remain to be determined, the function of IKKε in type I IFN signaling is to guide the transcriptional machinery to a subset of ISGs required for a direct antiviral response. By contrast, the IKKe-independent genes may function primarily in regulating the IFN signaling machinery, which is required for the integration of innate and adaptive immune systems. These results emphasize the importance of the interplay between local and systemwide antiviral mechanisms. Even when the systemwide antiviral response is intact, defects in the local response lead to an increase in viral load, ultimately overwhelming the immune defenses.

References and Notes
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**LRP6 Mutation in a Family with Early Coronary Disease and Metabolic Risk Factors**

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Coronary artery disease (CAD) is the leading cause of death worldwide and is commonly caused by a constellation of risk factors called the metabolic syndrome. We characterized a family with autosomal dominant early CAD, features of the metabolic syndrome (hyperlipidemia, hypertension, and diabetes), and osteoporosis. These traits showed genetic linkage to a short segment of chromosome 12p, in which we identified a missense mutation in LRP6, which encodes a co-receptor in the Wnt signaling pathway. The mutation, which substitutes cysteine for arginine at a highly conserved residue of an epidermal growth factor–like domain, impairs Wnt signaling in vitro. These results link a single gene defect in Wnt signaling to CAD and multiple cardiovascular risk factors.

Coronary artery disease (CAD) due to atherosclerosis results in myocardial infarction (MI) and is the leading cause of death worldwide (1). Epidemiologic studies and clinical intervention trials have established the key roles of specific risk factors for CAD, including smoking, hypertension, high low-density lipoprotein (LDL) cholesterol, high triglycerides, low high-density lipoprotein (HDL) cholesterol, and diabetes mellitus (2–4). Surprisingly, many of these risk factors cluster with one another more often than expected by chance (5, 6). This metabolic syndrome is recognized to be a common cause of CAD; however, the molecular mechanisms that unify their association have been obscure.

The marked increase in risk of early cardiovascular mortality to a second monozygotic twin when the first has died from early CAD provides evidence for a strong genetic effect and supports investigation of families with early disease (7). Such studies have the capacity to identify genes and pathways whose altered function impart large effects on CAD outcome; these may provide insight into basic mechanisms that are also involved in common forms of disease and that may be manipulated for health benefit.

From a screen of patients and families with CAD, we identified one extreme outlier kindred with an extraordinary prevalence of early CAD. Kindred CAD-100 is of Iranian ancestry, ascertained via Subject II-7 (table S1), who presented with MI at age 48. CAD risk factors included hypertension, hyperlipidemia, and diabetes mellitus; he had never smoked and his body mass index (BMI) was 24. Evaluation revealed critical stenosis of all three major coronary arteries, which led to coronary artery bypass grafting. His course was complicated by progressive atherosclerosis of the grafts and internal carotid arteries. At age 62, he suffered a low-impact hip fracture and was found to have very low bone mineral density of unknown cause (z score of –3.4 at the femoral neck of his intact hip). He died from a stroke at age 72.

Among 58 blood relatives of the index case, 28 were diagnosed with early CAD (MI, angina, or sudden cardiac death) at or before age 50 (men) or 55 (women) (Fig. 1). Of these, 23 have died from CAD (mean age of death, 52 years). In contrast, kindred members without early CAD died at a mean age of 81. This familial clustering is noteworthy given that early CAD and early CAD death are uncommon in the general population (8, 9).

Detailed clinical data were obtained for all available kindred members, including 13 affected with early CAD, 5 free of early CAD at or beyond the age threshold of 50 years (men) and 55 (women), and 9 younger asymptomatic members (CAD phenotype unknown; mean age 35 years) (table S1). Cardiac risk factors before or at presentation among affected subjects were surprisingly homogeneous, including high fasting LDL cholesterol in all (mean 176 ± 4 mg/dl, nl < 130 mg/dl), high fasting triglycerides in 90% (mean 240 mg/dl, nl < 150 mg/dl), marked hypertension in all (mean 175/103 mm Hg, nl < 140/90, typically diagnosed after age 40), and type II diabetes mellitus in 77% (fasting blood glucose > 126 mg/dl; typically diagnosed after hypertension and hyperlipidemia). Despite high triglycerides, HDL levels were normal in all, and only one had a history of smoking. Nearly all of the affected subjects met criteria of the NIH National Cholesterol Education Program for metabolic syndrome based on the presence of diabetes, high triglycerides, and hypertension (10).

Although obesity is strongly associated with metabolic syndrome and each of these risk factors,
it is noteworthy that obesity is absent among affected subjects (mean BMI 24.6, none greater than 26). In contrast to these affected subjects, the five unaffected family members all had normal levels of blood pressure (mean 116/81 mm Hg), LDL cholesterol (mean 97.8 mg/dl), and triglycerides (mean 60.75 mg/dl), and type 2 diabetes mellitus was absent. Finally, among younger subjects with CAD phenotype unknown, all nine had high LDL levels and seven had high triglyceride levels, whereas hypertension and glucose intolerance were less frequent (table S1).

The marked clustering of early CAD and risk factors in this kindred suggests a strong genetic component. Among sibships in which all subjects are beyond the age threshold for onset of CAD, the offspring of single affected parents yielded 17 affected and 15 unaffected subjects, and male-to-male transmission is present; in addition, the union of two affected first cousins yielded six affected and one unaffected offspring. Similarly, LDL levels are strongly bimodal in the kindred, with all members having either high levels (157 to 195 mg/dl) or low levels (92 to 105 mg/dl), and these levels cosegregate with early CAD. This extreme familial clustering and segregation of phenotypes within the kindred is unlikely to be explained by chance or multifactorial determination and provides strong evidence that early CAD is transmitted as a highly penetrant autosomal dominant trait.

Nineteen family members were available for genetic studies, including seven with early

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**Fig. 1.** Relationships of members of kindred CAD-100 are shown. The index case is indicated by the arrow. Numbered individuals correspond to those in table S1. Individuals with early CAD are indicated by black symbols; individuals without CAD who are beyond age 50 (men) or 55 (women) are shown as unfilled symbols; and individuals who are without symptomatic CAD, are below these ages, and have high LDL levels (range 157 to 192) are shown as half-black, half-gray symbols. 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. Genotypes of informative microsatellite and SNP markers are shown in their chromosomal order below the symbol for each individual and their distance in centimorgans and megabases from 12pter is indicated. Segments of the haplotype seen in the index case which segregate with CAD and/or high LDL levels are indicated by shaded boxes. The presence of the wild-type LRP6 or LRP6_1212C mutation is indicated by a plus sign or a red asterisk, respectively. Subjects III-6, III-7, and III-8 are offspring of a homozygous mutation carrier and hence obligate mutation carriers; subjects III-3, III-4, and III-5 are also statistically likely to be offspring of a homozygous carrier. This explains the high proportion of mutation carriers in generation III.
A genome-wide analysis of linkage was performed using Affymetrix 10K Gene Chips. We analyzed linkage using all single-nucleotide polymorphisms (SNPs) by applying two pre-specified models of the trait locus—a conservative model that specified 90% penetrance, 1% phenocopies, and allele frequency of 0.001 and a stringent model that specified 99% penetrance, 0.1% phenocopies, and allele frequency of 0.0001 (11). Results under both models demonstrated significant evidence of linkage to a segment of chromosome 12p, and no other interval yielded a logarithm (base 10) of the odds ratio (lod score) greater than 1.5. Under the stringent model, the maximum multipoint lod score was 4.4 for linkage of CAD within the 2.7-cM interval flanked by loci rs2213177 and rs747726 (Figs. 1 and 2A; odds ratio 25,000:1 in favor of linkage). Results of linkage under the conservative model were similar. 

Linkage was confirmed by genotyping 11 highly polymorphic di- and tetranucleotide repeat markers this interval of 12p (Fig. 1), and vir-}

tual indistinguishable results were obtained when all markers were combined in the analysis. The observed lod scores approximate the theoretical maximum under the specified models. It is worth noting that the index case, who was the offspring of affected first cousins, was homozygous across this interval, implying that he was homozygous for the underlying disease-causing mutation.

The lod-1 interval spans only 750,000 base pairs and contains only six annotated genes: ETY6, BCL2L14, LR6P, MANSC1, LOH12CRI, and DUSP16 (fig. S1). Among these, LR6P (LDL receptor–related protein 6) is noteworthy. LR6P and its close paralog LR5P serve as coreceptors with frizzled proteins (members of the G protein–coupled receptor family) for Wnt ligands (12). Mice deficient for LR5P develop hypercholesterolemia and impaired glucose tolerance on a high-fat diet (13), and LDL levels are markedly increased on the apolipoprotein E-deficient background (14). In addition, deficiency for either LR5P (in the human and mouse) or LR6P (in the mouse) results in early severe osteoporosis (15, 16). This latter observation recalls the unexplained results in early severe osteoporosis of the index case. Evaluation of kindred members identified two additional affected males with early hip fractures (ages 48 and 68) and low bone density in the three additional affected subjects studied; in contrast, bone density was normal in the one unaffected subject studied (table S1).

These findings motivated further evaluation of LR6P. Direct sequencing of all exons and intron-exon boundaries of LR6P in the index case revealed a single variant, a homozygous mutation that introduces a missense substitution, R611C (Fig. 2B). R611 lies in an epidermal growth factor (EGF)–like domain (fig. S2) and is conserved among LR6P orthologs ranging from Xenopus to human (Fig. 2C); it is also found in mammalian LR5P, LR2P, and LR3P. This mutation precisely cosegregated with early CAD in the kindred, was absent among 400 unrelated Iranian and 3600 U.S. Caucasian control chromosomes, and is predicted to be deleterious by the PolyPhen and Sift programs. Sequencing of the other five genes in the lod-1 interval identified no other missense or splice site mutations.

We next considered the impact of this mutation on CAD risk factors. Analysis revealed complete linkage of LR6Pbasic and high LDL, with a lod score of 5.5 (Fig. 2A, odds of 316,000:1 in favor of linkage). The difference in mean LDL levels between mutation carriers and noncarriers is significant (170 ± 12 mg/dl versus 98 ± 5 mg/dl, P = 6 × 10−6; Table 1). Because high LDL levels are found in all mutation carriers,
regardless of age, this trait can serve as a biomarker of the mutation in subjects too young to manifest CAD. Similarly, LRP6<sub>R611C</sub> imparts significant effects on triglyceride levels, blood pressure, fasting blood glucose, and prevalence of diabetes (Table 1). No significant effects were seen on HDL levels or body mass index. Finally, all five mutation carriers studied have low bone densities, each with values expected in less than 12.5% of the population (P < 0.001).

The functional significance of this LRP6 mutation was explored by expression in NIH3T3 cells (17) (Fig. 3). In these cells, expression of LRP6 potentiates Wnt signaling, assayed as LEF-1 mediated expression of luciferase. In the absence of added Wnt 3a, LRP6<sub>R611C</sub> showed a 49% reduction of induced signaling compared with that of wild-type LRP6 (P < 0.01). The addition of low doses of Wnt 3a also showed markedly reduced signaling with LRP6<sub>R611C</sub> (42% reduction, P < 10<sup>-5</sup>). At high doses of Wnt 3a, however, Wnt signaling through LRP6<sub>R611C</sub> does not differ significantly from that of the wild type (P = 0.48). Measurement of total LRP6 expression by Western blotting and cell surface expression measured by specific binding to Dkk-1 demonstrate similar levels of wild-type and mutant LRP6 (fig. S3, A and B). These findings are consistent with an impaired biochemical function of LRP6<sub>R611C</sub>.

Our findings establish a causal link between LRP6 mutation and early CAD with high LDL, high triglyceride levels, hypertension, diabetes, and low bone density. The evidence includes strong priori evidence of segregation of the disease as an autosomal dominant trait in this kindred, linkage of this trait and underlying risk factors to a single small genomic interval, identification of a single rare mutation in the linked interval that alters a highly conserved amino acid, biochemical evidence that the mutation impairs function of the encoded protein, and evidence from mouse models that mutations in orthologs commonly identify individuals with inherited or acquired impairment in Wnt signaling.

Because loss-of-function mutations in LRP5 (12, 15) and LRP6 (16) result in reduced bone density, the osteoporosis among LRP6<sub>R611C</sub> carriers lends further support to the functional significance of this mutation. Moreover, recent epidemiologic studies have found strong association of osteoporosis and CAD (22). Our observations suggest that osteoporosis and CAD can be pleiotropic consequences of impaired Wnt signaling, raising the question of whether the co-occurrence of osteoporosis and CAD might commonly identify individuals with inherited or acquired impairment in Wnt signaling.

Our findings underscore emerging evidence implicating effects of altered Wnt signaling on cardiovascular risk factors. Common intronic variants in the Wnt-responsive transcription factor TCF7L2 result in altered insulin secretion and type II diabetes mellitus (23). Similarly, rare mutations in other Wnt-related transcription factors cause maturity onset diabetes of youth (24, 25). The LRP6<sub>R611C</sub> mutation confers effects not only on many risk factors but on CAD outcomes as well. The ubiquitous expression of LRP6 (26) supports the possibility of pleiotropic effects in diverse tissues. Further investigation of Wnt signaling in patients with early CAD, metabolic syndrome, and its components may provide new insight into disease pathophysiology and approaches to prevention of these disorders.

Table 1. Comparison of phenotypes in carriers and noncarriers of LRP6<sub>R611C</sub>. Means ± standard deviation are shown for quantitative traits. All kindred members with measured values were included for LDL, triglyceride, HDL, and BMI measurements. For blood pressure, fasting blood glucose, and diabetes, results for subjects over age 40 are shown.

<table>
<thead>
<tr>
<th>Trait</th>
<th>LRP6&lt;sub&gt;R611C&lt;/sub&gt; carriers</th>
<th>Noncarriers</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL (mg/dl)</td>
<td>170 ± 12</td>
<td>98 ± 5</td>
<td>6 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>209 ± 7</td>
<td>68 ± 20</td>
<td>1 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>57 ± 8</td>
<td>56 ± 7</td>
<td>0.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3 ± 2.6</td>
<td>24.4 ± 1.6</td>
<td>0.13</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>168 ± 21</td>
<td>116 ± 5</td>
<td>8 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>100 ± 14</td>
<td>81 ± 7</td>
<td>0.0025</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>159 ± 43</td>
<td>80 ± 3</td>
<td>0.001</td>
</tr>
<tr>
<td>Diabetes (yes/no)</td>
<td>11/4</td>
<td>0/5</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Fig. 3. LRP6<sub>R611C</sub> impairs Wnt signaling. NIH3T3 cells were transfected with plasmids encoding wild-type (WT) or mutant hemagglutinin-tagged LRP6 and Wnt reporter genes and incubated with indicated concentrations of purified Wnt 3a protein followed by an assay of Wnt signaling (LEF-1–dependent expression of luciferase) (27). The results are shown as mean and standard error of the mean of quadruplicate experiments. RLU, relative light units.

References and Notes
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Emulating Membrane Protein Evolution by Rational Design

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How do integral membrane proteins evolve in size and complexity? Using the small multidrug-resistance protein EmrE from Escherichia coli as a model, we experimentally demonstrated that the evolution of membrane proteins composed of two homologous but oppositely oriented domains can occur in a small number of steps: An original dual-topology protein evolves, through a gene-duplication event, to a heterodimer formed by two oppositely oriented monomers. This simple evolutionary pathway can explain the frequent occurrence of membrane proteins with an internal pseudo–two-fold symmetry axis in the plane of the membrane.

Membrane protein evolution is often accomplished by gene-duplication and gene-fusion events (1), and high-resolution membrane protein structures have disclosed an unanticipated number of cases where homologous N- and C-terminal domains are related by an approximate two-fold symmetry axis either perpendicular to or in the plane of the membrane. In the former case, each domain has an even number of transmembrane helices and the two domains are oriented parallel to each other in the membrane, whereas in the latter case each domain has an odd number of transmembrane helices and the two domains are antiparallel. Representative examples of membrane proteins with parallel domains are LacY (2), GlpT (3), the Sav1866 ABC transporter (4), AcrB (5), EmrD (6), and the ADP/ATP carrier (7); among membrane proteins with antiparallel domains are LeuT (8), SecY (9), BtuCD (10), AQP1 (11), GlpF (12), AmtB (13), the CIC H+/Cl− exchange transporter (14), and NhaA (15).

A particularly notable mode of gene-duplication-based membrane protein evolution was suggested recently by an analysis of proteins in the small multidrug-resistance (SMR) family (16). The best-studied SMR protein is EmrE from E. coli, an inner-membrane drug-efflux pump with four transmembrane helices. EmrE likely has a dual topology with identical copies of the protein forming an antiparallel homodimer (or higher oligomer) composed of Nin-Cin and Nout-Cout monomers (16–20), although some data suggest a parallel Nin-Cin, Nout-Cout dimer (21, 22). Membrane protein topology is largely governed by the positive-inside rule (23)—i.e., loops rich in Lys and Arg residues tend to orient toward the cytoplasm.

EmrE has only a weak K+/R bias (24), as would be expected for a dual-topology protein, and it is encoded by a singleton gene with no homologous genes nearby on the chromosome. In contrast, many genes encoding SMR proteins, in both E. coli and other bacteria, appear as pairs on the chromosome, and the two encoded proteins have large but opposite K+/R biases and therefore likely insert into the membrane with opposite orientations (16). These findings immediately suggest an evolutionary scenario in which a singleton gene encoding a dual-topology protein undergoes a gene duplication, after which the two resulting proteins become fixed in opposite orientations by evolving opposite K+/R biases. Possibly, oppositely oriented proteins occasionally mutate back to a dual topology (25). In a final step, the two oppositely oriented proteins may fuse into a single polypeptide that folds into an antiparallel structure.

Materials and Methods

Supporting Online Material

www.sciencemag.org/cgi/content/full/315/5816/1278/DC1

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