Original Contribution

The Atherosclerosis Risk Variant rs2107595 Mediates Allele-Specific Transcriptional Regulation of *HDAC9* via E2F3 and Rb1

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Background and Purpose—Genome-wide association studies have identified the *HDAC9* (histone deacetylase 9) gene region as a major risk locus for atherosclerotic stroke and coronary artery disease in humans. Previous results suggest a role of altered *HDAC9* expression levels as the underlying disease mechanism. rs2107595, the lead single nucleotide polymorphism for stroke and coronary artery disease resides in noncoding DNA and colocalizes with histone modification marks suggestive of enhancer elements.

Methods—To determine the mechanisms by which genetic variation at rs2107595 regulates *HDAC9* expression and thus vascular risk we employed targeted resequencing, proteome-wide search for allele-specific nuclear binding partners, chromatin immunoprecipitation, genome-editing, reporter assays, circularized chromosome conformation capture, and gain- and loss-of-function experiments in cultured human cell lines and primary immune cells.

Results—Targeted resequencing of the *HDAC9* locus in patients with atherosclerotic stroke and controls supported candidacy of rs2107595 as the causative single nucleotide polymorphism. A proteomic search for nuclear binding partners revealed preferential binding of the E2F3/TFDP1/Rb1 complex (E2F transcription factor 3/transcription factor Dp-1/Retinoblastoma 1) to the rs2107595 common allele, consistent with the disruption of an E2F3 consensus site by the risk allele. Gain- and loss-of-function studies showed a regulatory effect of E2F/Rb proteins on *HDAC9* expression. Compared with the common allele, the rs2107595 risk allele exhibited higher transcriptional capacity in luciferase assays and was associated with higher *HDAC9* mRNA levels in primary macrophages and genome-edited Jurkat cells. Circularized chromosome conformation capture revealed a genomic interaction of the rs2107595 region with the *HDAC9* promoter, which was stronger for the common allele as was the in vivo interaction with E2F3 and Rb1 determined by chromatin immunoprecipitation. Gain-of-function experiments in isogenic Jurkat cells demonstrated a key role of E2F3 in mediating rs2107595-dependent transcriptional regulation of *HDAC9*.

Conclusions—Collectively, our findings imply allele-specific transcriptional regulation of *HDAC9* via E2F3 and Rb1 as a major mechanism mediating vascular risk at rs2107595. **(*Stroke*. 2019;50:00-00. DOI: 10.1161/STROKEAHA.119.026112.)**

Key Words: atherosclerosis ■ chromosome ■ coronary artery disease ■ proteome ■ transcription

Stroke is the leading cause of permanent disability and the second most common cause of death worldwide.[1] GWAS (Genome-wide association studies) have mapped > 35 genomic loci for stroke most residing in noncoding DNA.[2] However, at many loci the causal variant, gene, and mechanism remain undetermined[3] thus impeding the identification of novel pathways and possible targets for intervention. The *HDAC9* (histone deacetylase 9) gene region on chromosome 7p21.1 represents

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the strongest risk locus for atherosclerotic stroke (large artery stroke)[2] and has further been established as a major risk locus for myocardial infarction, coronary artery disease,[4] and peripheral artery disease,[5] thus implying a broader involvement in atherosclerosis and a major impact on human health.

rs2107595, the lead SNP (single nucleotide polymorphism) in recent GWAS for stroke[2,6] and coronary artery disease[4] resides in noncoding DNA 3′ to the *HDAC9* gene. rs2107595 colocalizes with DNase I hypersensitive sites and histone modification marks H3K27ac and H3K4me1 (ENCODE [Encyclopedia of DNA Elements],[7] genome build hg19) indicating a possible involvement in gene regulatory mechanisms.[8]

We and others recently provided evidence for a central role of *HDAC9* expression levels in atherogenesis and stroke: first, *HDAC9* deficiency attenuates atherogenesis in mouse models of atherosclerosis.[9,10] Second, *HDAC9* expression levels were found to be elevated in human atherosclerotic plaques.[11] Third, gene expression studies in peripheral blood mononuclear cells revealed an association between the rs2107595 risk allele and elevated levels of *HDAC9* mRNA expression with a gene dosage effect.[10] The same variant further associates with both carotid intima-media thickness and the presence of atherosclerotic plaques in the common carotid artery.[11,12] Collectively, these findings point to the possibility that the rs2107595 region mediates disease risk by influencing *HDAC9* expression levels.

In the current study, we aimed to elucidate the molecular mechanisms linking genetic variation in the rs2107595 region to *HDAC9* expression. For this, we employed targeted resequencing of the *HDAC9* locus, proteome-wide search for allele-specific nuclear binding partners, ChIP [chromatin immunoprecipitation], genome-editing, reporter assays, circularized chromosome conformation capture, and gain- and loss-of-function experiments in cultured human cell lines and primary vascular and immune cells. We provide evidence for a regulatory effect of rs2107595 on *HDAC9* expression involving a direct physical interaction between the rs2107595 region and the *HDAC9* promoter. We further demonstrate a role of the E2F3 (E2F transcription factor 3) and Rb1 (Retinoblastoma 1) proteins in mediating allele-specific effects of rs2107595 on *HDAC9* transcription.

Methods

All data and supporting materials have been provided with the published article. Tables and a detailed description of the methodology used for Targeted Resequencing, Proteome-Wide Analysis of SNPs, ChIP, Cell culture and Transfection, RNA isolation and cDNA synthesis, Protein isolation and Immunoblotting, Gene expression analysis, Cell cycle synchronization, the Isolation and Culture of HAoSMCs (Human Primary Aortic Smooth Muscle Cells) and human blood-derived MΦ (macrophages), Dual luciferase reporter assay, Generation of genome-edited Jurkat cell lines, Circular Chromosome Conformation Capture, and Cell proliferation assays is provided in the online-only Data Supplement.

Experiments in primary human cells were approved by the local institutional review board (project No. 17–693). Primary human blood-derived MΦ were obtained from healthy volunteers. Primary HAoSMC were obtained from Dr Civelek (University of Virginia).

Statistical Analysis

The Shapiro-Wilks Test was used to determine the distribution of data sets. Normally distributed data were statistically analyzed with the parametric *t* test, else a Wilcoxon rank-sum test or Wilcoxon signed-rank Test were applied. Data are represented as mean values and SEM unless specified otherwise. Significance is depicted as follows; **P*<0.05; ***P*<0.01; and ****P*<0.001. *HDAC9* regional plots (Figure 1A) were constructed using locuszoom. The upper panel uses data from the large artery stroke analysis of the MEGASTROKE collaboration.[2]

Results

Targeted Resequencing of the *HDAC9* Region Supports Candidacy of rs2107595 as the Causal Variant for Large Artery Stroke

rs2107595 gave the strongest signal in previous GWAS for atherosclerotic phenotypes,[2,6] (Figure 1A, upper panel) and had a >95% posterior probability of being the only causal SNP at this locus in the most recent stroke GWAS from the MEGASTROKE consortium.[2] To further examine the candidacy of rs2107595 as the causal variant at this locus while also capturing rare variants, low-frequency variants, and haplotypes, we performed targeted resequencing of the *HDAC9* gene region including the nearby *TWIST1* (twist basic helix-loop-helix transcription factor 1) and *FERD3L* (Fer3 like BHLH transcription factor) genes in 176 patients with large artery stroke and 176 stroke-free controls (Figure 1A, middle panel; Figure I in the online-only Data Supplement). Genotypes for rs2107595 showed 99.8% agreement with previously obtained microarray and TaqMan genotyping data demonstrating the reliability of our sequencing approach. Overall, we identified 9428 variants (8496 SNPs, 932 insertions/deletions) and 169 haplotype blocks but no rare or low-frequency variants in the rs2107595 haplotype block. Following correction for multiple testing, none of the variants or haplotypes significantly associated with large artery stroke thus arguing against variants with large effect sizes in this region. Next, we used variant-collapsing methods (SKAT [sequence kernel association test] and SKAT-O [optimized sequence kernel association test]) to analyze the conserved 2.5 kb sequence block around rs2107595, the intergenic region between *HDAC9* and *TWIST1*, and the *HDAC9*, *TWIST1*, and *FERD3L* genes (Figure 1A, lower panel). SKAT-O analyses revealed a significant association (*P*=0.017) for the conserved sequence block encompassing rs2107595, while all other equally sized sequence blocks showed higher *P* values. Of note, all proxy SNPs (r^2 with rs2107595 >0.8) localize outside the conserved sequence block. Collectively, these findings support rs2107595 as the causative variant at this locus. Hence, we focused on this variant in our functional analyses.

The rs2107595 Risk Variant Interferes With E2F3 Binding

The rs2107595 region shows enrichment for marks of regulatory chromatin (DNase I hypersensitive sites, H3K27ac, H3K4me1, and H3K9me3) in various cell types listed in HaploReg,[13] Roadmap Epigenomics,[14] and ENCODE[7] (Tables I and II and Figure IIA in the online-only Data Supplement) suggesting a potential involvement of rs2107595 in transcriptional regulation. To identify transcription factors with allele-specific binding at rs2107595 and hence a possible role in transcriptional regulation, we performed proteome-wide analysis of SNPs. This approach is based on the interaction of synthetic oligonucleotides with metabolically

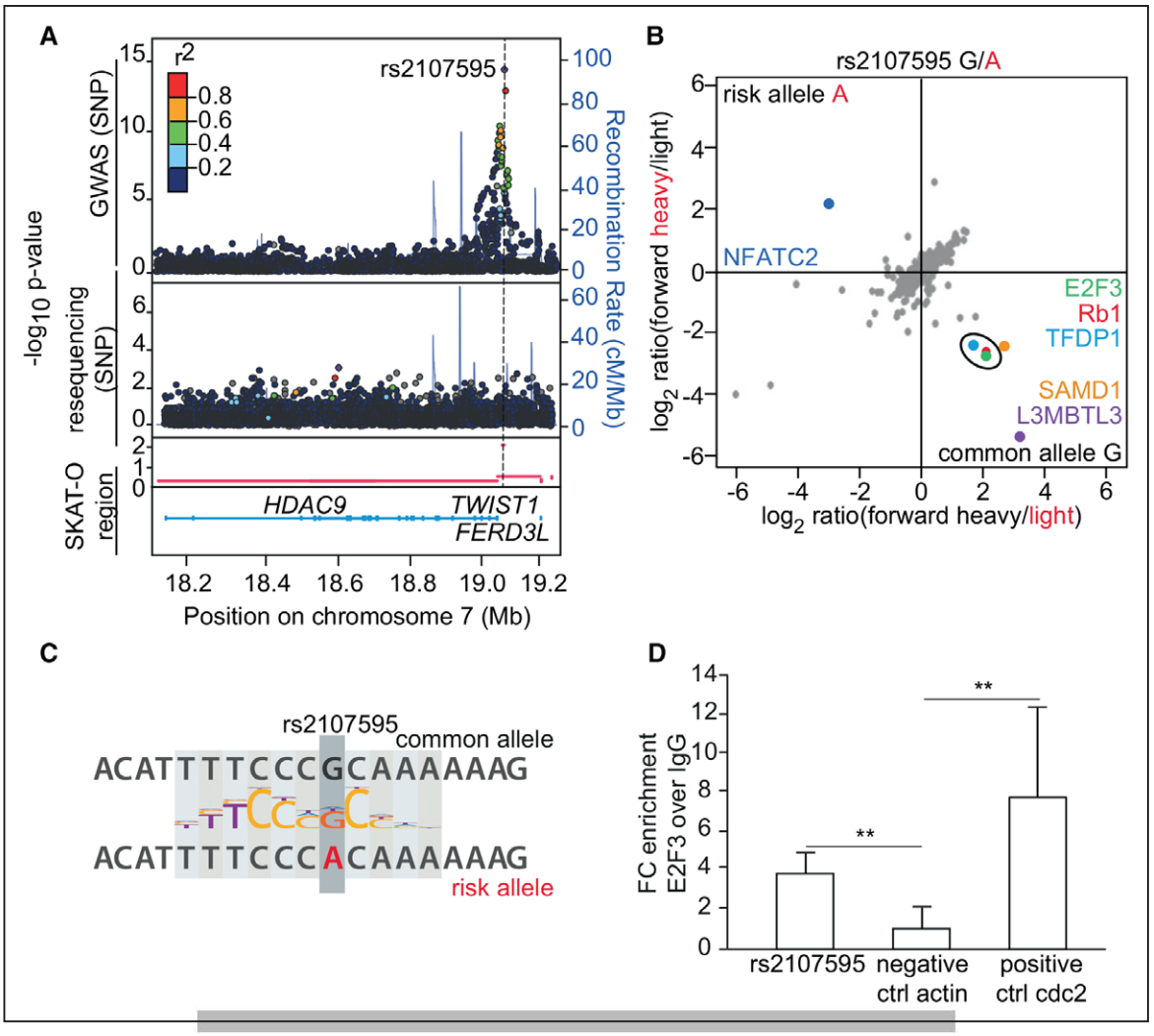

Figure 1. The rs2107595 risk variant interferes with E2F3 binding. **A**, **Top**: regional association plot of the *HDAC9* (histone deacetylase 9) gene region (18123000-19188000, GRCh37/hg19) showing association signals around rs2107595 for large artery stroke in the MEGASTROKE data set.[2] **Middle**: association plot of the same region showing variants identified by targeted resequencing. **Bottom**: $-\log_{10} P$ values for the conserved sequence element around rs2107595, the intergenic region between *HDAC9* and *TWIST1*, and the *HDAC9*, *TWIST1*, and *FERD3L* genes, calculated by variant-collapsing methods (SKAT and SKAT-O). The conserved 2.5 kb sequence block around rs2107595 (position marked by the dashed line) significantly associated with large artery stroke (P=0.017). **B**, Identification of allele-specific binding partners of rs2107595 using PWAS. E2F3, Rb1, TFDP1, SAMD1, and L3MBTL3 preferentially interacted with the common allele (G) whereas NFATC2 preferentially bound to the risk allele (A). **C**, Position Weight Matrix[20] for the consensus site of the human E2F3 protein aligned to the genomic sequence surrounding rs2107595. **D**, Chromatin immunoprecipitation experiments showing in vivo binding of E2F3 to the rs2107595 region in HeLa cells. (n=7–8, mean±SD. Wilcoxon signed-rank test). GWAS indicates Genome-wide association studies; and SNP, single nucleotide polymorphism.

labeled nuclear factors that are subsequently identified by mass spectrometry.[15] Forty-one-bp-SNP-centered oligonucleotides differing only at rs2107595 (Table III in the online-only Data Supplement) were incubated either with light or heavy isotope labeled nuclear factors from HeLa cells. A comparison of the heavy/light ratios of all binding proteins revealed 6 factors surpassing the predefined FDR of 0.01: NFATC2 (nuclear factor of activated T cells 2), a member of the nuclear factor of activated T-cell family,[16] L3MBTL3 (lethal (3) malignant brain tumor-like protein 3), a putative polycomb group protein functioning as transcriptional regulator in large protein complexes,[17] SAMD1 (sterile alpha motif domain containing 1), a protein with a potential role in immobilizing LDL (low-density lipoprotein) in the arterial wall,[18] and all constituents of the E2F3/TFDP1 (transcription factor Dp-1)/Rb1 complex (Figure 1B).

E2F3 and TFDP1 represent transcription factors of the E2F and DP1 families known to complex with Rb proteins.[19] The observed enrichment of E2F3 at the common allele is supported by the prediction of an E2F3 consensus site[20] within the common allele sequence, which is disrupted by the risk allele (Figure 1C). To validate the allele-specific binding of E2F3, we further incubated biotinylated synthetic oligonucleotides with nuclear extracts from HeLa cells and purified the assembled allele-specific nucleoprotein complexes by DNA pull-down. Subsequent immunoblotting revealed enriched binding of E2F3 to the common allele (Figure IIB in the online-only Data Supplement). Finally, we performed ChIP experiments in HeLa cells, which are homozygous for the rs2107595 common allele and thus suited to explore E2F3 binding in vivo. ChIP revealed a significant occupancy of E2F3 at rs2107595 (Figure 1D). Given these results and the known role of E2F and Rb proteins

in transcriptional regulation,[21,22] we considered these proteins to be strong candidates for regulating *HDAC9* expression.

E2F3 and Rb1 Regulate *HDAC9* Expression

To determine the effect of E2F and Rb proteins on endogenous *HDAC9* expression, we next conducted gain- and loss-of-function (Table IV in the online-only Data Supplement) experiments in HeLa cells. Overexpression of E2F3a resulted in a 6-fold increase in *HDAC9* mRNA levels compared with empty vector control. In contrast, overexpression of Rb1 led to a reduction in *HDAC9* expression (Figure 2A and Figure IIIA and IIIB in the online-only Data Supplement). siRNA mediated knockdown of E2F3, E2F4, or both resulted in a significant decrease of *HDAC9* mRNA compared with non-targeting control (Figure 2B and Figure IIIC and IIID in the online-only Data Supplement). In contrast, knockdown of Rb proteins caused a significant increase in *HDAC9* expression (Figure 2C and Figure IIIE and IIIF in the online-only Data Supplement).

E2F and Rb act as transcriptional regulators of cell cycle genes. At the G_1/S boundary repressive Rb proteins become phosphorylated by cyclin-dependent kinases and dissociate from E2F proteins, which then activate the expression of target genes.[21,22] Hence, we analyzed cell cycle-dependent variations in *HDAC9* expression. Synchronization of HeLa cells by hydroxyurea-induced cell cycle arrest at the G_1/S boundary led to a significant increase in *HDAC9* mRNA expression compared with untreated cells (Figure 2D). After release of the cell cycle arrest *HDAC9* mRNA expression further increased during progression through S phase and declined on reaching G2, thus paralleling the activity of E2F proteins across the cell cycle.[23]

The rs2107595 Risk Variant Is Associated With Elevated *HDAC9* Transcription

To examine the association between rs2107595 and *HDAC9* gene expression in cells relevant to atherosclerosis, we first examined primary MΦ and HAoSMCs with defined carrier status at rs2107595. Proinflammatory MΦ were isolated from peripheral blood mononuclear cells obtained from healthy donors (GG genotype: n=7; GA: n=7; AA: n=5, matched for age and sex) and differentiated in vitro (Figure IVA in the

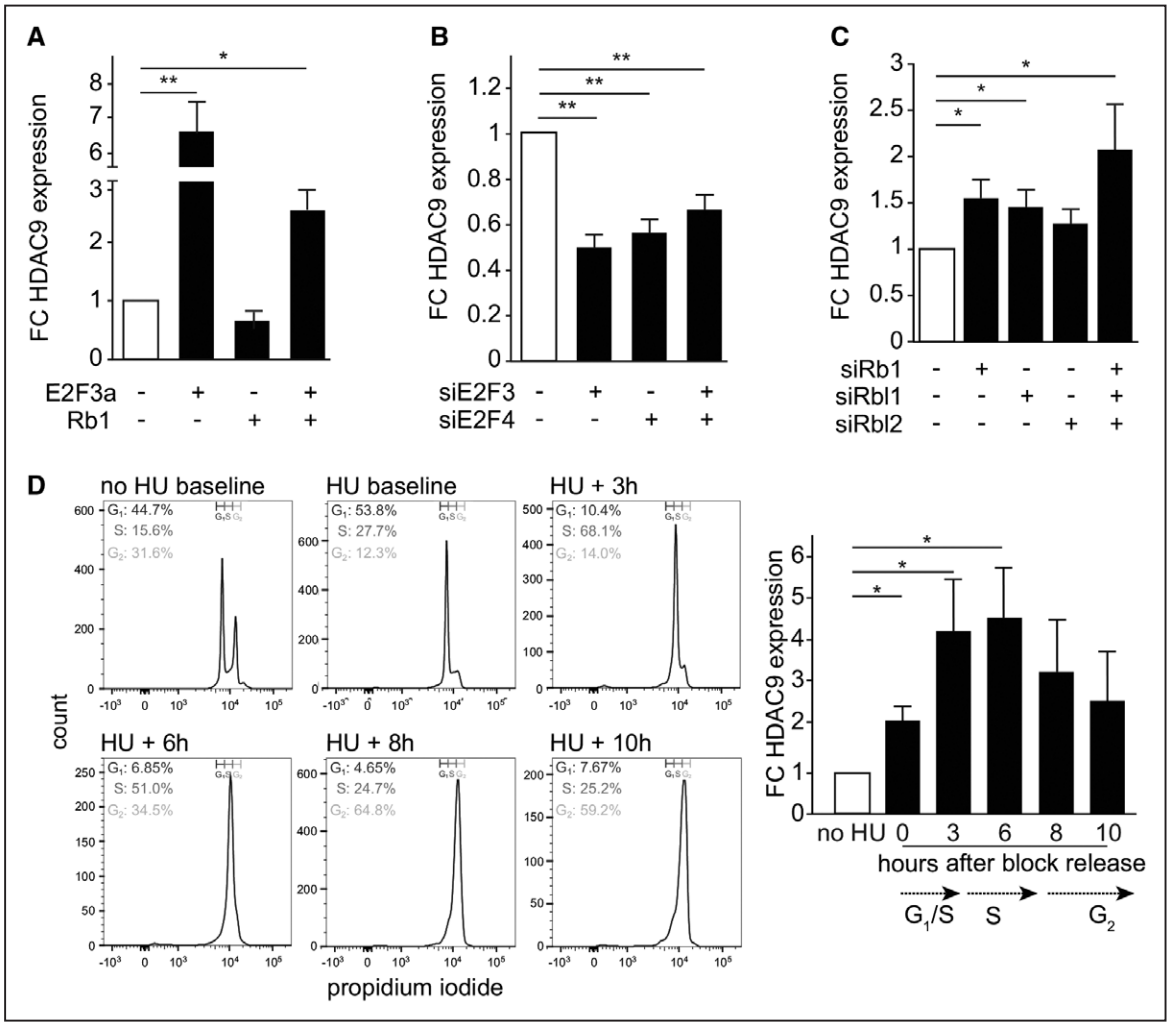

Figure 2. E2F3 and Rb1 regulate *HDAC9* (histone deacetylase 9) expression. **A**–**C**, Fold change (FC) in *HDAC9* mRNA expression assessed in HeLa cells after (**A**) overexpression of E2F3a and Rb1, (**B**) siRNA mediated knockdown of E2F3 and E2F4, and (**C**) siRNA mediated knockdown of Rb1, Rbl1, and Rbl2. n=7. **D**, Cell cycle analysis by flow cytometry and propidium iodide staining in HeLa cells after cell cycle arrest at the G1/S boundary by hydroxyurea. *HDAC9* expression is increased at the G_1/S boundary and during S phase. (n=6–7; FC mean±SEM. Wilcoxon signed-rank test).

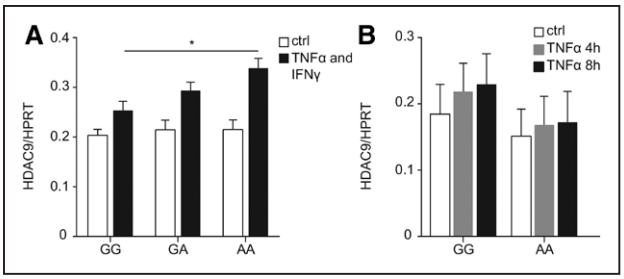

Figure 3. The rs2107595 risk variant is associated with elevated *HDAC9* (histone deacetylase 9) transcription in human primary MΦ. **A**, Human blood-derived monocytes were differentiated in vitro to proinflammatory MΦ. On TNFα (tumor necrosis factor-alpha) and IFNγ (interferon-γ) stimulation MΦ homozygous for the risk allele displayed significantly higher *HDAC9* expression levels compared with common allele carriers (GG: n=5; GA: n=5; AA: n=7). **B**, Cultured postmortem-derived HAoSMC showed no significant expression differences in unstimulated or TNFα-stimulated (4 h or 8 h) HAoSMCs. (GG: n=9; AA: n=6).

online-only Data Supplement). On stimulation with TNF-α (tumor necrosis factor-alpha) and IFN-γ (interferon-γ), MΦ homozygous for the risk allele showed significantly elevated *HDAC9* expression levels compared with MΦ homozygous for the common allele (Figure 3A). Gene expression analysis in cultured HAoSMC (GG genotype: n=9; AA: n=6) revealed no allele-specific differences in *HDAC9* expression before and after 4 or 8 hours of TNFα stimulation (Figure 3B). Also, there was no allele-specific effect on *TWIST1* expression in HAoSMCs and MΦ (Figure IVB in the online-only Data Supplement and results not shown).

To examine the effects of rs2107595 on transcriptional regulation, we further performed luciferase reporter assays in T-lymphoid Jurkat cells, THP-1 acute monocytic leukemia cell line, and PMA (phorbol 12-myristate 13-acetate)-induced THP-1 MΦ, HAoEC (human aortic endothelial cells), and HAoSMC. Forty-one bp-SNP centered fragments containing either the rs2107595 common or risk variant were cloned into a firefly luciferase reporter vector (Figure 4A) and tested for a *cis*-regulatory function by measuring luciferase activity after transient transfection. Transcriptional activity was significantly higher for the risk allele compared with the common allele both in Jurkat cells and PMA-induced THP-1 MΦ[24] (Figure 4A) whereas, we found no allele-specific differences in HAoEC, HAoSMCs, and THP-1 monocytes, (Figure VA through VC in the online-only Data Supplement).

Next, we specifically genome-edited rs2107595 in Jurkat cells using recombinant adeno-associated virus[25] resulting in isogenic cells differing solely at rs2107595. Jurkat cells were chosen because of (1) their immunologic origin, (2) the presence of open chromatin marks both in the rs2107595 region (Figure II in the online-only Data Supplement) and *HDAC9* promoter, and (3) their diploidy and heterozygosity for rs2107595[7,14,26] allowing a one-step editing procedure in either direction (Figure 4B). Cells homozygous for the risk allele exhibited 2-fold higher mRNA levels of *HDAC9* compared with cells carrying the common allele (Figure 4C). Heterozygous cells displayed intermediate mRNA levels compatible with a gene dosage effect. *TWIST1* and *FERDL3* expression levels were below detection limit in these cells (data not shown). Collectively, these results show that rs2107595 regulates *HDAC9* transcription in an allele-specific manner. We further examined allele-specific effects of rs2107595 on *HDAC9* transcription across the cell cycle. After synchronization at the G_1/S-boundary, *HDAC9* levels were significantly elevated in risk allele cells compared with common allele cells (Figure 4D) in accordance with the results obtained in unsynchronized cells (Figure 4C). This difference was sustained for 6 hours after release of the hydroxyurea block. Because of the allele-specific effects on cell cycle associated *HDAC9* expression, we analyzed the effect of rs2107595 on cell proliferation in genome-edited Jurkat cells. Pulse-chase labeling with the thymidine analog EdU and detection by flow cytometry revealed no allele-specific differences for rs2107595 (Figure VIA and VIB in the online-only Data Supplement).

The rs2107595 Region Physically Interacts With a *HDAC9* Promoter

Given the observed effect of rs2107595 on *HDAC9* transcription, we next tested for physical interactions of the rs2107595 region with the *HDAC9* promoter by circularized chromosome conformation capture in isogenic Jurkat cells. Based on Jurkat cell-specific open chromatin structure (DNase I hypersensitive sites) and promoter information (H3K4me3),[7] we selected the promoter viewpoint at nt ≈18 330 000. Mapping the circularized chromosome conformation capture-seq signals to the *HDAC9* gene region revealed a significant interaction between rs2107595 and the promoter region in common allele (GG in Figure 5) but not in risk allele cells (AA) indicating allele-specific differences in chromatin organization. Analyses for an alternative *HDAC9* promoter lacking detectable chromatin marks in Jurkat cells showed lower significance for allele-specific interactions at both viewpoints (Figure VII in the online-only Data Supplement). These results provide further mechanistic evidence for a role of the rs2107595 region in regulating *HDAC9* transcription.

E2F3 Mediates Allele-Specific Effects of rs2107595 on *HDAC9* Transcription

To determine whether the binding of E2F3 and Rb1 at rs2107595 observed in HeLa cells occurs in a truly allele-specific manner in vivo, we next performed ChIP experiments in genome-edited isogenic Jurkat cells. Since E2F3 and Rb1 control cell cycle progression at the G1/S boundary,[23] we arrested these cells with hydroxyurea. On synchronization, we found a significantly enriched occupancy of E2F3 and Rb1 proteins at the common allele compared with the risk allele (Figure 6A and 6B), which was not present in unsynchronized cells (Figure VIIIA and VIIIB in the online-only Data Supplement) possibly reflecting cell cycle-dependent binding of E2F3 and Rb1 to the common allele.

Finally, to examine whether the allele-specific effects on *HDAC9* transcription at rs2107595 are mediated by allele-specific binding of E2F3 and Rb1, we tested the influence of exogenous E2F3a and Rb1 expression in isogenic Jurkat cells. Compared with empty vector control, overexpression of E2F3a but not Rb1 resulted in a significant increase of the ratio between *HDAC9* expression in cells homozygous for the common allele versus cells homozygous for the risk allele (Figure 6C and Figure VIIIC in the online-only Data Supplement). Collectively, these

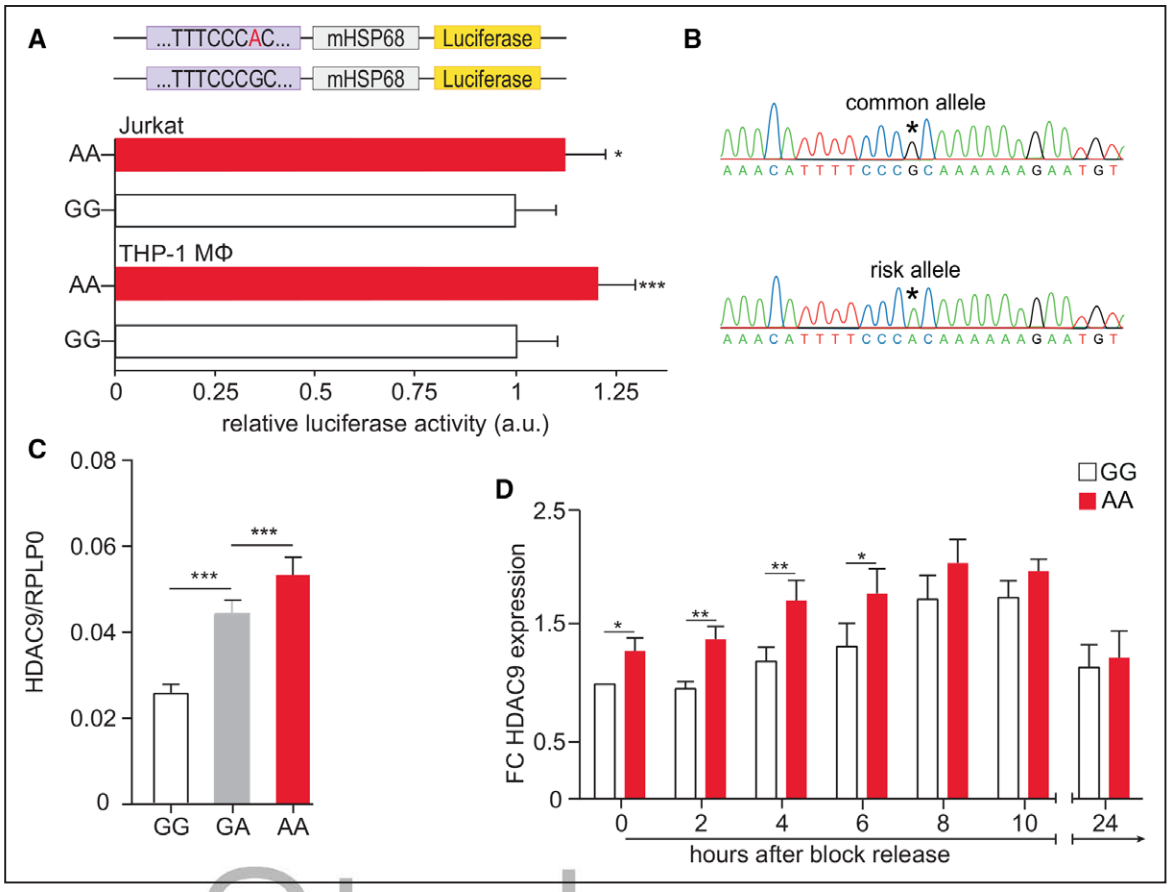

Figure 4. The rs2107595 risk variant is associated with elevated *HDAC9* (histone deacetylase 9) transcription in reporter assays and genome-edited Jurkat cells. **A**, Risk allele associated with a significant increase in luciferase activity compared with the common allele in T-lymphoid Jurkat cells and PMA-induced THP-1 MΦ. **B**, Sanger sequencing of genome-edited Jurkat cells containing either the (*) common allele (G) or risk allele (A). **C**, rs2107595 risk allele-dependent increase in *HDAC9* mRNA expression in genome-edited Jurkat cells. n=16 or 24, mean±SD, *t* test. **D**, Comparative expression analysis during cell cycle progression in isogenic Jurkat cells carrying either the common (G) or risk allele (A). *HDAC9* expression levels increased during the first 8 h after hydroxyurea removal. Risk allele carrying cells showed a significantly increased expression of *HDAC9* until 6 h. (mean±SD; *t* test).

results suggest allele-specific interactions between rs2107595 and the *HDAC9* promotor and show a mediating effect of E2F3 on *HDAC9* expression via rs2107595 (Figure 6D).

Discussion

We present a mechanism by which a noncoding variant at the large artery stroke and coronary artery disease risk locus 7p21.1 regulates *HDAC9* transcription. We show that rs2107595, the likely causal variant at this locus, has allele-specific transcriptional capacity and associates with elevated *HDAC9* expression in cell types relevant to atherosclerosis. We further identify a physical interaction of the rs2107595 region with the *HDAC9* promoter, demonstrate preferential binding of the E2F3/TFDP1/Rb1 cell cycle complex to the common allele, and show that E2F3 mediates *HDAC9* transcription in an allele-specific manner. This novel mechanism for transcriptional regulation of *HDAC9* by E2F3/Rb1 complexes provides a plausible mechanistic link between genetic variation at rs2107595 and disease risk.

Several lines of evidence point to rs2107595 as the causal variant mediating vascular risk: rs2107595 was the lead SNP in GWAS for stroke[2,6] and coronary artery disease,[4] it was the only variant contained in the 95% credible SNP set in the MEGASTROKE,[2] and here using targeted sequencing and SKAT-O analyses we find no variants with large effect sizes in the *HDAC9* region. A transcriptional effect of rs2107595 on *HDAC9* expression is demonstrated by our results in genome-edited T-lymphoid Jurkat cells and in primary proinflammatory MΦ, and is further substantiated by the circularized chromosome conformation capture results, which showed a physical interaction between the rs2107595 region and the *HDAC9* promoter. The directionality of the transcriptional effect was consistent with results from luciferase assays in Jurkat cells and PMA-induced THP-1 MΦ. It was further consistent with the effects on *HDAC9* transcription reported previously for peripheral blood mononuclear cells[10] in that the risk allele was associated with higher *HDAC9* expression levels. Of note, however, our earlier observations in peripheral blood mononuclear cells did not allow attributing allele-specific effects to a specific genetic variant. As such, the current findings represent a major advance.

Our results suggest that the effects of rs2107595 on *HDAC9* expression might be cell-type dependent. While the rs2107595 risk allele was associated with higher *HDAC9* expression levels in proinflammatory human MΦ and genome-edited T-lymphoid Jurkat cells, we found no indication for an allele-specific effect in cultured HAoSMC. Similarly,

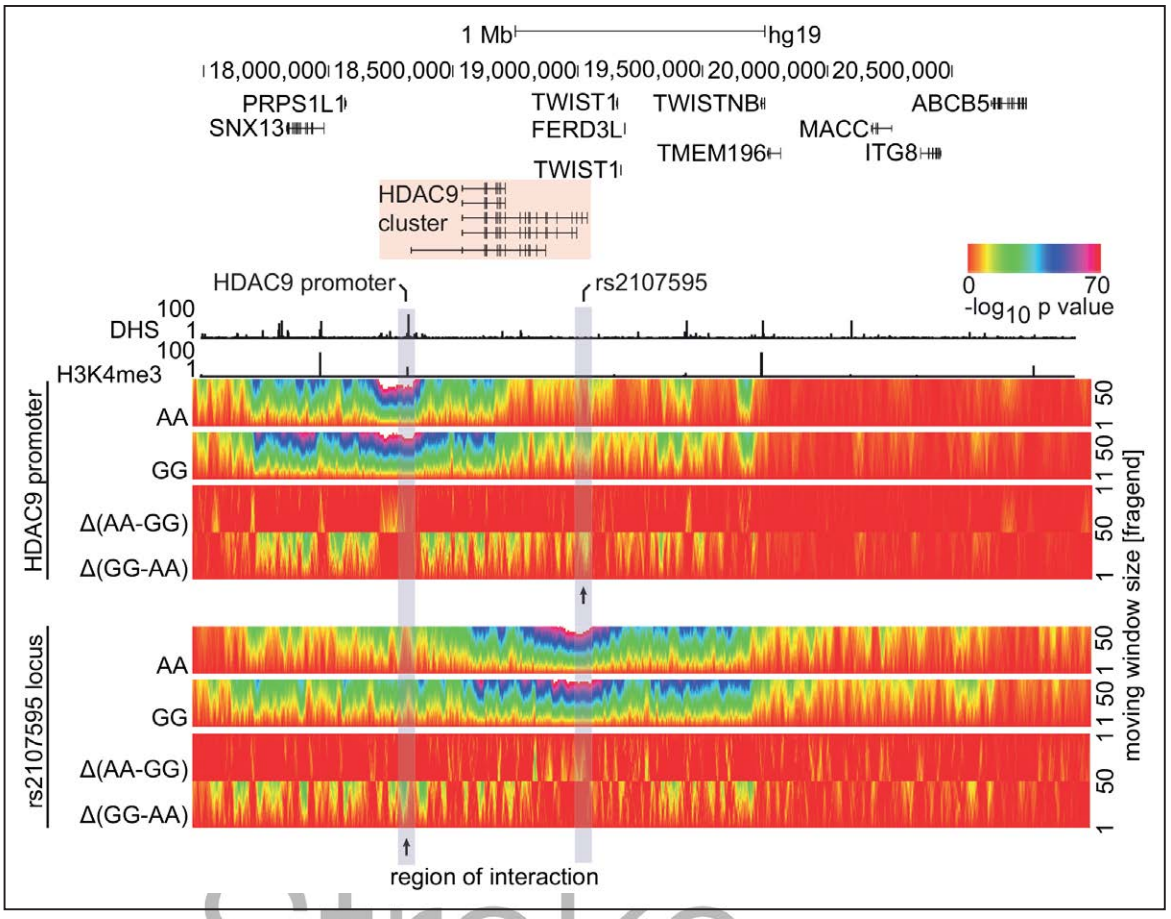

Figure 5. The rs2107595 region physically interacts with a *HDAC9* (histone deacetylase 9) promoter. Domain plot of the circularized chromosome conformation capture (4C)-seq results obtained in isogenic Jurkat cells homozygous for the common (G) or risk allele (A). Shown are the significance levels of the 4C-seq signal coverage with viewpoints in the *HDAC9* promoter (**top**) and rs2107595 region (**bottom**). For both viewpoints, results for individual alleles are depicted in the upper panels with difference plots depicted below. Region of interactions (arrows) are defined by an enrichment of covered fragends within a running window of 1 to 50 fragends. Gray boxes represent the location of the 4C viewpoints. DNase I hypersensitive sites and H3K4me3 histone marks are displayed at the **top**.

luciferase assays showed a higher transcriptional activity with the risk allele in Jurkat cells and proinflammatory THP-1 MΦ but not in undifferentiated THP-1 monocytes, HAoSMCs and HAoECs. Genome-edited Jurkat cells showed a constitutive allele-specific effect on *HDAC9* expression, but an inflammatory stimulus was required to uncover a risk allele-dependent increase in *HDAC9* expression in THP-1 cells and proinflammatory human MΦ. This might be due to a cell-type-specific chromatin conformation affecting the accessibility of transcription factors and thus gene expression.[14] Despite a cell cycle-dependent *HDAC9* expression and the proposed role of *HDAC9* in cell proliferation and cancer,[27–30] we found no allele-specific effect on cell proliferation in isogenic Jurkat cells. However, this might relate to Jurkat cells lacking functional p53,[31] which is transcriptionally regulated by *HDAC9*.[30] Additional work is needed to determine a possible role of rs2107595-mediated control of *HDAC9* in cell proliferation.

An allele-specific interaction between rs2107595 and E2F3/Rb1 complexes is supported by 4 independent lines of evidence: our proteome-wide analysis of allele-specific binding partners, DNA pull-down experiments in combination with immunoblotting, ChIP, and the presence of a consensus-binding site for E2F3 at the common allele. Importantly, the directionality was consistent across all approaches in that the risk allele disrupted binding to E2F3. There is evidence for a role of Rb in atherosclerosis.[32] MΦ specific deletion of Rb has previously been shown to enhance atherosclerosis in ApoE deficient mice. Aside from their crucial function in cell cycle regulation, Rb and E2F proteins cooperatively regulate transcriptional programs for development, metabolism, and cell differentiation.[21] For instance, both proteins are required for proper myeloid cell development[33,34] and control migration and senescence of vascular SMC in human atherosclerotic lesions.[35,36] E2F and Rb further induce foam cell formation through the mTor/SREBP-2 pathway on inflammatory stress.[37] Hence, loss of Rb/E2F binding at the rs2107595 risk variant and the associated increase in *HDAC9* expression might provide a proinflammatory environment promoting atheroprogression. *HDAC9* is known to act as a proinflammatory factor,[9,10,38–40] and a recent gene expression study in large atherosclerotic stroke patients found the rs2107595 risk allele to be associated with enhanced IL-6 (interleukin 6) signaling in peripheral blood.[41] However, *HDAC9* also mediates cholesterol efflux in mouse MΦ.[9] Thus, there may be a synergistic effect of Rb/E2F-mediated *HDAC9* expression on both cholesterol

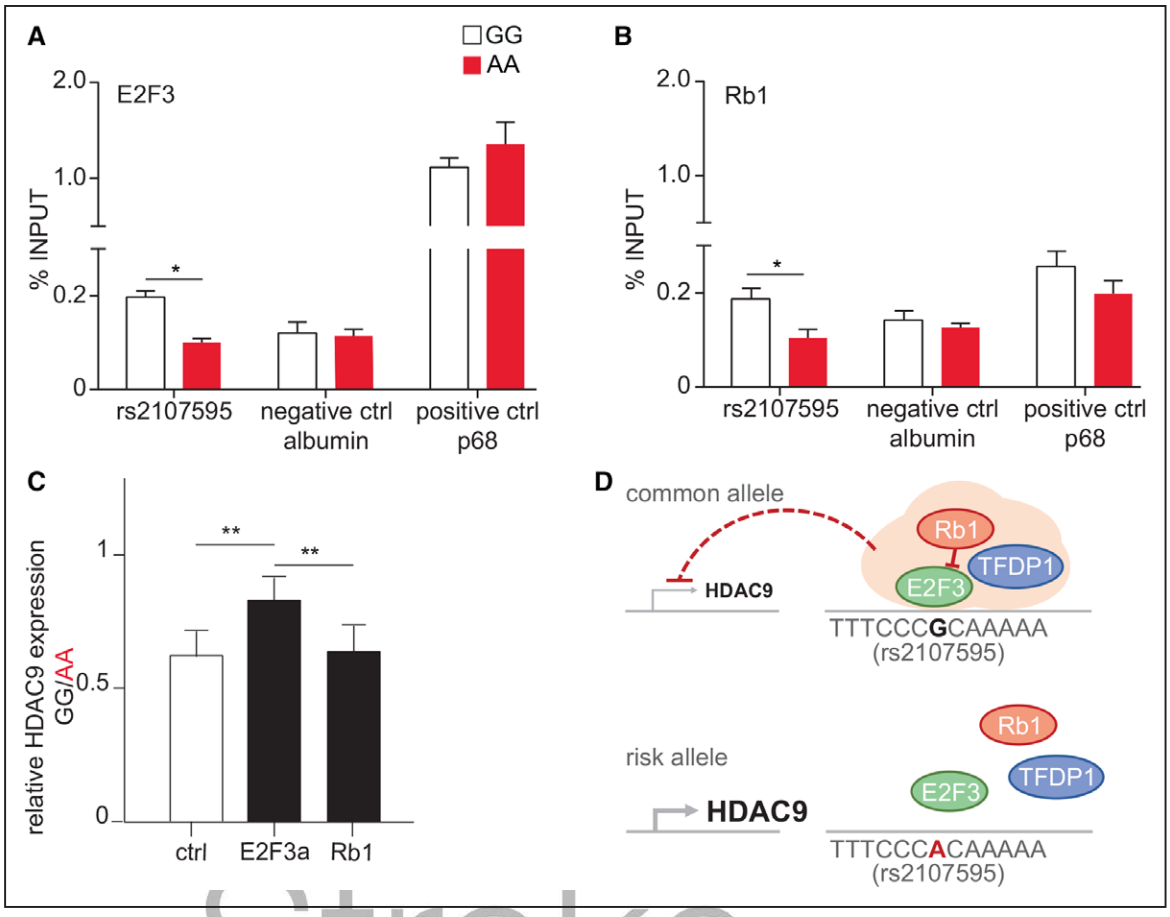

Figure 6. E2F3 mediates allele-specific effects of rs2107595 on *HDAC9* (histone deacetylase 9) transcription. **A** and **B**, Comparative chromatin immunoprecipitation experiments in isogenic Jurkat cells homozygous for the common (G) or risk allele (A). G1/S boundary arrested cells showed an enriched E2F3 (**A**) and Rb1 (**B**) occupancy in common vs risk allele cells at rs2107595. (n=6, mean±SEM, Wilcoxon rank-sum test). **C**, Overexpression of E2F3a resulted in a significant increase of the ratio between *HDAC9* expression in cells homozygous for the rs2107595 common allele (G) vs cells homozygous for the risk allele (A). (n=8–10, mean±SD, *t* test). **D**, Proposed model for the regulatory effect of rs2107595 on *HDAC9* expression by allele-specific binding of the E2F3/Rb1/TFDP1 complex. In the presence of the common allele (G) the E2F3/Rb1/TFDP1 complex is recruited to the rs2107595 region and mediates a repressive effect on *HDAC9* transcription. The risk allele (A) disrupts binding of the E2F3/Rb1/TFDP1 complex, thus resulting in elevated *HDAC9* expression.

metabolism and inflammation in mediating atherosclerosis risk.[39,41,42] Our proteome-wide experiment identified differential interactors aside from E2F3 and Rb proteins, and we cannot exclude a role of these factors in mediating allele-specific effects.[17,43] Yet, the binding of 3 proteins belonging to the same complex (E2F3, TFDP1, and Rb1) together with our functional results strongly support a major role of E2F3/Rb1 in mediating the effects of rs2107595 on *HDAC9* expression.

HDAC9 has emerged as a potential target for drug development. For one, there is evidence from different mouse models of atherosclerosis that lowering *HDAC9* expression may attenuate atherogenesis.[9,10] Second, rs2107595 has been associated with early stages of atherogenesis,[11,12] which makes *HDAC9* an attractive target for early intervention. Third, recent drug discovery programs have resulted in the development of selective class IIa HDAC inhibitors with reasonable specificity and inhibitory activity against *HDAC9*.[44] Interest in *HDAC9* further emerges from the observation that the *HDAC9* locus is implicated in 3 major manifestations of atherosclerosis: stroke, coronary artery disease, and peripheral artery disease. More work is needed to better understand the mechanisms linking genetic variation in the rs2107595 region to atherosclerosis and stroke.

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Disclosures

None.

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