BRIEF REPORT

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Rare Nonconservative *LRP6* Mutations Are Associated with Metabolic Syndrome

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ABSTRACT: **A rare mutation in *LRP6* has been shown to underlie autosomal dominant coronary artery disease (CAD) and metabolic syndrome in an Iranian kindred. The prevalence and spectrum of *LRP6* mutations in the disease population of the United States is not known. Two hundred white Americans with early onset familial CAD and metabolic syndrome and 2,000 healthy Northern European controls were screened for nonconservative mutations in *LRP6*. Three novel mutations were identified, which cosegregated with the metabolic traits in the kindreds of the affected subjects and none in the controls. All three mutations reside in the second propeller domain, which plays a critical role in ligand binding. Two of the mutations substituted highly conserved arginines in the second YWTD domain and the third substituted a conserved glycosylation site. The functional characterization of one of the variants showed that it impairs Wnt signaling and acts as a loss of function mutation.**

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KEY WORDS: ***LRP6*; metabolic syndrome; mutation; coronary artery disease**

Epidemiological studies have identified the metabolic syndrome as a major risk factor for cardiovascular morbidity and mortality. Despite high degree of heritability, very few genes have been identified that may underlie the disease pathogenesis. Study of genetic causes of this trait is hindered by uncertainty about its underlying mode of inheritance, number of alleles that influence the trait, and the magnitude of the effect imparted by any single locus. We recently reported a single missense mutation (MIM #610947) in Wnt coreceptor $LRP6_{R611C}$ that underlies autosomal dominant early onset coronary artery disease (CAD) and multiple metabolic risk factors including hypertension, high-serum low-density lipoprotein (LDL) cholesterol and triglyceride (TG) levels, osteoporosis, and diabetes in a very large outlier Iranian kindred [Mani et al., 2007]. This finding suggested that mutations with large effects may occasionally underlie pathogenesis of most complex quantitative traits. This discovery generated a paradigm shift, implicating Wnt signaling in pathogenesis of metabolic syndrome and provided a valuable opportunity to investigate mechanisms that underlie the association of its diverse and complex metabolic phenotypes [Mani et al., 2007].

The spectrum and prevalence of disease-causing *LRP6* mutations in the disease population within the United States was not known. We have ascertained a large cohort of white Americans enriched for early onset CAD and multiple metabolic phenotypes and here present the results of screening 200 of these subjects for nonconservative and potentially deleterious *LRP6* (DQ047155.1) mutations.

Index cases were Caucasian subjects from across the United States, either referred to us by their primary physicians or recruited by our group at the Yale New Haven Hospital for their extreme phenotypes of angiographically documented early onset CAD (males age under 55, female under 60 years of age), presence of at least three risk factors of the metabolic syndrome and strong family history of CAD and metabolic traits. CAD was defined based on AHA criteria. All participants underwent a detailed interview and physical examination. In addition, relevant clinical and laboratory data including fasting glucose and lipids were obtained. All index cases had hyperlipidemia (TG > 150 mg/dl, LDL > 120 mg/dl), diabetes (fasting glucose ≥126 mg/dl), or impaired glucose tolerance (fasting glucose ≥100 and <126 mg/dl), and hypertension (systolic blood pressure [BP] > 139, diastolic BP > 90), and hence met three from five criteria for metabolic syndrome. The kindred of each index case with a novel and deleterious *LRP6* mutation was examined for cosegregation. For comparison, we assessed the mutation burden of the *LRP6* gene in an exome database of 2,000 healthy Northern Europeans with no signs or symptoms of CAD, hypertension, hyperlipidemia, and diabetes. For more information about the methods please refer to the Supporting Information.

The research protocol was approved by the institutional review boards of all participating institutions. All study participants provided written informed consent for clinical and genetic studies.

Three novel nonconservative missense mutations predicted to be deleterious were identified among cases.

The index case of the pedigree in Figure 1A (arrow) was a white American with elevated serum TG (270 mg/dl), diabetes, hypertension (170/105), osteoporosis, modestly elevated LDL (127 mg/dl), and early onset CAD (coronary artery bypass surgery at age 54). He was overweight (BMI 29 kg/m^2) and had low HDL cholesterols (32 mg/dl). A missense mutation (c.1418G>A (p.Arg473Gln)) was

Additional Supporting Information may be found in the online version of this article.

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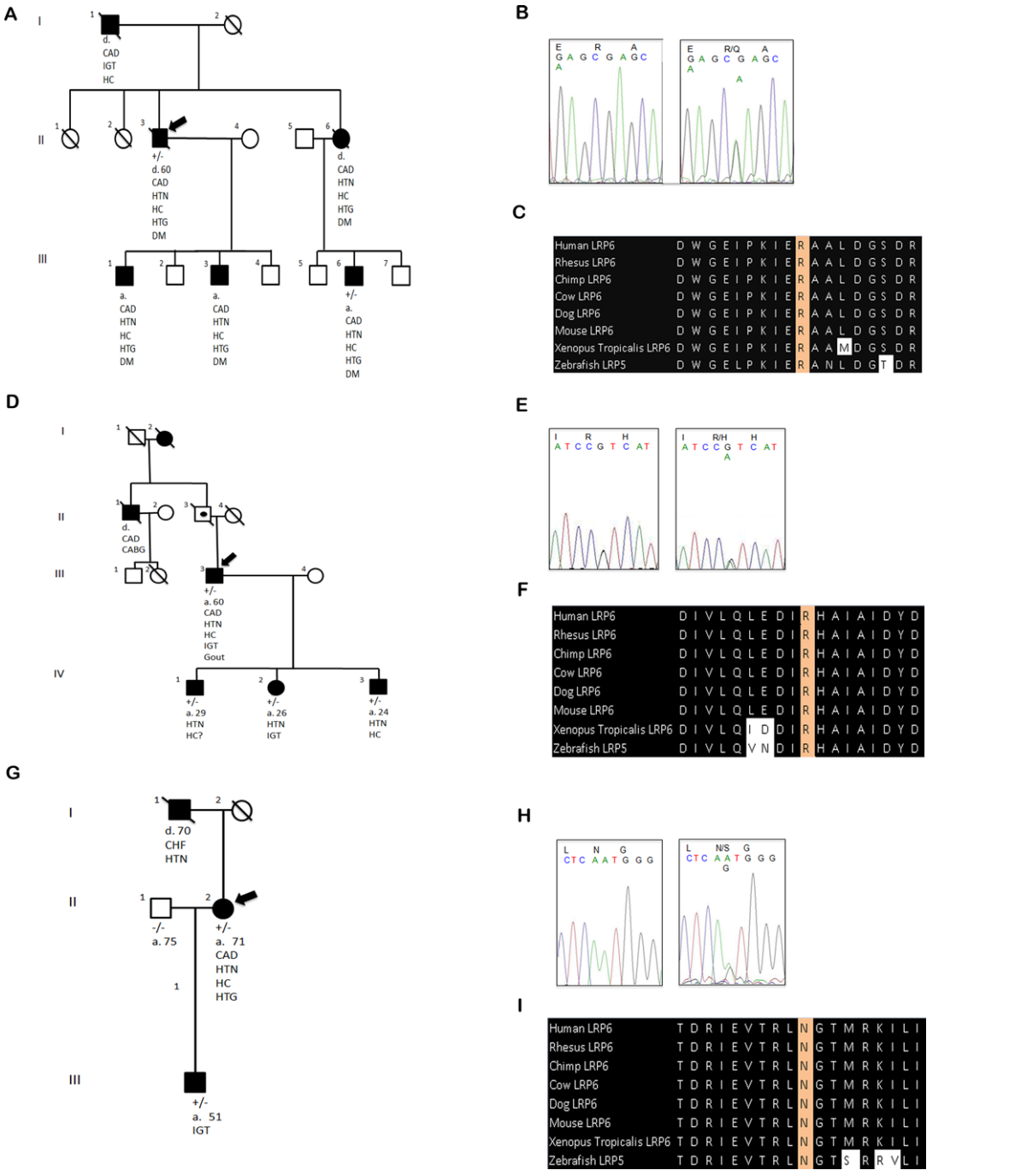

Figure 1. **A–I:** Three Pedigree structures of three kindreds with novel nonconservative *LRP6* mutations: R473Q, R360H, and N433S (**A, D, G**). Pedigree structure for each kindred is shown with the index case indicated by the arrow. Individuals with early CAD or metabolic syndrome traits are indicated by black symbols; individuals. Individuals who were not studied are indicated by symbols with dots. Circles represent females; squares represent males. Symbols with a slash through them indicate deceased subjects. **B, E, H:** Exon sequences from an unaffected family member (left) and affected family member (right), demonstrating sequence area of interest are shown. A portion of the amino-acid sequence of the second beta-propeller domain of *LRP6* is shown from diverse vertebrate species (**C, F, I**). These segments are completely conserved in orthologs from Human to Zebrafish. MI, myocardial infarction; CAD, coronary artery disease; CABG, coronary artery bypass grafting; HTN, hypertension; HC, hypercholesterolemia; HTG, hypertriglyceridemia; DM, type II diabetes; IGT, impaired glucose tolerance; CHF, congestive heart failure.

identified in exon 7 of *LRP6*, which leads to glutamine for arginine substitution (Fig. 1B) and is predicted to be deleterious by PolyPhen and SIFT. The substituted residue is conserved in orthologs from human to zebrafish (Fig. 1C), and lies in the highly conserved second propeller domain. This mutation was not present in dbSNP, the NHLBI Exome Variant Server, or the entire Yale database of 5,000 exomes (which includes 2,000 healthy Northern Europeans). He had a sister who had died from myocardial infarction and two sons with CAD and metabolic syndrome. His affected nephew, who was known to have hypertension, hyperlipidemia, diabetes, and CAD and later died from myocardial infarction, carried the same mutation.

The index case of the pedigree in Figure 1D (arrow) had CAD, hypertension, elevated serum TG and LDL cholesterol levels. He had a normal bone density. Although he was not known to have impaired glucose tolerance, a 2 hr OGTT at Yale Human Research Unit revealed abnormal fasting (114 mg/dl) and elevated post glucose ingestion serum glucose levels (1 hr glucose 270 mg/dl). He also had history of gout and was treated for high uric acid levels. His father had died from cancer in young age and was only known to have early onset osteoporosis, but his paternal uncle and grandmother had died from CAD. He had three children (daughter age 26, two sons age 24 and 29), who were too young for having sign or symptoms CAD. However, they all had early onset hypertension despite normal BMIs. The two sons had both elevated LDL cholesterols (LDL > 130 mg/dl), and the daughter had impaired glucose tolerance (FBS > 100 mg/dl).

Genetic screening revealed that the index case and all his children carried an *LRP6* missense mutation (c.1079G>A (p.Arg360His)) in exon 6, which led to a histidine for arginine substitution (Fig. 1E). The substituted residue is conserved in orthologs and paralogs from human to zebrafish (Fig. 1F), lies in the second propeller domain, and is predicted to be deleterious by both PolyPhen and SIFT. The R360H variant is reported in two subjects with unknown phenotypes in the NHLBI Exome Variant Server (allele frequency 0.015%), but is absent in the Yale Exome database. Since Exome Variant Server consists of exome data from large clinical studies of patients with CAD, finding a disease variant with very low allele frequency was expected.

The index case in Figure 1G had CAD, diabetes, hypertension, high LDL (>180), hypertriglyceridemia, since age 52. Her father was a semiprofessional boxer, who was excluded from athletic competition for uncontrolled hypertension, a "heart condition," and later died from heart failure. Her 41 years old son had impaired glucose tolerance. The mutations identified in these subjects (c.1298T>C (p.Asp433Ser) in exon 6 (1H) substituted asparagine 433 for serine within a highly conserved glycosylation site (Fig. 1I) that confers stability to the protein and protects it against decay. The mutation is absent in 1000 Genomes, NHLBI Exome Variant Server and Yale Exome database.

In comparison, no novel nonconservative *LRP6* mutation was present in the 2,000 healthy Northern European controls, indicating low mutation burden of *LRP6* gene in healthy populations.

These novel mutations have been reported to a locus-specific database and the report of the mutations can be found at http://www.lovd.nl/LRP6.

We have previously shown that $LRP6_{R611C}$ allele is a loss of function mutation, which impairs Wnt signaling. We explored the functional significance of R473Q mutation using a Lef1 reporter TOPFlash assay. NIH3T3 cells were transfected with pCMV vectors containing wild-type *LRP6*, $LRP6_{R473Q}$ or empty vectors and their effect on Lef1 promoter was assessed by expression of the luciferase gene. Cells expressing $LRP6_{R473Q}$ showed a 40% reduction in Lef1-mediated expression of luciferase when stimulated with Wnt-3a conditioned medium compared to wild-type *LRP6* (Fig. 2A). This finding once again implied that disease-causing *LRP6* alleles are often loss of function mutations.

Metabolic syndrome is a highly heritable trait, however, its underlying genetic causes is not understood. As a cluster of quantitative traits, it is likely a complex disorder caused by multiple mutations and gene–environment interactions. A number of quantitative traits like hypertension and hypercholesterolemia have been shown to be significantly influenced by rare alleles with major effects [Cohen et al., 2004; Ji et al., 2008]. The strong heritability of the metabolic syndrome similarly suggests contribution of genes with moderate to large effects. We have previously identified a rare *LRP6* allele (R611C) that underlies an autosomal dominant form of early onset atherosclerosis and metabolic syndrome. This finding shifted the existing paradigm and underscored the role of alleles with large effects in pathogenesis of metabolic syndrome and CAD. Wnt signaling was implicated in pathogenesis of metabolic syndrome, type II diabetes and atherosclerosis and led to discovery of novel pathways that govern them [Go and Mani, 2012; Keramati et al., 2011; Liu et al., 2008; 2012; Singh et al., 2013; Ye et al., 2012]. The finding raised the question as to whether rare LRP6 alleles with large effects contribute to metabolic syndrome and/or early onset CAD in disease populations, enriched for extreme forms of CAD and metabolic syndrome, in the United States. The spectrum of these mutations was previously not known.

In screening of the cohort of 200 white Americans with CAD and metabolic syndrome, we identified three novel, nonconservative, and predictably deleterious mutations in *LRP6* gene. In comparison, no novel variant was present in exome database of 2,000 healthy Northern Europeans. This difference in mutation burden surpassed genome-wide significance (P<2.37E-06) and was strongly suggestive of disease association of the identified alleles. The finding was further confirmed by the cosegregation analysis. All three independent mutations cosegregated with metabolic phenotypes in individual kindreds. This distinguishes these mutations from other potentially innocent mutations in this gene that may have no effects on the metabolic traits. Most, if not all mutation carriers had hypertension and hypercholesterolemia; a significant number had impaired glucose tolerance or diabetes and most subjects older than 50 years old had CAD.

LRP6 is a Wnt coreceptor in both canonical and noncanonical pathways [Schweizer and Varmus, 2003]. The canonical Wnt signaling pathway consists of cascades of events that initiate after Wnt proteins bind to the cell-surface receptor frizzled and its coreceptors LRP5/6 (Fig. 2B). These trigger translocation of Axin to the cell surface and CDY-dependent phosphorylation of LRP6 by CK and GSK3-β leading to inactivation of GSK3-β and stabilization of β-catenin [Schweizer and Varmus, 2003]. The final steps include translocation of β-catenin from cytoplasm to the nucleus where it interacts with Tcf/Lef family of transcription activators to promote gene expression. This pathway was initially known for its role in diverse processes during development, including segmentation, CNS patterning, and control of asymmetric cell divisions, cell polarity and tissue polarity during morphogenesis [Wodarz and Nusse, 1998]. Most recently, common genetic variations in Wnt5B [Kanazawa et al., 2004] and TCF7L2 (a member of TCF family of transcription factors) have been associated with susceptibility to type 2 diabetes [Sladek et al., 2007]. The effects imparted by the common variants are inherently too small to be detectable in biological system. The *LRP6* alleles identified in patients with metabolic syndrome and CAD are all rare nonconservative mutations, which impart large effects on Wnt signaling. These effects are readily verifiable in vivo and in vitro and have reproducibly shown to cause loss of function by independent groups [Berendsen et al., 2011; Mani et al., 2007].

Strikingly, all three novel mutations reside in the second propeller domain (Fig. 2C), which has a critical role in ligand binding. The previously identified R611C allele resides in second EGF domain [Go and Mani, 2012; Mani et al., 2007]. The crystal structure of LRP6 has revealed the importance of intermolecular interactions between EGF and propeller domain for the tertiary structure of the protein. For instance, the positively charged arginine 611 in the second EGF domain is proposed to build a salt bridge with negatively charged glutamic acid 477 in the propeller domain [Cheng et al., 2011]. Cysteine substitution is predicted to disrupt the salt bridge, leading

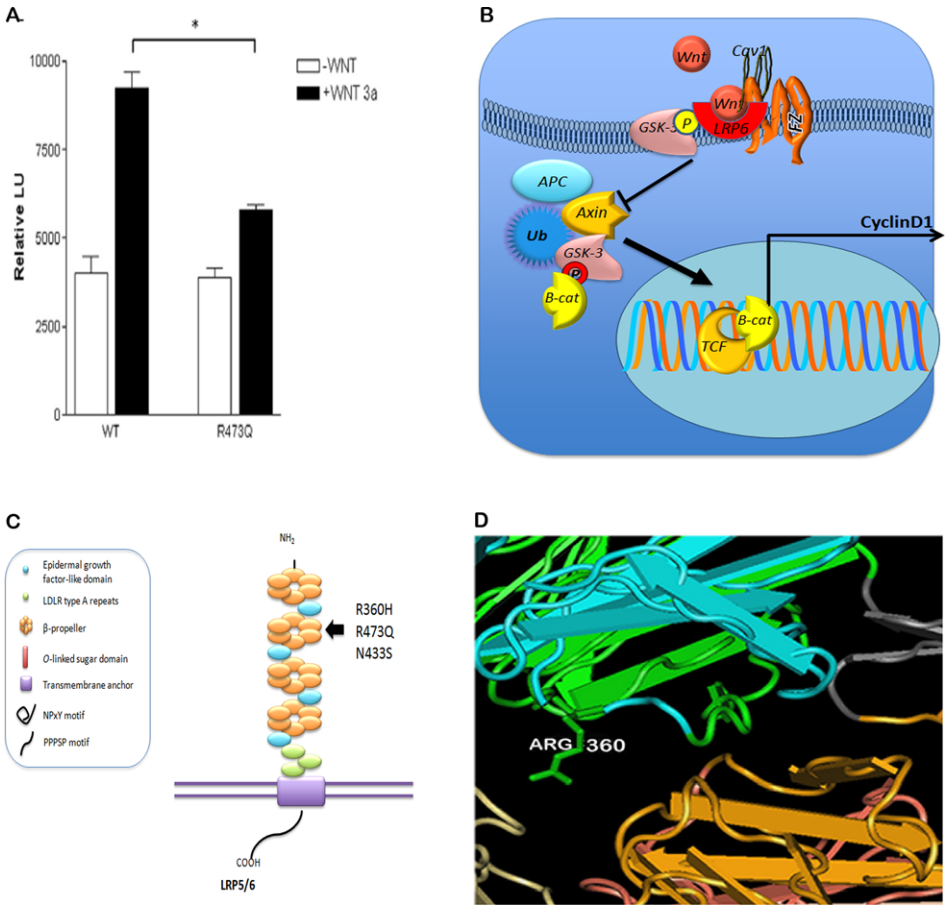

Figure 2. **A:** *LRP6*$_{R473Q}$ impairs Wnt signaling. NIH3T3 cells were transfected with plasmids encoding HA-tagged wild-type (WT) or *LRP6*$_{R473Q}$. Wnt signaling was assessed using a TopFash assay under control of Lef1 promoter. The results are showed as the mean and standard of error of the mean of three experiments. RLU, relative light units. **B:** Schematic of the canonical Wnt signaling pathway. **C:** Schematic of the *LRP6* structure. *LRP6* protein consists of four propeller structures, each followed by an epidermal growth factor-like repeat (blue) and three low-density lipoprotein (LDL) receptor-like ligand binding domains (green). These are followed by a single transmembrane domain and C-terminal cytoplasmic tail (purple). All three novel *LRP6* mutations lie in the second beta-propeller domain of the gene. The position of R611C allele in the second EGF domain is also shown. **D**: Crystal structure of *LRP6* depicting R360 at the surface of second beta-propeller domain.

to instability of the protein and impaired ligand binding. Arginine 473 is positioned on the surface of the molecule (Fig. 2D) and its substitution is expected to similarly impair the protein structure and ligand binding. Accordingly, the TOPFlash assay revealed that the 473Q allele impairs Wnt signaling. The functional characterization of R360 substitution was not carried out, but based on its position on the surface it likely affects ligand binding. Asparagine 433 has shown to be a site of glycosylation [Cheng et al., 2011], a function that is also critical for protein stability.

Our study confirms an association between single rare and non-conservative mutations in *LRP6* and the cluster of metabolic risk factors, and underscores the role of altered Wnt signaling in metabolic syndrome and atherosclerosis. Although not defined as a component of metabolic syndrome, most *LRP6* mutation carriers have elevated LDL cholesterol levels. LRP6 is a member of the LDL receptor (LDLR) family of proteins with unique characteristics [Go and Mani, 2012]. Our studies have shown that LRP6 is involved in binding and internalization of lipoproteins [Go and Mani, 2012] and plays an important role in LDLR-dependent LDL uptake [Ye et al., 2012]. Our studies in primary human and mice cells and different cell lines have revealed that LRP6 colocalizes with LDLR, forms a complex with LDLR and ARH and is required for clathrin-mediated endocytosis of the LDLR and LDL–LDLR complex [Ye et al., 2012]. Macrophages and skin fibroblasts of *LRP6*$_{R611C}$ mutation carriers exhibit impaired LDL clearance [Liu et al., 2008]. These findings suggest that like most other inherited lipid disorders, defective LDL-dependent LDL clearance is a major contributor to elevated LDL levels [Hobbs et al., 1992].

The role of Wnt/LRP6 in atherosclerotic processes in humans is not understood. Postmortem studies have underscored the key role of vascular smooth muscle cell (VSMC) proliferation in development of CAD and plaque erosion in young subjects, women and patients with diabetes. PDGF signaling plays a critical role in recruitment and proliferation of VSMCs. Our studies have shown that LRP6 and PDGF receptor β (PDGFR-β) are excessively expressed and colocalize in tunica intima and adventitia of the atheromatous plaques of atherosclerotic human coronary arteries [Keramati et al., 2011]. Pathway-based gene expression studies of the hematopoietic cells of the LRP6 mutation carriers have identified PDGF signaling as one of the most significantly altered pathways. Our studies have shown that LRP6 forms a complex with PDGFR-β, enhances its lysosomal degradation and reduces VSMC proliferation in response to PDGF; these functions are severely impaired by LRP6$_{R611C}$ mutation [Keramati et al., 2011]. Accordingly, PDGF signaling was abrogated

by wild-type LRP6 but was significantly activated by $LRP6_{R611C}$ compared to empty vector in human aortic VSMC. These findings implicated LRP6 as a critical inhibitor of PDGF-dependent cell cycle activity in VSMCs and its altered function as a major inducer of VSMC proliferation.

Altered Wnt signaling has been implicated in type II diabetes by numerous studies [Singh et al., 2013]. Common variants of *TCF7L2* have been associated with type II diabetes in most ethnic groups around the globe [Scott et al., 2007]. While the effects imparted by common variant are inherently small and not detectable in biological systems, our studies of the rare nonconservative *LRP6* mutations in outlier kindreds have led to groundbreaking discoveries. These studies have shown that Wnt/LRP6 activation of TCF7L2 enhances insulin receptor transcription while it inhibits mTOR activation [Singh et al., 2013]. These findings imply that impaired Wnt signaling, either due to *LRP6* mutation or genetic variations in *TCF7L2*, contributes to the metabolic syndrome and diabetes via altered function of both nutrient sensing and insulin signaling pathways.

Taken together, our study demonstrates that rare independent variants with large effects may underlie the association of diverse components of the metabolic syndrome, and motivates future genetic studies for rare disease alleles with moderate to large effects in patients with CAD and metabolic syndrome.

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