Loss-of-Function Mutations in *YY1AP1* Lead to Grange Syndrome and a Fibromuscular Dysplasia-Like Vascular Disease

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Fibromuscular dysplasia (FMD) is a heterogeneous group of non-atherosclerotic and non-inflammatory arterial diseases that primarily involves the renal and cerebrovascular arteries. Grange syndrome is an autosomal-recessive condition characterized by severe and early-onset vascular disease similar to FMD and variable penetrance of brachydactyly, syndactyly, bone fragility, and learning disabilities. Exome-sequencing analysis of DNA from three affected siblings with Grange syndrome identified compound heterozygous nonsense variants in *YY1AP1*, and homozygous nonsense or frameshift *YY1AP1* variants were subsequently identified in additional unrelated probands with Grange syndrome. *YY1AP1* encodes yin yang 1 (YY1)-associated protein 1 and is an activator of the YY1 transcription factor. We determined that YY1AP1 localizes to the nucleus and is a component of the INO80 chromatin remodeling complex, which is responsible for transcriptional regulation, DNA repair, and replication. Molecular studies revealed that loss of YY1AP1 in vascular smooth muscle cells leads to cell cycle arrest with decreased proliferation and increased levels of the cell cycle regulator p21/WAF/CDKN1A and disrupts TGF-β-driven differentiation of smooth muscle cells. Identification of *YY1AP1* mutations as a cause of FMD indicates that this condition can result from underlying genetic variants that significantly alter the phenotype of vascular smooth muscle cells.

Introduction

Fibromuscular dysplasia (FMD [MIM: 135580]) is a poorly understood arterial disease that affects primarily women and results in arterial stenosis or occlusion, and less commonly, arterial dissection or aneurysm formation.[1] FMD can affect almost any artery but most commonly affects the renal arteries, presenting as hypertension, and the carotid and vertebral arteries, leading to ischemic stroke, transient ischemic attacks, headaches, and pulsatile tinnitus. On pathologic analysis, the arterial lesions are distinct from atherosclerotic lesions in that FMD lesions do not contain inflammatory cells or lipids. Instead, the pathology of FMD is characterized by either intimal fibroplasia, with neointimal lesions of cells and matrix deposition, or medial fibroplasia, in which there is loss of smooth muscle cells (SMCs) and increased deposition of collagen and proteoglycans in the medial layer.[2,3] With angiographic imaging, the majority of individuals with FMD have the typical "string of beads" appearance along the artery due to stenoses and aneurysms, which is termed multifocal FMD.[4] A less common angiographic appearance is characterized by arteries that show focal tubular stenosis, termed unifocal FMD. Approximately 7%–10% of individuals with FMD have an affected family member, supporting a genetic basis for the disease.[1,5] Familial cases show distinct patterns of affected vascular beds and tend to be more severe with bilateral and multivessel involvement.[5] However, no causative genes have been identified to date.[6]

Vascular disease similar to FMD can also occur as part of a genetic syndrome, as illustrated by Grange syndrome (MIM: 602531).[7] This syndrome was originally described in a family in which four out of nine siblings had variable occlusion or stenosis of arteries, including renal artery lesions associated with chronic hypertension and cerebral artery lesions, leading to transient ischemic attacks. Occlusion of abdominal and coronary arteries was also described. In addition, the affected siblings had brachydactyly and syndactyly of the hands and feet, increased bone fragility with multiple fractures, mild learning disabilities, and variable penetrance of other cardiovascular defects, including patent ductus arteriosus, bicuspid aortic valve, and ventricular septal defect. Additional cases of Grange syndrome have been described with similar vascular complications.[8–10] The steno-occlusive

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arterial lesions and involvement of the cerebral and renal arteries led to the classification of the vascular disease in Grange syndrome as FMD, and both unifocal and multifocal lesions have been described in affected individuals. Interestingly, the intracranial location of the cerebrovascular occlusions and the formation of compensatory collateral vessels at the base of brain in individuals with Grange syndrome are similar to those observed in Moyamoya disease. Since rare genetic syndromes have the potential to provide insight into the pathogenesis of specific disorders, we sought to determine the genetic basis of Grange syndrome to provide insight into the molecular pathogenesis of vascular diseases like FMD.

Material and Methods

Case Recruitment

Blood or saliva samples from affected and unaffected family members with Grange syndrome or FMD or control subjects were collected after obtaining approval from the institutional review board at the University of Texas Health Science Center at Houston, University of Michigan, or Cleveland Clinic and informed consent was obtained from the participants. In the study design, healthy control subjects are matched to the case subjects (with 1:1 ratio) by gender, ethnicity, and age (±5 or 10 years).

Exome and Sanger Sequencing Assay

Using genomic DNA from individuals of Grange syndrome-affected families, the exome sequences were captured by SeqCap EZ exome v.1.0 (Roche). Enriched libraries were then sequenced on an Illumina GAIIx according to manufacturer's protocols. Reads were mapped to the reference human genome (UCSC hg19) with Burrows-Wheeler Aligner and variants called using SAMtools. Insertion-deletion (indel) variants affecting the coding sequence were identified after a Smith-Waterman realignment of the BWA calls. Single-nucleotide variants (SNVs) and indels were filtered to >8× and quality >30. Annotation of variants was performed using the SeattleSeq server (see Web Resources).[11]

For FMD exome analysis, gene burden testing using VT method (Variable Threshold Test with adaptive permutation) was used, which was implemented in EPACTS (Efficient and Parallelizable Association Container Toolbox) software pipeline developed by the University of Michigan Center for Statistical Genetics. The variable threshold approach does not require researchers to preset the allele frequency threshold as other rare variant gene burden tests do. Instead, it searches for the optimal threshold that maximizes the difference between trait distributions for subjects with and without rare variants. The gene-wise burden test focused only on non-synonymous and loss-of-function genetic variants.

Bidirectional Sanger DNA sequencing was performed on DNA fragments amplified with intron-based, exon-specific primers from genomic DNA to validate exome-sequencing results and check segregation of *YY1AP1* variants in families with Grange syndrome.

Mammalian Cell Culture

Human aortic smooth muscle cells (SMCs) from transplant donor controls were grown in SmGM-2 media (Lonza, #CC-3182) with 20% FBS (Atlanta). For induction of smooth muscle differentiation, cells were serum starved in 1% FBS for 24 hr followed by treatment with TGF-β1 at 2 ng/mL (R&D Systems). Human HeLa cells and HEK293T cells were grown in DMEM media (Hyclone) with 10% FBS. HEK293 cells and HEK293 cells stably producing Flag-tagged INO80E were kindly gifted by Dr. Joan W. Conaway from Stowers Institute for Medical Research and maintained in DMEM media with 10% FBS.[12] To immortalize human primary vascular SMCs, primary human aortic SMCs (11023 and 10975) were transduced with sterile filtered supernatants of passage 3 of the PA317LXSNHPV16E6E7 packaging cell line (CRL2203, ATCC) in the presence of polybrene at 8 μg/mL (Sigma) followed by selection with G-418 (100 μg/mL) (Sigma).[13] The established immortalized SMC lines were grown in SmGM-2 complete media. The levels of major smooth muscle proteins were analyzed by western blot with antibodies against smooth muscle α-actin (Sigma), calponin1 (Abcam), and SM22α (Abcam).[14]

Identification of YY1AP1 Isoforms in SMCs and Aortic Tissue

More than ten *YY1AP1* splicing isoforms are documented in the Ensembl database with lengths between 424 and 888 amino acid residues. To identify the major *YY1AP1* isoform expressed in SMCs, total RNA was extracted from control human primary SMCs (#11023) and an aortic sample using the Trizol reagent (Invitrogen) and cDNA was synthesized with Iscript cDNA synthesis kit (Bio-Rad, #170-8691). RT-PCR and Sanger sequencing were performed and only the isoforms ENST00000295566 (043) and ENST00000404643 (046) were detected in the RNA isolated from cultured SMCs or aortic tissue (Figure S1). To further identify the isoform expressed in the SMCs, real-time qPCR (Roche LC96) was performed using primers to differentiate between the 043 and 046 isoforms: 43F1: 5′-CCT CGA TCC CCT CGC CGC-3′; 46F: 5′-GCG GTT GGT GGC CGT GCG-3′; 43/46R: 5′-CTC CCG CTT GTC AGG AGG C-3′. The mRNA level of *GAPDH*, *ACTB*, and the 18S rRNA gene served as internal controls.

Subcellular Fractionation, Immunoblots, and Immunofluorescence

Mammalian cells, including HEK293T, HeLa, and SMCs, were lysed and fractionated using the cell fractionation kit (Cell Signaling, #9038). The fractionated proteins were separated by SDS-PAGE followed by western blot with Lamin A/C (Thermo) and GAPDH (Cell Signaling) as nuclear and cytoplasmic markers, respectively. Subcellular localization of YY1AP1 was analyzed with antibodies from Millipore (ABC477). To directly visualize the subcellular localization of YY1AP1, vascular smooth muscle cells and HEK293T cells were fixed in 4% paraformaldehyde, and immunofluorescence was assessed by Leica DMi8 SPE confocal microscopy.

Co-immunoprecipitation Experiments

To test whether YY1AP1 is a component of the INO80 chromatin remodeling complex, HEK293 cells and HEK293 cells producing Flag-INO80E were lysed in 50 mM Tris-Cl (pH 7.5) and 1% Triton X-100 with protease inhibitor cocktails (Sigma) and immunoprecipitated with Flag beads (Sigma). The Flag-INO80E bound proteins were analyzed by western blot for proteins that bound to INO80E, including YY1 (Cell Signaling), YY1AP1 (Millipore), and TIP49A (Abcam).

To perform co-immunoprecipitation in SMCs, nuclear proteins from immortalized SMC11023 cells were extracted with CelLytic NuCLEAR Extraction kit (Sigma) per manufacturer's manual, followed by immunoprecipitation in 10 mM HEPES (pH 7.9), 150 mM

NaCl, 0.1 mM EGTA, 20% glycerol, and 0.2% NP-40 with normal rabbit IgG as negative control (Santa Cruz) or antibodies against YY1AP1 (Millipore) for 4 hr at 4°C. The agarose beads designed to bind immunoglobulins (Protein A/G PLUS-Agarose; Santa Cruz) were added and the incubation at 4°C continued another 4 hr. Assessment to determine whether YY1 and INO80C were associated with YY1AP1 was done by immunoblot analysis of the immunoprecipitated proteins.

Knockdown of YY1AP1 in HEK293T Cells and SMCs

To knock down the expression of *YY1AP1*, five lentiviral shYY1AP1 constructs were purchased (GE Dharmacon). Previous analyses determined that shYY1AP1 C2 and C6 resulted in the most efficient knockdown.[15] To confirm knockdown efficiency, shYY1AP1-C2 and C6 were co-transfected with Flag-YY1AP1 in HEK293T cells, and the lysates were subjected to western blot analysies for the Flag protein.

To generate stable *YY1AP1* knockdown HEK293T cell lines, control pLKO.1 with a scrambled shRNA, pLKO.1-shYY1AP1-C2 and pLKO.1-shYY1AP1-C6 were transfected in the packaging cell line HEK293T together with the packaging plasmids pSPAX2 and pMD2.G using Lipofectamine 3000 (Life Technologies) and incubated for 48 hr in a 37°C incubator. The supernatants containing lentivirus from the transfected packaging cells were filter sterilized. HEK293T cells were infected and screened with puromycin at 2 ng/mL. The knockdown efficiency was analyzed by western blot and real-time qPCR in Roche LC96 real-time PCR machine (Roche) with Taqman probe (Life Technologies) and qPCR master mix (Clontech). *GAPDH*, *ACTB*, and 18S rRNA were used as internal controls.

To knock down the expression of YY1AP1 in SMCs, the lentiviral constructs were modified; the puromycin-resistant gene on the lentiviral constructs of control vector pLKO.1 with the scrambled shRNA, pLKO.1-C2, and pLKO.1-C6 was replaced with an enhanced green fluorescent protein (EGFP) gene using KpnI and BamHI restriction sites. The modified lentiviral constructs were used to generate lentivirus in HEK293T packaging cells as described above. The supernatants were then passed through 0.22 μm of filter (Millipore) and concentrated with LENTI-X concentrator (Clontech) according to the manufacturer's instructions. The concentrated lentiviruses were used to infect immortalized human SM cell line 11023 with polybrene at 8 μg/mL. After 48 hr of infection, GFP-positive cells were selected through cell sorting with MoFlo Astrios in the Flow Cytometry & Cellular Imaging Facility at M.D. Anderson Cancer Center. GFP-positive cells were seeded in 6-well plates for further experiments.

The method of cell cycle analysis was performed as previously described.[16] In brief, 2×10^6 of control shRNA and YY1AP1-C2,YY1AP1-C6 knockdown HEK293T, or SM cells were trypsinized, washed once in DPBS, and fixed in 70% ethanol overnight. After treatment with PBS-0.25% Triton X-100, cells were incubated with propidium iodide (Sigma) at 37°C for 15 min. Cell cycle was analyzed with Beckman Coulter Gallios analytical flow cytometer in the Flow Cytometry & Cellular Imaging Core Facility at M.D. Anderson Cancer Center.

Results

Identification of *YY1AP1* Loss-of-Function Variants as the Cause of Grange Syndrome

Exome sequencing was pursued using DNA from the unaffected parents and two affected children of the first family described with Grange syndrome, DVD047 (Figure 1A).[7] Gene variants identified by whole-exome sequencing were filtered based on the following: (1) variants that alter amino acids in proteins, including nonsynonymous, stop-loss, stop-gain, coding indel, frameshift, or splice site variants; (2) variants with a minor allele frequency (MAF) less than 0.5% in the NHLBI Exome Sequencing Project (ESP) database;[17] and (3) variants heterozygous in parents and homozygous or compound heterozygous in the affected children. The only variants that met these criteria in family DVD047 were two *YY1AP1* (MIM: 607860; GenBank: NM_001198903.1) nonsense variants: c.724C>T (p.Gln242*) and c.2390T>A (p.Leu797*) (Figure 1B). These variants were not found in 121,000 chromosomes in the ExAC database. Variant p.Gln242* is predicted to cause nonsense-mediated mRNA decay, whereas p.Leu797* is predicted to produce an 80 kDa truncated protein with 91 residues deleted. Sanger sequencing was used to validate the *YY1AP1* variants and confirm that the third affected child in the family had both variants.

YY1AP1 was sequenced in two unrelated probands with FMD and features of Grange syndrome; both of these cases were previously reported.[8,9] Two different *YY1AP1* homozygous loss-of-function mutations (c.1903_1906delTCTG [p.Glu636Profs*13] and c.2401G>T [p.Glu801*]) were identified in the probands of DVD097 and DVD093, respectively, and in both families, the parents were heterozygous for the *YY1AP1* mutations. A third child with features of Grange syndrome was determined to be homozygous for the c.664C>T (p.Gln222*; DVD112) variant through clinical exome sequencing and her parents were heterozygous for this variant. None of these *YY1AP1* variants are present in the ExAC database.

Clinical Features of Affected Individuals with *YY1AP1* Mutations

The phenotype of the affected siblings in family DVD047 was included in the first report of Grange syndrome.[7] The proband is currently 47 years of age and was previously reported to have bilateral stenoses of the vertebral and internal carotid arteries, along with occlusive lesions in the superior mesenteric artery and celiac artery complicated by bowel ischemia and requiring surgery. At the age of 32 years, she also developed coronary artery disease, involving branches of the left anterior descending artery. She has a history of patent ductus arteriosus requiring ligation and bicuspid aortic valve and long QT syndrome requiring an implantable cardioverter defibrillator. Her affected brother is 46 years old with a bicuspid aortic valve and ascending aortic aneurysm (maximal diameter 4.9 cm). He has stenosis of the origin of the celiac axis and renal arteries, along with moderate to severe narrowing of the internal carotid arteries and segmental narrowing of the middle cerebral arteries and their branches and the posterior cerebral arteries with collateral vessel formation. Additionally, hyperintense lesions were noted within the periventricular white matter distribution suggestive of

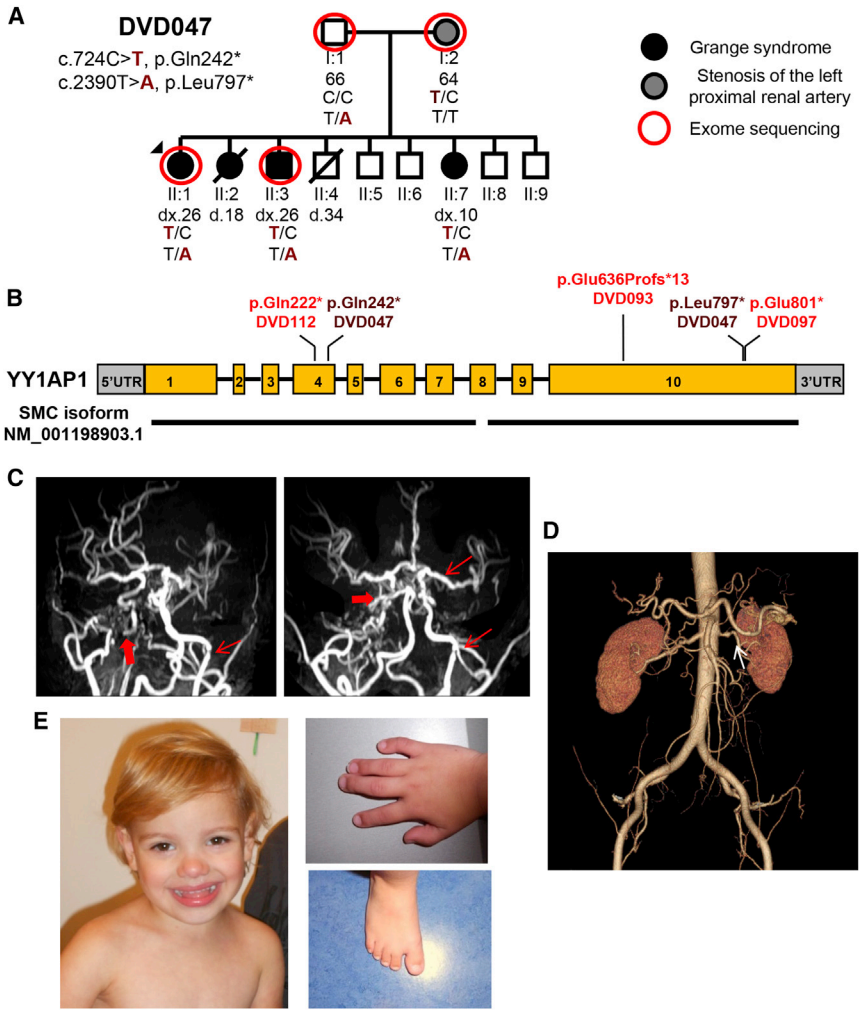

Figure 1. Identification of Homozygous *YY1AP1* Mutations in Grange Syndrome
(A) Pedigree of family DVD047 with Grange syndrome. The age at diagnosis (dx) or death (d) in years is shown below the individual symbols. A red circle indicates the individuals who underwent exome sequencing.
(B) Schematic of *YY1AP1* with exons 1 through 10 (orange boxes) and the untranslated regions (UTRs; gray boxes). The *YY1AP1* mutations identified in this study are indicated above the gene diagram. The brown letters indicate compound heterozygous mutations and red letters indicate homozygous mutations. The black line at the bottom of the diagram indicates the SMC isoform of *YY1AP1* identified to be expressed in SMCs.
(C) MR angiogram (oblique and anterior-posterior views) of individual II:7 (family DVD047) showing severe abnormalities of the cerebral vasculature with focal stenoses (thin arrow) and frank areas of discontinuity consistent with severe stenoses (thick arrow).
(D) CT angiogram of individual I:2 (family DVD047) with a heterozygous YY1AP1 p.Gln242* variant showing stenosis of the left proximal renal artery.
(E) Photographs of the proband of DVD112 with the homozygous YY1AP1 p.Gln222* variant showing features of Grange syndrome, including hypertelorism, brachydactyly of the fingers and toes, and fifth finger clinodactyly and mild cutaneous syndactyly of the second and third toes.

small vessel ischemic disease. The proband's sister is 34 years of age and has severe abnormalities of the cerebrovasculature with areas of focal and diffuse stenoses (Figure 1C). Recent imaging also showed narrowing near the origin of the celiac axis and external iliac arteries bilaterally. Their 67-year-old mother, heterozygous for the YY1AP1 p.Gln242* variant, has difficult-to-control hypertension, chronic renal insufficiency, and stenosis of the proximal left renal artery (Figure 1D).

The proband of DVD093 was reported to have cerebral, renal, and celiac artery stenosis[8] and has subsequently had two ischemic strokes. The proband of DVD097 was previously reported to have renal artery stenosis and underwent a repeat angioplasty at the age of 7 years due to renal artery stenosis (Figure S2).[9] At the age of 15 years, brain imaging showed stenoses of the internal carotid arteries, collateral vessel formation, and multiple periventricular hyperintense lesions. The proband of DVD112 was evaluated for borderline intellectual disability and clinical diagnostic exome sequencing identified homozygous *YY1AP1* c.664C>T (p.Gln222*) variants. In addition, she has mild facial dysmorphia and brachydactyly and clinodactyly of the hands and feet but no evidence of bone fragility (Figure 1E). Echocardiography was normal and CT angiography found no stenoses of the cerebral, renal, or other arteries.

Heterozygous *YY1AP1* Variants May Be a Rare Predisposing Allele for FMD

To determine whether *YY1AP1* variants contribute to medial fibroplasia or multifocal type FMD, exome-sequencing data from 282 individuals with FMD and renal artery, carotid disease, and mesenteric artery disease and 286 age-, gender-, and race/ethnicity-matched controls were analyzed for rare heterozygous or homozygous variants in *YY1AP1* predicted to disrupt protein function (Table S1). There was no increased burden of *YY1AP1* variants in the FMD-affected case subjects (Table S2). Only one heterozygous frameshift variant predicted to lead to nonsense-mediated decay was identified in the FMD cohort and none in the control subjects. The female subject with this variant had a long-standing history of FMD, involving both the renal and extracranial carotid arteries, and no family history of FMD. She presented with severe hypertension and underwent angioplasty of the right renal artery and surgical repair of a left renal artery

aneurysm. Taken together with the parent of a Grange syndrome proband with a heterozygous *YY1AP1* mutation and renal artery stenosis, these findings raise the possibility that heterozygous *YY1AP1* mutations may be a rare predisposing allele for FMD in the general population.

Characterization of YY1AP1 in SMCs and Arteries

YY1AP1 encodes YY1 associated protein 1, which was identified as an activator of the transcriptional activity of yin yang 1.[18] Although the function of YY1AP1 in SMCs is not known, YY1AP1 levels are increased due to somatic copy number alterations in hepatocellular cancer cells and acts cooperatively with YY1 as an oncogenic driver in these tumors.[15] Since *YY1AP1* is not present in the genome of rodents, human cells and tissues were used to further characterize its function. Nine isoforms of *YY1AP1* have been identified, and sequencing and quantitative assays of RNA from human aortic tissue and cultured SMCs determined that the major isoform in SMCs is a 2391 bp isoform, predicting an 88 kDa protein (Figure S1). Immunoblot analysis of fibroblasts from a control and the affected proband of Grange-affected family DVD047 showed the expected 88 kDa YY1AP1 protein in the control fibroblasts but no evidence of a full-length or truncated protein in the proband's fibroblasts (Figure S1A).

In arteries, SMCs are fully differentiated and characterized as quiescent with high cellular levels of contractile proteins (e.g., SM α-actin, calponin, and myosin heavy chain), and this differentiation is driven by TGF-β1.[19] Primary explants of human aortic SMCs are difficult to transfect or infect; therefore, immortalized aortic SMCs with human papilloma virus-16 E6/E7 were used to define a role of YY1AP1 in SMCs. Analysis of two immortalized SMC lines confirmed differentiation based on the induction of SMC contractile proteins after exposure to TGF-β1 (Figure S3).[20] Lysates from these SMCs, along with HeLa cell lysates, were fractionated to separate nuclear and cytoplasmic proteins, and YY1AP1 was detected in the nuclear fraction (Figure 2B). Immunofluorescence confirmed the nuclear localization of YY1AP1 in SMCs and demonstrated staining throughout the nucleoplasm, along with localized staining that overlapped with immunostaining of a nucleolus marker, NOP1p (Figure 2C). Immunohistochemical staining of a control human carotid artery showed that YY1AP1 was present in the nuclei of the spindle-shaped SMCs between elastin lamellae (Figure 2D). The cells in neointimal lesions in the lumen and the endothelial cells in the vasa vasorum also demonstrated nuclear staining of YY1AP1 (Figure 2D).

YY1AP1 Is a Component of the INO80 Chromatin Remodeling Complex

YY1 is a component of the ATP-dependent INO80 chromatin remodeling complex, and the INO80 complex is required for YY1 activation of gene transcription.[12] To determine whether YY1AP1 is also a component of the INO80 complex, we used HEK293T cells stably producing Flag-tagged INO80E that were previously used to characterize the components of the INO80 complex.[12] The INO80 complex was immunoprecipitated from these cells with a Flag antibody and known proteins in the multiunit INO80 complex were detected, including TIP49A and YY1. YY1AP1 was also immunoprecipitated with INO80E, suggesting that YY1AP1 is a component of the INO80 complex (Figure 3A). Immunofluorescent staining of YY1AP1 and YY1 in HEK293T cells showed that YY1AP1 and YY1 were both located in the nucleoplasm (Figure 3A). To determine whether YY1AP1 was associated with YY1 and INO80 in SMCs, SMC lysates were immunoprecipitated using an antibody against YY1AP1. Both YY1 and INO80C (a subunit of INO80) were precipitated with YY1AP1, supporting the conclusions that YY1AP1 associates with the INO80 complex and YY1 in the nucleus of SMCs (Figure 3C). Immunofluorescence showed that both YY1AP1 and YY1 are located in the nucleoplasm of SMCs, but only YY1AP1 was located in the nucleolus (Figure 3D).

Loss of YY1AP1 Leads to Increased p21/WAF/CDKN1A Levels and Cell Cycle Arrest

To determine how deficiency of YY1AP1 altered cellular functions, lentiviral short hairpin shRNA constructs previously reported to decrease levels of YY1AP1 in hepatic cancer cells were used (C2 and C6).[15] Hepatic cancer cells (HepG2) transfected with these two shRNAs specifically reduced the mRNA levels of *YY1AP1* and had no effect on *GON4L* (MIM: 610393), a gene that shares 97% amino acid sequence identity with YY1AP1 in a limited region.[15] We confirmed that both of the shRNA constructs decreased cellular levels of YY1AP1 in HEK293T cells (Figure S4A). HEK293T cells were then infected with lentivirus containing either YY1AP1 shRNA (C2) or a control shRNA, both with a selective antibiotic marker. After selection of cells with stable integration of the shRNA into the genome, western blot analyses confirmed decreased levels of YY1AP1 (Figure S4B). Decreased cellular levels of YY1AP1 was associated with decreased cell proliferation and increased levels of the cyclin-dependent kinase inhibitor, p21/WAF/CDKN1A (denoted p21; Figures S4B and S4C). Flow cytometric analysis of the cell cycle with propidium iodide staining indicated that decreased YY1AP1 in HEK293T cells leads to G1 and G2 growth arrest without evidence of increased apoptosis (Figure S4D).

To explore the function of YY1AP1 in SMCs, we investigated whether YY1AP1 levels are altered when SMCs are differentiated into quiescent, non-proliferative cells with high cellular levels of contractile proteins after exposure to TGF-β1. After TGF-β1 treatment, YY1AP1 levels increased, peaking earlier than the SMC differentiation markers smooth muscle α-actin (SM α-actin) and calponin (Figure 4A). Blocking TGF-β1 signaling with SB431542, a TGF-β type I receptor kinase inhibitor, prevented the increased levels of both YY1AP1 and SMC differentiation markers (Figure S5). To determine whether knockdown of

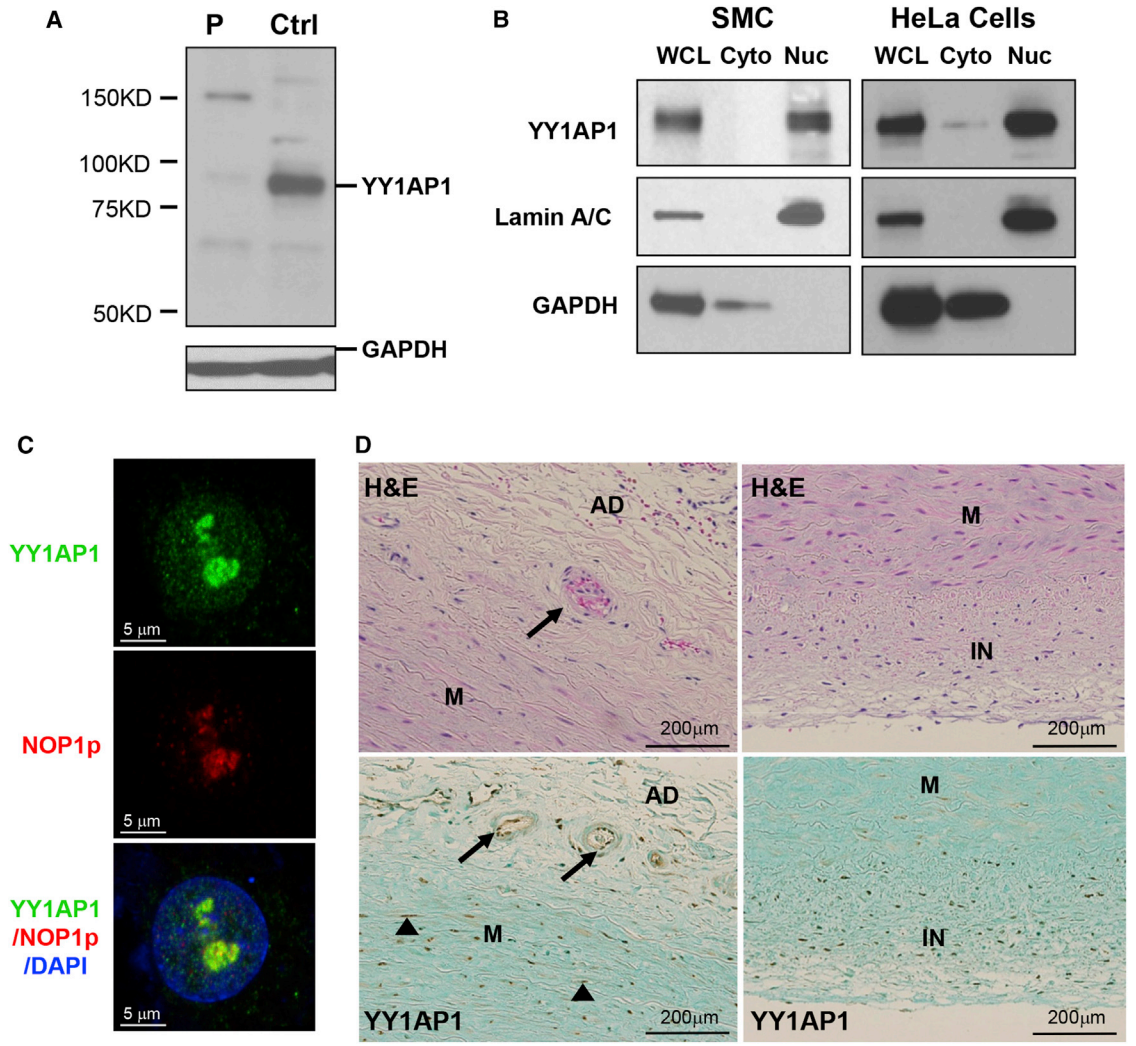

Figure 2. Characterization of YY1AP1 Protein Level and Localization in Human Smooth Muscle Cells
(A) YY1AP1 is identified as an approximately 90 kDa protein in control fibroblasts; no full-length or truncated YY1AP1 protein (predicted to be 80 kDa) is present in fibroblasts explanted from an individual with Grange syndrome (DVD047; II:7).
(B) YY1AP1 has a nuclear localization in SMCs and HeLa cells based on cell fractionation studies. Abbreviations: WCL, whole cell lysate; Cyto, cytoplasmic lysate; and Nuc, nuclear lysate.
(C) Immunofluorescence staining with YY1AP1 antibody (green), NOP1p antibody (red), and nuclear DAPI (blue) demonstrates that in SMCs, YY1AP1 is in the nucleoplasm but also co-localizes with NOP1p in the nucleolus.
(D) Hematoxylin and eosin (H&E) stain and immunohistochemical staining of YY1AP1 in control carotid artery shows YY1AP1 present in the nuclei of the SMCs between the elastic lamellae (arrowheads). Cells within the neointimal lesions in the lumen of the carotid artery (IN) and endothelial cells in the vasa vasorum (arrows) also have nuclear staining of YY1AP1. Abbreviations: IN, intima; M, media; AD, adventitia. Magnification 200×.

YY1AP1 affected the phenotype of human SMCs, green fluorescent protein (GFP) was linked to the shRNA in the construct, and the infected SMCs were sorted based on GFP to select for stable integration of the shRNA. Deficiency of YY1AP1 after transfection with both the C2 and C6 shRNA was confirmed by immunoblot analyses (Figure 4B). Loss of YY1AP1 disrupted the differentiation of SMCs, blocking the increased cellular levels of SM α-actin and SM22α after exposure to TGF-β1 (Figure 4B). Similar to the HEK293T cells, deficiency of YY1AP1 in SMCs also increased the levels of p21 (Figure 4B) and decreased cell proliferation compared with SMCs infected with control shRNA (Figure 4C). Flow cytometry showed that these changes are associated with G2 cell cycle arrest without evidence of apoptosis (Figure 4D).

Discussion

To provide initial insight into the pathogenesis of vascular diseases like FMD, we identified homozygous and compound heterozygous loss-of-function mutations in *YY1AP1* in individuals with FMD and Grange syndrome. These results confirm that a vascular phenotype with similarities to FMD can result from mutations in a single gene. The vascular phenotype associated with *YY1AP1* mutations

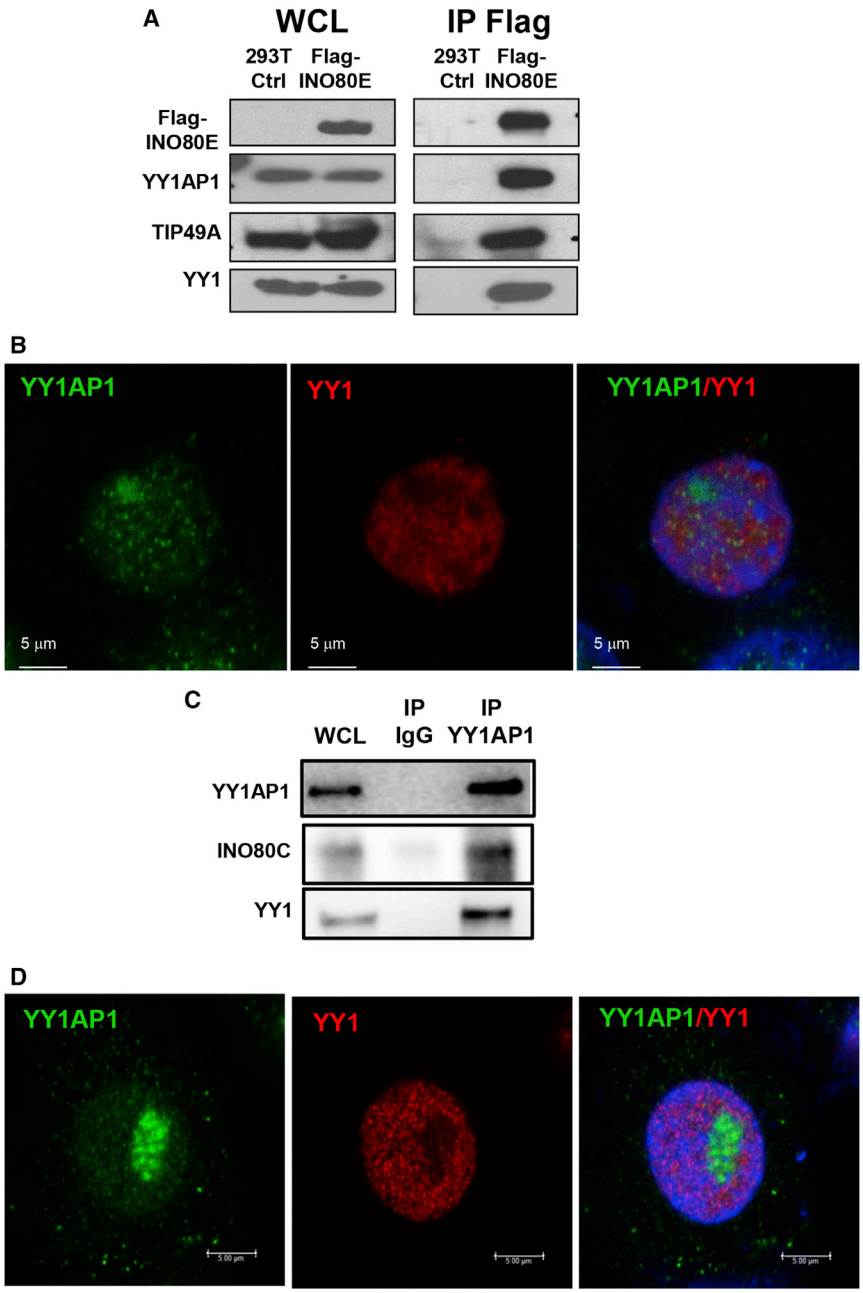

Figure 3. YY1AP1 Interacts with the INO80 Chromatin Remodeling Complex
(A) Western blots of whole cell lysates (WCLs) and lysates immunoprecipitated with Flag antibody (IP-Flag) from HEK293T and HEK293T cells with constitutive production of Flag-INO80E show pulldown of YY1AP1, TIP49A, and YY1 with Flag-INO80. These studies demonstrate that both YY1 and YY1AP1 associate with the INO80 complex in these cells.
(B) Immunofluorescence staining of YY1AP1 and YY1 shows that both are present in the nucleoplasm of HEK293T cells.
(C) Western blot of whole cell lysates (WCLs) and lysates immunoprecipitated with IgG or a YY1AP1 antibody from SMCs show pulldown of INO80C and YY1 with YY1AP1. These results demonstrate that YY1AP1 associates with the INO80 complex and YY1 in SMCs.
(D) Immunofluorescence staining of YY1AP1 and YY1 shows that both are present in the nucleoplasm of SMCs, but only YY1AP1 is located in the nucleolus.

is characterized by highly penetrant stenosis of the renal and intracranial arteries with variable involvement of the abdominal and coronary arteries. No arterial specimens are available for pathologic classification but the angiographic appearance of the majority of the affected arteries is consistent with unifocal FMD. At the same time, an affected member of DVD047 was noted to have a beaded appearance of her right renal artery,[7] and the proband of DVD093 was noted to have irregular wall contours, the typical "string and bead" appearance, of the left vertebral artery.[8] Thus, these individuals can present with both angiographic appearances of FMD.[4,21] Clinically, the vascular disease in Grange syndrome is more consistent with unifocal FMD, which affects more males than multifocal FMD and has a younger age of onset of hypertension.[4] It is notable that a parent of a Grange syndrome proband with a heterozygous *YY1AP1* variant had late-onset renal artery stenosis, and exome sequencing identified a novel heterozygous *YY1AP1* haploinsufficiency variant in an unrelated individual with FMD. These findings raise the possibility that heterozygous loss-of-function *YY1AP1* mutations are a rare predisposing allele associated with reduced penetrance for FMD in the general population.

Loss of YY1AP1 causes profound changes in SMCs characterized by decreased differentiation and proliferation, raising the question as to how these SMC phenotypic changes could lead to both stenoses and aneurysms along an artery. Similar intracranial occlusive lesions observed in Moyamoya disease result from inappropriate SMC hyperplasia due to underlying mutations, including *ACTA2* (MIM: 102620) and *NF1* (MIM: 162200).[14,22–24] De-differentiation of SMCs is associated with increased cell proliferation,[25] but the increase in p21 that occurs with loss of YY1AP1 appears to override the proliferation typically associated with undifferentiated SMCs. At the same time, de-differentiation and cell cycle arrest could prime the YY1AP1-deficient SMCs to excessively proliferate with exposure to mitogens. Therefore, spatially restricted cues within the arteries may result in both decreased differentiation and cycle arrest of YY1AP1-deficient SMCs, along with inappropriate hyperplasia of these same cells. Alternatively,

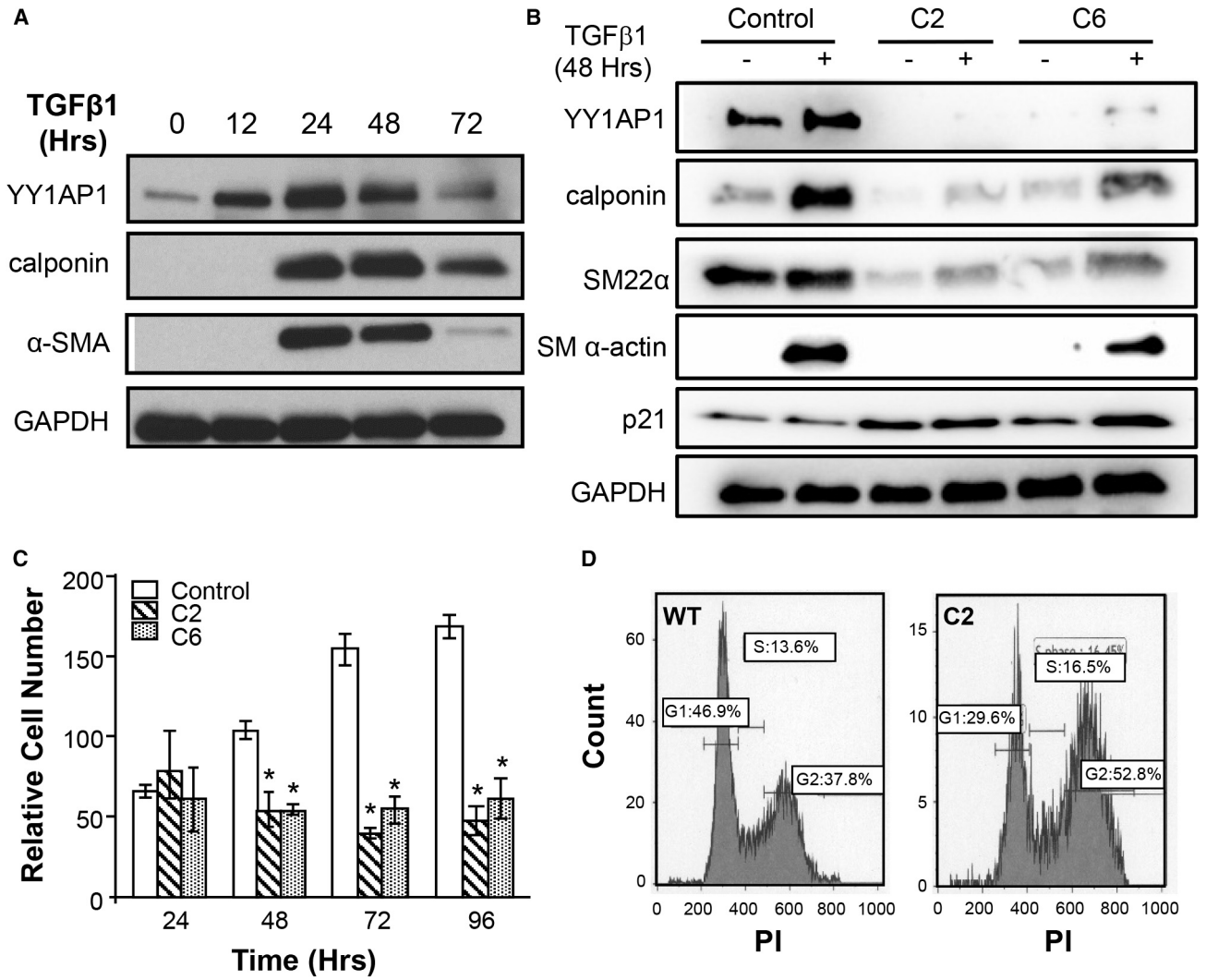

Figure 4. YY1AP1 Protein Levels Are Increased after Exposure to TGF-β1 and Decreased Cellular Levels of YY1AP1 Suppress SMC Differentiation and Proliferation

(A) Cellular levels of YY1AP1 are increased in SMCs in response to TGF-β1 exposure, with maximal levels at 24 hr after exposure.

(B and C) The shRNAs directed against YY1AP1, C2 and C6, effectively decrease YY1AP1 levels in SMCs. Knockdown of YY1AP1 prevents increased cellular levels of SMC differentiation markers, including calponin, SM22α, and SM α-actin, after TGF-β1 treatment. Decreasing YY1AP1 in SMCs increases cellular levels of the cyclin-dependent kinase inhibitor, p21 (B), and suppresses SMC proliferation (C). Error bars represent standard deviation.

(D) Flow cytometry data indicate that loss of YY1AP1 results in G2 cell cycle arrest of SMCs when compared to the control SMCs (WT).

loss of YY1AP1 could disrupt the differentiation of precursor cells into SMCs such that some cells never fully differentiate and aberrantly proliferate, while others commit to differentiation but fail to proliferate and differentiate sufficiently to properly populate arteries. These two SMC outcomes with differentiation could lead to both occlusion and aneurysms of an involved artery. Such a scenario has been observed in chimeric mice lacking both the retinoblastoma protein and a related protein, p107.[26] These mice form both retinal tumors and accelerated retinal degeneration due to altered retinal precursors that either aberrantly proliferate and form tumors, or commit to differentiation and undergo apoptosis, leading to degeneration.

The packaging of nuclear DNA into nucleosomes is required to maintain the compaction of DNA in the nucleus and control the transcription of genes. Chromatin modifying complexes are large, multiunit complexes that open up the DNA to allow for transcription of a gene.[27] INO80 is one of the human ATP-dependent chromatin remodeling complexes and shares eight core subunits with the Ino80 complex in yeast, including the subunits of INO80, actin, and actin-related proteins (Arp4, Arp5, and Arp8). INO80 also interacts with other proteins, including YY1. Our data indicate that YY1AP1 is also a component of INO80 complexes. Previously, Zhao and colleagues determined that YY1AP1 levels are increased in $EpCAM^{+}AFP^{+}$ hepatocellular cancers and acts cooperatively with YY1 to serve as an oncoprotein.[15] When YY1AP1 was depleted in hepatoblastoma cells (HepG2), the chromatin landscape was altered and cell cycle arrest and apoptosis occurred

in these cells. Interestingly, previous studies determined that individually silencing other components of the INO80 complex, including INO80 and Arp8, also leads to decreased cellular proliferation with cell cycle arrest and increased p21 protein level.[28] Our data align with the previous studies, leading to the conclusion that the INO80 complex and YY1AP1 are involved in maintaining cell cycle progression and proliferation.

Nuclear ATP-dependent chromatin remodeling complexes including INO80 also contain monomeric actin.[29] Mutations disrupting the SMC-specific isoform of actin encoded by *ACTA2* predispose to thoracic aortic disease, but a subset of mutations also predispose to occlusive vascular diseases, including Moyamoya-like cerebrovascular disease. We have demonstrated that SM α-actin can localize to the nucleus in SMCs (unpublished data), raising the possibility that the subset of *ACTA2* mutations that cause occlusive cerebrovascular disease disrupt the nuclear localization of SM α-actin, leading to defects in the transcriptional program of SMCs.[22,23] Thus, these data, along with the identification of *YY1AP1* mutations as a cause of FMD occlusive lesions, suggest that alterations in the INO80 chromatin complex may underlie major changes in the epigenetic and transcriptional programs in SMCs, thus predisposing to vascular diseases. Supporting this hypothesis is a recent report of two siblings with aortic hypoplasia, early-onset and calcific occlusive arterial lesions, and hypertension. Whole-exome sequencing identified only a single genetic variant as the cause of the vascular diseases in these siblings, a homozygous missense mutation in a gene for a subunit of INO80, *INO80D*.[30]

To conclude, homozygous *YY1AP1* mutations predispose to vascular lesions characteristic of FMD, thus indicating that FMD can result from mutations in a single gene. Grange syndrome is also associated with intellectual disability and bone abnormalities, and additional studies are needed to see how deficiency of YY1AP1 leads to these phenotypic features. It is notable that *YY1* was one of the first genes identified by trio-based exome sequencing as a candidate gene for intellectual disability.[31] The studies reported here indicate that YY1AP1 is a component of the nuclear INO80 chromatin remodeling complex and that deficiency of this protein profoundly alters SMC phenotype. Thus, these data provide insight into the pathogenesis of FMD, an unusual and poorly understood vascular disease.

Supplemental Data

Supplemental Data include five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.11.008.

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Web Resources

dbNSFP v.2.0, https://sites.google.com/site/jpopgen/dbNSFP
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
Depletion http://cadd.gs.washington.edu/score
Ensembl Genome Browser, http://www.ensembl.org/index.html
ExAC Browser, http://exac.broadinstitute.org/
GenBank, http://www.ncbi.nlm.nih.gov/genbank/
NCBI Nucleotide, http://www.ncbi.nlm.nih.gov/nuccore/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
SeattleSeq Annotation 137, http://snp.gs.washington.edu/SeattleSeqAnnotation137/

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