CELIA2A mutations predispose to early-onset atherosclerosis and metabolic syndrome and affect plasma insulin and platelet activation

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Factors that underlie the clustering of metabolic syndrome traits are not fully known. We performed whole-exome sequence analysis in kindreds with extreme phenotypes of early-onset atherosclerosis and metabolic syndrome, and identified novel loss-of-function mutations in the gene encoding the pancreatic elastase chymotrypsin-like elastase family member 2A (CELIA2A). We further show that CELIA2A is a circulating enzyme that reduces platelet hyperactivation, triggers both insulin secretion and degradation, and increases insulin sensitivity. CELIA2A plasma levels rise postprandially and parallel insulin levels in humans. Loss of these functions by the mutant proteins provides insight into disease mechanisms and suggests that CELIA2A could be an attractive therapeutic target.

Metabolic syndrome is a cluster of inherited risk factors for coronary artery disease (CAD)1–4, which in outlier kindreds with early-onset CAD may be caused by single-gene mutations5–7. In this study, we present a cohort of 30 North European index cases with early-onset CAD and metabolic syndrome. Combined linkage and gene burden analyses led to the identification of multiple independent mutations in CELIA2A, which encodes the chymotrypsin-like elastase family member 2A (CELIA2A).

CELA2A was primarily known as an ‘exocrine’ pancreatic elastase that preferentially cleaves A-acyetyl-L-alanyl-L-alanyl-L-alanine/proline methyl ester and forms a sodium dodecyl sulfate (SDS)-resistant complex with alpha-1-antitrypsin (A1AT). The physiological function of CELIA2A outside the exocrine pancreas was not known. Here, we characterize the CELIA2A protein in vitro and in vivo, and explore the effects of human mutations on its diverse metabolic functions. Using systems biology, we discovered that CELIA2A is a circulating protein that impacts diverse biological processes, including insulin secretion, degradation and sensitivity. Our analyses show that impaired regulation of plasma insulin is a major consequence of disease-inducing CELIA2A mutations. The potential to exploit disease pathways makes CELIA2A an appealing target for treating diabetes and its complications.

Results
Clinical characterization of individuals and families with early-onset CAD and metabolic syndrome. We recruited 30 index cases with early-onset CAD (age of onset: at or before 30 years in men and 35 years in women) and extended their kindreds. This led to the identification and recruitment of a multiplex kindred with 25 affected individuals that we named CAD-2001 (Fig. 1a). The index case was an American female of European ancestry with an extensive family history of CAD, and first ST elevation myocardial infarction at age 28 years (arrow). Her CAD risk factors included hypertension (HTN), type 2 diabetes (T2D), hypertriglyceridemia (HTG) and obesity. She underwent a coronary artery angiography and percutaneous intervention of two major coronary arteries. Among 53 extended blood relatives of the index case, 25 were diagnosed with early-onset CAD (a myocardial infarction diagnosis by enzyme and electrocardiogram, angiographic diagnosis of CAD, or sudden cardiac death) with a median age of 43 years, and 11 had died from CAD (mean age of death: 52 years). All affected individuals traced their ancestry to a common ancestor, and male-to-female transmission of the phenotype was present.

Detailed clinical data were available for all 11 living family members with CAD, two younger family members (mean age: 30 years) with unknown CAD status, and 12 living unaffected family members (Supplementary Table 1). All affected individuals met the National Cholesterol Education Program criteria for metabolic syndrome with surprisingly homogeneous risk factors, including markedly elevated triglycerides (mean 287.1 mg dl−1; NI < 150 mg dl−1), HTN, low high-density lipoprotein (HDL) levels; mean 35.1 mg dl−1; NI > 50 mg dl−1) and T2D (fasting blood glucose > 126 mg dl−1 or on glucose-lowering drugs). In contrast, all 12 unaffected family members had normal triglycerides (mean: 100.5 mg dl−1) and normal HDL levels, and none had T2D. The two younger family members with unknown CAD status had both HTG and HTN. The familial clustering and pattern of inheritance of these clinical
features were consistent with the effect of a highly penetrant autosomal dominant trait.

Whole-exome sequencing identifies mutations in CELA2A underlying CAD. We carried out a gene-burden analysis using whole-exome data of all 30 index cases. An independent genetic analysis was carried out in the CAD-2001 pedigree (Fig. 1a), using 35 DNA samples of the affected and unaffected family members. The unbiased analysis of whole-exome sequence data from affected family members of CAD-2001 with angiographically diagnosed CAD led to the identification of a single shared 4.8-megabase-pair chromosomal interval (11,102,837–15,933,568) on chromosome 1, flanked by two single nucleotide polymorphisms: rs225874 and rs848210 (Fig. 1b). Within the linked interval, we identified a single non-conservative missense mutation (1:15,789,885G>A; hg19) in exon 6 of CELA2A. The mutation results in substituting asparagine (p.D121N) and perfectly segregates with CAD in all 12 affected family members. No other non-conservative segregating mutations were identified, and the presence of a shared structural variant was excluded by whole-genome sequencing in two remotely related affected individuals (II-10 and IV-2). The p.D121N amino acid substitution was predicted to be damaging by Polymorphism Phenotyping version 2 (PolyPhen-2; score: 1.00) and Sorting Intolerant From Tolerant (SIFT; score: 0).
assessments\(^4\). A two-point linkage analysis revealed a logarithm of the odds score of 6.9 (odds ratio: 7,758,879:1 in favor of linkage) between CAD and the CELA2A locus. The individuals with HTG and HTN and unknown CAD status were also carriers of p.D121N. Hence, we considered the impact of this mutation on CAD risk factors. The linkage between the CELA2A locus and HTN was stronger compared with CAD (logarithm of the odds score = 7.48; Supplementary Table 2). The difference in mean triglyceride levels between mutation carriers and non-carriers was also significant (Supplementary Table 3). Because HTN and HTG are found in most mutation carriers (Supplementary Table 1 and Supplementary Fig. 1a), these traits could serve as biomarkers of the mutation in individuals too young to manifest CAD. Similarly, p.D121N was associated with body mass index (BMI; Supplementary Fig. 1b) and T2D (Supplementary Table 3). There were lower plasma cortisol levels in mutation carriers versus non-carriers, while plasma glucagon-like peptide 1 and globular adiponectin levels were not significantly different (Supplementary Fig. 1c–e).

Strikingly, the analysis of whole-exome sequencing in all 30 index cases showed the highest burden of novel non-conservative mutations in the CELA2A gene. Two CELA2A missense mutations and one splice site mutation were identified in three unrelated index cases. All mutations were novel and non-conservative, and the missense mutations were predicted to be deleterious by SIFT and PolyPhen-2. No other gene was identified with more than two mutations in all 30 exomes. Only two predicted deleterious CELA2A mutations were identified in 4,000 control chromosomes of healthy individuals of European ancestry (Yale control exome database). Statistical analysis by Mann–Whitney U-test showed that recurrent mutations in this gene in 30 subjects are unlikely to have occurred by chance (\(P < 3.1 \times 10^{-10}\); Monte Carlo simulation).

The mutation 1:15,789,253C>A, which results in p.L85M substitution, was found in a female of European ancestry (Fig. 1c) with a strong family history of CAD, who had undergone percutaneous coronary intervention to the left anterior descending artery at 34 years. This female had HTG (triglycerides > 234 mg dl\(^{-1}\)) and low HDL (26 mg dl\(^{-1}\)). The third mutation was found in a 24-year-old male of European ancestry with CAD, as well as his uncle (Fig. 1d), who had developed CAD in his early 40s. Both individuals and the deceased father of the index case had HTG (triglycerides > 350 mg dl\(^{-1}\)), HTN and T2D. This mutation was a single nucleotide substitution one base pair upstream of the splice site for exon 6 (splice donor c.639+1, 1:15,792,640G>C). Based on predictive programs and functional analysis (see below), this mutation leads to an alternative splice site and generates a stop codon (Fig. 1e).

Interestingly, the common intronic CELA2A variants rs1042010 and rs3820068 have been associated with elevated systolic blood pressure by independent genome-wide association studies\(^{10-12}\) (\(P < 3 \times 10^{-13}\) and \(P < 1 \times 10^{-12}\), respectively). Further analysis revealed that the rs1042010(G) allele is in disequilibrium with the expression single nucleotide polymorphism rs3753326(A) (\(D'\) normalized coefficient of linkage disequilibrium) = 0.8674; \(r^2\) (square of the correlation coefficient) = 0.7134; \(X^2 = 3,572.8956\); \(P < 0.0001\), which is reportedly located in an open chromatin region (http://www.regulomedb.org/snp/chr1/15790973) and is associated with reduced CELA2A transcription in the adrenal gland (GTEx database; \(P = 2.4 \times 10^{-5}\)).

Human CELA2A mutations abolish its elastase activity. Wild-type (WT) and mutant CELA2A constructs were expressed in HEK293T cells, and the cell lysate and conditioned media were examined for CELA2A protein expression. Western blot analysis of the conditioned media showed the expected 25-kDa band and a 75-kDa band that was resistant to denaturing conditions before loading (Fig. 2a), was found to be enriched in CELA2A and few other proteins by proteomic analysis (Supplementary Table 4), and was absent in p.D121N and p.T70M (lanes 3 and 4). Expression of the CELA2A construct containing the splice site variant led to the generation of a weak band of somewhat lower molecular weight in cell lysate that was undetectable in the conditioned medium.

We then examined the elastase activities of the WT versus mutant CELA2A proteins. It is notable that D121 constitutes one of the three amino acids of the catalytic triad (Fig. 2b), which forms a low-barrier hydrogen bond between its \(\beta\)-carboxyl group and N delta 1 of His73, stabilizes the positive charge on its active site, and plays a crucial role in the nucleophilic activity of Ser216 (ref. \(^2\)). Accordingly, the elastase activity of p.D121N-CELA2A on a synthetic substrate containing the canonical cleavage site\(^2\) was considerably lower than that of WT-CELA2A (Fig. 2c). Purified p.L85M, p.T70M and splice variant CELA2A proteins also exhibited a dramatic reduction in elastase activity. The elastase activities of co-expressed WT and mutant CELA2A proteins were significantly less than the sum of their individual activities, consistent with a dominant, negative effect of the mutant proteins (Fig. 2d). Western blot analysis of purified A1AT treated with the purified CELA2A proteins showed that, in contrast with WT-CELA2A\(^7\) (75-kDa band; Fig. 2e, lane 2), p.D121N-, p.T70M- and splice variant CELA2A form no complex, and p.L85M-CELA2A forms a small complex with A1AT (lane 6).

**CELA2A is a circulating protein.** The array of traits associated with the CELA2A mutations suggested a systemic effect of the encoded protein, and led to examination of its tissue distribution. The specificity of the antibody for Western blot analysis was verified by preblocking with rCELA2A (recombinant human CELA2A) and testing the levels of secreted CELA2A from CELA2A-transfected HEK293T cells or serum fractions (Supplementary Fig. 2a,b), and by immunohistochemistry via the staining of mouse skeletal muscle before and after rCela2a injection (Supplementary Fig. 2c).

The enzyme-linked immunosorbent assay (ELISA) kit was validated for cross-reactivity between the human and mouse using vehicle and different titers of purified human WT- and p.D121N-CELA2A and rCela2a (Supplementary Fig. 2d). In mice, the highest messenger RNA and protein levels were found in the pancreas (Fig. 3a,b). We further examined the tissue localization of Cela2a by immunohistochemistry; strong signals were also observed in the adrenal gland and small intestine (Supplementary Fig. 3a–c). We then examined the expression of CELA2A in human surgical specimens (Fig. 3c). The most intense staining was observed in exocrine secretions of the pancreas. A small fraction of cells in pancreatic islets also stained positive for CELA2A. In addition, adrenal cortex, intestinal glands and colonic lymphoid follicles stained positive for CELA2A. CELA2A of human cadaveric pancreas, liver and white adipose tissue were further verified by western blot analysis (Supplementary Fig. 3d).

Interestingly, data extracted from the Gene Expression Omnibus showed elevated expression of CELA2A in disease states in tissues that
have otherwise no or low expression levels of CELA2A, including skeletal muscle of insulin-resistant obese subjects\(^1\), and \(\beta\) cells of subjects with T2D\(^2\) (Supplementary Fig. 4a,b).

We subsequently examined and verified the presence of CELA2A in human plasma. Western blot analysis of human serum with CELA2A antibody recognized 75- and 25-kDa bands that were absent when Cela2a antibody was preblocked with rCEL-A2A (Fig. 3d). Plasma CELA2A and glucagon levels were assayed by validated ELISA and were found to be higher in p.D121N- than WT-CELA2A carriers (Fig. 3e and Supplementary Fig. 4c). However, the total plasma elastase activity was reduced by greater than 1.75-fold in mutation carriers compared with non-carriers (Fig. 3f), indicating a substantial contribution of CELA2A to total circulating elastase activity.

Plasma CELA2A levels rise postprandially and parallel plasma insulin levels. Since T2D was a common trait among mutation carriers, we posited that CELA2A might regulate insulin secretion. We first measured baseline and postprandial plasma CELA2A, insulin and C-peptide levels in healthy controls (mean age: 35 years) by ELISA (Fig. 3g–i). The responses to a meal for plasma CELA2A, insulin and C-peptide were virtually identical (\(R^2\) coefficients: 0.90 and 0.88, respectively) (Fig. 3j,k), while glucagon levels showed an inverse relationship with plasma CELA2A (Supplementary Fig. 4d,e; \(R^2\) coefficient: \(−0.72\)). The parallel surge of CELA2A and insulin raised the possibility that CELA2A acts as an insulinotropic peptide.

We subsequently compared the effect of oral versus intravenous glucose on plasma CELA2A levels in obese non-diabetic individuals subjected to a hyperglycemic clamp and oral glucose tolerance test (OGTT). A hyperglycemic clamp designed to keep glucose levels clamped around 200 mg d\(\text{l}^{-1}\) led to higher plasma insulin and CELA2A levels (Fig. 3l). The OGTT of the same individuals showed a parallel rise of plasma CELA2A and insulin (Fig. 3m). The plasma CELA2A-to-glucose ratios at 60 min were similar between the OGTT and hyperglycemic clamp studies (Supplementary Fig. 4f).
Fig. 3 | Mouse and human Cela2a tissue expression, human CELA2A plasma levels and activities. a, b, Cela2a messenger RNA (a; means ± s.e.m.) and protein levels (b) in different mouse tissues (n = 6 mice). c, Immunohistochemical staining of human tissues using CELA2A-specific antibody. Controls without primary antibodies are also shown (n = 1). Scale bars: 100 μm. d, Western blot analysis of CELA2A in human serum (75- and 25-kDa Cela2a bands). Both bands are absent in the parallel western blot performed on human serum using CELA2A-specific antibody preblocked with rCela2a (n = 2 replicated experiments). The whole blot is shown in Supplementary Fig. 7. e, f, Total plasma CELA2A levels (e) and elastase activity (f) (means ± s.e.m.) measured in p.D121N carriers versus non-carriers, measured by validated ELISA assay (n = 7 in each group). Significance was determined by two-tailed Student’s t-test (**P < 0.01). g–i, Baseline and postprandial plasma CELA2A (g), insulin (h) and C-peptide (i) levels (means ± s.e.m.; n = 8). Significance was determined by two-tailed Student’s t-test (**P = 0.0009; **P < 0.0001 and **P = 0.0006, respectively). j, k, Correlation of baseline and postprandial plasma insulin (j) and C-peptide (k) with plasma CELA2A in random healthy subjects (n = 8; age: 20–30 years), presented as values. Correlation coefficients for j and k were calculated using GraphPad Prism 8. The dashed lines denote the 95% confidence area of the graph. l, m, Plasma glucose and CELA2A levels (means ± s.e.m.) during hyperglycemic clamps (l) and OGTT (m) in obese non-diabetic subjects (n = 5).
There was an inverse relationship between plasma CELA2A and glucagon levels in both studies (Supplementary Fig. 4g,h).

**CEL&A** promotes glucose-induced calcium ion-dependent insulin secretion. To explore the in vivo effect of CELA2A on glucose and triglyceride homeostasis, 3-month-old male hyperlipidemic and diabetic Ldlr<sup>−/−</sup> mice on a Western diet were injected intravenously with exogenous endotoxin-free (<0.1 EU ml<sup>−1</sup>; L00338; GenScript) mouse rCela2a (2.5 mg kg<sup>−1</sup>) and to form a complex with the truncated α<sub>1</sub>-subunit and protein kinase A<sup>18,19</sup>, leading to their activation. Proteomics/phosphoproteomics analysis of INS-1 cells (rat insulinoma cell line) treated with rCela2a exhibited detectable activation of protein kinase A and higher levels of CACNA1A (calcium channel subunit alpha-1A) and CACNA1D (calcium channel subunit alpha-1D) phosphorylation compared with the vehicle alone or untreated cells (Supplementary Table 5). Accordingly, intracellular Ca<sup>2+</sup> transient was dramatically increased after rCela2a treatment of INS-1 cells in the presence of 9 mM glucose compared with vehicle (Fig. 4c)<sup>19</sup>.

**Fig. 4** Cela2a induction of insulin secretion in vivo and in vitro. a–c. Plasma glucose (a), C-peptide (b) and the insulin-to-glucose ratio (c) (means±s.e.m.) in hyperlipidemic and hyperglycemic Ldlr<sup>−/−</sup> mice injected with rCela2a (recombinant mouse Cela2a) or vehicle (n=5 mice in each group in two replicated experiments). Significance was determined by one-way ANOVA (**P<0.01**) in a, **P=0.0096; **P=0.0196** in b and c, respectively. d. Insulin responses (means±s.e.m.) of rat islets to rCela2a (50 nM) or vehicle at 2.5 and 9 mM glucose concentrations. KCl was used as a positive control and test of viability (n=3 replicated experiments). Significance was determined by two-tailed Student’s t-test (**P<0.01**). e. Area under the curve (AUC), assessed by percentage fluorescence multiplied by time of Ca<sup>2+</sup> transient (means±s.e.m.) on treatment of INS-1 cells with rCela2a, showing greater calcium transient in rCela2a-treated cells compared with 9 mM glucose medium served as a control or vehicle-treated cells (n=3 replicated experiments). Significance was determined by two-tailed Student’s t-test (**P=0.0045**). Cmpd, compound; KCl30, 30 mM.
Fig. 5 | Insulin secretion, degradation and sensitivity of WT-CELA2A and p.D121N-CELA2A proteins. a, b, Insulin (a) and C-peptide (b) secretion of rat islets in response to WT-CELA2A (50 nM), p.D121N-CELA2A or vehicle at 2.5 and 9 mM glucose concentrations, represented as violin plots showing mean, minimum and maximum values (n = 4 in two replicated experiments). **P < 0.01 by one-way ANOVA. KCl was used as a positive control and test of viability. c, Calcium transient representations of INS-1 cells treated with WT- versus p.D121N-CELA2A. d, AUC of Ca^{2+} transient in the cells shown in c, represented as dot plots (means ± s.e.m.; n = 3 biologically independent experiments). Panels c and d show greater calcium transient in WT-CELA2A-treated cells. Significance in d was determined by one-way ANOVA with a 95% CI (**P < 0.0001). e, Insulin content (means ± s.e.m) in the media of rat islets treated with WT- and p.D121N-CELA2A for 48 h, represented as dot plots (n = 3 replicated experiments). Significance was determined by two-sided Student’s t-test with a 90% CI (*P < 0.05). f, UPLC analysis of insulin digestion by WT-, p.D121N-, p.T70M- and p.L85M-CELA2A (n = 2 replicated experiments). Arrows show fragmented insulin. g, Western blot analysis of insulin/mammalian target of rapamycin signaling pathways in 3T3L1 cells treated with insulin, WT- or p.D121N-CELA2A and predigested insulin with WT- or p.D121N-CELA2A (n = 2 biologically independent experiments; quantification in Supplementary Fig. 6; whole blot in Supplementary Fig. 7). IRS and pIRS denote insulin receptor substrate and its phosphorylation, respectively. pAKT denotes phosphorylated AKT, also known as protein kinase B (PKB).
In addition, there was upregulation of proteins involved in vesicle transport and insulin secretion. Accordingly, gene enrichment analysis of proteins/phosphoproteins increased by \( >1.5\)-fold after rCela2a treatment (adjusted \( P \leq 0.01\)) identified ‘regulation of insulin signaling’ as one of the three top-ranked gene sets (Supplementary Table 6).

**Impaired insulin degradation and sensitivity of mutant CELA2A proteins.** We compared the effect of WT-CELA2A versus p.D121N-CELA2A on insulin secretion in rat islets. Rat islets treated with WT-CELA2A showed only a trend towards higher secretion of insulin but significantly higher C-peptide secretion at the 9 mM glucose concentration compared with p.D121N-CELA2A (Fig. 5a,b, second phase). Accordingly, WT-CELA2A triggered greater intracellular \( \text{Ca}^{2+} \) transients in INS-1 cells compared with p.D121N-CELA2A at 9 mM glucose (Fig. 5c,d). These findings indicated that p.D121N substitution impairs the activity of CELA2A in augmenting glucose-dependent calcium receptor activation and insulin secretion. However, the absence of a substantial difference in insulin as opposed to C-peptide secretion between WT-CELA2A and p.D121N-CELA2A may be explained by the possibility that CELA2A proteolytically degrades insulin and loss of this function by p.D121N-CELA2A may contribute to the elevated insulin levels observed in non-diabetic CELA2A mutation carriers (IV-2 and IV-3 in the D121 kindred had documented fasting insulin levels of 142 and 79.5 mg dl\(^{-1}\), respectively). Insulin secretion and degradation have been shown for insulin-degrading enzymes\(^1\) and some pancreatic proteases\(^2\). Thus, we measured intact insulin levels in rat islets treated with WT- and p.D121N-CELA2A for 48 h in the presence of 9 mM glucose. Strikingly, after 48 h, there were lower insulin levels in the medium of rat islets treated with WT- versus p.D121N-CELA2A (Fig. 5e). We verified the effect of purified WT and different mutant CELA2A proteins on insulin degradation using ultra performance liquid chromatography (UPLC). The analysis showed that WT but not mutant CELA2A proteins (with the exception of p.L85M) cause insulin fragmentation (Fig. 5f).

It has been shown that loss of the insulin-degrading peptide Carcinoembryonic antigen-related cell adhesion molecules (CEACAM1) causes hyperinsulinemia and triggers insulin resistance\(^3\). Therefore, we examined the effect of WT- and p.D121N-CELA2A (300 pg ml\(^{-1}\)) on the insulin signaling pathway in 3T3L1 cells after 24 h starvation at 0, 15, 30 and 45 min. Western blot analysis showed that WT-CELA2A but not p.D121N-CELA2A enhances insulin signaling activity after 15 min (Fig. 5g; quantification in Supplementary Fig. 6a–e). Insulin pre-incubated with WT-CELA2A showed lower signaling activity compared with insulin alone and insulin pre-incubated with p.D121N-CELA2A, consistent with its proteolytic degradation by WT-CELA2A.

**CELA2A inhibits platelet activation.** In screening for proteins with extracellular CELA2A target site(s)\(^4\), we came across integrin A2B, which binds integrin \( \beta_3 \) to generate the GPIIb/IIa receptor complex. On binding of soluble fibrinogen, the receptor complex triggers platelet aggregation/activation. The effect of purified WT and mutant CELA2A (300 pg ml\(^{-1}\)) proteins on human platelet aggregation (\( n = 4 \) healthy volunteers; age 30–60 years; with collagen as the agonist) was measured using a Lumi-Aggregometer (Chrono-Log). WT-CELA2A reproducibly reduced while p.D121N-CELA2A increased the platelet aggregation compared with vehicle (Fig. 6a). Platelet aggregation was also visualized by a platelet adhesion assay using confocal microscopy (Fig. 6b). The degree of aggregation and size of platelet aggregates were dramatically increased after treatment with p.D121N-CELA2A compared with WT-CELA2A. We carried out a PAC1 (platelet \( \alpha IIb \beta 3 \) activation specific antibody) binding assay to examine the structural changes in the platelet GPIIb/IIa complex that mark their activation in response to WT-versus p.D121N-CELA2A\(^2\). WT-CELA2A reduced while p.D121N-CELA2A increased PAC1 binding compared with vehicle-treated platelets (Fig. 6c). The apoptotic tendency of platelets treated with WT- or p.D121N-CELA2A was assessed based on the measurement of mitochondrial inner membrane potential (\( \Delta \Psi_m \)) using tetra-methylrhodamine ethyl ester dye (TMRE)\(^2\). TMRE-negative cells were reduced by WT-CELA2A treatment by about 3.5–5.0%, and increased by p.D121N-CELA2A between 3 and 5% (Fig. 6d), suggesting enhanced platelet apoptosis by the mutation.

Treatment of purified GPIIb/IIa integrin with WT-CELA2A but not p.D121N-CELA2A resulted in cleavage of the ITGA2B component and generation of a smaller 75-kDa band (Fig. 6e). The mass spectrometry-based analysis of the 75-kDa band showed that it was composed of CELA2A and ITGA2B fragments, and this was further confirmed by western blot analysis (Fig. 6f). Platelet aggregation was also increased by p.T70M- and p.L85M-CELA2A proteins (Fig. 6g). Together, these findings suggest that WT-CELA2A binds to and cleaves ITGA2B, thus limiting the activation of platelets. In contrast, mutant CELA2A promotes hyperactivation and aggregation of platelets.

**Discussion**
Our study establishes an association between mutations in the CELA2A gene and early-onset CAD and metabolic syndrome. Our success in identifying single-gene mutations underlying CAD and metabolic traits is the result of a unique approach to ascertain kindreds with clustering of extreme phenotypes, including early age of onset of CAD and HTG. The evidence for disease causality of CELA2A variants includes identification of multiple independent non-conservative mutations in disease subjects, perfect disease segregation of a novel loss-of-function mutation in a large
multiplex kindred, unraveling the disease relevant functions of the protein, and strong biochemical data indicating loss of function of the mutant proteins.

Most carriers of the CELA2A mutations had early-onset CAD, HTG, HTN and T2D, and met the criteria for the metabolic syndrome. These observations indicate the broad and important effect of CELA2A mutations, and suggest that the clustering of the individual risk factors imparts extremely high cardiovascular risk to mutation carriers. Very little is known about the function of CELA2A and its role in human disease. The demonstration of CELA2A as a circulating protein in humans and mice, its expression in diverse tissues, and the reduced plasma elastase activity in mutation carriers versus non-carriers are all indicative of its important systemic effects.
Our results indicate that WT-CELA2A proteolytically degrades GPIIb/IIIa and reduces platelet aggregation. This is in contrast with the proteolytic degradation of GPIIb/IIIa by chymotrypsin, which results in the generation of different fragments and enhanced platelet aggregation\textsuperscript{26}. This function, which is impaired by CELA2A mutations, extends the potential therapeutic application of this protein.

Elastases target different components of the extracellular matrix\textsuperscript{28} and may facilitate the regeneration of elastin fibers in the aorta\textsuperscript{29}. Association between decreased serum elastase activity, increased serum elastase inhibitor activity and increased accumulation of carotid plaque has been shown in longitudinal human studies\textsuperscript{30}. These findings differ from those related to neutrophil elastases and their putative roles in atherogenesis\textsuperscript{31} and insulin resistance\textsuperscript{32}, and may be attributed to the structural and biological differences between distinct elastases, their tissue-specific activities or the relatively narrow range of their physiological concentrations.

In conclusion, our genetic studies associate mutations in CELA2A with a clinical phenotype that is characterized by early-onset CAD, as well as metabolic syndrome. In addition, our in vivo and in vitro studies identify CELA2A as a peptide with systemic functions and as a potential candidate for developing novel therapeutics for treating atherosclerosis, thrombosis and impaired glucose tolerance.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41588-019-0470-3.

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Author contributions
F.E. contributed primarily to designing and performing the experiments, as well as to preparing the figures and manuscript. J.S.B., R.C., T.T., A.S., N.U., M.V.M., B.A., S.C., M.E. and A.A. were involved with performing the experiments. S.M., R.P.L., M.H.N., J.H., M.S.-T. and R.G.K. were involved in the design and supervision of certain aspects of the project. G.K., E.W., J.B. and E.S. were involved in patient recruitment and clinical characterizations. R.B.-D. carried out all of the OGTT and hyperglycemic clamp studies. N.U. was involved in the analysis of the genetic data. F.S.G. was involved in the design and supervision of aspects of the project, and participated in manuscript writing. A.M. designed the study and oversaw its implementation, supervised all aspects of the project from performing the experiments to the analysis of all data, and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Methods

Ascertainment and recruitment of cases. The study protocol was approved by the institutional review board of Yale University, and written informed consent was obtained from all study participants. The index cases had been identified as individuals with early-onset CAD, defined as a first-time angiographic diagnosis of CAD at or before the age of 30 years in men and 35 years in women, with only modest hyperlipidemia and without familial hypercholesterolemia. All available family members of the index cases willing to participate in the study were recruited, and their medical records were reviewed. The kindreds examined were of European ancestry, with all members living in the United States. Venous blood samples were obtained from all index cases, and members of their extended kindred and were used for DNA extraction, routine blood chemistry, plasma lipids and metabolomics. Participants were considered hypertensive if they had blood pressures greater than 140/90 mmHg or were receiving antihypertensive medications, and were considered diabetic or to have impaired glucose tolerance if they were taking glucose-lowering medications or had a fasting blood sugar greater than 126 mg/dl-1 or 100 mg/dl-1, respectively. Participants were considered overweight if they had a BMI > 25 kg/m² and obese if they had BMI > 30 kg/m². Plasma lipids were considered as continuous variables.

Whole-exome and whole-genome sequencing. Exome data for 30 index cases and the extended family members with a diagnosis of early-onset CAD and metabolic syndrome, as well as the Yale control exome database, were all generated at the W. M. Keck Facility of Yale University, as described. The Roche NimbleGen 2.1 M Human Exome Array covers 34.0 megabase pairs of genomic sequence and about 1000 exons from 103 protein-coding genes. Briefly, extracted DNA was fragmented, ligated to linkers and fractionated by gel electrophoresis. Extracted DNA was amplified by polymerase chain reaction (PCR) and hybridized to the capture arrays. The resulting bound DNA was eluted, purified and amplified by ligation-mediated PCR. The PCR products were purified and sequenced on an Illumina DNA sequencing platform. Captured data were analyzed on an Illumina genome analyzer, followed by image analysis and base calling. The resulting sequences were mapped to the reference genome hg19 and processed using MAQ Software. SAMtools software was used to detect single nucleotide variants. These were then filtered out as described. Filters were applied against a published database. The frequency, pathogenicity and location of mutations occurring in National Heart, Lung, and Blood Institute (NHLBI), ExAC and Yale control databases were examined. Variants were filtered for allele frequencies greater than 0.001% in the ExAC database. A Perl-based computer script was used to annotate variants based on protein effect, novelty, conservation and tissue expression. Mutation pathogenicity was assessed using PolyPhen-2 and SIFT predicting software and filtered if considered not damaging by either software.

A two-point parametric analysis of linkage was carried out using data obtained from the members of the largest kindred, specifying CAD as an autosomal dominant trait. Disease allele frequency was specified as 10⁻⁵ to 10⁻³, and the phenocopy rates as 10⁻³. To assess the prevalence of identified mutations in disease and control populations, exome data from the Yale control, NHLBI and ExAC databases were searched for variants in the CELA2A gene. The NHLBI exome database36, accessed on 20 October 2013, consisted of 6,503 CAD case and control samples from multiple Exome Sequencing Project cohorts. The Yale control exome database, including exome data from a control population of 2,000 Northern European individuals free of CAD and metabolic conditions.

To validate the association, two DNA samples from independent mutation carriers were subjected to whole-genome sequencing. The Integrative Genomics Viewer was used to assess copy number variation, using Variant Effect Predictor and WGS Annotator.

Antibodies, recombinant proteins and ELISA kits. Purified GP2B3A complex (MBS135714), mouse rCEL2A protein (MBS124687), human rCEL2A (MBS109662) and the CELA2A ELISA kit (MBS932150) were purchased from MyBioSource. CELA2A antibody (SABI104798) and the elastase substrate N-Succinyl-Ala-Ala-Pro-Leu-p-nitroanilide (S8511) were purchased from Sigma–Aldrich. The fluorescein isothiocyanate conjugated PAC1 antibody (2285S) and Annexin V-FITC (640905; BioLegend), CD41-FITC (303703; BioLegend) were purchased from Abcam. A human C-peptide ELISA kit (80-CPTHU-E01.1; ALPCO), Human Glucagon ELISA kit (10-1271-01; Mercodia), Rat Insulin ELISA kit (80-INSMU-E01; ALPCO), Human Glucagon ELISA kit (211-BLHU-E01; ALPCO) and Rat Insulin ELISA kit (80-INPRT-E01; ALPCO) were applied for C-peptide, glucagon and insulin measurements.

Tissue expression of CELA2A. For quantitative reverse transcription PCR analysis, RNA was extracted using TRI reagent (Sigma–Aldrich) according to the company's protocol. To synthesize complementary DNA (cDNA), 2 μg of RNA was used with oligo(dT), RNase OUT and SuperScript II Reverse Transcriptase (Invitrogen) in a total volume of 20 μl, and 0.3 μl of cDNA was used for further amplification by quantitative reverse transcription PCR. Amplification was performed with primers F (5’-CTGGCACCATTCTCCGTCCAGAA-3’) and R (5’-GTTGCCGATGTTCAACCCAGCAG-3’) using Platinum Taq DNA Polymerase (Invitrogen) and 40 cycles consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

Protein expression. CELA2A protein expression was examined in mouse tissues using western blot analysis. Tissue lysates were prepared from C57BL/6 mice, and the protein content was measured in lysates by Bradford assay. Lysate dilutions of equal protein concentration were separated by SDS polyacrylamide gel electrophoresis (PAGE) and processed for immunoblot analysis to assess the CELA2A protein level. Immunoblotting was performed using a mouse anti-CEL2A antibody (Sigma–Aldrich) and visualized with Anti-Mouse IgG HRP (Invitrogen) and 40 cycles consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

Validation of the CELA2A antibody and the ELISA kit. Protein levels were assessed by a validated CELA2A-specific antibody (Sigma–Aldrich), with a double band representing the zymogen and active form of the protein. This antibody recognizes both human and mouse CELA2A. Its specificity and cross-reactivity were confirmed using recombinant mouse CELA2A (rCel2a) protein, and the supernatant was collected from the 293T cells overexpressing human CELA2A and preblocking the antibodies with rCel2a protein (Supplementary Fig. 2a,b). The antibody specificity was confirmed by blocking 1 μg antibody (SABI104798; Sigma–Aldrich) with 2.5 μg rCel2a, followed by western blot analysis. To test the specificity of the antibody for immunohistochemistry, skeletal muscle tissue was used as a negative control for CELA2A labeling, and non-immune IgG was used as an antibody control. The skeletal muscle tissues of the C57Bl/6 mice after intravenous injection with the rCel2a protein were used as a positive control.

To test the specificity of the ELISA (MyBioSource) for human CELA2A, 1 and 2 μl of purified human WT- or p.D12IN-CEL2A, with protein concentrations measured previously by Bradford assay, were assayed for concentration by ELISA (Supplementary Fig. 2d). The consistency between the ELISA and Bradford measurements was confirmed.

Serum fractionation. First, 200 μl of mouse serum was separated by size exclusion chromatography on a 1.5 cm×31.5 cm column comprised of Sepharose 6 beads (fractionation range: 10–1000 kDa). The column buffer was 5 mM Hepes + 50 mM NaCl + 0.1 mM CaCl₂ at a pH of 7.4; 450 μl fractions were collected. Fraction number 65 was used for CEL2A2 detection (bands observed at 25 and 75 kDa).

Human plasma CELA2A measurement and associated elastase activity assay. CELA2A plasma levels were measured by ELISA. Briefly, 50 μl of plasma was incubated in CELA2A antibody-coated 96-well plates for 1 h at 37 °C, washed three times, then incubated with the horseradish peroxidase-conjugated secondary antibody and developed. Absorbance was measured at 450 nm and compared with a standard curve.

Plasma elastase activities of family members were assessed by first diluting the sample 1:5 in 0.2 M Tris/HCl buffer containing 0.2 M NaCl with 10 mM MgCl₂ and 10 mM CaCl₂. Succinyl-trialanyl-nitroanilide was used as the substrate; this was dissolved in 0.92% (v/v) N-methyl pyrrolidone at 12.5 mM. Then, 100 μl of substrate solution was added to 7 μl of diluted samples and incubated for 1 h, at which time the assays were stopped by adding 50 μl glacial acetic acid, and absorbance was measured at 410 nm. Purified porcine pancreatic elastase was used to generate a standard curve.

Generation and purification of WT and mutant CELA2A. Complementary DNA generation, site-directed mutagenesis and insertion of the WT and mutant constructs in pcDNA3.1-CEL2A- His plasmids were carried out per routine. Briefly, HEK293T cells were seeded in a six-well plate using Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS) and antibiotics. Cells were transfected by 24 h later with the plasmids and 10 μl Lipofectamine 2000, rinsed 48 h later and covered with 2 ml Opti-MEM. All transfections were performed in duplicates. Next, 50 μl of the collected conditioned media was loaded on a 4–15% gradient SDS–polyacrylamide gel and visualized by Coomassie Brilliant Blue staining.

CELA2A purification was carried out as described. Briefly, the WT and mutant CELA2A constructs with a His-tag at the C-terminus were generated and inserted in pcDNA3.1 vectors, under cytomeglovirus promoter, and expressed in HEK293T cells. HEK293T cells were cultured in DMEM (Thermo Fisher Scientific) with 10% FBS (Thermo Fisher Scientific) and supplemented with 4 mM l-glutamine at 37 °C, under 5% CO₂, to about 80–90% confluence. Subconfluent cells were transfected with plasmid DNA and incubated for 16–20 h, and subsequently washed and covered with Opti-MEM medium (Thermo Fisher Scientific). After 48 h incubation, the conditioned media were extracted and purified using Ni-affinity chromatography on a 1 ml Ni-NTA Superflow Cartridge (#30210; Qiagen). The column was equilibrated and 720 CC medium was loaded.
Articles

Nature Genetics

40 islets that were size matched by microscopy were picked for each condition before sample collection under a 5% CO2/95% air and 37 °C constant environment.

CO2/95% air before the insulin study.

indicated. Human donor islets were cultured for 48 h at 37 °C and under 5% secretory (2.5 mM glucose), followed by 2.5 mM glucose with vehicle control, with buffer NPI-250 (50 mM NaH2PO4, 300 mM NaCl and 250 mM imidazole). After this, the column was washed with the NPI-20 buffer, zymogens were eluted (1.5 µl) followed by 2.5 mM glucose with vehicle control, activated WT-CEL2a (1.5 µl−1), activated p.D121N-Cela2a (1.5 µl−1) or no treatment were applied to the cells as indicated in the individual graphs. Excitation was provided by a 488 nm laser, and emission was collected with a 510 nm long pass filter. Images were obtained using Image J software (National Institutes of Health).

Fluorescence data are expressed as percentages of baseline over time according to the following equation: F/F0 × 100, where F is the fluorescence intensity at any given time and F0 is the baseline fluorescence based on an average of the five fluorescence values for each cell. Each tracing corresponds to an individual cell.

Platelet aggregation assay.

Platelet aggregation was recorded as the percentage increase in light transmission after adding collagen (Chrono-Log) at a rate of 100 µl min−1 to verify modifications of the raw tandem mass spectrometry fragmentation spectra for every assigned precursor mass containing the phosphorylation site. The areas under the isotopic envelope were calculated for M, M + 1 and M + 2 isotopic peaks for all variants of each phosphorylated peptide for each sample, and the ratio of phosphorylated to total protein (phosphorylated + non-phosphorylated) was calculated for the M, M + 1 and M + 2 ions for each sample. The data are expressed as the ratio of phosphorylated/total recovered peptide. Statistical analyses were performed using one-way analysis of variance (ANOVA; SigmaPlot), and significantly altered phosphorylated peptides (P < 0.05) were used as an input for Gene Ontology enrichment analysis using platiGenes. Gene Ontology terms shared by the input genes were compared with the background distribution of the list annotated to that term. P values represent the probability for n number of input genes to the total number of annotated genes in any given Gene Ontology term, based on the proportion of genes in the whole genome that are annotated to that Gene Ontology term. Pathways were ranked based on fold enrichment for pathways with adjusted P < 0.05.

Isolation and preparation of human platelet suspension.

Venous blood was drawn from healthy adult volunteers (n = 3) who had taken no medications for at least 2 weeks. Platelet-rich plasma (PRP) was prepared from blood freshly drawn into citrate tubes by centrifugation at 250 RCF for 12 min at 25 °C. The platelet count in PRP was adjusted, if required, to 2–3 × 1011 platelets ml−1 by the platelet-poor plasma of the same individual. The PRP was incubated at 37 °C for 90 min with purified WT- and p.D121N-CEL2A proteins at a final concentration of 1.5 µg ml−1. The PRP tubes were used either for aggregation assay or for preparing washed platelets. Washed platelets were prepared by centrifugation of PRP at 700 RCF for 8 min. To avoid activation due to processing, PGI2 (20 ng ml−1) was added to the PRP just before spinning it for washed platelet preparation. The platelet pellet was resuspended in a calcium-free washing buffer (100 mM NaCl, 5 mM KCl, 1 mM MgCl2, 5 mM glucose, 36 mM citric acid and 0.35% bovine serum albumin (pH 6.5)). These washed platelets were used either for flow cytometer-based assays or for imaging by confocal microscopy.

Platelet aggregation assay.

Platelet aggregation was performed on a dual-channel Lumi-Aggregometer (Model 700; Chrono-Log) by the optical method, following the manufacturer's instructions. Platelet aggregation was recorded as the percentage increase in light transmission after adding collagen (Chrono-Log).
in human PRP at a final concentration of 2μg ml⁻¹ with constant stirring (1,100 r.p.m.). The channels were tested for confirmation of identical functioning, to avoid any variations due to variation in channel performance. The maximum amplitude was used for recording the aggregation data, and aggregation curves were merged using AGGRO/LINK8 software (Chrono-Log).

Platelet surface activation and apoptosis assay. The PAC1 assay was used to analyze activation of the GP2B3A receptor on platelet surfaces, as described²⁴. This assay uses an antibody that binds specifically to the activated conformation of the human GP2B3A receptor, but not to its resting form²⁴. The pretreated washed platelets were used for this assay. The FITC-conjugated PAC1 antibody (340507; BD Biosciences) was added to washed platelets and after 20 min incubation before acquiring the events on a flow cytometer. For the apoptosis assay, the pretreated washed platelets were incubated with 1μM TMRE reagent (T669; Thermo Fisher Scientific) at 37°C for 15 min followed by staining with annexin V for 15 min in apoptosis assay buffer, as described⁴¹. This assay quantifies the cells with depolarized mitochondrial membrane and exposed phosphatidyl serine on the platelet surface. For both of the assays, 3×10⁴ events were acquired using special-order BD LSR II Cell Analyzer (BD Biosciences), and data were analyzed by FlowJo version 10 (FlowJo).

OGTT and hyperglycemic clamp. Participants arrived at the Yale New Haven Hospital Research Unit in the morning of the study after at least 8 h of overnight fasting. Baseline measurements of BMI and vital signs (blood pressure, heart rate and respiratory rate) were measured. Females were assessed for pregnancy with a urine human chorionic gonadotropin test. After obtaining baseline hormone and glucose levels, subjects ingested 7.5 oz of glucola, which contains 75 g of glucose in orange-flavored water. Blood samples were taken at −15, 0, 10, 20, 30, 60, 90 and 120 min for the measurement of plasma glucose. Insulin and glucagon levels were measured 60 and 120 min after ingesting glucola.

The same participants returned to the Hospital Research Unit for the hyperglycemic clamp visit. An intravenous catheter was placed in the antecubital vein in each arm (one for glucose and insulin infusion and the other for blood draws). A baseline blood sample was drawn for glucose and hormone measurements (that is, insulin and glucagon). Blood draws were drawn for glucose every 5–10 min. Insulin and glucagon were measured at baseline, as well as 30 and 60 min after starting the bolus followed by continuous infusion of intravenous 20% dextrose solution. Dextrose solution was given at a variable infusion rate using an Alaris pump to maintain a glucose serum level of ~200 mg dl⁻¹ until the end of the hyperglycemic clamp.

Statistical analyses. In vivo studies included at least five mice in each group. In vitro studies were carried out in more than three independent experiments. The preparation of graphs and all statistical analyses, including two-tailed Student’s t-tests, one-way ANOVA (SigmaPlot) and testing for equal variance, were carried out using GraphPad Prism 8.1 Project software (GraphPad Software). Fisher’s exact test was carried out for the continuous variables. P<0.05 was considered significant. Data are presented as means±s.e.m. Fluorescence images were evaluated using Image J software (National Institutes of Health).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability Human variants and phenotypes have been reported to ClinVar under accession numbers SCV000916382, SCV000916383, SCV000916384 and SCV000916385. The data have also been reported to NIH with other identified variants in the Yale Center for Mendelian Genomics. Proteomics data are available on request.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

The computers used for genetic data are all encrypted and localized in a room designated for patients data.

Data analysis

Exome data for 30 index cases and the extended family members with the diagnosis of early onset CAD and metabolic syndrome, and the Yale control exome database were all generated at the W. M. Keck Facility of Yale University, using Roche/Nimble-Gen 2.1M Human Exome Array. Captured data was analyzed on the Illumina genome analyzer IIx system. The sequencing data were processed with the use of MAQ22 software Reference genome used was hg38 and processed using Mq software. SAMtools software was used to detect single nucleotide variants (SNVs). Mutation pathogenicity was assessed using PolyPhen-2 and SIFT predicting software9 A two-point parametric analysis of linkage was carried out using data obtained from the members of the largest kindred, specifying coronary artery disease as an autosomal dominant trait, using Linkage 3.0. Mann-Whitney test was used for non-parametric analysis of mutation burden analysis. GraphPad Prism 8 was used for statistical analysis and graph generation. AGGR/O/LINK5 was used for aggregation analysis. ImageJ/Image Studio was used for image processing.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

The power was calculated based on LOD score in a large kindred, using a threshold of 3.0 and Mann-Whitney test was used for genetic burden analysis.

Data exclusions

Two subjects too young to have the CAD trait were excluded from analysis of LOD score for CAD. Both subjects were included in all other analyses. These exclusions were preestablished.

Replication

Except for human studies all experiments were carried out at least in triplicate. All replications that are included in the results were reproducible.

Randomization

Patients were grouped in affected and unaffected. For the large kindred, unaffected family members used as controls and for burden analysis 2000 subjects with no known CAD or risk factor associated with the disease were used as controls. Covariants were the risk factor for the disease and tested by linkage analysis and/or Student t-test.

Blinding

The mutation screening was done blinded. The in vitro experiments, in vivo rodent and human physiological studies could not be performed in blinded fashion.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants
- Clinical data

Methods

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

The FITC conjugated PAC1 antibody: 1:20 dilution (340507, lot#793931 BD biosciences, San Jose, CA), Annexin V-FITC (640905, lot#241872, Biologend, San Diego, CA), CD41-FITC (HIP8) (303704, lot#B208419, Biologend, San Diego, CA). Cell-Based Assay TMRE (600331, lot#484102-1) was purchased from Cayman, Ann Arbor, MI. Rat AKT (#9277, lot#27), aPAKT (S473) (#9271s, lot#14), pAKT (T308) (#9275s, lot#21), total S6k (#9202, lot#20), pS6k (T389) (#9206, lot#22), total IRS (#2382, lot#10), pIRS (Y608) (#2385s, lot#21), pIRS (S636/S639) (#2388s, lot#14), GADPH (#14C10) (#2118, lot#10), Actin (13E5) (#4970, lot#14) and ITGA2B (D8V7H) (138075, lot#1) antibodies (Dilution of 1:500) were purchased from Cell Signaling Technologies, Danvers, MA. Recombinant A1AT (alpha1 antitrypsin, ab91136, Lot#GR 108058), A1AT antibody (G11) (ab9400, Lot#146649-11) and were purchased from Abcam, Cambridge, MA. Recombinant Mouse Cela2a (MBS1246487,
Validation

Except for CELA2A antibody all other antibodies have been previously validated. The CELA2A (18-29) antibody (SAB1104798, lot#012M2775V, Sigma, St Louis, MO) recognizes both human and mouse CELA2A and its specificity and cross-reactivity were confirmed using recombinant mouse CELA2A (rCela2a) protein, the supernatant collected from the 293T cells overexpressing human CELA2A and by pre-blocking the antibodies with rCela2a protein (Supplementary Fig. 2a,b). The antibody specificity was confirmed by blocking 1μg of antibody (Sigma, SAB1104798) with 2.5 μg of rCela2a (MBS11246487, Lot#03528, MyBiosource), followed by Western blot analysis. To test the specificity of the antibody for immunohistochemistry, skeletal muscle tissue was used as a negative control for rCela2a labeling and none-immune IgG as an antibody control. The skeletal muscle tissue of the C57Bl/6 mice after intravenous injection with the rCela2a protein was used as positive control.

All Cell Signaling antibodies are Verified for specificity, sensitivity, and reproducibility (https://www.cellsignal.com/contents/our-approach/cst-antibody-validation-principles/ourapproach-validation-principles).

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- Gapdh (D16H11) (#5174) cited in Experimental and Therapeutic Medicine 2019, Chen, D., Huang, X., et al., Experimental and Therapeutic Medicine 2019, Batulu, H., Du, G. J., et al., Oncology Reports, 2019 He, F., Fang, L., et al.
- Actin (13E5) (#4970) cited in Nature Communications on 4 April 2019 by Lim, S., Hermance, N., et al., Experimental and Therapeutic Medicine on 1 April 2019 by Huo, L., Wang, B., et al., Experimental and Therapeutic Medicine 2019, Zhang, X., Chen, D., et al., Cancer Research 2019, Chen, T., Wang, T., et al.
- A1AT antibody (ab0400) cited in Cancer Res 71:134-42, J Virol 87:5141-50 (2013), J Thromb Haemost 14:2023-2026 (2016)
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Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) 293T, 3T3L1, and INS-1 cell lines purchased from ATCC

Authentication 293T, 3T3L1, and INS-1 cells were purchased as authenticated cell lines from ATCC.

Mycoplasma contamination All Cell lines are routinely tested for mycoplasma and they were negative for mycoplasma.

Commonly misidentified lines None were used

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals C57/b6 wildtype and and homozygous Ldlrtm1Her (8-12 weeks) were purchased from JAX and the progenies were genotyped in the lab. Four month old mice of both genders were used
Wild animals | No wildtype animal was used in the study
Field-collected samples | No animal was collected from the field
Ethics oversight | Human studies were approved by Yale IRB

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Human research participants

#### Policy information about studies involving human research participants

**Population characteristics**

All patients were white Caucasians. Their kindreds were extended as described. We recruited 30 index cases with early onset CAD (age (yr) of onset at or before 30 yr in men and 35 yr in women), and extended their kindreds. This led to the identification and recruitment of a multiplex kindred with 25 affected subjects that we named CAD-2001 (Fig. 1a). The index case was a white American female with extensive family history of CAD, and first ST elevation myocardial infarction (STEMI) at age 28 yr (arrow). Her CAD risk factors included hypertension (HTN), type 2 diabetes (T2D), hypertriglyceridemia (HTG), and obesity. She underwent a coronary artery angiography and percutaneous intervention of two major coronary arteries. Among 53 extended blood relatives of the index case, 25 were diagnosed with early onset CAD (MI diagnosis by enzyme and EKG, angiographic diagnosis of CAD, or sudden cardiac death) with median age of 43 yr, and 11 had died from CAD (mean age of death of 52 yr). All affected subjects traced their ancestry to a common ancestor and male to female transmission of the phenotype was present.

Detailed clinical data were available for all 11 living subjects with CAD, two younger subjects (mean age 30 yr) with unknown CAD status and 12 living unaffecteds (Supplementary Table 1). All affected subjects met the NCEP criteria for metabolic syndrome with surprisingly homogeneous risk factors, including markedly elevated triglycerides (TG) (mean 287.1 mg/dl, Nl <150 mg/dl), hypertension, low HDL (mean 35.1 mg/dl, Nl >50 mg/dl) and T2D (fasting blood glucose > 126 mg/dl; or on glucose lowering drugs). In contrast, all twelve unaffected family members had normal TG (mean 100.5 mg/dl), near normal HDL levels and none had T2D. The two younger subjects with unknown CAD status had both HTG and HTN. The familial clustering and pattern of inheritance of these clinical features were consistent with the effect of a highly penetrant autosomal dominant trait.

**Recruitment**

Participants were identified by primary physicians at Yale and recruited by informed consents. The selection was biased toward kindreds with clustering of early onset CAD. In these kindreds the disease was inherited in an autosomal dominant fashion with complete penetrance. Metabolic syndrome in kindreds with late onset of disease is likely multifactorial and the effect of the disease genes is likely modified by other genetic variants. Thus, identified genetic mutations maybe subjected to modification in other kindreds as seen for all other disease genes. Our study was also biased toward identification of disease genes with large effect size. Accordingly, common variants with smaller effect size in Cela2a are associated with high blood pressure. I addition, other mutation in this gene may have no effect or be protective against CAD and its associated risk factors.

**Ethics oversight**

Yale

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Clinical data

**Policy information about clinical studies**

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

**Clinical trial registration**

Not applicable.

**Study protocol**

Note where the full trial protocol can be accessed OR if not available, explain why.

**Data collection**

Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.

**Outcomes**

Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.

### Flow Cytometry

**Plots**

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.
Methodology

| Sample preparation | platelet collected from healthy individuals |
|--------------------|---------------------------------------------|
| Instrument         | FACSAria                                    |
| Software           | flowjo                                      |
| Cell population abundance | Platelet rich plasmas were collected from control health individuals. A normal platelet count ranges from 150,000 to 450,000 platelets per micro liter of blood. |
| Gating strategy    | IgG stained platelets were used as negative control. |

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.