Chromatin Conformation Links Putative Enhancers in Intracranial Aneurysm–Associated Regions to Potential Candidate Genes

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**Background**—We previously showed that intracranial aneurysm (IA)–associated single-nucleotide polymorphisms are enriched in promoters and putative enhancers identified in the human circle of Willis, on which IAs develop, suggesting a role for promoters and enhancers in IAs. We further investigated the role of putative enhancers in the pathogenesis of IA by identifying their potential target genes and validating their regulatory activity.

**Methods and Results**—Using our previously published circle of Willis chromatin immunoprecipitation and sequencing data, we selected 34 putative enhancers in IA-associated regions from genome-wide association studies. We then used a chromatin conformation capture technique to prioritize target genes and found that 15 putative enhancers interact with the promoters of 6 target genes: SOX17, CDKN2B, MTAP, CNNM2, RPEL1, and GATA6. Subsequently, we assessed the activity of these putative enhancers in vivo in zebrafish embryos and confirmed activity for 8 putative enhancers. Last, we found that all 6 target genes are expressed in the circle of Willis, on the basis of RNA sequencing data and in situ hybridization. Furthermore, in situ hybridization showed that these genes are expressed in multiple cell types in the circle of Willis.

**Conclusions**—In 4 of 6 IA-associated genome-wide association study regions, we identified 8 putative enhancers that are active in vivo and interact with 6 nearby genes, suggesting that these genes are regulated by the identified putative enhancers. These genes, SOX17, CDKN2B, MTAP, CNNM2, RPEL1, and GATA6, are therefore potential candidate genes involved in IA pathogenesis and should be studied using animal models in the future. (J Am Heart Assoc. 2019;8:e011201. DOI: 10.1161/JAHA.118.011201.)

**Key Words:** circle of Willis • gene regulation • intracranial aneurysm • single-nucleotide polymorphism • zebrafish

Rupture of an intracranial aneurysm (IA) results in an aneurysmal subarachnoid hemorrhage. The consequences of an aneurysmal subarachnoid hemorrhage are enormous because of the relatively young age at which it occurs (mean age, 50 years) and the high case fatality and morbidity.¹

Genome-wide association studies (GWASs) have identified single-nucleotide polymorphisms (SNPs) associated with IA that are located within 6 genomic risk loci (4q31.23, 8q11.23-q12.1, 9p21.3, 10q24.32, 13q13.1, and 18q11.2).²⁻⁴ These SNPs may not be causal, but rather may be in linkage disequilibrium with the causal variants. Therefore, the entire genomic regions spanning the SNPs in strong linkage disequilibrium with the IA-associated SNPs should be investigated in search of the causal variants.

We recently showed that IA-associated SNPs are enriched in promoters and putative enhancers of the human circle of Willis (CoW), on which IAs develop, suggesting a role for such regulatory regions in IAs.⁵ In this study, we further investigated the role of these putative enhancers in the pathogenesis of IA by identifying their potential target genes and validating their regulatory activity. Specifically, we first aimed to select those putative enhancers that physically interact with the promoter of a nearby gene, thereby also identifying
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RNA was isolated and used to generate cDNA for in situ (CoW3) was also further use for the 4C experiments. The third CoW specimen

The genes that these enhancers interact with are expressed in the circle of Willis.

These genes are potential candidates to study intracranial aneurysm pathogenesis.

What Are the Clinical Implications?

- Further studying the identified potential candidate genes will reveal new insights into intracranial aneurysm pathogenesis.
- This may lead to the development of new therapies that prevent aneurysm development or inhibit growth or rupture.

the potential target genes of these putative enhancers, using chromatin conformation capture technology (4C; a screening method for DNA interaction) on human CoW specimens. Next, we aimed at assessing the intrinsic enhancer activity of these putative enhancers in vivo in zebrafish embryos, followed by gene expression analysis of the identified target genes.

Methods

The data sets discussed in this publication have been made publicly available in the National Center for Biotechnology Information Gene Expression Omnibus Series accession number GSE115304.

Human Material

Four CoW specimens were obtained from the Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. All material was collected from donors whose written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by the Netherlands Brain Bank (http://www.brainbank.nl). Approval for the study was obtained from the institutional review board of the Netherlands Brain Bank. Donor and sample characteristics can be found in Table S1.

The 4 CoW specimens were used for 3 different experiments (Table S1). Two CoW specimens (CoW1 and CoW2) were flash frozen in liquid nitrogen and stored at −80°C until further use for the 4C experiments. The third CoW specimen (CoW3) was also flash frozen in liquid nitrogen, after which RNA was isolated and used to generate cDNA for in situ hybridization (ISH) antisense probe synthesis. The fourth CoW specimen (CoW4) was fixed in 4% paraformaldehyde for 20 hours, washed in PBS twice, dehydrated in ethanol, embedded in paraffin, and divided into 4-μm-thick sections for ISH.

We used DNA from Dutch participants in the GWAS on IA2–4 to amplify putative enhancer sequences for in vivo enhancer activity testing.

Selection of Putative Enhancers and Their Potential Target Genes on the Basis of Physical Contact

For the purpose of this study, we selected all putative enhancers within the 6 GWAS-identified regions most strongly associated with IA: 4q31.23, 8q11.23-12.1, 9p21.3, 10q24.32, 13q13.1, and 18q11.2.2–4 These regions were defined as the entire genomic region spanning the SNPs in strong linkage disequilibrium (r² ≥0.8, using HaploReg,6 on the basis of the 1000 Genomes Project) with the IA-associated SNPs (genomic regions in Table 1).

Within these IA-associated regions, putative enhancers to use for 4C were selected on the basis of H3K27ac enrichment in our previously published chromatin immunoprecipitation and sequencing (ChiP-seq) data,5 using the peak calling algorithms MACS2 (enhancer peaks from Laarman et al5) and OccuPeak (using P=10−12).7 Because putative enhancers did not need to directly overlap with an IA-associated SNPs to be selected for the current study, this approach resulted in more putative enhancers than in our previous study.5 In addition, we also added putative enhancers to the selection if we observed an enrichment of reads in 4C experiments for other putative enhancers or promoters in this study. These putative enhancers were not in the initial selection because the H3K27ac ChiP-seq enrichment was not called by one of the peak calling algorithms. On the basis of the 4C experiments, however, there was contact with other putative enhancers or a promoter, suggestive of interaction. Therefore, these putative enhancers were added to the selection.

Table 1. Coordinates of IA-Associated Regions

| Locus      | Genomic Coordinates of IA-Associated Regions |
|------------|---------------------------------------------|
| 4q31.23    | Chromosome 4: 148364920-148414652            |
| 8q11.23-q12.1 | Chromosome 8: 55288149-55328345            |
| 9p21.3     | Chromosome 9: 22072264-22125504             |
| 10q24.32   | Chromosome 10: 104683480-104958901         |
| 13q13.1    | Chromosome 13: 33672530-33725688            |
| 18q11.2    | Chromosome 18: 20145379-20280647            |

IA indicates intracranial aneurysm.
To measure contact frequencies between our selected set of putative enhancers and promoters of nearby candidate target genes, we used 4C (Figure 1A). In short, bait fragments, or baits, from where chromatin interactions with other DNA regions are investigated, were designed for all putative enhancers to identify interacting DNA fragments for each putative enhancer. Subsequently, baits were designed for candidate target promoters for which the 4C results from the putative enhancers suggested there was physical contact with at least one of the selected putative enhancers (primers in Table S2). We used 2 CoWs (Table S1) for 4C.

The 4C experiments were conducted as described previously. Frozen (~80°C) CoW tissue was homogenized by disrupting it using a Mixer Mill MM 400 tissue disrupter (Retsch GmbH) twice at 30 Hz for 45 seconds, with refreezing in liquid nitrogen in between. Tissue powder was cross-disrupting it using a Mixer Mill MM 400 tissue disrupter (Retsch GmbH) twice at 30 Hz for 45 seconds, with refreezing in liquid nitrogen in between. Tissue powder was cross-linked and further processed, as described previously, using DpnII and Csp6I as restriction enzymes. The primers used for amplification of the genomic regions (bait fragments) used to search for interaction partners of the selected enhancers can be found in Table S2. Amplified fragments were sequenced on the Illumina HiSeq2000 or the Illumina NextSeq500 genome sequencer. Sequence reads were mapped to the human genome (hg19). Reads mapping to multiple fragment ends were removed. 4C coverage was computed by averaging mapped reads in running windows of 21 fragment ends. Quality assessment was done, as previously described. Only data sets that showed a cis/total ratio of mapped 4C captures of at least 40%, and 4C fragment end coverage in a 200 kilobase (kb) window around the viewpoint of at least 35% were included in the analysis (quality assessments of 4C data sets in Table S3).

On the basis of visual inspection of the results from the 4C experiments in a genome browser, we selected those putative enhancers that showed localized increased 4C contact frequencies with potential target promoters suggestive of 3-dimensional spatial interaction.

### Assessment of Enhancer Activity

The intrinsic activity of the putative enhancers thus selected using 4C was tested using an enhancer reporter assay in zebrafish embryos. Putative enhancers were amplified using DNA of participants in the IA GWAS that carried the reference allele in the locus of the amplified putative enhancer. Consequently, we tested the activity of the reference sequences. Primers used for amplification are described in Table S4. The amplified putative enhancers were cloned into an enhancer reporter vector containing the E1b minimal promoter, followed by the green fluorescent protein (GFP) gene (E1b-GFP-Tol2-Gateway; Addgene plasmid No. 37846), using Gateway technology (Life Technologies BV).

The resulting reporter constructs were injected in the cell of wild-type zebrafish embryos at the 1-cell stage, at a final concentration of 10 ng/μL plasmid and 25 ng/μL TOL2 transposase RNA (for incorporation into the zebrafish genome). Embryos were kept at 28.5°C, and GFP expression was assessed between 2 and 5 days after fertilization using a Leica M165 FC fluorescent stereomicroscope (Leica Microsystems GmbH).

### Expression of Target Genes

To analyze the expression of the potential target genes identified with 4C, we first investigated whether these genes are expressed in the CoW using our previously published RNA sequencing data. We subsequently performed ISH on 4-μm CoW paraffin sections to determine the expression patterns within the CoW vessel wall. Antisense RNA probes were designed against the human cDNA sequence of the target genes and selected control genes for 3 different cell types in the CoW: endothelial cells (EDN1 and VWF), vascular smooth muscle cells (VSMCs; ACTA2 and TAGLN), and fibroblasts (FN1). The primers used to generate these probes are described in Table S5. RNA from CoW3 (Table S1) was used to generate cDNA for probe synthesis. ISH was performed as described previously.

### Results

#### Selection of Putative Enhancers and Their Potential Target Genes on the Basis of Physical Contact

We selected 30 putative enhancers within the genomic regions spanning the SNPs in strong linkage disequilibrium with the IA-associated SNPs (hereafter referred to as IA-associated regions; Table 1), on the basis of our previously published ChIP-seq data. Four putative enhancers were added to the selection on the basis of an enrichment of reads in 4C experiments using other putative enhancers or promoters as baits: the enhancers with bait numbers 13, 14, and 17 were added on the basis of an enrichment of reads in the 4C from promoters CDKN2B (baits 13, 14, and 17) and MTAP (only bait 17; Table S2 and Figure S1), the enhancer with bait number 44 was added on the basis of an enrichment of reads in the 4C experiment from the enhancers with bait numbers 39, 42, 43, and 45 (Table S2 and Figure S1). This resulted in a total of 34 selected putative enhancers to be used in the 4C experiments (Table S2). Table S2 also describes the basis on which each putative enhancer was selected: it shows with which peak calling algorithm the enrichment of ChIP-seq reads was identified or if the putative enhancer was identified on the...
Figure 1. Chromatin conformation capture technology (4C) identifies physical interaction between putative enhancers in intracranial aneurysm (IA)–associated regions and nearby promoters. A, Graphical representation of the experimental setup. Putative enhancers were selected within IA-associated regions, on the basis of our previously published chromatin immunoprecipitation and sequencing (ChIP-seq) data in the circle of Willis (CoW).5 4C experiments were performed for the selected putative enhancers to identify physical interactions between these putative enhancers and nearby promoters. B, Genome browser view of a 900 kilobase example region, on chromosome 10, that contains an IA-associated region. This graph shows our previously published ChIP-seq data5 for histone modification H3K27ac, including the enhancer peaks identified using peak calling algorithms MACS25 and OccuPeak,7 the baits used for 4C (promoters in blue, putative enhancers in green), the results of 3 4C experiments (1 in which a selected putative enhancer was used as the bait, and 2 in which a promoter was used as the bait [CNNM2 and RPEL1]), and our previously published RNA-seq (RNA sequencing) data.5 Within this locus, 8 putative enhancers were selected for which an interaction with a promoter of a gene expressed in the CoW was observed. C, Overview of the 6 IA-associated regions, the number of baits used for 4C (including both putative enhancers and promoters), the genes for which an interaction was observed between the promoter and a putative enhancer, and the number of putative enhancers selected for further investigation.
basis of enrichment of reads in another 4C experiment in this study.

To systematically study 3-dimensional spatial contacts and identify potential functionally important links between our selected set of putative enhancers located in IA-associated regions and their target genes, we implemented the following 4C strategy. We designed baits for both the 34 selected putative enhancers and a set of 12 promoters for which the 4C results from the putative enhancers suggested there was physical contact with at least one of the selected putative enhancers (basis for selection of each promoter in Table S2). Of these 46 4C experiments, 40 were successful and met our quality criteria (29 putative enhancers and 11 promoters; Figure S2 and Table S3). The experiments for 7 baits were performed in 2 CoW samples, and the results from these replicated experiments were comparable (panels for the replicated experiments are incorporated in Figures S3 through S6).

On the basis of visual inspection for increased contact frequencies in the 4C results, we found evidence of physical contact between 12 putative enhancers and the promoters of 6 genes: SOX17 (8q11.23-q12.1), CDKN2B (9p21.3), MTAP (9p21.3), CNNM2 (10q24.32), RPEL1 (10q24.32), and GATA6 (18q11.2) (Figure 1B and 1C; Table 2). Most of these interactions were reciprocally confirmed by looking both from the enhancer and from the gene promoter (Table S6). We did not find convincing evidence of physical interaction between the putative enhancers in the IA-associated regions on chromosomes 4 and 13 and the promoters of any nearby genes. Therefore, these putative enhancers were not investigated further.

Visual representations of the 4C results and the selected putative enhancers in all 6 IA-associated regions are shown in Figures 2 through 7.

Together, these results indicate that 12 of 34 putative enhancers show an increased contact frequency with nearby genes.

### Table 2. Summary of 4C, ISH, and Enhancer Activity

| IA-Associated Region | Putative Enhancer Baits | Interaction With Promoter of Genes | Expression in the CoW on the Basis of ISH | Selected Putative Enhancers That Interact With Promoter | Intrinsically Active Putative Enhancers |
|----------------------|-------------------------|-----------------------------------|------------------------------------------|--------------------------------------------------------|----------------------------------------|
| Chromosome 4: 148364920-148414652 | 3 | – | – | – | – |
| Chromosome 8: 55288149-55328345 | 4 | SOX17 | Intima, media, and adventitia | 1 | 1 |
| Chromosome 8: 55407312-55462325 | 0 | – | – | – | – |
| Chromosome 9: 22072264-22125504 | 6 | MTAP and CDKN2B | Intima and media for MTAP; CDKN2B not tested because of technical failure | 3 | 1 |
| Chromosome 10: 104638480-104958901 | 9 | CNNM2 and RPEL1 | Intima, media, and adventitia for both genes | 8 for CNNM2; 7 for RPEL1 | 5 (2 not tested because of technical failure) |
| Chromosome 13: 33672530-33725688 | 4 | – | – | – | – |
| Chromosome 18: 20145379-20280647 | 8 | GATA6 | Intima and media | 3 | 1 (2 not tested because of technical failure) |

4C indicates chromatin conformation capture technology; CoW, circle of Willis; IA, intracranial aneurysm; ISH, in situ hybridization.
promoters in our 4C data, suggestive of 3-dimensional spatial interaction, and can therefore be prioritized as functional enhancers involved in regulating levels of transcription levels of the genes that they are in contact with: **SOX17**, **CDKN2B**, **MTAP**, **CNNM2**, **RPEL1**, and **GATA6**. These putative enhancers and genes were, therefore, selected for further investigation.

**Assessment of Enhancer Activity**

We used our previously published ChIP-seq data and online available ENCODE (Encyclopedia of DNA Elements) data sets (H3K27ac ChIP-seq and transcription factor ChIP-seq) to determine the genomic coordinates of the 12 selected putative enhancers more precisely (coordinates in Table 3). For 2 of these putative enhancers, the CoW ChIP-seq H3K27ac enrichment was broad (≈10 kb) and seemed to consist of multiple separate enrichment peaks. Therefore, these 2 putative enhancers were divided into multiple putative enhancers for enhancer activity assessment: one putative enhancer was divided into putative enhancers 6, 7, and 8 (Figure S7A), and the other enhancer was divided into putative enhancers 11 and 12 (Figure S7B). This resulted in a total of 15 putative enhancers for enhancer activity assessment (Table 3).

An initial search for available information on enhancer activity in the VISTA Enhancer Browser showed that none of the enhancers on chromosomes 8, 10, and 18 were present in the database, and the 2 enhancers on chromosome 9 (enhancers 2 and 4) that were present in the database did not result in a detected reproducible activity at embryonic day 11.5 of mouse development. However, because this database only contains information for a single time point during mouse development, this does not mean that these enhancers are not active.

We successfully cloned 11 of the 15 selected putative enhancers into the E1b enhancer reporter vector for further functional analysis in zebrafish embryos (Figure 8A). For the other 4 putative enhancers, the cloning did not work because of technical failure. Although injection of the empty control vector in zebrafish embryos did not result in any clear fluorescence, injection of 8 of the 11 enhancer vectors resulted in clear GFP.
expression in the embryo in the first 5 days after fertilization (Figure 8B and 8C; Figure S8; Table 2), suggesting that these putative enhancers are functional in vivo.

These results demonstrate that >70% of the putative enhancers that were selected on the basis of our criteria display enhancer activity in vivo.

**Expression of Target Genes**

To assess the expression of the identified potential candidate genes for IA, we first looked into our previously published CoW RNA sequencing data. On the basis of these data, 5 of the 6 genes are expressed in the CoW. Expression of the sixth gene, RPEL1, was found in only 1 of the 4 CoWs; and this expression was low (Figure 9A).

To assess the expression pattern of the candidate genes in the vascular wall of the CoW, we compared the ISH-stained paraffin sections of the CoW (Figure 9A and 9B; Table 2) with those of selected control genes known to be expressed in endothelial cells (VWF and EDN1), VSMCs (ACTA2 and TAGLN), and fibroblasts (FN1; Figure S9). Most of the CoW sections that were used contained a thickened intima suggestive of atherosclerosis. For 1 of the 6 candidate genes, CDKN2B, we were unable to generated a probe to assess the expression pattern because of technical failure.

The expression pattern of SOX17 appeared similar to that of endothelial cell markers VWF and EDN1. There was staining for SOX17 in all 3 layers of the CoW vascular wall, although only sparsely in the adventitia. The expression pattern of MTAP looked most similar to that of fibroblast marker FN1, but the staining for MTAP was weaker and there was no staining in the adventitia. The expression patterns of CNNM2 and RPEL1 were most similar to those of VSMC markers ACTA2 and TAGLN, and some of the smaller stained foci looked similar to the staining of fibroblast marker FN1. The expression pattern of GATA6 looked similar to the expression patterns of VSMC markers ACTA2 and TAGLN and of fibroblast marker FN1. These results suggest that our IA candidate genes are expressed in all 3 major cell types of the vessel wall.

**Discussion**

We used 4C to identify the potential target genes for 8 intrinsically active putative enhancers, residing in 4 of the 6
known genomic risk loci associated with IA (being 4q31.23, 8q11.23-q12.1, 9p21.3, 10q24.32, 13q13.1, and 18q11.2). The likely target genes of these putative enhancers, SOX17 (8q11.23-q12.1), CDKN2B (9p21.3), MTAP (9p21.3), CNNM2 (10q24.32), RPEL1 (10q24.32), and GATA6 (18q11.2), are all expressed in various cell types of the CoW and are, thus, potential candidate genes for IA pathogenesis.

In all IA-associated regions in which we investigated intrinsic enhancer activity, we found that at least one of the selected putative enhancers was capable of inducing GFP expression.
Figure 5. Chromosomal interactions around the intracranial aneurysm (IA)–associated region on chromosome 10. Genome browser view of a 950 kilobase example region on chromosome 10, which contains an IA-associated region. This graph shows a combination of multiple types of data. First, the localization of the IA-associated region on the chromosome is shown. Then, our previously published chromatin immunoprecipitation and sequencing data for histone modification H3K27ac are shown, including the enhancer peaks called using peak calling algorithms MACS2 and OccuPeak. Next, the chromatin conformation capture technology (4C) baits that were selected by combining the results of the 2 peak calling algorithms are shown. Then, the 4C results from this region for those of the 11 baits that were chosen for which the 4C experiment was successful are shown. Bait 19 is the promoter of the gene CNNM2, and bait 29 is the promoter of the gene RPEL1. Next, our previously published RNA sequencing data are shown. Last, the graph shows the 8 putative enhancers that were selected in this region because of their interaction with the promoters of CNNM2 and RPEL1.
expression in vivo in zebrafish embryos, confirming their intrinsic activity. Because our 4C data show that these putative enhancers are in contact with the promoter of a nearby gene, this suggests that a SNP in these putative enhancers could alter the expression of these gene(s). This potentially links the identified IA-associated SNPs to altered gene expression involved in IA pathogenesis.

We identified physical contact between the promoter of SOX17 and an active putative enhancer within the IA-associated region 8q11.23, 60 kb upstream of SOX17. SOX17 is a likely candidate gene for IA because it is expressed in endothelial cells, and a SOX17 deficiency in mice induces IAs.14

We did not find evidence of physical contact between any putative enhancer within the IA-associated region on chromosome 9 and the promoter of previously suggested candidate gene CDKN2B-AS or ANRIL, a long-noncoding RNA gene.15 In contrast, we did find evidence of contact between an active putative enhancer within this IA-associated region and 2 other genes that are expressed in the CoW, MTAP and CDKN2B (putative enhancers 75 kb upstream of CDKN2B and 280 kb downstream of MTAP), suggesting...
involvement of these genes in IA. MTAP homozygous-deficient mice die early during embryogenesis, whereas MTAP heterozygous mice have a reduced lifespan with defects in multiple organs. The role of MTAP in the vasculature, however, is thus far unknown. Loss of CDKN2B increases VSMC apoptosis and results in the formation of aortic aneurysms in mice. The loss of VSMCs in CDKN2B knockout mice may explain its involvement in IAs because IAs have decreased vessel wall integrity related to a loss of VSMCs.

Our results provide evidence of physical contact between the promoter of CNNM2 (locus 10q24.32) and multiple enhancers on chromosome 18. This interaction is supported by genome browser views and chromatin conformation capture technology (4C) data, showing the promoter of GATA6 as a putative enhancer in this region.

Figure 7. Chromosomal interactions around the intracranial aneurysm (IA)–associated region on chromosome 18. Genome browser view of a 1300 kilobase example region on chromosome 18, which contains an IA-associated region. This graph shows a combination of multiple types of data. First, the localization of the IA-associated region on the chromosome is shown. Then, our previously published chromatin immunoprecipitation and sequencing data for histone modification H3K27ac are shown, including the enhancer peaks called using peak calling algorithms MACS2 and OccuPeak. Next, the chromatin conformation capture technology (4C) baits that were selected by combining the results of the 2 peak calling algorithms are shown. Then, the 4C results from this region are shown for those of the 11 baits that were chosen for which the 4C experiment was successful. Bait 36 is the promoter of the gene GATA6. Next, our previously published RNA sequencing data are shown. Last, the graph shows the 3 putative enhancers that were selected in this region because of their interaction with the promoter of GATA6.
Intrinsically active putative enhancers within the IA-associated region on this chromosome, between 120 and 200 kb downstream of the CNNM2 promoter. CNNM2 is a transmembrane protein that plays an important role in magnesium homeostasis. Mutations in CNNM2 lead to hypomagnesemia, a low concentration of magnesium in the blood. In addition, a GWAS links a SNP just 3' of CNNM2 to blood pressure and hypertension. Therefore, changes in CNNM2 expression, induced by changes in enhancer sequences, could alter magnesium levels in the blood and lead to hypertension. Because hypertension is a strong risk factor for IAs and aneurysmal subarachnoid hemorrhages, this might explain the involvement of CNNM2 in IA. We have also found evidence of physical contact between multiple intrinsically active putative enhancers in this locus and the gene RPEL1 (between 125 and 210 kb upstream of the promoter), which encodes a ribulose-5-phosphatase-3-epimerase. However, the role of this gene in IA remains unknown.

We found evidence of physical contact between the promoter of GATA6 (locus 18q11.2) and an active putative enhancer 440 kb downstream. GATA6 is expressed in VSMCs, and a conditional knockout of GATA6 in VSMCs results in perinatal mortality from a spectrum of cardiovascular defects. Other studies have suggested that GATA6 plays a role in the contractility of VSMCs. A loss of VSMC contractility in the CoW could lead to a weakening of the vessel wall and, thereby, increase the likelihood of IA development. Previous studies have already shown loss of VSMCs and changes in VSMC proliferation in IAs, further underlining the potential role of VSMCs in IA.

The ISH experiments suggest that, together, the candidate target genes are expressed in all 3 major cell types of the vascular wall, indicating that multiple cell types in the CoW vessel wall may be involved in IA. In addition, we also saw that all genes are expressed in the intima. Although the intima consists mainly of endothelial cells, cells from other layers can infiltrate the intima and endothelial cells can transition to mesenchymal cells, such as fibroblasts, especially in atherosclerotic lesions. Because most of the CoW sections that were used in this study contained a thickened intima, suggestive of atherosclerosis, the intimal expression found for some genes may be attributable to cells from other layers infiltrating the intima.

We have used 4C technology as a method to identify preferred contacts between putative enhancers and promoters. Physical contacts between putative enhancers and genes do not automatically imply that they functionally interact, but 4C technology enables prioritizing genes and putative enhancers for further analysis. We used the 4C results to further investigate whether the prioritized putative enhancers were able to induce GFP expression in a zebrafish reporter assay and whether the prioritized target genes were expressed in the CoW, additional criteria to evaluate their relevance. More important, 8 of 11 tested enhancers were able to induce GFP expression and all 6 prioritized genes are expressed in the CoW. Unambiguous demonstration that they are functionally wired and that variants in the enhancers affect the expression of these genes needs to come from editing experiments that delete or modify these enhancers in their natural context.

The role of regulatory DNA in disease is a subject of growing interest. With this study, we started investigating the role that enhancers, a subset of regulatory regions, play in IA. We identified putative enhancers in IA-associated regions that contact nearby promoters and display intrinsic enhancer activity in vivo. Future studies should further investigate the role of these putative enhancers in IA and the effect of SNPs on their intrinsic activity, by comparing the in vivo activity of these putative enhancers obtained from

### Table 3. Selected Enhancers for Testing in Enhancer Reporter Vector in Zebrafish

| Putative Enhancer No. | Genomic Coordinates | Interaction With Promoter of Gene |
|-----------------------|---------------------|----------------------------------|
| Chromosome 8:         | 55306508-55309340   | SOX17                            |
| Chromosome 9:         | 22079012-22081464   | MTAP and CDKN2B                   |
| Chromosome 9:         | 22083188-22085645   | MTAP and CDKN2B                   |
| Chromosome 9:         | 22117140-22119074   | MTAP and CDKN2B                   |
| Chromosome 10:        | 104780000-104783499 | CNNM2                            |
| Chromosome 10:        | 104797968-104799025 | CNNM2 and RPEL1                  |
| Chromosome 10:        | 104799792-104803919 | CNNM2 and RPEL1                  |
| Chromosome 10:        | 104804093-104805772 | CNNM2 and RPEL1                  |
| Chromosome 10:        | 104813022-104815607 | CNNM2 and RPEL1                  |
| Chromosome 10:        | 104828907-104831256 | CNNM2 and RPEL1                  |
| Chromosome 10:        | 104880183-104882307 | CNNM2 and RPEL1                  |
| Chromosome 10:        | 104884027-104887002 | CNNM2 and RPEL1                  |
| Chromosome 18:        | 20138725-20140673   | GATA6                            |
| Chromosome 18:        | 20183875-20186066   | GATA6                            |
| Chromosome 18:        | 20188536-20190356   | GATA6                            |
Selected human putative enhancers display intrinsic enhancer activity in vivo. 

**A**, Graphical representation of experimental setup. Plasmids in which the selected putative enhancer is followed by a minimal promoter and a green fluorescent protein (GFP) reporter gene were injected in the cell of a 1-cell stage zebrafish embryo. After 2 to 5 days of development, enhancer activity was assessed through GFP expression in these embryos. 

**B**, GFP fluorescence and bright-field images of injected zebrafish embryos between 2 and 5 days postfertilization (dpf) are shown. Enhancer activity resulted in clear GFP expression for enhancer 3 (Enh3; heart; arrowheads), Enh10 (eye; arrowheads), Enh11 (between the eye and ear; arrowheads), and Enh15 (multiple tissues), but no clear GFP expression was detected in embryos injected with the empty vector. 

**C**, Table that summarizes the findings for all injected putative enhancers in each of the intracranial aneurysm–associated regions on the different chromosomes. For 4 putative enhancers, cloning was unsuccessful because of technical failure.

**Figure 8.**
healthy individuals with the activity of the same enhancers obtained from patients carrying the risk allele. Finally, the role of the identified potential candidate genes (SOX17, CDKN2B, MTAP, CNNM2, RPEL1, and GATA6) in IA pathogenesis should be studied with the use of animal models for these genes.

**Summary**

In this study, we identified potential candidate genes that may play a role in IA: SOX17, CDKN2B, MTAP, CNNM2, RPEL1, and GATA6. This is based on the identification of putative enhancers in IA-associated regions that exhibit intrinsic enhancer activity in vivo and physical contact between these putative enhancers and the promoters of the suggested candidate genes. These candidate genes are expressed in the CoW vessel wall. The suggested candidate genes should be studied in animal models to further elucidate their involvement in IA pathogenesis.

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Disclosures
De Laat is founder of and shareholder of Cergentis.

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SUPPLEMENTAL MATERIAL
Table S1. Characteristics of CoW donors and use of CoW tissue.

| Sample | Sex     | Age | Post-mortem delay (hours) | Cause of death                                | Relevant pathological findings                      | Experiments |
|--------|---------|-----|---------------------------|------------------------------------------------|---------------------------------------------------|-------------|
| CoW1   | Female  | 92  | 06:35                     | Heart failure                                  | -                                                  | 4C          |
| CoW2   | Female  | 54  | 08:00                     | Acute renal failure                            | -                                                  | 4C          |
| CoW3   | Female  | 60  | 08:25                     | Metastasized non-small cell lung carcinoma, euthanasia | Adenocarcinoma metastases in cerebro               | ISH probe synthesis |
| CoW4   | Female  | 89  | 13:00                     | Voluntary euthanasia                           | -                                                  | ISH         |

CoW: circle of Willis, ISH: in situ hybridization, 4C: chromatin conformation capture based technique
Table S2. Primers for 4C experiments.

| Bait | Putative enhancer / promoter | Selected based on | Reading primer | Non-reading primer | Fragment size |
|------|-------------------------------|-------------------|---------------|-------------------|---------------|
| 1    | Putative enhancer             | OccuPeak          | TGTAAGAATTCTCTTGATGC | CTGACTGTTGACAAACATGC | 266          |
| 2    | Putative enhancer             | OccuPeak          | TTTTGATACTATGCTTGATGC | CCACTGCTATGAACAAATTGA | 218          |
| 3    | Putative enhancer             | MACS2 and OccuPeak | TGAAAACATGTAAGGAGAGATGC | CAAAACACCTCACAGACAC | 1108         |
| 4    | Promoter EDNRA                | Enrichment in 4C from bait 2 and located in IA-associated region | TAGTGCAAGGCAGATTTGATGC | AGTTTTTCTTTTCGTGCGAG | 339          |
| 5    | Promoter TEMEM184C            | Enrichment in 4C from bait 4 | TAGGCCGCTTTGTTATTTCTT | GCACCTGCTTTCTTTCTAA | 361          |
| 6    | Putative enhancer             | MACS2 and OccuPeak | TTTCTGAGGCAGATTTGATGC | CAGTTTTGTCCTTCGTATTTA | 417          |
| 7    | Putative enhancer             | OccuPeak          | AGAAATTAACACAGAAGATGC | AGGCAAGGTATTTGAACAGA | 231          |
| 8    | Putative enhancer             | OccuPeak          | AGATTTAGGAGGAATGATGC | CACTCACCCTTTGTATCAC | 221          |
| 9    | Putative enhancer             | OccuPeak          | TGAGATTTCTTATGTCAGATGC | TAAAAGTGGTGCAAAACATG | 209          |
| 10   | Promoter SOX17                | Enrichment in 4C from bait 8 and 9 and located between both IA-associated regions | ATTAAACCTTGTTTTCATGC | AAGGCTCACCTGTACTGAA | 290          |
| 11   | Promoter MTAP                 | Enrichment in 4C from bait 15, 17 and 18 | AATAATGGGTGGCAAGGATGC | GAGTTTGCAAGGCTCAAC | 350          |
| 12   | Promoter CDKN2B               | Enrichment in 4C from bait 13, 14, 15 and 16 | CTAAGCTTCACACTTGATGC | ACCTGACAAAGTGTTTGA | 1150         |
| 13   | Putative enhancer             | Enrichment in 4C from promoter CDKN2B | ATCTGAGGGTGGAATGTGACATGC | AATCTGCTGTTCCTCAAG | 349          |
| 14   | Putative enhancer             | Enrichment in 4C from promoter CDKN2B | GGCTGGTTAGATAAATGG | TCAATGAGAAAGATGGGAATC | 188          |
| 15   | Putative enhancer             | MACS2 and OccuPeak | TGTTTGGATTGTCAGATGC | TGATAAGAAAGGCTCACAAGC | 519          |
|   | Putative enhancer | MACS2 and OccuPeak | ACAGCAAAATAAGAGGCGATC | AGTTTCATTTGGAAATGGACA | 479 |
|---|-------------------|---------------------|------------------------|------------------------|-----|
| 17 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 18 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 19 | Promoter CNNM2    | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 20 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 21 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 22 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 23 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 24 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 25 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 26 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 27 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 28 | Promoter NT5C2   | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 29 | Promoter RPEL1   | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 30 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 31 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 32 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
| 33 | Putative enhancer | MACS2 and OccuPeak | GCAAGAGTTGGAGAGAGATC | AAAGAAATGAGGTAAGGCC | 687 |
|   | Promoter     | Enrichment in 4C from bait | Sequence 1 | Sequence 2 |   |
|---|--------------|----------------------------|------------|------------|---|
| 34 | Promoter STARD13 | Enrichment in 4C from bait 31 | TCTAAGCGAAGGAGACAGTG | CATGCAAGGCTAAGTAACAA | 409 |
| 35 | Promoter STARD13 | Enrichment in 4C from bait 32 | AACCAGGCACACCATTGATC | CCACACGTTTACCATGAATA | 847 |
| 36 | Promoter GATA6 | Enrichment in 4C from bait 40 and 42 | TTTCTCTCCTCCCCCTGATC | CTGACCTGTGGGAACTTTAA | 603 |
| 37 | Promoter CTAGE1 | Enrichment in 4C from bait 39 | AAGCCGACTCTGAACTGATC | CTTTTGCAAGAAGCTGAAGT | 564 |
| 38 | Putative enhancer | MACS2 and OccuPeak | GAGAAGACCGTCTAGGATC | AGCTGGTTGATTGCTTTTAG | 639 |
| 39 | Putative enhancer | MACS2 and OccuPeak | CCAGCTACCTAGTAAGAGT | GAAGAAGGTATGAATCGCTG | 320 |
| 40 | Putative enhancer | MACS2 and OccuPeak | GGGTGTCAGGGAAGAGGATC | AAACCCAGAAGAAGGAAGT | 305 |
| 41 | Putative enhancer | OccuPeak | TGAAGAGTGCTAAATGTGATC | AGTTGCAATTCAACGTCGT | 371 |
| 42 | Putative enhancer | MACS2 and OccuPeak | TTGGGCTCAAGTCCAGATC | TACTCCAAATGTATCCCTGG | 322 |
| 43 | Putative enhancer | MACS2 and OccuPeak | GCCTCTCATCCGTCAGGATC | AAAAGAGAAACTTCCCAC | 484 |
| 44 | Putative enhancer | Enrichment in 4C from putative enhancers 39, 42, 43 and 45 | TAGTTTCACGCAGGGAGATC | TCAAAATATTTTGACCTGC | 616 |
| 45 | Putative enhancer | OccuPeak | GGAAGGAGAGCAGTGTGATC | TAAGTGTTTCTTTGGGCAC | 435 |
| 46 | Putative enhancer | MACS2 and OccuPeak | AGGTCACATTTTTGGGATC | TGGGAAATGTCTTCTTGTT | 545 |

4C: chromosome conformation capture based technique, MACS2 and OccuPeak: two different peak calling algorithms.
Table S3. Quality assessment of 4C datasets.

| Experiment | Mapped reads (#) | Unique reads (#) | Unique cis reads (#) | % cis/total reads | % fragends covered in 200kb around bait | Illumina sequencer used |
|------------|------------------|-------------------|----------------------|------------------|---------------------------------------|------------------------|
| Bait1      | 755              | 511               | 388                  | 75.93            | 12.58                                 | HiSeq2000              |
| Bait2      | 985845           | 511817            | 261177               | 51.03            | 83.64                                 | HiSeq2000              |
| Bait3      | 784762           | 101070            | 72139                | 71.38            | 77.71                                 | HiSeq2000              |
| Bait4      | 1912946          | 1094979           | 787000               | 71.87            | 48.76                                 | NextSeq500             |
| Bait5      | 1371544          | 776302            | 567720               | 73.13            | 71.74                                 | NextSeq500             |
| Bait6      | 155042           | 93765             | 50533                | 53.89            | 70.78                                 | HiSeq2000              |
| Bait7      | 5882             | 4452              | 2739                 | 61.52            | 70.38                                 | HiSeq2000              |
| Bait9      | 444991           | 237082            | 119489               | 50.40            | 69.66                                 | HiSeq2000              |
| Bait10     | 550123           | 346810            | 259534               | 74.83            | 36.39                                 | NextSeq500             |
| Bait11     | 1332622          | 244454            | 166923               | 68.28            | 41.69                                 | NextSeq500             |
| Bait12     | 518001           | 349686            | 231756               | 66.28            | 54.77                                 | NextSeq500             |
| Bait13     | 548898           | 252950            | 170981               | 67.59            | 75.00                                 | HiSeq2000              |
| Bait14     | 878700           | 231613            | 158023               | 68.23            | 67.99                                 | NextSeq500             |
| Bait15     | 1498882          | 725451            | 378666               | 52.20            | 92.44                                 | HiSeq2000              |
| Bait16     | 38918            | 16078             | 11064                | 68.81            | 63.16                                 | HiSeq2000              |
| Bait17     | 623503           | 462548            | 250970               | 54.26            | 89.80                                 | HiSeq2000              |
| Bait18     | 1268250          | 818036            | 595339               | 72.78            | 75.91                                 | NextSeq500             |
| Bait19     | 1268062          | 460813            | 295385               | 64.1             | 52.99                                 | NextSeq500             |
| Bait20     | 290323           | 99313             | 62033                | 62.46            | 79.06                                 | HiSeq2000              |
| Bait21     | 102019           | 51655             | 30592                | 59.22            | 61.68                                 | HiSeq2000              |
| Bait22     | 1144578          | 236495            | 144550               | 61.12            | 68.00                                 | NextSeq500             |
| Bait23     | 301913           | 151624            | 93774                | 61.85            | 73.93                                 | HiSeq2000              |
| Bait24     | 71441            | 35944             | 23058                | 64.15            | 65.42                                 | HiSeq2000              |
| Bait25     | 76077            | 40561             | 24203                | 59.67            | 73.65                                 | HiSeq2000              |
| Bait26     | 21673            | 12350             | 7206                 | 58.35            | 63.30                                 | HiSeq2000              |
| Bait27     | 1064460          | 585079            | 329590               | 56.33            | 78.13                                 | NextSeq500             |
| Bait28     | 1431445          | 704882            | 423212               | 60.04            | 75.71                                 | HiSeq2000              |
| Bait29     | 1444299          | 267995            | 167132               | 62.36            | 74.18                                 | NextSeq500             |
| Bait30     | 1443579          | 326316            | 223077               | 68.36            | 80.18                                 | HiSeq2000              |
| Bait31     | 519997           | 324978            | 221857               | 68.27            | 82.57                                 | HiSeq2000              |
| Bait32     | 227740           | 177263            | 121110               | 68.32            | 78.40                                 | HiSeq2000              |
| Bait33     | 762741           | 416211            | 286082               | 68.73            | 77.71                                 | HiSeq2000              |
| Bait34     | 1209762          | 558368            | 386610               | 69.24            | 73.21                                 | NextSeq500             |
| Bait35     | 737368           | 434569            | 293285               | 67.49            | 56.02                                 | NextSeq500             |
| Bait36     | 779627           | 559613            | 343459               | 61.37            | 42.36                                 | NextSeq500             |
| Bait37     | 999865           | 482525            | 71314                | 14.78            | 67.82                                 | NextSeq500             |
| Bait38     | 3704             | 1750              | 1176                 | 67.2             | 28.19                                 | HiSeq2000              |
| Bait39     | 745333           | 541270            | 350656               | 64.78            | 72.97                                 | HiSeq2000              |
| Bait40 | 778315 | 588192 | 378495 | 64.35 | 72.30 | HiSeq2000 |
|-------|--------|--------|--------|-------|-------|-----------|
| Bait42 | 220089 | 59960  | 38067  | 63.49 | 59.11 | HiSeq2000 |
| Bait43 | 692046 | 244779 | 161096 | 65.81 | 68.89 | HiSeq2000 |
| Bait44 | 1656784 | 274341 | 178126 | 64.93 | 65.11 | NextSeq500 |
| Bait45 | 751997 | 524306 | 358965 | 68.46 | 80.77 | HiSeq2000 |
| Bait46 | 32925  | 11263  | 7430   | 65.97 | 56.85 | HiSeq2000 |

cis reads: reads on the same chromosome as the bait, fragends: fragment ends, 4C: chromatin conformation capture based technique, red numbers do not meet quality criteria
### Table S4. Primers used for testing enhancer activity in zebrafish.

| Putative enhancer number | Forward primer             | Reverse primer             | PCR product size |
|--------------------------|----------------------------|----------------------------|------------------|
| 1                        | CTGGCCATGTGAGGAGCAAT       | AGCTGGAGACTTCCGAGCTA       | 3109             |
| 2                        | TCTTTTGGGTITTCCTATGTCT    | TGTCAGACCCACAAGCTTGTTC     | 2703             |
| 3                        | GCCATGCCCTGTACTAAGAACTA   | AGTGACTCCAAGATACCTGAGAT    | 2681             |
| 4                        | ATGGAAAGCTGGGAGTGTGAA     | AATTGGAATGCTTGCCCTTTGA     | 2307             |
| 5                        | CGAACATGTGTCTACTCGTGTGG   | AGGTTATGTGGAAGAATGTGCA     | 3982             |
| 6                        | TCGATGTCTCGGTAGGGTAGACCA  | TGCCCTGCAAGTCTTCCGGAC     | 1301             |
| 7                        | GAGGGTGCCATCAAGCTAGG      | GCAGAAAAGCGACTCTGGGA       | 4441             |
| 8                        | TCGTCTTCCAGAGCTCCTT       | TACCCAGCAAGACACTGCC        | 1920             |
| 9                        | Technical failure         | Technical failure          | -                |
| 10                       | AGTAAGGCCCCCTCTCTTTTC     | AGGAATACAGGGAGGCACAAGA     | 2829             |
| 11                       | AAGAAGAAATGCTAAAGCAGACGT  | AAGTCCGGAGGTGAGATGGA       | 2893             |
| 12                       | Technical failure         | Technical failure          | -                |
| 13                       | Technical failure         | Technical failure          | -                |
| 14                       | Technical failure         | Technical failure          | -                |
| 15                       | GTCACCCGCTCCTTACCGGG      | TAGCAAATAGCTGACGGGCAA      | 2501             |

PCR: polymerase chain reaction
### Table S5. Primers used to make antisense RNA probes for in situ hybridization.

| Gene   | Control gene / gene of interest | Forward primer | Reverse primer | PCR product size |
|--------|---------------------------------|----------------|----------------|-----------------|
| EDN1   | Control - endothelium           | GGCTGAAGGATCGCTTTGAG | TGGGAAGCCAGTGAGATGGT | 953             |
| VWF    | Control - endothelium           | AGGTGTCACTGCTGCTGATTAT | ATTCAACCCTGCTCCATGT | 890             |
| ACTA2  | Control - smooth muscle         | TGCCCTGGTGTGTAAGAATG | CGATGAAGGATGGCTGGAAC | 785             |
| TAGLN  | Control - smooth muscle         | GGCTGGTGAGTGATGCATGA | CAGTGACAGAGCCCTCAGACT | 649             |
| FN1    | Control - fibroblasts           | AACGAAGGCTTTGAACCAACC | TCGAAGCAGACAGGCAATG | 807             |
| CDKN2B | Gene of interest                | Technical failure | Technical failure | -               |
| CNNM2  | Gene of interest                | CCAGCTCAATTCTTCCCTCC | CCCACAGTGATGACAGACT | 878             |
| GATA6  | Gene of interest                | TGTGCAATGGTCTGTGACT | CTCAGGCCTCCAGAGATGTT | 863             |
| MTAP   | Gene of interest                | GATTCAAGCCCGCGATATG | GGGAGAAGAGATGGTGTG | 827             |
| RPEL1  | Gene of interest                | GTCGGGCTGCAAGATTGG | GATGGGAAGTGGCTCCTCA | 501             |
| SOX17  | Gene of interest                | GCATGACTCCGTTGTAATC | TGCCACTCTCCAAGGTGTAA | 927             |

PCR: polymerase chain reaction
Table S6. Identified contacts between putative enhancers and promoters for those enhancers selected for activity testing.

| Selected enhancer | Enhancer bait # | Promoter bait # (gene) | Reciprocal confirmation of interaction |
|-------------------|-----------------|------------------------|----------------------------------------|
| 1                 | 8               | 10 (SOX17)             | No, 4C from bait 8 was unsuccessful, but increased contact frequency seen from SOX17 promoter (bait 10) |
| 2                 | 13              | 12 (CDKN2B)            | Yes, but strongest increase of contact frequency seen from CDKN2B promoter (bait 12) |
| 3                 | 14              | 12 (CDKN2B)            | Yes, but strongest increase of contact frequency seen from CDKN2B promoter (bait 12) |
| 4                 | 17/18           | 11 (MTAP)              | Yes |
|                   | 17/18           | 12 (CDKN2B)            | No |
| 5                 | 21              | 19 (CNNM2)             | Yes |
|                   | 21              | 29 (RPEL1)             | No |
| 6-8               | 22              | 19 (CNNM2)             | Yes |
|                   | 22              | 29 (RPEL1)             | Yes |
| 9                 | 23              | 19 (CNNM2)             | Yes |
|                   | 23              | 29 (RPEL1)             | Yes |
| 10                | 24              | 19 (CNNM2)             | Yes |
|                   | 24              | 29 (RPEL1)             | Yes |
| 11-12             | -               | 19 (CNNM2)             | Not determined |
|                   | -               | 29 (RPEL1)             | Not determined |
| 13                | 39/40           | 36 (GATA6)             | Yes |
| 14-15             | 42/43           | 36 (GATA6)             | Yes, but strongest increase of contact frequency seen from GATA6 promoter (bait 36) |

4C: chromatin conformation capture based technique
Figure S1. Selection of four additional putative enhancers based on chromatin conformation capture (4C) results from this study.

Both A and B show the intracranial aneurysm (IA)-associated region in each chromosome, the H3K27ac chromatin immunoprecipitation and sequencing (ChIP-seq) experiments in the CoW from our previous publication, including the enhancer peaks called using peak calling algorithms MACS21 and Occupeak. Next are the 4C baits that were selected. A, Selection of putative enhancers with bait numbers 13, 14 and 17. Bait 13 and 14 (green bar) were selected based on an enrichment of reads in the 4C experiment using the promoter of CDKN2B (bait 12) as a viewpoint, and based on a slight enrichment of reads in the H3K27ac (ChIP-seq) experiments. Bait 17 (blue bar) was selected based on an enrichment of reads in the 4C
experiments using MTAP (bait 11) and CDKN2B (bait 12) as a viewpoint, and based on a slight enrichment of reads in the H3K27ac (ChIP-seq) experiments. B, Selection of putative enhancer with bait number 44. Bait 44 (pink bar) was selected based on an enrichment of reads in the 4C experiments using putative enhancers with bait numbers 39, 42, 43 and 45 as a viewpoint, and based on a slight enrichment of reads in the H3K27ac (ChIP-seq) experiments.
Each dot represents a dataset with the quality values for reads in *cis* versus total reads (%) on the y-axis and the percentage of covered fragends in the 200kb around the bait. Cut-off values of 40% for the reads in cis versus total reads and 35% for the percentage of covered fragends were used to include datasets in the study. From the 45 datasets, 4 datasets were removed from the analysis based because they did not meet the quality criteria.
Figure S3. Chromosomal interactions around the intracranial aneurysm (IA)-associated region on chromosome 4, including replicate.

Genome browser view of a 1,100 kb example region on chromosome 4, that contains an IA-associated region. This graph shows a combination of multiple types of data. First, the localization of the IA-associated regions on the chromosome. Then, our previously published chromatin immunoprecipitation and sequencing (ChIP-seq) data for histone modification H3K27ac, including the enhancer peaks called using peak calling algorithms MACS2 and Occupeak. Next are the chromatin conformation capture (4C) baits that were selected by combining the results of the two peak calling algorithms. Then, the 4C results from this region for those of the 5 baits that were chosen for which the 4C experiment was successful, including a replicate (green). Baits 4 and 5 are promoters of the genes EDNRa and TMEM184C. Next are our previously published RNA sequencing data. No putative enhancers were selected in this region since there was no evidence of interactions between enhancers and promoters.
Figure S4. Chromosomal interactions around the intracranial aneurysm (IA)-associated region on chromosome 8, including replicates.

Genome browser view of a 735 kb example region on chromosome 8, that contains an IA-associated region. This graph shows a combination of multiple types of data. First, the localization of the IA-associated region on the chromosome. Then, our previously published chromatin immunoprecipitation and sequencing (ChIP-seq) data for histone modification H3K27ac,1 including the enhancer peaks called using peak calling algorithms MACS21 and Occupeak.2 Next are the chromatin conformation capture (4C) baits that were selected by combining the results of the two peak calling algorithms. Then, the 4C results from this region for those of the 5 baits that were chosen for which the 4C experiment was successful, including replicates (green). Baits 10 is the promoter of the gene SOX17. Next are our previously published RNA sequencing data.1 Lastly, the graph shows the putative enhancer that was selected in this region, because of its interaction with the promoter of SOX17.
Figure S5. Chromosomal interactions around the intracranial aneurysm (IA)-associated region on chromosome 9, including replicates.

Genome browser view of a 800 kb example region on chromosome 9, that contains an IA-associated region. This graph shows a combination of multiple types of data. First, the localization of the IA-associated region on the chromosome. Then, our previously published
chromatin immunoprecipitation and sequencing (ChIP-seq) data for histone modification H3K27ac, including the enhancer peaks called using peak calling algorithms MACS2 and Occupeak. Next are the chromatin conformation capture (4C) baits that were selected by combining the results of the two peak calling algorithms. Then, the 4C results from this region for those of the 8 baits that were chosen for which the 4C experiment was successful, including replicates (green). Baits 11 and 12 are promoters of the genes MTAP and CDKN2B respectively. Next are our previously published RNA sequencing data. Lastly, the graph shows the 3 putative enhancers that were selected in this region, because of their interaction with the promoters of MTAP and CDKN2B.
Figure S6. Chromosomal interactions around the intracranial aneurysm (IA)-associated region on chromosome 10, including replicates.
Genome browser view of a 950 kb example region on chromosome 10, that contains an IA-associated region. This graph shows a combination of multiple types of data. First, the localization of the IA-associated region on the chromosome. Then, our previously published chromatin immunoprecipitation and sequencing (ChIP-seq) data for histone modification H3K27ac, including the enhancer peaks called using peak calling algorithms MACS2 and Occupeak. Next are the chromatin conformation capture (4C) baits that were selected by combining the results of the two peak calling algorithms. Then, the 4C results from this region for those of the 11 baits that were chosen for which the 4C experiment was successful, including replicates (green). Baits 19 is the promoter of the gene CNNM2, baits 29 is the promoter of the gene RPEL1. Next are our previously published RNA sequencing data. Lastly, the graph shows the 8 putative enhancers that were selected in this region, because of their interaction with the promoters of CNNM2 and RPEL1.
Two larger enhancer regions were divided into smaller individual putative enhancers (putative enhancers 6, 7 and 8 in panel A; putative enhancers 11 and 12 in panel B) based on H3K72ac chromatin immunoprecipitation and sequencing (ChIP-seq) results from 4 circles of Willis (CoWs)\textsuperscript{1} (including the enrichment peaks called by the MACS21 and the OccuPeak2 algorithms), H3K27ac ChIP-seq from 7 cell types from ENCODE (derived from the UCSC genome browser), DNaseI hypersensitive clusters from 125 cell types from ENCODE (derived from the UCSC genome browser) and transcription factor ChIP-seq for 161 transcription factors from ENCODE (derived from the UCSC genome browser).
Figure S8. GFP fluorescence and brightfield images of injected zebrafish embryos between 3 and 4 days post fertilization (dpf).
Enhancer activity results in clear green fluorescent protein (GFP) expression for Enh1 (heart; arrowheads), Enh6 (heart; arrowheads), Enh7 (heart; arrowheads) and Enh8 (heart; arrowheads), but no clear GFP expression was detected in embryos injected with the empty vector. Scale bars are 200µm.
In situ hybridization (ISH) on CoW paraffin section shows the expression patterns of five genes in the CoW: VWF and EDN1 (endothelium), ACTA2 and TAGLN (smooth muscle) and FN1 (fibroblasts). VWF and EDN1 are the only genes that are expressed in endothelial cells (arrowheads) lining the lumen of the blood vessel. We have also detected (lower) expression in other cells throughout the intima and media of the CoW vessel wall for VWF and EDN1. ACTA2 and TAGLN are strongly expressed in the media, where smooth muscle cells are mainly present. ACTA2 is also expressed in the thickened intima, these cells are likely also smooth muscle cells that have infiltrated the intima. FN1 is expressed throughout the thickened intima and media. Intima thickening likely results in other cells, such as smooth muscle cells and fibroblasts, infiltrating the intima3-7 in the microphotographs of VWF, EDN1, ACTA2 and FN1. Scale bars are 100µm.
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