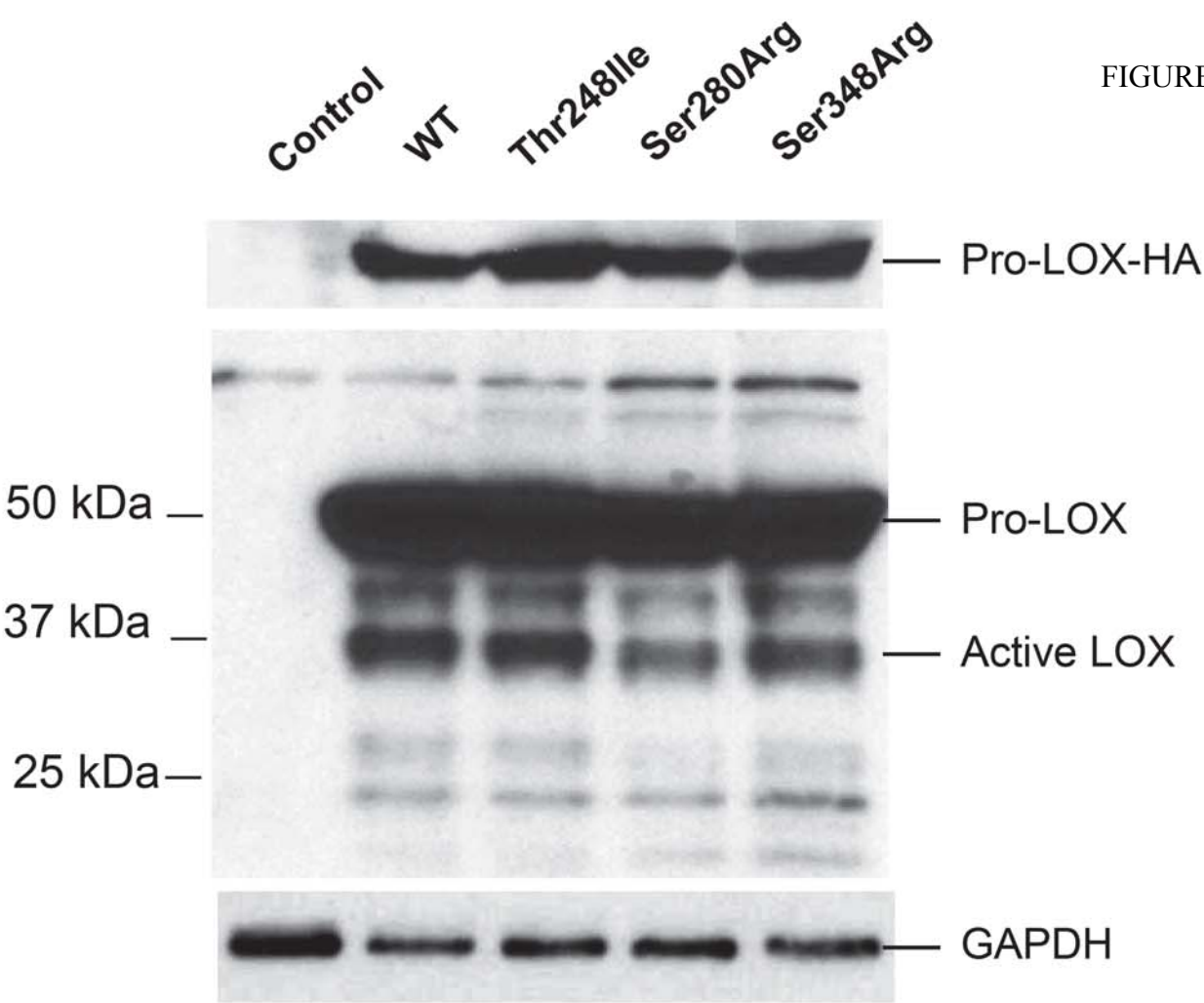
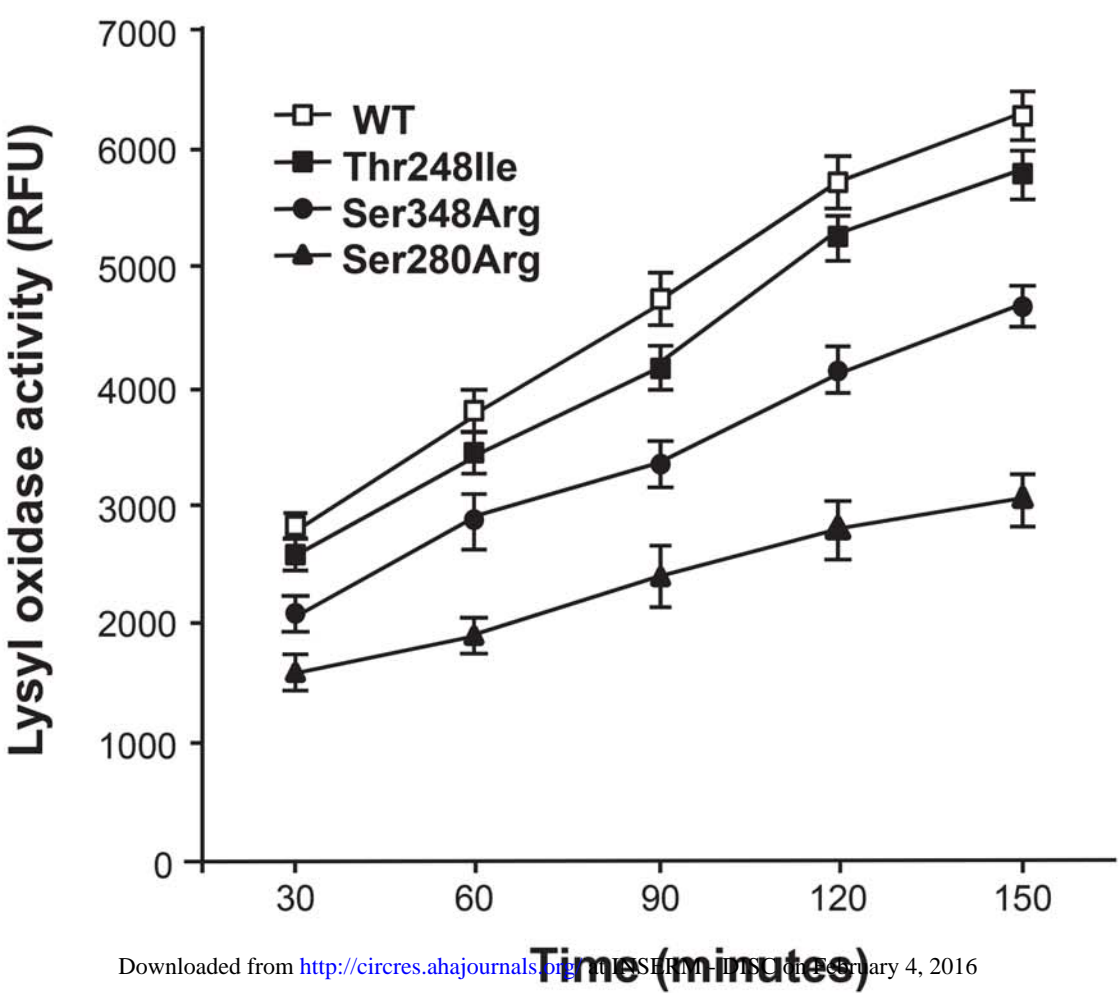


FIGURE 3

**A**



**B**



## **Supplemental Material**

### ***LOX* Mutations Predispose to Thoracic Aortic Aneurysms and Dissections**

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## Materials and Methods

### Exome and Sanger Sequencing

Genomic DNA was extracted from peripheral blood lymphocytes using standard protocols. Five micrograms of DNA from three affected individuals in family TAA602 were used for construction of the shotgun sequencing library as described previously using adaptors for paired-end sequencing. Exome sequences were captured by SeqCap EZ. Exome probes version 2.0 (Roche) and recovered according to manufacturer's directions. Enriched libraries were then sequenced on an Illumina GAIIX using manufacturer protocols. Reads were mapped to the reference human genome (UCSC hg19) with BWA (Burrows-Wheeler Aligner), and variant detection and genotyping are performed using the UnifiedGenotyper (UG) tool from GATK. Annotation of variants was performed using the SeattleSeq server (<http://gvs.gs.washington.edu/SeattleSeqAnnotation/>).<sup>9</sup> Sanger DNA sequencing assay was performed to validate exome sequencing results and test the segregation of variants with TAAD in families. *LOX* mutations were reported based on the RefSeq codes NM\_002317.5.

### Construction of *LOX* recombinant plasmids:

WT-*LOX* was amplified by using pcDNA-*LOX* (GenScript, Inc., Piscataway Township, NJ) with primers 5'-gcgaattcatgcgcttcgcctggaccgt-3' and 5'-acggtcgacatacggcgaaattgtgcagcct-3' (IDT, Inc., Coralville, Iowa). Three *LOX* mutants were generated by PCR-directed site mutagenesis with HiFi HotStart DNA polymerase (KAPA Biosystems, Wilmington, MA) and corresponding primers sets as follows: Ile248-Fw, 5'-ctgtctggccagtatagcatcacagggcaga-3', Ile248-Rv, 5'-tctgccctgtatgctatactggccagacag-3', Arg280-Fw, 5'-cagatttcttacccatccgaccaagatatcc-3' Arg280-Rv, 5'-ggaatatcttggtcggtgggtaagaaatctg-3', Arg348-Fw, 5'-cacagggttagAcctggctgttatg-3', and Arg348-Rv, 5'-cataacagccaggtctcaatccctgtg-3'. The construct and mutations were verified by

Sanger sequencing. The cDNA of LOX-WT, -Ile248, -Arg280 and -Arg348 were inserted into pcDNA3 vector by digesting with EcoRI and SalI.

### **Lysyl Oxidase Assay and Western blot analysis**

HeLa cells ( $10^5$ ) were seeded into six-well plates. The recombinant plasmids were transfected into HeLa cells respectively using Lipofectamine 2000 reagent. After 72 hours, the dishes were scraped and the lysate homogenized (RIPA lysis buffer and protease inhibitor cocktail (Sigma-Aldrich) on ice). The lysates were spun and 50  $\mu$ l of supernatant was assayed in triplicate. LOX activity was measured with a commercially available fluorimetric assay (AAT Bioquest, Sunnyvale, CA), which utilizes a proprietary LOX substrate that releases hydrogen in a horseradish peroxidase-coupled reactions. The assay was done following the manufacturer's recommendations. The reaction was incubated at 37°C for 30min, 60min, 90min, 120min, and 150min. Its signal was read by a fluorescence microplate reader at Ex/Em=540/590 nm.

Lysates (20  $\mu$ g) were separated by 4-15% SDS polyacrylamide gel electrophoresis (TGX Mini Protean, Bio-Rad) with Tris-glycine running buffer. The proteins were transferred to a nitrocellulose membrane using the iBlot dry blotting system (Life Technologies). The membrane was blocked with 5% skim milk and incubated with rabbit polyclonal primary anti-LOX diluted 1:1,000 (abcam). The membrane was also incubated with rabbit monoclonal anti-GAPDH (1:5,000) to confirm equal loading of cellular proteins. The immune complexes were visualized using enhanced chemiluminescence reagent (Western Lightning Chemiluminescence ECL Pro, Perkin Elmer, Waltham, MA) and detected with ImageQuant LAS4000 (GE Healthcare).

### **Pathology Analysis**

Aorta from patients and healthy control were sectioned and stained with Movat pentachrome and picro sirius red.



Supplementary Table I. Candidate genes identified by exome sequencing in the index family (TAA602).

Gene	Amino acid substitution	mRNA level <sup>a</sup>	Conservation <sup>b</sup>	Cscore	Predicted effect on function <sup>c</sup>	MAF in ExAC
<i>LOX</i>	S280R	3873.7	1	17.36	5/7	1.65E-05
<i>ACER1</i>	G41R	147.6	0.939	18.5	5/7	2.47E-05
<i>ARHGAP33</i>	P705L	144.5	0.002	2.809	1/7	0
<i>HPGD</i>	D80G	126.4	1	15.55	3/7	0
<i>ISM1</i>	D140N	126.3	1	22.9	3/7	1.69E-05
<i>PCDHGA11</i>	H650Q	159.5	0	14.61	3/6	0
<i>PCSK1</i>	S332N	176.3	1	29.5	4/7	0
<i>RNF150</i>	I395F	158.7	0.995	8.702	0/7	0
<i>UBR5</i>	R994C	341.6	1	14.42	5/6	0
<i>ZNF609</i>	R240H	211.1	1	19.75	5/7	2.56E-05

<sup>a</sup> The average level of mRNA expression in four smooth muscle cell lines explanted from control aorta. The levels of mRNA expression were quantified based on an arbitrary unit that was detected by the Illumina Sentrix Human Ref8 Beadchip Expression Array.

<sup>b</sup> PhastCons conservation score

<sup>c</sup> LRT, MutationTaster, Polyphen2 HDIV, Polyphen2 HVAR, SIFT, PROVEAN, and MutationAssessor.

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*Circ Res.* published online January 12, 2016;

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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