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Id3 is a Novel Atheroprotective Factor Containing a Functionally Significant SNP Associated With IMT in Humans

Amanda C. Doran[1,2], **Allison B. Lehtinen, PhD**[4,5], **Nahum Meller, PhD**[1,2], **Michael J. Lipinski, MD**[1,2], **R. Parker Slayton, MD**[1,2], **Stephanie N. Oldham, MS**[1,2], **Marcus D. Skaflen**[1,2], **Joseph Yeboah, MD, MS**[2], **Stephen S. Rich, PhD**[2,3], **Donald W. Bowden, PhD**[4,5,6], and **Coleen A. McNamara, MD**[1,2]

[1]Cardiovascular Research Center, University of Virginia, Charlottesville, Virginia

[2] Division of Cardiovascular Medicine/Department of Medicine, University of Virginia, Charlottesville, Virginia

[3]Center for Public Health Genomics, University of Virginia, Charlottesville, Virginia

[4]Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, North Carolina

[5]Center for Human Genomics, Wake Forest University School of Medicine, Winston-Salem, North Carolina

[6]Department of Internal Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina

Abstract

Rationale—The gene encoding the helix-loop-helix transcription factor, Id3, is located within atherosclerosis susceptibility loci of both mice and humans, yet its influence on atherosclerosis is not known.

Objective—The present study sought to determine if polymorphisms in the *ID3* gene were associated with indices of atherosclerosis in humans and if loss of Id3 function modulated atherogenesis in mice.

Methods and Results—Six tagging SNPs (tagSNPs) in the human *ID3* gene were assessed in participants of the Diabetes Heart Study. One tagSNP, rs11574, was independently associated with carotid intima-media thickness (IMT). The human *ID3* variant at rs11574 results in an alanine to threonine substitution in the C-terminus. To determine the effect of this polymorphism on the basic function of Id3, site-directed mutagenesis of the human *ID3* gene at rs11574 was performed. Results demonstrated a significant reduction in co-immunoprecipitation of the known E-protein partner, E12, with Id3 when it contains the sequence encoded by the risk allele (Id3105T). Further, Id3105T had an attenuated ability to modulate E12-mediated transcriptional activation compared to Id3 containing the ancestral allele (Id3105A). Microarray analysis of vascular smooth muscle cells from WT and *Id3*$^{-/-}$ mice revealed significant modulation of multiple gene pathways

Correspondence to: Coleen A. McNamara, University of Virginia, P.O. Box 801394, Charlottesville, VA 22908, Phone: (434)924-5500, Fax: (434)924-2828, cam8c@virginia.edu.

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implicated in atherogenesis. Moreover, *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice developed significantly more atherosclerosis in response to 32 weeks of Chow or Western diet feeding than *Id3*$^{+/+}$*ApoE*$^{-/-}$ mice.

Conclusions—Taken together, results provide novel evidence that Id3 is an atheroprotective factor and link a common SNP in the human *ID3* gene to loss of Id3 function and increased IMT.

Keywords

atherosclerosis; diabetes mellitus; genetics

Introduction

Atherosclerosis is a chronic, inflammatory disease in which lipids, cells and fibrous elements accumulate in the intimal layer of large arteries[1, 2]. This process begins in adolescence and follows a variable clinical course that may ultimately result in myocardial infarction (MI) or stroke[3, 4]. Because of this, it is estimated that atherosclerosis is the underlying cause of 50% of all deaths in Westernized society[1]. Despite the magnitude of this problem, understanding mechanisms whereby specific genes or gene pathways modulate atherosclerosis in humans remains challenging. This is largely attributed to the fact that atherosclerosis, in its common form, is a multifactorial disorder. While recent studies have identified potential candidate genes in humans, few studies have been able to determine the impact of specific human gene variants on protein function and disease modulation.

Recent studies have identified susceptibility loci for atherosclerosis in both humans and mice[5]. Using genomewide linkage analysis in a Caucasian American population, Wang et al. reported a novel significant susceptibility locus for premature myocardial infarction at 1p34-36 (12-40 Mbp)[6]. Welch et al., used the LDLR$^{-/-}$ mouse in an interspecific genetic cross to identify a murine atherosclerosis susceptibility locus on mouse chromosome 4 (27-154 Mbp), which they named Athsq1[7]. Intriguingly, comparison of human 1p34-36 with the Athsq1 locus in mice reveals that one major gene common to both loci is Inhibitor of Differentiation-3 (Id3).

Id3 is a member of the basic helix-loop-helix (bHLH) family of proteins. While Id3 contains the HLH domain required for protein:protein dimerization, it lacks the basic DNA-binding domain possessed by other members of the family, making it incapable of binding to DNA. Instead, Id3 binds to another subset of bHLH factors known as the E-proteins, including E12 and E47, thereby preventing their dimerization with tissue-specific bHLH factors and inhibiting subsequent DNA binding. These Id:E-protein dimers have been demonstrated to regulate many genes in a variety of cell types including B cells, T cells, adipocytes and smooth muscle cells[8-10]. Id3 itself has been implicated in the pathobiology of vascular disease and has been detected in vascular lesions of rodents, pigs and humans[11-13]. While it is undetectable in normal arteries, Id3 is expressed in response to wire endothelial denudation of the carotid artery in rodents[12] and enhances vascular smooth muscle cell (VSMC) growth[11, 14-16], implicating Id3 in promoting the neointimal response to injury. Animals fed a high fat diet leading to hyperlipemia express higher levels of Id3 in the atherosclerotic vessel wall compared with their normolipemic non-diseased controls[13]. Yet, the pathobiology and genetic determinants of injury-induced neotintimal formation and diet-induced atherosclerosis are quite distinct[17, 18]. Whether Id3 expressed in atherosclerotic lesions activates gene pathways to limit or promote atherosclerosis is unknown.

Results of the present study have identified an association between polymorphism at rs11574 in the *ID3* gene and carotid intima-medial thickness (IMT) in participants from the Diabetes Heart Study (DHS). Mutation of the major allele of the human *ID3* gene at rs11574 to the risk allele resulted in attenuated Id3 function. Moreover, deletion of the *ID3* gene

resulted in a significant increase in atherosclerosis formation in Western-fed *ApoE*$^{-/-}$ mice. Together, these results provide evidence that Id3 is an important atheroprotective factor in mice and in humans.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://www.circres.ahajournals.org.

Human subjects

The Diabetes Heart Study (DHS) is an affected sib-pair study of subclinical atherosclerosis and its risk factors, consisting of families with two or more siblings with a diagnosis of T2D and lacking advanced renal insufficiency. Ascertainment and recruitment have been described previously[19, 20]. Details of the protocols for clinical data acquisition, IMT measurement, isolation of human genomic DNA and genotyping, and statistical analysis are provided in the online supplement.

Functional analysis of Id3105A vs. Id3105T

Plasmid constructs expressing products of the ancestral (Id3105A) and variant (Id3105T) alleles of *ID3* were generated and analyzed for differences in expression, co-immunoprecipitation with E12, and dominant negative antagonism of E12 function as detailed in the online supplement.

Studies using Id3 null mice

Detailed methods for microarray analysis of VSMC from WT (C57BL/6) and *Id3*$^{-/-}$ mice and quantitation of atherosclerosis in *Id3*$^{+/+}$*ApoE*$^{-/-}$ *vs. Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice are provided in the online supplement.

Results

Analysis of ID3 SNPs in a Diabetic Human Population: Association with Subclinical Atherosclerosis

Two previous studies have identified regions of atherosclerosis susceptibility in mice and men which contain the *ID3* gene, suggesting that Id3 may be a candidate gene for association with CVD[6,7]. We sought to determine whether polymorphisms in the human *ID3* gene were associated with subclinical markers of atherosclerosis in humans by assessing tagSNPs in the participants of the DHS. The subjects for this analysis were all European American sib-pairs with T2D (n=780). Clinical characteristics of the sample are consistent with a population of subjects with T2D: older age (mean 62 yrs), increased BMI (32.4 kg/m^2), elevated systolic blood pressure (139.8 mmHg). However, participants did not have overly aberrant lipid measures (LDL 104 mg/dL, HDL 42.7 mg/dL), likely due to the extensive use of lipid-lowering therapy in this group (44.7%)(Supplementary Table I). As a measure of subclinical atherosclerosis, intima-media wall thickness (IMT, mean 0.68 mm) was obtained on participants in this sample.

ID3 is a small gene, spanning only three exons, the first two of which are coding exons. Six SNPs were identified within the gene which captured all eight alleles and tagged haplotypes with a mean r^2 of 0.967 (Figure 1A)[21]. All six tagSNPs were successfully genotyped in the samples from the DHS: rs1555026 and rs1555025 (5′ of the gene), rs11574 (exon 2), rs2920 and rs1050096 (exon 3) and rs2071495 (3′ to the gene). The total distance between rs155026 and rs2071495 is 2.9kb. The region consists of two blocks of linkage disequilibrium, the first two SNPs in one block and the last four SNPs in the other (Figure 1B).

To determine whether an association exists between any of the *ID3* tagSNPs and subclinical atherosclerosis, the affected sib-pair families were subjected to a quantitative trait locus (QTL) association analysis. This analysis was performed in a model without covariates as well as in one that incorporated the effects of known risk factors (age, sex, BMI, systolic blood pressure, LDL and HDL). Of the six SNPs that were used for this analysis, one (rs11574) showed evidence of significant association with subclinical atherosclerosis as measured by IMT (Table 1, p=0.01). Incorporating the aforementioned covariates into the model, the association of rs11574 with IMT was shown to be independent of these risk factors (Table 1, p=0.005). When rs11574 was included in the model of IMT as a covariate, no other SNP was significantly associated with the residual phenotype (next most associated: rs2920, p=0.09). Pedigree-wide regression analyses demonstrated that there was significant heritability (h^2) for IMT independent of the effects of known risk ($h^2 = 0.26 \pm 0.12$; p=0.02). Interestingly, there is a stepwise increase in mean IMT associated with the minor (variant) allele of rs11574. Subjects who are homozygous for the major (ancestral) allele (GG) have a mean IMT of 0.66mm, while mean IMT was 0.69mm for those who are heterozygous (GA) and 0.72mm for those homozygous for the minor allele (AA). Studentized range statistic for the 3 groups demonstrated these differences were significant (p<0.01)(Table 2).

SNP rs11574 Does Not Affect the Expression of Id3

The rs11574 SNP is a nonsynonymous change, resulting in an alanine (ancestral) to threonine (variant) substitution at amino acid 105. In addition, this SNP is located in the second exon encoding the C-terminal portion of Id3, which has previously been shown to be essential for dominant negative function[22]. To determine functional significance of polymorphism at rs11574, the coding region of human *ID3* (Id3105A) was cloned into a mammalian expression vector and site-directed mutagenesis was employed to generate a construct expressing the variant encoded by the minor allele (Id3105T). NIH3T3 fibroblasts were transfected with either expression vector and analyzed by Western blotting (Figure 2A). To control for potential differences in transfection efficiency, expression of Id3 was normalized to an internal control, the ShBle protein, which is expressed from the same plasmid. The results demonstrate no significant difference in the expression of Id3105A compared with Id3105T (Id3105A/ShBle ratio = 1.13 (95% CI 0.88 to 1.38), Id3105T/ShBle ratio = 1.19 (95% CI 0.91 to 1.47), p=0.62). β-actin levels were not significantly different between samples.

SNP rs11574 Reduces the Binding of Id3 to E12

To determine whether differences exist in the ability of Id3105A and Id3105T to dimerize with typical Id protein partners, co-immunoprecipitation (co-IP) studies were employed. NIH3T3 fibroblasts were transfected with either Id3105A or Id3105T and FLAG-E12 and harvested after 48 hours. Lysates were then immunoprecipitated with sepharose G beads that were pre-conjugated with a monoclonal Id3 antibody generated within the lab (see methods). Id3105T demonstrated a robust 92% reduction in E12 co-IP versus Id3105A (Id3105A binding ratio = 2.58 ± 0.70, Id3105T binding ratio = 0.20 ± 0.20, p=0.0002) (Figure 2B). Quantitation of all of the co-IP experiments (n=3 in triplicate) is shown in Figure 2C.

Id3105T has a reduced ability to inhibit transcription compared with Id3105A

To determine if the reduced binding of Id3105T to its E12 partner may have functional consequences, promoter-reporter studies were undertaken. As a readout of transcriptional activity, the smooth muscle alpha actin (SMaA) promoter, which has previously been shown to be activated by E12, in an E-box dependent manner, was used[9, 23]. As anticipated, transfection of E12 resulted in activation of the SMaA promoter, an effect which was inhibited by Id3105A in a concentration-dependent manner. In contrast, the inhibitory ability

of Id3105T was significantly attenuated, yielding only partial inhibition of E12-mediated SMaA promoter activity even at the highest concentration (Figure 3). Western blotting confirmed that the observed differences between Id3105A and Id3105T activity were not due to differences in expression.

Loss of Id3 alters expression of genes in multiple pathways linked to atherogenesis

The attenuated ability of Id3105T to antagonize E12-mediated SMaA promoter activation provided proof of concept that, in addition to attenuating dimerization with E12, Id3 polymorphism at rs11574 had functional consequences on gene expression. Yet, atherosclerosis is a complex process involving many pathways and cell types, and it is not likely that transcriptional regulation of a single gene would result in the magnitude of difference in IMT seen in the DHS population. To determine if loss of Id3 has a significant impact on other genes in major atherogenic pathways, we performed microarray analysis of primary aortic VSMCs from WT (C57/BL6) or *Id3*$^{-/-}$ mice. Gene expression was assessed using the Affymetrix MG430A GeneChip representing ~ 22,600 transcripts in duplicate samples in each group to confirm reproducibility. After quality of the array data was confirmed, the data set was analyzed using Ingenuity Pathway Analysis software and pathways known to be involved in atherogenesis were assessed. Results demonstrate that VSMCs from *Id3*$^{-/-}$ mice have significant modulation of genes involved in adhesion, immune trafficking, apoptosis, cellular proliferation, and cell movement (Table 3). Genes from these pathways with the greatest modulation and $p<0.05$ (WT vs. *Id3*$^{-/-}$) are listed in Supplementary Tables II and III.

ApoE$^{-/-}$ Mice Null for Id3 Develop Significantly More Atherosclerosis than Control Mice

To evaluate the impact of loss of Id3 not only on atherogenic pathways, but on atherogenesis in the intact animal, *Id3*$^{-/-}$ mice were bred with atherosclerosis-prone *ApoE*$^{-/-}$ mice. The resulting *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice were placed on either a standard chow or Western diet and analyzed by *en face* analysis after 16 and 32 weeks of diet. While no significant amount of atherosclerosis was detected in chow-fed mice of either genotype after 16 weeks of chow diet, *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice had significantly more atherosclerosis than *Id3*$^{+/+}$*ApoE*$^{-/-}$ mice after 32 weeks on a chow diet. Similarly, *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice had two-fold more atherosclerosis than *Id3*$^{+/+}$*ApoE*$^{-/-}$ mice after 32 weeks of Western feeding (Figure 4). To confirm and extend these findings, cross-sectional analysis of the ascending aorta was also employed. In agreement with the results of the *en face* analysis, *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice had significantly more lesion in the ascending aorta than *Id3*$^{+/+}$ *ApoE*$^{-/-}$ mice after 16 weeks of Western feeding (2.0-fold)(Figure 5A and 5B). Consistent with a role for Id3 in regulating genes in many pathways involved in atherogenesis, while the absolute amount of plaque was larger in the *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice, the gross characteristics of the plaques were similar. The proportion of cellular to acellular area is similar between the two groups (Figure 5C and 5D).

Atherosclerosis is known to be influenced by lipid levels, obesity and diabetic status. Western feeding induced the expected increase in total cholesterol, LDL cholesterol, glucose and insulin, but no significant differences were found in any of the lipid measures, insulin levels or body weights when Western or Chow fed *Id3*$^{+/+}$ *ApoE*$^{-/-}$ were compared with the more atherogenic *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice (Supplementary Table IV). Interestingly, glucose levels were lower in the more atherogenic Western-fed *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice compared with *Id3*$^{+/+}$ *ApoE*$^{-/-}$ mice but not in the Chow-fed group.

Discussion

The development of advanced technologies that allow sequencing and analysis of the human genome has led to the identification of a number of SNPs that are associated with disease;

however a multitude of confounding factors hamper progress in identifying their functional significance. Few studies have been able to relate variants in candidate genes to a disease phenotype in an animal model or to understand how polymorphisms in the gene alter protein function and ultimately affect disease. In this study, we analyzed the functional significance of a human *ID3* gene polymorphism at rs11574 that was associated with carotid IMT in DHS and found loss of a major functional property of Id3 when the specific polymorphic nucleotide at rs11574 was mutated from the major (Id3105A) to the risk (Id3105T) allele, and a marked increase in atherosclerosis with a loss of function approach in a mouse model of atherosclerosis. Together, these data suggest that Id3 may be an important upstream regulatory factor in atherogenesis.

Genotypes of rs11574 were associated with a 9% change in IMT between individuals who were homozygous for G versus homozygous for A (Table 2). Intima-media thickness of the carotid artery is a surrogate quantitative measure of atherosclerosis that has a graded, predictive relationship to overt CVD[24]. IMT serves as an indicator of atherosclerotic burden[25], and a predictor of subsequent cardiovascular events[26]. IMT changes of the magnitude of those seen in association with *ID3* polymorphism have been associated with significant increases in the relative risk of cardiovascular events[24, 27], suggesting that in addition to being statistically significant, the percent change in IMT associated with *ID3* polymorphism is clinically significant. A large percentage of individuals in the DHS study were on lipid-lowering therapy (44.7%) which is associated with reduced IMT in humans. The average LDL cholesterol in the studied population was only 104.1 with a SD of 32.4. It is possible that an even greater reduction in IMT could be seen in those without the disease allele in a group with high lipids. While lipid values (LDL and HDL) were adjusted for in the covariate analysis and the association of rs11574 with IMT was independent of these variables ($p<0.005$), the present study does not have sufficient patients with high lipid levels to test this hypothesis directly. Confirmation of the association of *ID3* polymorphism with IMT and other indices of atherosclerotic burden in cohorts with a broad range of cholesterol levels and with and without diabetes needs to be performed. Nonetheless, results from the DHS population provided support for Id3 as a candidate gene modulating atherosclerosis and raised the interesting possibility that polymorphism in *ID3* at rs11574 alters Id3 function.

Polymorphism at rs11574 results in an amino acid substitution in the C-terminus of Id3, a region of the protein that has been shown to have functional significance. For example, a variant of human Id3, which has an alternate C-terminus generated by retention of an intron (Id3L), was unable to abrogate binding of E-protein to its consensus E-box site in a DNA mobility shift assay[28]. Consistent with these data, here we have shown that a SNP in this essential domain attenuated E-protein interaction (Figure 2B). Further support for a loss of function phenotype for rs11574 was demonstrated by promoter-reporter assays in which Id3105T had a diminished ability to inhibit E12-mediated promoter activation, confirming that this substitution alters the ability of Id3 to regulate transcription (Figure 3). Interestingly, the inhibition of luciferase activity with the highest concentration of Id3105T appears greater than what might be predicted based on co-IP results (Figure 2B and 2C). Our luciferase results suggest that at high Id3:E12 ratios, Id3105T has some capacity, although significantly less than Id3105A, to inhibit E12 activation of the SMaA promoter (Figure 3). It is possible that the alteration in Id3:E12 interaction for Id3105T is not an all or none phenomenon. The amino acid change at Id3105 may alter affinity for E12, may change the interaction of Id3 with an obligate co-factor or result in a change in partnering with other bHLH factors in the cell. Further detailed biochemical characterization of binding constants and competition assays will be needed to determine the mechanism behind this effect.

The multifactorial nature of atherosclerosis suggests that, for a polymorphism in a single gene to be associated with a change in IMT of the magnitude seen in our study, it must regulate multiple pathways or cell types. Ids exert their dominant negative effect by inhibiting bHLH factors such as the E-proteins from binding to their cognate consensus sites (E-boxes)[29]. bHLH factors are broadly expressed in many cell types and regulate a wide range of genes that play a role in atherosclerosis and T2D, including C-reactive protein [30], cholesterol synthesis genes[31, 32], p21[33], plasminogen activator inhibitor type-1 (PAI-1)[34], fatty acid synthase[35, 36] and insulin[37]. Recently, we have demonstrated that E-proteins interact with SREBP-1c to activate the adiponectin promoter and by sequestering E-proteins, Id3 can inhibit SREBP-1c activation of transcription[8]. SREBP-1c has been implicated in the development of obesity, T2D, dyslipidemia and atherosclerosis in humans (reviewed in 38), thus Id3 regulation of SREBP-1c may contribute to atherogenesis. In the present study, pathway analysis of microarray data from VSMC derived from WT and *Id3*$^{-/-}$ mice reveal that loss of Id3 results in significant modulation of multiple genes within atherogenic pathways (Table 3 and Supplementary Tables II and III). In addition to potential upstream regulation of many VSMC genes involved in atherogenesis, Id3 may regulated atherogenic genes in other cell types known to be important in atherosclerosis, including T cells[39], B cells[40] and endothelial cells[41]. Thus, the potential of Id3 to regulate many genes in many cell types may amplify the effects of the rs11574 SNP. Identification of E12 target genes in cells involved in atherogenesis may provide valuable insights into the pathways whereby *ID3* polymorphisms and loss of function lead to vascular disease phenotypes.

Deletion of the *Id3* gene in *ApoE*$^{-/-}$ mice, a more dramatic phenotype than attenuated Id3 function due to a SNP, provides further evidence that loss of Id3 function increases atherosclerosis development. *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice were found to have significantly more atherosclerotic lesion than *Id3*$^{+/+}$*ApoE*$^{-/-}$ mice after 16 or 32 weeks of Western feeding. Furthermore, even in the setting of less hyperlipidemia, the *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice maintained on a chow diet for 32 weeks also developed more atherosclerosis than the *Id3*$^{+/+}$ *ApoE*$^{-/-}$ mice (Figure 4). *Id3*$^{+/+}$*ApoE*$^{-/-}$ and *Id3*$^{-/-}$ *ApoE*$^{-/-}$ animals had similar lipid values whether chow fed or Western fed despite significantly different amounts of atherosclerosis, providing evidence that the impact of Id3 on atherogenesis is not mediated by alterations in lipid values (Supplementary Table IV). As *en face* analysis does not provide information on depth or composition of the lesion, cross-sectional lesion area analysis was also performed. Consistent with our *en face* findings, *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice were found to have significantly more cross-sectional lesion area than *Id3*$^{+/+}$*ApoE*$^{-/-}$ mice in the proximal aorta after 16 weeks of Western diet (Figure 5A and 5B). While the overall size of the lesions was significantly greater in the *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice, the gross lesion composition was similar between groups (Figure 5C and D). The finding that the size of the lesion, not lesion characteristics, were increased in mice null for *Id3* is consistent with our array findings that Id3 is an upstream regulator of multiple processes and pathways involved in atherogenesis and consistent with the finding that a single SNP in a single gene could be associated with IMT in humans.

These data are the first to demonstrate that loss of Id3 increases atherosclerosis in mice. Moreover, results provide the first report of a SNP in the *ID3* gene associated with IMT in humans that leads to the expression of an Id3 protein with attenuated function. Taken together, results underscore the importance of identifying pathway regulators like Id3 and their partner proteins that may be important therapeutic targets in the prevention of atherogenesis.

Novelty and Significance

What is known?

- 1p34-36, a susceptibility locus for premature myocardial infarction in humans, and Athsq1, a murine atherosclerosis susceptibility locus, both contain the gene encoding the helix-loop-helix factor Id3.
- Id3 has been shown *in vitro* to regulate many pathways in many cell types implicated in atherogenesis, but the impact of modulating the expression or function of Id3 on atherogenesis *in vivo* is unknown.

What new information does this article contribute?

- The present study is the first to identify a single nucleotide polymorphism in the *ID3* gene (at rs11574) that is associated with carotid intima-media thickness (a surrogate marker of atherosclerotic plaque burden and clinical cardiovascular events) in humans.
- The Id3 protein encoded by the minor (disease-associated) allele has attenuated function as an inhibitor of gene expression.
- Consistent with these findings, *ApoE*$^{-/-}$ mice null for *Id3* have a significant increase in atherosclerosis.

Genomics has emerged as a powerful method with the potential to identify gene loci and gene variants (such as single nucleotide polymorphisms or SNPs) that may provide novel pathophysiological insights and serve as useful biomarkers of complex diseases. Recent studies have identified gene loci and SNPs associated with atherosclerosis in humans, yet little is known about the impact of these genes on atherosclerosis or if these SNPs alter function of the protein encoded by the gene variant. The present study is the first to report a SNP in the *ID3* gene (at rs11574) associated with carotid intima-media thickness (IMT) in humans. Moreover, our results demonstrate that the protein encoded by this disease-associated *ID3* variant has significantly attenuated function as an inhibitor of gene activation. Loss of Id3 resulted in modulation of many genes in pathways involved in atherogenesis, highlighting the potential magnitude of the impact of altered Id3 function. Consistent with these findings, *Id3* gene deletion in *ApoE*$^{-/-}$ mice resulted in a marked increase in atherosclerosis formation. Taken together, these results provide evidence for a pathophysiological role of attenuated Id3 function in promoting atherogenesis and suggest that *ID3* polymorphism at rs11574 may be a biomarker of atherosclerotic burden in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non Standard Abbreviations and Acronyms

ApoE	Apolipoprotein E
bHLH	Basic helix-loop-helix
CAT	Chloramphenicol acetyltransferase

Co-IP	Co-immunoprecipitation
CVD	Cardiovascular disease
DHS	Diabetes Heart Study
Id3	Inhibitor of Differentiation-3
IMT	Intima-media thickness
LDLR	LDL receptor
MI	Myocardial infarction
oxLDL	Oxidized LDL
QTL	Quantitative trait locus
PREST	Pedigree Relationship Statistical Test
SmaA	Smooth muscle alpha actin
T2D	Type 2 diabetes
tagSNP	Tagging SNPs
VSMC	Vascular smooth muscle cell

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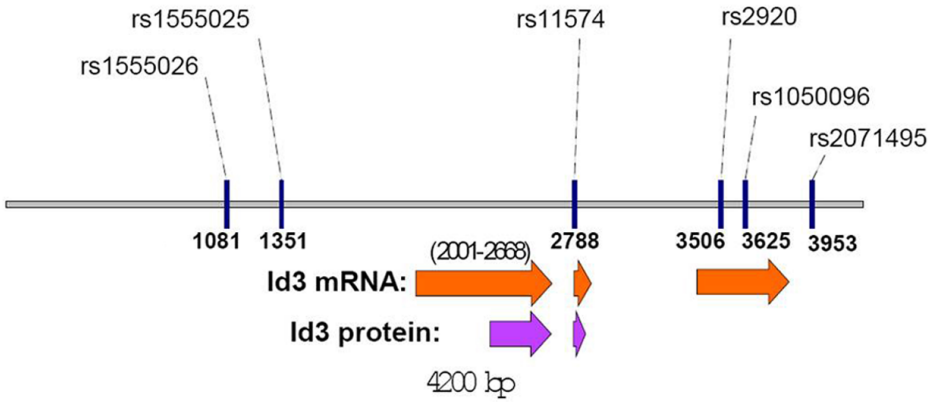
A.



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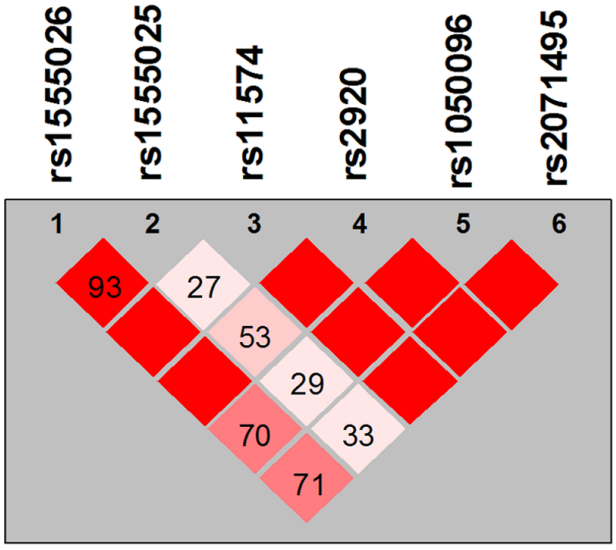

Figure 1. Tagging SNPs within the human *ID3* gene

A, Schematic depicting the six tagging SNPs in the human *ID3* gene. Approximate sites of RNA transcripts and protein products are noted beneath the gene sequence. ***B,*** LD plot depicting the two haplotype blocks encompassing the six *ID3* tagging SNPS in the DHS analysis. Numbers reflect D' values.

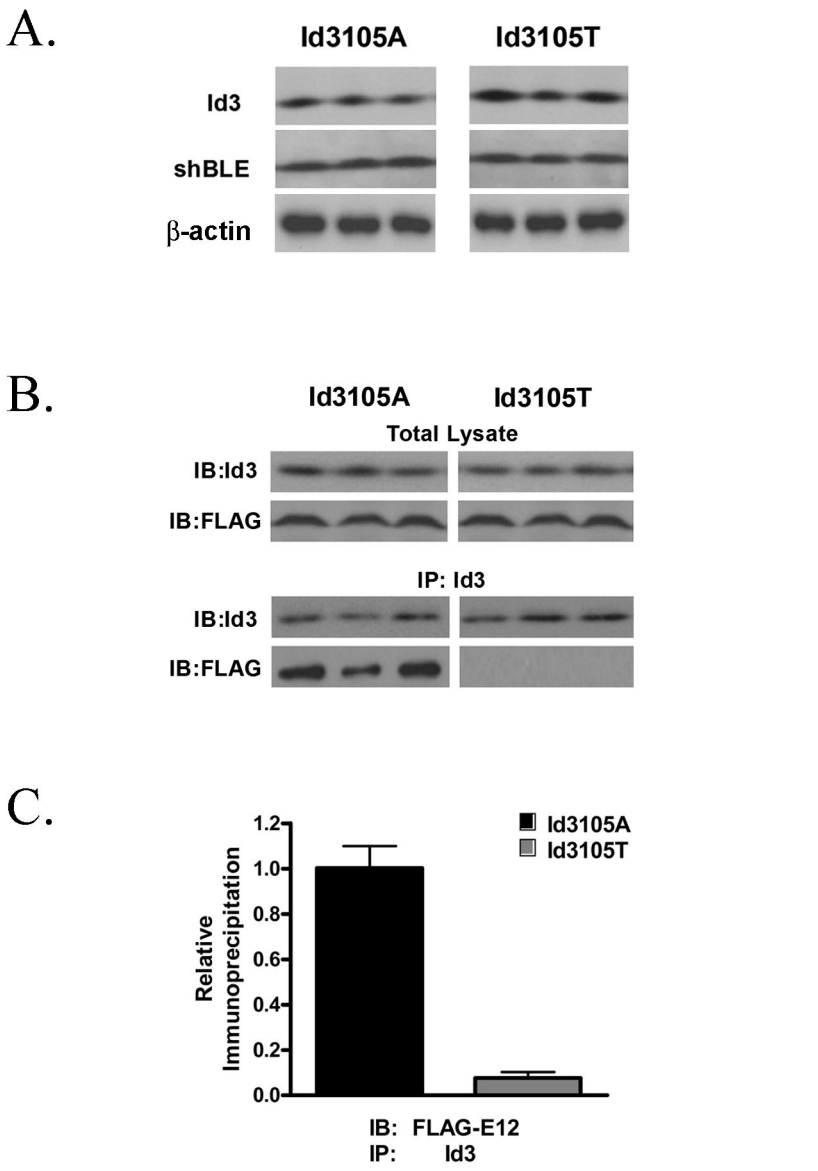

Figure 2. The variant *ID3* allele at rs11574 demonstrates decreased binding to E12

A, NIH3T3 cells were transfected with either the pEF4-Id3105A or pEF4-Id3105T plasmids and analyzed by Western blotting 48 hours after transfection. To control for potential differences in transfection efficiency between Id3105A and Id3105T, expression of Id3 was normalized to expression levels of ShBle in the same samples. β-actin was used as a loading control. Results are representative of three independent experiments in triplicate. ***B,*** NIH3T3 fibroblasts were transfected with either Id3105A or Id3105T constructs and FLAG-E12. Forty-eight hours after transfection, total lysates were precipitated using G sepharose beads that had been pre-conjugated to an Id3 antibody. Immunoprecipitates and total lysates were separated by SDS-PAGE and immunoblotted with FLAG or Id3 antibodies. Results are representative of three independent experiments. ***C,*** Quantitation of co-IP experiments in B. p=0.0002.

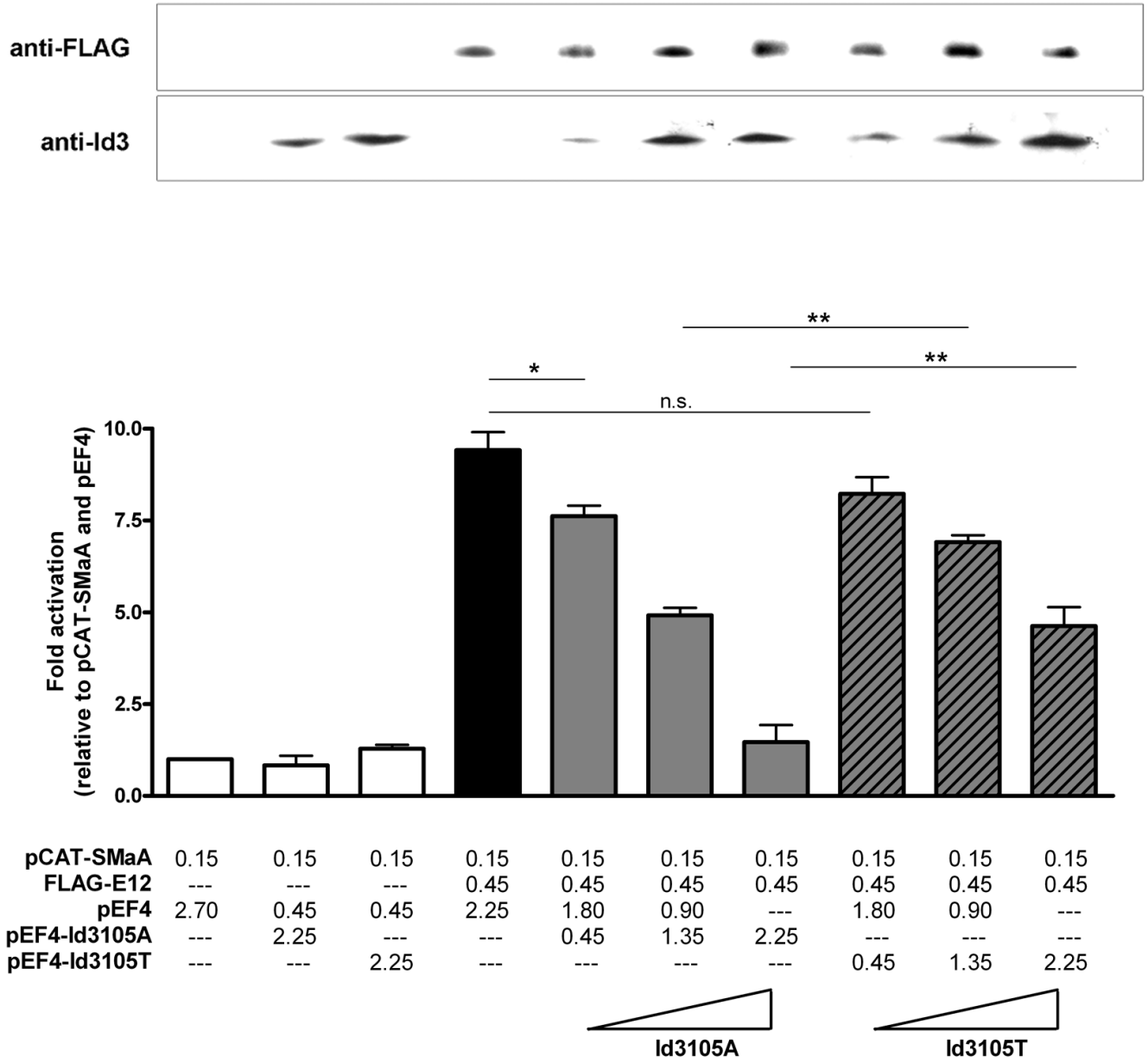

Figure 3. Id3105T has a decreased ability to inhibit transcription compared with Id3105A

NIH3T3 cells were transfected in triplicate with a smooth muscle alpha actin promoter-reporter (pCAT-SMaA) as well as with Id3 and FLAG-E12 expression vectors as indicated (values in μg). Thirty-six hours after transfection, cells were harvested and assayed for CAT activity. CAT activity was normalized to protein concentration and is presented as fold activation relative to the first group (promoter plus vector only). Samples were immunoblotted with anti-FLAG and anti-Id3 antibodies in parallel to confirm the expected expression patterns. Representative Western blots are shown above their corresponding samples. *:p=0.034. **:p=0.002.

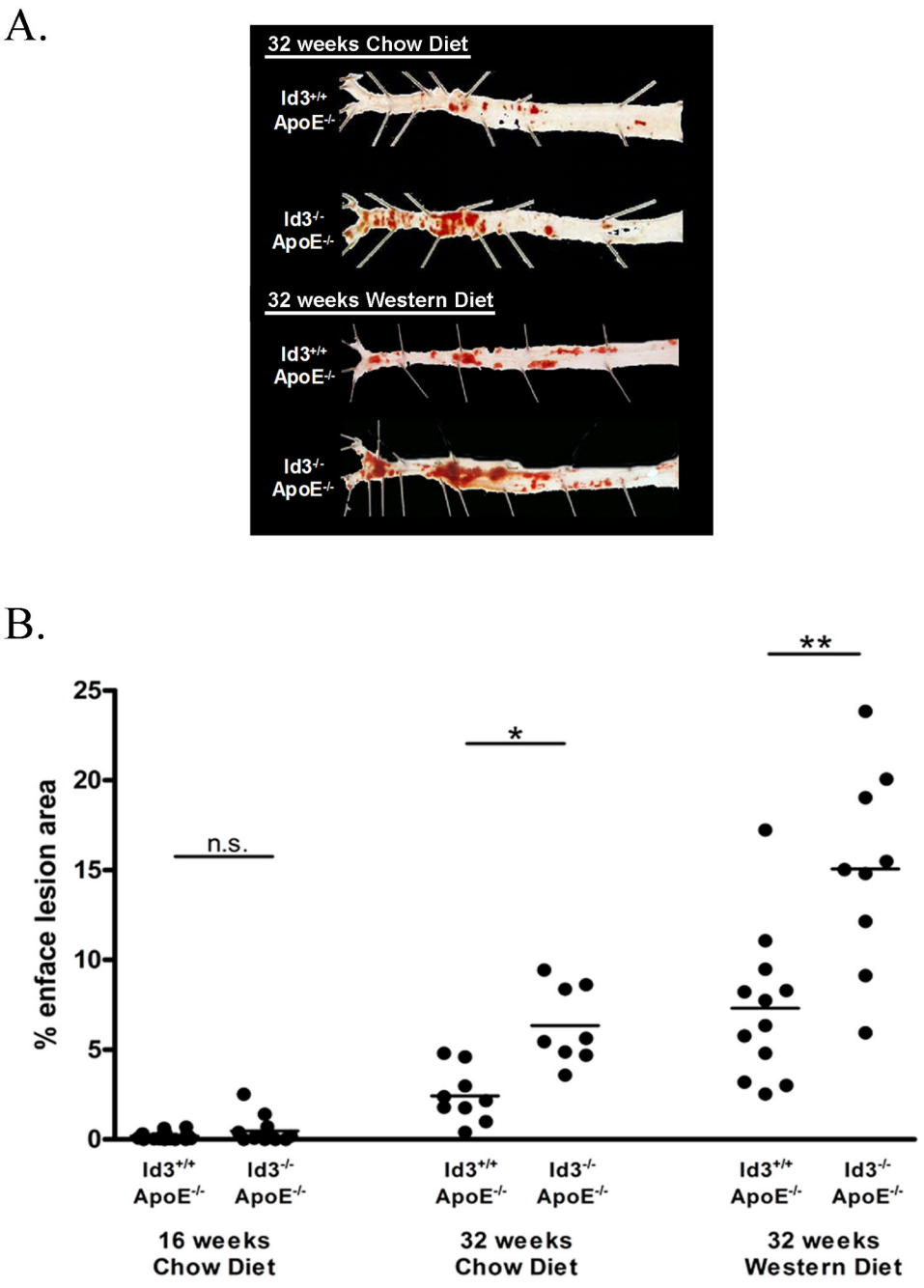

Figure 4. *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice have significantly more atherosclerosis than *Id3*$^{+/+}$ *ApoE*$^{-/-}$ mice in the descending aorta

Beginning at eight weeks of age, *Id3*$^{+/+}$ *ApoE*$^{-/-}$ and *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice were fed a chow or Western diet for 16 or 32 weeks. Aortas were perfused with paraformaldehyde, harvested, opened longitudinally and stained with Sudan IV. *En face* lesion area was quantitated using Image-Pro 5.0 software. ***A,*** Representative vessels from *Id3*$^{+/+}$ *ApoE*$^{-/-}$ and *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice. Regions shown are of the descending aorta from the bifurcation of the iliac arteries to the bifurcation of the left subclavian (left to right). ***B,*** Quantitation of lesion area by *en face* analysis in *Id3*$^{+/+}$ *ApoE*$^{-/-}$ and *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice after 16 weeks of chow diet, 32 weeks of

chow diet or 32 weeks of Western diet. Each point represents one animal. *: p = 0.001. **: p = 0.005.

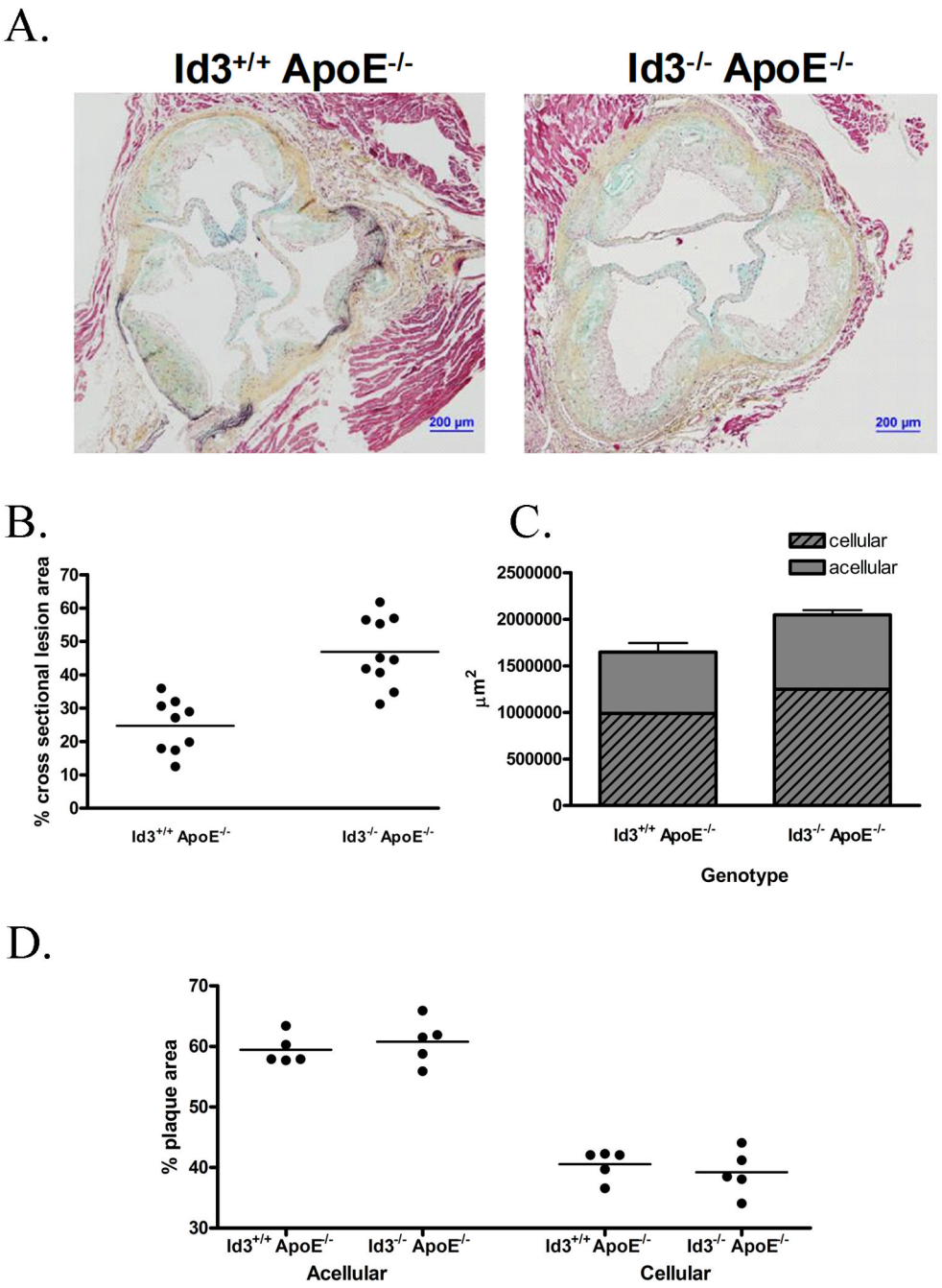

Figure 5. *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice have significantly more atherosclerosis than *Id3*$^{+/+}$ *ApoE*$^{-/-}$ mice in the aortic arch

Id3$^{+/+}$ *ApoE*$^{-/-}$ and *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice were fed a chow or Western diet for 16 weeks. Aortas were perfused with paraformaldehyde and the ascending portions from the aortic cusp to the bifurcation of the brachiocephalic artery were removed and paraffin embedded. Embedded tissue was sectioned into five µm thick intervals. Ten sections, 150 µm apart were stained by the Movat method and analyzed using Image-Pro 5.0 software. ***A,*** Representative cross sections from matched regions of the aortas of *Id3*$^{+/+}$ *ApoE*$^{-/-}$ and *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice after 16 weeks of Western feeding. Quantitation of: ***B*** atherosclerosis in *Id3*$^{+/+}$ *ApoE*$^{-/-}$ and *Id3*$^{-/-}$

ApoE$^{-/-}$ mice after 16 weeks of Western diet for cross sectional lesion area (*:p = 0.0002), ***C,*** cellular vs. acellular content, and ***D,*** percent plaque that is cellular vs. acellular.

Table 1
Association of *ID3* SNPs with IMT in the Diabetes Heart Study

	Without Covariates		With Covariates	
SNP	**Effect**	**p-value**	**Effect**	**p-value**
rs1555026	0.011	0.17	0.008	0.28
rs1055025	0.002	0.59	0.000	0.79
rs11574	0.011	0.01	0.011	0.005
rs2920	0.003	0.47	0.003	0.55
rs1050096	0.007	0.10	0.006	0.10
rs2071495	-0.004	0.49	-0.004	0.34

Table 2

IMT increases stepwise with the minor allele at rs11574

Genotype	Resulting Amino Acid	Number of Patients	Mean IMT (mm)	Standard Deviation	Median IMT (mm)
GG	Ala/Ala	419	0.66	0.13	0.64
GA	Ala/Thr	296	0.69	0.14	0.66
AA	Thr/Thr	49	0.72	0.12	0.71
XX	---	16	0.64	0.10	0.61

$p < 0.01$ for comparison of GG vs. GA vs. AA

Table 3

Loss of *Id3* alters expression of genes in multiple pathways linked to atherogenesis

Atherogenic Process	p-value
Cell to cell signaling and interaction (adhesion of cells)	3.9×10^{-6}
Immune cell trafficking (cell movement of leukocytes)	1.8×10^{-6}
Cell death (apoptosis of eukaryotic cells)	8.5×10^{-11}
Cellular growth and proliferation (proliferation of eukaryotic cells)	6.3×10^{-7}
Cellular movement (cell movement of eukaryotic cells)	7.4×10^{-9}