miR-103 promotes endothelial maladaptation by targeting IncWDR59

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Blood flow at arterial bifurcations and curvatures is naturally disturbed. Endothelial cells (ECs) fail to adapt to disturbed flow, which transcriptionally direct ECs toward a maladapted phenotype, characterized by chronic regeneration of injured ECs. MicroRNAs (miRNAs) can regulate EC maladaptation through targeting of protein-coding RNAs. However, long non-coding RNAs (lncRNAs), known epigenetic regulators of biological processes, can also be miRNA targets, but their contribution on EC maladaptation is unclear. Here we show that hyperlipidemia- and oxLDL-induced upregulation of miR-103 inhibits EC proliferation and promotes endothelial DNA damage through targeting of novel lncWDR59. MiR-103 impedes IncWDR59 interaction with Notch1-inhibitor Numb, therefore affecting Notch1-induced EC proliferation. Moreover, miR-103 increases the susceptibility of proliferating ECs to oxLDL-induced mitotic aberrations, characterized by an increased micronucleic formation and DNA damage accumulation, by affecting Notch1-related β-catenin co-activation. Collectively, these data indicate that miR-103 programs ECs toward a maladapted phenotype through targeting of IncWDR59, which may promote atherosclerosis.
Arterial endothelial cells (ECs) are exposed to different types of blood flow patterns and shear stresses, which determine the endothelial phenotype. Indeed, laminar flow results in high shear stress and favors a quiescent EC phenotype characterized by a low proliferation rate and a reduced inflammatory activation. In contrast, disturbed flow at arterial branching points and curvatures constantly induces low-grade injury to ECs resulting in apoptosis and inflammation. Moreover, the injury triggers a repair process by EC proliferation, which is essential to maintain the endothelial lining; however, because disturbed flow-induced EC injury is physiologic, endothelial repair remains ineffective. Hence, EC maladaptation generates the Achilles heel of the arterial system, vulnerable to additional damages by hyperlipidemia, which inhibits EC proliferation and sustains chronic endothelial wound healing.

Different types of shear stresses regulate the expression of specific microRNAs (miRNAs), which play key roles in EC injury and proliferation by negatively regulating post-transcriptional gene expression. Indeed, endothelial Dicer affects inflammation and promotes angiogenesis partly by miR-103-mediated suppression of Krüppel-like factor-4 (Klf4), which control the expression of 172 out of 2926 lncRNAs (IncRNAs), which can reprogram ECs toward a more adaptive phenotype.

Let-7b and miR-103 target IncRNAs. Dicer knockout in ECs reduces the expression of 20 miRNAs, including miR-103 and let-7b, in atherosclerotic arteries. To study whether miRNAs target IncRNAs in ECs during atherosclerosis, the sequences of 44 annotated IncRNAs upregulated in EC-Dicer<sup>lox/lox</sup> mice were also expressed in MAoECs (Supplementary Table 2). In addition, read fragments of 5 out of the 58 non-annotated IncRNAs upregulated in EC-Dicer<sup>lox/lox</sup> mice, including IncWDR59, were identified by de novo transcript assembly combined with chromatin-state maps (Fig. 1C–E and Fig. 1c). Among these five IncRNAs, the sequence of one new IncRNA, here called Leonardo, was fully determined by RNA-seq (Fig. 1c, Supplementary Figure 1D and Supplementary Notes). Leonardo gene is 1.317 kb long, consists of 5 exons and 4 introns, and is located between Gm29666 and nucleoporin 50 (Nup50) genes on the negative strand of chromosome 15. Analysis of the read fragments showed that the Leonardo transcript is spliced into a 7.92 bp long mature IncRNA, which is predicted to fold into a thermodynamically stable secondary structure (Fig. 1c and Supplementary Figure 2). In contrast to Leonardo, the sequence of IncWDR59 was only partially determined by RNA-seq. To discover the IncWDR59 full sequence, we performed a modified RACE-PCR (rapid amplification of cDNA ends–polymerase chain reaction) (Supplementary Figure 1E and Supplementary Notes). The IncWDR59 transcript was 1.61 kb long, derived from one exon located on chromosome 8 between the fatty acid 2-hydroxylase (fa2h) and the wdr59 genes (Fig. 1c and Supplementary Figure 1E). Similarly as Leonardo, IncWDR59 transcript showed a thermodynamically stable secondary structure with characteristic functional IncRNA loops (Supplementary Figure 2 and Supplementary Table 3). Moreover, Coding Protein Calculator analysis showed a low protein-coding ability for IncWDR59, similar to Malat1 (used as positive control).

Results
Dicer regulates IncRNA expression in ECs during atherosclerosis. After a 12-week high fat diet (HFD) feeding period, the expression of 172 out of 2926 IncRNAs was differentially regulated in atherosclerotic arteries from EC-Dicer<sup>lox/lox</sup> mice compared with EC-Dicer<sup>WT</sup> mice (fold change (FC) ≥ 1.5, P < 0.05, n = 2 mice per group) as determined by genome-wide microarray analysis (Fig. 1a). Among the differentially regulated IncRNAs, the expression of 97 and of 75 IncRNAs was increased and decreased, respectively, in EC-Dicer<sup>lox/lox</sup> mice (Fig. 1a). Among the 97 upregulated IncRNAs, 6 were downregulated while 5 were also upregulated in the atherosclerotic arteries from Apoe<sup>−/−</sup> mice after Dicer knockout in myocardial cells (Supplementary Table 2). For 39 upregulated IncRNAs, including Jpx, Dklas1, and Sist3os, which play a role in cell viability and differentiation, the transcript sequence was determined (Supplementary Table 2). To determine the sequences of the other 58 upregulated IncRNAs, including the novel most significantly upregulated IncRNA IncWDR59, we performed a strand-specific RNA sequencing (RNA-seq) of the RNA isolated from murine aortic ECs (MAoECs). In addition to 15,199 protein-coding genes (fragments per kilobase million (FPKM) > 1), like the endothelial-specific genes Cdh5, Nos3, and Pecam1 (Supplementary Figure 1A, B), we found that 34,524 IncRNA genes, including 18,478 annotated IncRNAs such as Malat1, Neut1, and Fendrr, and 16,046 novel IncRNA genes were expressed in MAoECs (Supplementary Figure 1A,B). All 39 annotated IncRNAs upregulated in EC-Dicer<sup>lox/lox</sup> mice were also expressed in MAoECs (Supplementary Table 2). In addition, read fragments of 5 out of the 58 non-annotated IncRNAs upregulated in EC-Dicer<sup>lox/lox</sup> mice, including IncWDR59, were identified by de novo transcript assembly combined with chromatin-state maps (Fig. 1c). Among these five IncRNAs, the sequence of one new IncRNA, here called Leonardo, was fully determined by RNA-seq (Fig. 1c, Supplementary Figure 1D and Supplementary Notes). Leonardo gene is 1.317 kb long, consists of 5 exons and 4 introns, and is located between Gm29666 and nucleoporin 50 (Nup50) genes on the negative strand of chromosome 15. Analysis of the read fragments showed that the Leonardo transcript is spliced into a 7.92 bp long mature IncRNA, which is predicted to fold into a thermodynamically stable secondary structure (Fig. 1c and Supplementary Figure 2). In contrast to Leonardo, the sequence of IncWDR59 was only partially determined by RNA-seq. To discover the IncWDR59 full sequence, we performed a modified RACE-PCR (rapid amplification of cDNA ends–polymerase chain reaction) (Supplementary Figure 1E and Supplementary Notes). The IncWDR59 transcript was 1.61 kb long, derived from one exon located on chromosome 8 between the fatty acid 2-hydroxylase (fa2h) and the wdr59 genes (Fig. 1c and Supplementary Figure 1E). Similarly as Leonardo, IncWDR59 transcript showed a thermodynamically stable secondary structure with characteristic functional IncRNA loops (Supplementary Figure 2 and Supplementary Table 3). Moreover, Coding Protein Calculator analysis showed a low protein-coding ability for IncWDR59, similar to Malat1 (used as positive control).
Moreover, inhibition of miR-103 upregulated lncWDR59, Gm4258, Jpx, Inc064083 and Inc039159, but not Inc002669 and Inc073657 (Fig. 1f).

To assess whether let-7b and miR-103 target lncRNAs in the RISC (RNA-induced silencing complex) of ECs, GW182 immunoprecipitation was performed in MAoECs treated with let-7b or miR-103 mimics. Treatment with let-7b mimics enriched the transcripts of Leonardo, lnc002669, and lnc039159 in the RISC, but not those of Jpx and lnc064083 (Fig. 1g). MiR-103 mimics enriched lncWDR59, Gm4258, Inc064083, and Jpx transcripts in the RISC (Fig. 1g). These results indicate a prominent role of miR-103 in the targeting of lncRNAs in ECs.

miR-103 targets lncWDR59. Next, the effect of pro-atherogenic conditions on the expression of the miR-103 targets lncWDR59,

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**Figure Legends**

**a** Intron length distribution of IncRNA genes in the mouse genome. **b** Number of IncRNAs per intron length in the mouse genome. **c** Detected regions of IncWDR59, Gm4258, and Jpx. **d** Detected regions of IncWDR59, Gm4258, and Jpx. **f** FC (let-7b inh/ctrl) and FC (miR-103 inh/ctrl) of IncRNAs. **g** Fold enrichment of IncRNAs in the RISC.
Gm4258, Jpx, and lnc064083 was studied in ECs and murine arteries. In contrast to lnc064083, the expression of LncWDR59, Jpx, and Gm4258 was higher in MAoECs than in bone marrow-derived macrophages (BMDM) (Fig. 2a). Moreover, lncWDR59 levels were similar between ECs and murine aortic smooth muscle cells (MAoSMCs), whereas miR-103 expression was slightly lower in MAoECs than in ECs and BMDM (Supplementary Figure 4A). Low shear stress, which increases endothelial miR-103 expression, reduced lncWDR59 and increased lnc064083 compared to high shear stress, whereas Jpx and Gm4258 expression was similar in MAoECs exposed to low or high shear stress (Fig. 2b). Treatment of MAoECs with oxLDL, (100 μg ml⁻¹) upregulated miR-103 and decreased lncWDR59, lnc064083, and Jpx, but not Jpx, and their expression was restored by inhibition of miR-103 in oxLDL-treated ECs (Supplementary Figure 4A, B and Fig. 2c). Notably, oxLDL treatment did not affect lncWDR59 and miR-103 expression in BMDM and MAoSMCs (Supplementary Figure 4A).

In mice, the expression of LncWDR59, Jpx, lnc064083, and Gm4258 was substantially higher in athero-protected thoraco-abdominal aortas than in athero-prone aortic arches (Fig. 2d), in opposite to miR-103 expression (Supplementary Figure 4C). Moreover, HFD feeding strongly downregulated LncWDR59, lnc064083 and Gm4258 and upregulated miR-103 in the aorta of Apoe⁻/⁻ mice, whereas Jpx was not affected (Fig. 2e and Supplementary Figure 4D). In addition, IncRNA expression was studied in laser-microdissected ECs and macrophages from atherosclerotic lesions from Apoe⁻/⁻ mice (Supplementary Figure 4E). LncWDR59 and Jpx expression was higher in ECs than in macrophages, whereas the Lnc064083 and Gm4258 expression levels were similar in both groups (Fig. 2f). In opposite to the expression of miR-103, in situ hybridization showed that IncWDR59 was more prominently expressed in ECs from EC-Dicer⁰ mice than in those from EC-DicerWT mice (Fig. 2g).

Taken together, these data show that, in contrast to Gm4258, Jpx, and lnc064083, atherogenic conditions such as disturbed flow and hyperlipidemia consistently suppress IncWDR59 in ECs, which is in opposite to the regulation of miR-103.

miR-103 affects EC proliferation by targeting IncWDR59. To study the functional role of miR-103 and IncWDR59 interaction, an anti-sense oligonucleotide (target site blocker (TSB)) was designed to specifically block the binding of miR-103 to its predicted IncWDR59 target site (Fig. 2h). Treatment of MAoECs with TSB increased the expression of IncWDR59, but not that of Jpx, Gm4258, lnc064083, or Klf4 (Fig. 2h). This result confirms the miR-103 target site located at nucleotides 1266–1291 of IncWDR59 transcript (Supplementary Figure 2).

miR-103 promotes pro-inflammatory chemokine expression by targeting KLF413. Although inhibition of miR-103 suppressed Ccl2 and Cxcl1 in MAoECs, silencing lncWDR59 or treatment with TSB did not affect the expression of these chemokines (Fig. 3a and Supplementary Figure 4F). Moreover, treatment of HFD-fed Apoe⁻/⁻ mice with TSB that block the interaction between miR-103 and Klf4 did not alter IncWDR59 expression in arteries (Supplementary Figure 4G). Hence, IncWDR59 does not mediate the pro-inflammatory effects of miR-103 in ECs.

In addition to endothelial inflammation, inhibition of EC proliferation by hyperlipidemia at athero-prone sites promotes atherosclerosis (Supplementary Figure 4H). Accordingly, atheroprotective EC-Dicer knockout restored endothelial proliferation (Supplementary Figure 4I). In vitro, treatment of MAoECs with miR-103 inhibitors or TSB increased EC proliferation and reduced cell cycle-dependent kinase inhibitor 1a (Cdkn1a) expression, whereas IncWDR59 inhibition reduced endothelial replication and upregulated Cdkn1a (Fig. 3b). IncWDR59 inhibition did not alter the expression of Fa2h and Wdr59, indicating that IncWDR59 and not its neighboring genes promotes EC proliferation (Supplementary Figure 4J). Moreover, TSBs promoted cell cycle progression of ECs from the G0 phase to the G1 and S+M phases (Supplementary Figure 4K) and increased proliferation of ECs subjected to low shear stress, but not to high shear stress (Fig. 3c). By contrast, TSB treatment did not affect MAoSMC proliferation (Supplementary Figure 4L). Taken together, these data show that miR-103 inhibits EC proliferation and cell cycle progression by targeting IncWDR59, which may enhance atherosclerosis.

Notch1 and β-catenin in IncWDR59-induced EC proliferation. In addition to Klf4, genes related to Wnt and Notch signaling pathways, which can increase cell proliferation14,15, were most strongly regulated in atherosclerotic arteries from EC-Dicer⁰ mice12. Accordingly, EC-Dicer knockout increased endothelial Notch1 and β-catenin activation in atherosclerotic arteries (Supplementary Figure 5A). Feeding a normal or HFD increased and decreased endothelial Notch1 and β-catenin activation and EC proliferation at predilection sites compared to non-predilection sites (Supplementary Figure 5B-C). In vitro, treatment with the Notch1-inhibitor DAPT or silencing Cnmb1 reduced EC proliferation (Fig. 3d and Supplementary
Fig. 2 Screening of miR-103 target lncRNAs expression and identification of IncWDR59 as target of miR-103. aPCR for miR-103 target lncRNAs expression in different cell types or conditions, such as a MAoECs and BMDM (n = 3–5 per group), b MAoECs under high or low shear stress (HSS or LSS) conditions for 48 h (n = 3 per group), c MAoECs treated with oxidized LDL (oxLDL) for 24 h (n = 4 per group), d aortic arches (Arch) and thoraco-abdominal (Thoracoabd) aortas from 12-week normal diet-fed (ND) or high-fat diet (HFD) (n = 3–5 per group), and e ECs and plaques isolated from HFD mice (−/−) mice fed 4 weeks an HFD using the laser microdissection system (n = 3–7 per group). g In situ hybridization for miR-103 and IncWDR59 on 12 weeks HFD-fed EC-Dicerlox mice. vWF and DAPI were used to stain ECs and nuclei, respectively (n = 4 mice per group). h The 6-mer seed predicted binding site for miR-103 on IncWDR59 transcript predicted using RNAhybrid and the sequence of the competitive TSB, which speciﬁcally inhibits the binding of miR-103 on IncWDR59 transcript (n = 3–8 per group). Non-classical Watson and Crick interactions between A and U were represented with a dot. B2m was used as housekeeping gene for relative quantiﬁcation. EC endothelial cells, MAoECs murine aortic ECs, BMDM bone marrow-derived macrophages, TSB target site blocker. Data are represented as mean ± SEM of the indicated number (n) of repeats. *P < 0.05, **P < 0.01, and ***P < 0.001 by Student’s t-test. Scale bar: 25 μm

Figure 5D,E), indicating that Notch1 and Wnt signaling promote athero-protective EC regeneration.

Next, we studied whether Notch1 and β-catenin play a role in IncWDR59-mediated EC proliferation. Inhibition of miR-103 and TSB signiﬁcantly promoted whereas IncWDR59 knockdown reduced the activation of Notch1 and β-catenin (Fig. 3e, f). TSB-induced EC proliferation was prevented by DAPT, indicating that Notch activation acts downstream of IncWDR59 on endothelial proliferation (Fig. 3g). Surprisingly, silencing Ctnnb1 enhanced the proliferation of TSB-treated ECs (Fig. 3g).

Moreover, DAPT reduced the proliferation of ECs treated with siCtnnb1 and TSB, demonstrating that β-catenin can inhibit EC proliferation by limiting Notch1 activation (Fig. 3h). Accordingly, DAPT decreased β-catenin activation in ECs treated with or without TSBs, whereas silencing Ctnnb1 reduced Notch1 activation (Notch intracellular domain (NICD)) in control-treated ECs (Fig. 3i, j). Moreover, silencing Ctnnb1 increased Notch1 activation in TSB-treated ECs (Fig. 3i, j). These data show that the interaction between miR-103 and IncWDR59 inhibits EC proliferation due to reduced IncWDR59-mediated...
Notch1 and β-catenin activation. Moreover, blocking miR-103-mediated repression of IncWDR59 induced a negative feedback loop in which β-catenin limited Notch1 activation and EC proliferation.

**IncWDR59 blocks Numb to promote Notch1 activation.** To study the mechanisms by which IncWDR59 regulates Notch1 activation, the subcellular localization of IncWDR59 was analyzed. Treatment of MAoECs with miR-103 inhibitors increased the cytosolic levels of IncWDR59 (Fig. 4a), indicating that IncWDR59 may bind to Notch1 or to cytosolic proteins related to the Notch1 signaling pathway. Therefore, we determined the interaction propensity between IncWDR59 and NICD, deltex4, Numb, anterior pharynx-defective 1 (aph-1), presenilin-1 and -2 (psen-1 and -2), and nicastrin using the catRAPID prediction software. IncWDR59 had no binding sites predictable in the amino acid sequence of NICD. Accordingly, IncWDR59 was not enriched in NICD-immunoprecipitated (IP) samples from MAoECs treated with control LNAs or miR-103 inhibitors (Fig. 4b and Supplementary Figure 6A), indicating that IncWDR59 does not bind to NICD. Among the Notch1 pathway-related proteins, Numb contained sites with the highest propensity to interact with IncWDR59 (Fig. 4c and Supplementary Figure 1F). To study whether IncWDR59 binds to Numb, we performed Numb-IP, which revealed that IncWDR59 was not
enriched (Supplementary Figure 6B). However, NICD was high in the Numb-IP fraction, whereas it was low in the input fraction (Fig. 4d, e and Supplementary Figure 6C, D). To test the existence of a possible competitive binding between lncWDR59 and NICD to Numb, Numb-IP was performed in MAoECs after miR-103 inhibition. LncWDR59 was highly enriched in the Numb-IP compared to the input fraction. In addition, miR-103 inhibition reduced NICD levels in the Numb-IP fraction and increased those in the input (Fig. 4d, e and Supplementary Figure 6B–D). Interestingly, one of the four predicted Numb-interaction sites on lncWDR59 transcript comprises miR-103 BS, and involves the phosphotyrosine-binding domain of Numb, through which Numb interacts and induces Notch1 degradation. Hence, these data suggest that binding of miR-103 on lncWDR59 prevents

Fig. 4 LncWDR59 interaction with Notch1-inhibitor Numb. a Nuclear and cytoplasmic lncWDR59 levels in MAoECs treated with miR-103 or control-LNA inhibitors for 24 h. B2m was used as quality marker of nucleus and cytoplasmic RNA isolation (n = 3 per group), expressed as percentage of total b2m RNA expression. Gapdh was used for relative quantification. b LncWDR59 expression in MAoECs before (input) and after NICD-immunoprecipitation (IP) analyzed by qPCR and loaded on 2% agarose gel (lncWDR59 amplicon of 92 bp). Efficiency of NICD-IP was tested by western blot (n = 3 per group). IgGs were used as IP-negative controls. c Heat-maps representing the interaction propensity analysis between lncWDR59 and Numb. The y- and x-axes represent the index of the protein and lncWDR59 sequences, respectively. The colors indicate the interaction score of individual nucleotide and amino acid pairs (rank ± 4). The interaction strength with respect to a training set is represented by the interaction score and the discriminative power values. d, e LncWDR59 expression in MAoECs treated with miR-103 or control-LNA inhibitors for 24 h analyzed by qPCR, following Numb-IP. Amplification products were loaded on 2% agarose gel (lncWDR59 amplicon of 92 bp), together with PCR products from lncWDR59 expression analyzed before IP (d). Numb-IP efficiency was evaluated by western blot (d) and NICD binding to Numb quantified (n = 3 per group) (e). IgGs were used as IP-negative controls. FC fold change of control-LNA inhibitors of the corresponded subcellular fraction, IP immunoprecipitate, NICD Notch intracellular domain, aa amino acid, nt nucleotides. *P < 0.05 by Student’s t-test

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IncWDR59 and Numb interaction, allowing Numb interaction to NICD.

**IncWDR59 reduces oxLDL-induced DNA damage.** EC proliferation regenerates low shear stress-induced damage of ECs. However, additional injuries, for example oxLDL-induced DNA damage, may exhaust the EC repair capacity and enhance atherosclerosis. In line, treatment of MAoECs for 24 h with oxLDL dose-dependently increased DNA damage and reduced EC proliferation determined by immunostaining of phosphor-histone H2AX (γH2AX foci) and Ki67, respectively (Fig. 5a). Moreover, we performed combined γH2AX and CD31 immunostaining of en face prepared aortas from ApoE−/− mice by image stack and three-dimensional (3D) reconstruction microscopy to determine the effect of HFD on endothelial DNA damage. Whereas endothelial DNA damage was not different between predilection and non-predilection sites in ND-fed mice, a 12-week HFD increased γH2AX in ECs at predilection sites, but not at non-predilection sites of atherosclerosis (Fig. 5b). This result indicates that low shear stress increases the

**Fig. 5** Role of IncWDR59 on hyperlipidemia-mediated DNA damage and MN formation in ECs. 

- **a** Immunofluorescence analysis of Ki67 or phosphorylated gamma H2AX histone (γH2AX) in MAoECs treated for 24 h with 25 or 100 μg ml−1 oxLDL. Data are represented as percentage of total number of cells (n = 4 per group).
- **b** The en face 3D reconstructed arch and thoracic aorta from ApoE−/− mice fed 12 weeks of ND or HFD and stained for γH2AX and CD31. The graph represents the number of CD31+ cells with a positive γH2AX staining in the nucleus, normalized on total number of CD31+ cells and expressed in percentage (n = 4 mice per group).
- **c** Analysis of nuclear γH2AX+ vWF+ in the root of EC-DicerWT mice compared to EC-DicerWT mice, fed 12 weeks of HFD, and expressed as percentage of total vWF+ cells (n = 3 mice per group).
- **d** Analysis of nuclear γH2AX+ MAoECs treated with TSBs for 24 h, and stimulated with or without 25 μg ml−1 oxLDL. Data are normalized on total number of ECs and expressed in percentage (n = 4–6 per group).
- **e** MAoECs were treated for 24 h with DAPT or siCtnnb1 to analyze the nuclear γH2AX+ staining. The same treatments were performed in 25 μg ml−1 oxLDL-treated MAoECs, alone or in combination with TSB, to analyze the nuclear γH2AX+ staining. Data are normalized on total number of cells and expressed in percentage (n = 3–5 per group).
- **f** Analysis of micronucleated CD31+ cells (MN cells) and CD31− cells with γH2AX+ micronuclei (γH2AX+ MN) on en face 3D reconstructed arch and thoracic aorta from ApoE−/− mice fed 12 weeks of ND or HFD (n = 3 mice per group).
- **g** Analysis of MN formation and γH2AX− MN from micronucleated MAoECs (γH2AX+ MN) treated for 24 h with 25 or 100 μg ml−1 oxLDL (n = 3 per group). Micronucleated MAoECs treated or not with 25 μg ml−1 oxLDL, captured using confocal microscope. Transversal section shows γH2AX staining around the heterochromatin of principal nucleus and inside of the MN.
- **h** Analysis of MN formation and γH2AX− MN in micronucleated MAoECs treated with TSBs for 24 h, and stimulated with or without 25 μg ml−1 oxLDL. Data are normalized on total number of ECs and expressed in percentage (n = 4–10 per group).
- **i** Analysis of MN formation and γH2AX− MN MAoECs treated for 24 h with DAPT or siCtnnb1, in combination with 25 μg ml−1 oxLDL (n = 3–10 per group). DMSO dimethyl sulfoxide, DAPT γ-secretase inhibitor. Data are represented as mean ± SEM of the indicated number (n) of repeats. *P < 0.05, **P < 0.01, and ***P < 0.001 by Student’s t-test. #P < 0.05 versus all other groups by one-way ANOVA and two-way ANOVA. Scale bar: 25 μm.
susceptibility of ECs to oxLDL-induced DNA damage. Hence, hyperlipidemia may promote atherosclerosis by limiting EC proliferation and by increasing endothelial DNA damage. Notably, endothelial Dicer knockout reduced endothelial γH2AX in hyperlipidemic ApoE−/− mice (Fig. 5c), indicating a role of miRNAs in hyperlipidemia-induced endothelial DNA damage. To study whether miR-103 affects endothelial oxLDL-mediated DNA damage by targeting IncWDR59, γH2AX was analyzed in MAoECs treated with TSBs and oxLDL. TSBs reduced γH2AX in MAoECs treated with or without oxLDL (Fig. 5d). Blocking Notch1 by DAPT or silencing Ctnnb1, alone or in combination with TSB, reduced γH2AX in oxLDL-treated ECs (Fig. 5e). However, in the absence of TSB silencing, Ctnnb1 but not DAPT increased γH2AX in ECs treated with or without oxLDL (Fig. 5e and Supplementary Figure 7A). Taken together, these data indicate that the protective effect of IncWDR59 on endothelial DNA damage is mediated by Notch1 and β-catenin activation.

**IncWDR59 reduces oxLDL-mediated micronuclei formation.**

During proliferation the chance of mitotic errors is high and can lead to the generation of extranuclear chromatin bodies, called micronuclei (MN), where double-strand breaks accumulate.31,32

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**Fig. 6** Role of Sox17 on β-catenin activation, MN formation, and micronuclear DNA damage. a qPCR analysis of Sox17 expression in MAoECs treated with TSB, alone or in combination with 100 μg ml−1 oxLDL for 24 h (n = 4 per group). b qPCR analysis of Sox17 expression in MAoECs treated for 24 h with DAPT or siCtnnb1, alone or in combination with TSB (n = 4 per group). B2m was used for relative quantification. c, d Analysis of nuclear (stars) and perinuclear (arrowheads) β-catenin, activated Notch intracellular domain (NICD) (e), or nuclear Ki67 staining (d) in MAoECs treated for 24 h with Sox17 gapmers, alone or in combination with TSB. Data are normalized on total number of cells and expressed in percentage (n = 4 per group). e, f Analysis of γH2AX+ cells, MN formation, γH2AX MN from micronucleated cells (γH2AX MN) (e), and cdkn2a expression (f) in MAoECs treated for 24 h with Sox17 gapmers, alone or in combination with TSB (n = 4–6 per group). B2m was used for relative quantification. Data regarding MN formation are normalized on total number of cells and expressed in percentage. Data regarding γH2AX MN are normalized on total number of micronucleated cells, following normalization on total number of cells, and expressed in percentage. TSB target site blocker, DMSO dimethyl sulfoxide, DAPT γ-secretase inhibitor, G Sox17 Sox17 gapmers, cdkn2a cyclin-dependent kinase inhibitor 2a. *P < 0.05, **P < 0.01, and ***P < 0.001 by Student’s t-test. By two-way ANOVA and one-way ANOVA. Scale bar: 25 μm
In line, the number of MN was high in proliferating ECs (Supplementary Figure 7B).

Whereas damaged DNA is efficiently repaired in the nucleus, persistent DNA damage in the MN may further impair transcriptional activity in MN and compromise cell function32. Because in vitro oxidative stress can induce MN in ECs, we analyzed the effect of hyperlipidemia on MN formation in ECs in arterial en face prepared aortas. In contrast to the thoracic aorta, where MN-containing ECs were less than in the aortic arch under ND, HFD feeding increased MN formation in aortic arch ECs (Fig. 5f). Moreover, in ND-fed mice micronuclear γH2AX in aortic arch ECs was more frequent than in thoracic ECs (Fig. 5f). HFD feeding increased the micronuclear γH2AX in aortic arch but not thoracic ECs (Fig. 5f). In vitro, oxLDL increased MN formation and micronuclear DNA damage in ECs at predilection sites of atherosclerosis.

**Fig. 7** MiR-103/lncWDR59 conserved function in vivo during atherosclerosis. Apoe<sup>−/−</sup> mice were fed 12 weeks of HFD and were injected four times (once per week) with TSBs or control-LNA oligonucleotides (0.5 mg per 20 g). Paxgene-fixed/paraffin-embedded roots were sectioned and used for (a) elastic van Gieson (EVG) staining, (b) Ki67, and (c) γH2AX immunofluorescence staining. a EVG-stained aortic roots were used to quantify the plaque area per valve, the necrotic core area (expressed as percentage of total plaque area), and the fibrous cap thickness (n = 5 mice per group, 3–4 slides per mouse). Scale bar: 200 μm. b,c The number of Ki67<sup>+</sup>/ and γH2AX<sup>+</sup>/vWF<sup>+</sup> cells was divided to the total number of vWF<sup>+</sup> cells and expressed as percentage (n = 5 mice per group, 3 slides per group). Ctrl control-LNA, TSB target site blockers. *P < 0.05 and **P < 0.01 by Student’s t-test. Scale bar: 25 μm
Next, we studied the role of miR-103-mediated suppression of lncWDR59 on oxLDL-induced MN formation in ECs. TSBs abolished oxLDL-induced MN formation and micronuclear γH2AX (Fig. 5h), an effect prevented by blocking Notch1 or Ctnnb1 silencing (Fig. 5i). Moreover, in the absence of TSB, silencing Ctnnb1 but not DAPT increased MN formation and micronuclear γH2AX in EC treated with or without oxLDL (Fig. 5i and Supplementary Figure 7C, D). Hence, these results suggest that suppression of lncWDR59 by miR-103 in ECs increases MN formation and micronuclear DNA damage in response to oxLDL by inhibiting Notch1 and β-catenin. Moreover, hyperlipidemia may promote atherosusceptibility of predilection sites by DNA damage accumulation in the endothelial MN.

Analysis of cyclin-dependent kinase inhibitor 2a (cdkn2a, p16INK4A) expression showed that treatment of MAoECs with TSB reduced Cdkn2a, whereas DAPT treatment or Ctnnb1 silencing increased and reduced Cdkn2a, respectively (Supplementary Figure 7E). Moreover, DAPT treatment or Ctnnb1 silencing abolished and enhanced TSB-mediated effect on Cdkn2a expression (Supplementary Figure 7E), suggesting that lncWDR59 might protect ECs from DNA damage-associated premature senescence.

lncWDR59 activates β-catenin through Notch1-mediated Sox17 expression. To determine the mechanism by which lncWDR59 promotes Notch1-mediated β-catenin activation, we studied genes related to the Wnt and Notch1 signaling pathways differentially regulated in HFD-fed EC-Dicerlox/lox mice, such as lymphoid enhancer binding factor 1 (Lef1), dickkopf WNT signaling pathway inhibitor 2 (Dkk2), sex determining region Y-box (Sox) 17 (Sox17), deltex E3 ubiquitin ligase 4 (Dtx4), Sox4, and Numb (Supplementary Figure 7F). Inhibition of miR-103 increased Lef1, Dkk2, Sox17, and Dtx4 in MAoECs, but did not affect Sox4 and Numb (Supplementary Figure 7F). LncWDR59 inhibition reduced Sox17, Dtx4, and Sox4 and increased Dkk2, whereas Lef1 and Numb expression was unchanged (Supplementary Figure 7F). Notably, TSBs increased Sox17 expression in MAoECs treated with or without oxLDL, and prevented the oxLDL-induced downregulation of Sox17 (Fig. 6a). Although Sox17 expression was decreased by both DAPT and Ctnnb1 silencing, TSBs prevented only the effect of Ctnnb1 silencing on Sox17 expression (Fig. 6b). Silencing Sox17 abolished the activation of β-catenin induced by TSB, whereas TSB-mediated Notch1 activation was slightly increased after silencing Sox17 (Fig. 6c).

Inhibition of Sox17 enhanced EC proliferation, cdkn2a expression, γH2AX, MN formation, and micronuclear γH2AX in TSB-treated MAoECs (Fig. 6d-f). Taken together, these data indicate that lncWDR59 promotes β-catenin activity by upregulating Notch1-mediated Sox17 expression. Moreover, like β-catenin, Sox17 limits EC proliferation and reduces micronucleic DNA damage.

Targeting of lncWDR59 by miR-103 promotes atherosclerosis. To assess the role of the interaction between miR-103 and lncWDR59 in vivo, Apoe−/− mice were treated with lncWDR59-TSBs or control LNA-modified oligonucleotides (0.5 mg per 20 g per injection) during the last 4 weeks of a 12-week HFD feeding program. Compared with mice treated with control LNAs, treatment with lncWDR59-TSBs reduced atherosclerosis, the necrotic core area, and endothelial DNA damage, and increased EC proliferation (Fig. 7a-c), whereas the fibrous cap thickness and the Cxcl1-expressing ECs (Supplementary Figure 7H) were not affected (Fig. 7a). Taken together, these data indicate that miR-103 enhances atherosclerosis and impairs EC regeneration partly by suppressing lncWDR59.

Conserved function of human lncWDR59 in ECs. In humans, a homolog of lncWDR59 (hsa-lncWDR59) exists expressed from a conserved genomic location between the FA2H and WDR59 genes on human chromosome 16 (Fig. 8a). MiR-103 mimics enriched hsa-lncWDR59 in the GW182-IP fraction from human aortic ECs (HAoECs) compared to control mimics (Fig. 8b). Transfection of HAoECs with hsa-lncWDR59 inhibitors downregulated hsa-lncWDR59 expression (Supplementary Figure 7G), reduced EC proliferation, and MN formation (Fig. 8c).

In human atherosclerotic lesions, in situ hybridization indicated that endothelial expression of miR-103 and lncWDR59 was high and low, respectively, in human atherosclerotic lesions compared with control vessels (Fig. 8d). Analysis of hsa-lncWDR59 expression in human atherosclerotic lesions indicated that hsa-lncWDR59 and SOX17 expression levels were negatively correlated with necrotic core area and positively with endothelial proliferation, whereas the levels of Cdkn2a were correlated with increased necrotic core area (Fig. 8e and Supplementary Figure 7I). Moreover, the levels of hsa-lncWDR59 correlated with increased SOX17 levels (Fig. 8e). Taken together, these data indicate that hsa-lncWDR59 shows a conserved function in human ECs and might also play an athero-protective role in humans.

Discussion

Chronic, low-grade endothelial injury and regeneration is a characteristic feature of endothelial maladaptation to disturbed flow at arterial bifurcations. We found that miR-103 inhibits endothelial proliferation by targeting lncWDR59, which resulted in reduced activation of Notch1 signaling. Moreover, suppression of lncWDR59 by miR-103 increased the susceptibility of proliferating ECs to oxLDL-mediated DNA damage and mitotic errors by reducing Notch1-mediated β-catenin activation. Hence, miR-103-mediated suppression of lncWDR59 promotes endothelial maladaptation by impairing EC regeneration and increasing mitotic aberrations during hyperlipidemia (Supplementary Figure 8).

Endothelial Dicer promotes arterial susceptibility to atherosclerosis by generating miRNA9,10,12. In addition to miRNAs, miRNAs can interact with other RNA species, like lncRNA transcripts. This interaction can inhibit miRNA function, because lncRNAs can act as molecular decoys or sponges of miRNAs or mediate the silencing of lncRNAs. Our data show that Dicer suppressed the expression of multiple lncRNAs in ECs, indicating that targeting of lncRNAs by miRNAs plays a role in EC maladaptation. The miRNAs miR-103 and let-7b silenced lncRNAs, such as the novel intergenic lncRNAs lncWDR59 and Leonardo, respectively, which contain mainly non-canonical miRNA binding sites. We confirmed that miR-103 targets lncWDR59, which was downregulated at predilection sites and by hyperlipidemia, via a 6-mer binding site in ECs. Whereas miR-103 promotes endothelial inflammation by silencing KLF4, the interaction between miR-103 and lncWDR59 in ECs inhibited cell proliferation and, in response to oxLDL, increased micronucleic DNA damage. Hence, miR-103 might promote an EC maladaptive phenotype by the synergistic silencing of KLF4 and lncWDR59. In line, our data indicate that lncWDR59 limits the increased susceptibility of proliferating ECs to oxLDL-induced DNA damage and micronuclei formation. Moreover, miR-103-induced silencing of lncWDR59 may mediate the effect of endothelial Dicer on disturbed flow-induced EC proliferation and...
Fig. 8 Human IncWDR59 conserved role in vitro and in human plaques. a Gene locus and full transcript of human IncWDR59 (hsa-incWDR59) sequence on chromosome 16 between FA2H and WDR59 genes. b Enrichment of hsa-incWDR59 transcripts in the RISC complex after transfection of human aortic ECs (HAoECs) with miR-103 or control mimics together with a mutant form of Ago2, following an immunoprecipitation of GW182 protein (GW182-IP). Results of three experiments are shown. GAPDH was used as housekeeping gene and for relative expression analysis. c Analysis of Ki67 immunostaining and MN formation in HAoECs transfected for 48 h with hsa-incWDR59 gapmers (hGlncWDR59). Data are normalized and expressed as percentage of the total number of cells (n = 4 per group). d In situ hybridization for miR-103 and IncWDR59 on human plaques. Areas lacking of lesions were used as control areas. vWF and DAPI were used to stain ECs and nuclei, respectively (n = 4 per group). e Correlation of the relative expression levels of hsa-incWDR59 in human carotid lesions with necrotic core area, SOX17 expression, and Ki67 endothelial staining (Ki67+ vWF+), (n = 17–20 per group). *P < 0.05 by Student’s t-test and a compute nonparametric Spearman’s rho correlation analysis. Scale bar: 25 μm
hyperlipidemia-induced EC injury and, together with the targeting of Ki67, promote atherosclerosis. Previous studies indicate that endothelial Notch1 signaling triggers endothelial differentiation,38–39, promotes proliferation of adult ECs, and prevents athero-progression. Notably, endothelial Dicer suppresses EC proliferation and Notch1 signaling pathway at predilection sites of atherosclerosis,12, and miR-103 targeting of IncWDR59 reduced EC proliferation by inhibiting Notch1 activation. Therefore, these findings support the hypothesis that miRNA silencing of lncRNAs, e.g., IncWDR59 silencing by miR-103, may promote endothelial maladaptation and atherosclerosis. Notably, IncWDR59 promoted Notch1 activation by binding to Numb, which mediates NICD degradation,39, therefore impeding Numb interaction with Notch1. Therefore, disturbed flow promotes Notch1 activation not only via miR-126–5p-mediated suppression of Notch1-inhibitor Dllk144, but also through IncWDR59 targeting of Notch1-inhibitor Numb. Taken together, down-regulation of miR-126–5p and upregulation of miR-103 may both contribute to EC maladaptation by inhibiting Notch1 activity.44 Proliferating ECs are vulnerable to mitotic aberrations and DNA damage, e.g., by oxidative stress,39 due to the accumulation of damaged DNA in MN, which can lead to mitotic catastrophe.42,43,44 Although MN frequency was increased in proliferating ECs, IncWDR59 protected proliferating ECs from micronucleic DNA damage accumulation via β-catenin signaling, activated through Notch1-induced Sox17 expression. Accordingly, β-catenin can promote cell cycle checks and DNA damage repair processes in response to DNA damage in proliferating cells.44 Moreover, the activation of β-catenin generated a negative feedback loop that limited Notch1 and protected ECs from aberrant proliferation. Hence, IncWDR59 plays a dual role in EC maladaptation by promoting EC proliferation through Notch1 activation and protecting ECs during proliferation from micronucleic DNA damage accumulation through β-catenin activation. Decreasing the effect of IncWDR59 by hyperlipidemia- and oxLDL-mediated upregulation of miR-103 increased the vulnerability of proliferating ECs to mitotic aberrations, which may limit EC regeneration and promote atherosclerosis.

Targeting of lncRNAs by miRNAs may represent a new mechanism by which ECs are reprogrammed toward a maladapted phenotype under disturbed flow.

Methods

In vivo TS8 treatment and staining. Following 8 weeks of HFD feeding, ApoE–/– mice (The Jackson Laboratory, Bar Harbor, ME, USA) were randomized to the different experimental groups and tail vein injected once weekly for four consecutive weeks with IncWDR59 LNA-TSB or control LNA oligonucleotides (0.5 mg per 20 g; miRCURY LNA Target Site Blocker, in vivo use; Exiqon). During the injection period, mice were fed with a HFD comprising 21% crude fat, 0.15% cholesterol, and 19.5% protein. Tissues were harvested 1 week after the last injection. Paxgene-fixed/paraffin-embedded aortic roots were stained with Elastic van Gieson stain. A bright-field microscope (Leica DM6000B; Leica Microsystems) connected to a CCD camera (Leica DFC365) was used to obtain the images. The lesion area was quantified with ImageJ. Immunostaining of Ki67, γH2AX, C-X-C motif chemokine ligand 1 (Cxcl1), and von Willebrand factor (vWF) was also performed using a Leica DM6000B fluorescence microscope. To detect the fluorescent signal in en face prepared tissues, 2 stacks of two-dimensional images were recorded, and deconvolution was performed using a mathematical algorithm to remove out-of-focus information (AF6000 3D deconvolution software module, Leica Microsystems). The 3D rendering was performed using the LAS X 3D algorithm module (Leica) to consider the staining and MN within CD31+ cells. For quantification, en face were entirely quantified. Data regarding MN formation were expressed as number of CD31+ cells containing MN on total number of CD31+ cells, in percentage. Data regarding γH2AX immunostaining in the MN were expressed as number of CD31+ cells showing γH2AX+ MN normalized on total number of micronucleated CD31+ cells (previously normalized on total number of cells), expressed in percentage. The analysis of the staining was performed in a blinded manner.

Laser-capture microdissection. Root sections were collected on ultraviolet-sterilized and RNase-free polyester-membrane 0.9 μm FrameSlides (Leica). ECs and plaques were collected using a laser microdissection system (LMD7900, Leica) in RNase-free tubes. RNA was isolated with the PAXgene RNA MinElute kit (Qiagen) and followed by pre-amplification and reverse transcription with Ovation Picoscribe WT System II V2 (NuGEN) following manufacturer’s instructions.

Cell culture and transfection. Primary MAOECs (passage 3; PELOBiotech GmbH, Planegg, Germany) and primary HAOECs (passages 2, 3; Promocell, Heidelberg, Germany) were cultured using endothelial cell complete growth medium (Promocell) containing gentamicin (0.05 mg ml−1; Thermofisher). To grow the cells under different shear stresses, MAOECs were cultured in collagen-coated perfusion chambers (μ-Slides VI44, ibidi GmbH, Martinsried, Germany) and exposed to high shear stress (10 dyne cm−2) or low shear stress (5 dyne cm−2) for 48 h generated by the perfusion with EC complete medium (ibidi Pump System, ibidi GmbH) containing 5-ethynyl-2′-deoxyuridine (EdU, 10 μM final conc., Click-it® EDU Alexa Fluor® 488 Imaging Kit, Life Technologies) and TSBs (TSBs in vivo ready) or control LNAs (both 50 nM final conc., Exiqon). MAOECs were transfected with antisense oligonucleotides to block the interaction between miR-103 and IncWDR59 (TSB; 50 nM, miRCURY LNA™ microRNA Target Site Blockers; Exiqon) or scrambled controls. To inhibit IncWDR59 function, murine and human ECs were transfected with IncWDR59 GapmeRs (50 nM, LNA™ GapmeR; Exiqon) that strongly induce the degradation of the IncWDR59 transcript in the nucleus of the ECs.

MicroRNA target identification and quantification system (MiTrap). MAOECs were co-transfected with miR-103-mimics, let-7b mimics, or scrambled controls, and pMiTrap Vector using the XfectTM MicroRNA Transfection Reagent in combination with Xfect Polymer for 24 h (all from Clontech). The pMiTrap Vector expressed a DYKDDDDK-tagged GW182 protein, member of the active RISC complex, which enabled locking of the miRNA/mRNA complex into the RISC. Cell lysates were collected and separated in two parts: one was used as input RNA and extracted using the NucleoSpin RNA XS Kit (Macherey-Nagel GmbH & Co. KG), the other fraction was incubated with anti-DYKDDDDK-conjugated magnetic beads. IP and subsequent RNA isolation was performed using the NucleoSpin RNA XS Kit. Reverse transcription of input and IP samples was performed using a competitive RNA isolation protocol with RNAse-free cDNA (cDNA reverse transcription kit (Life Technologies), followed by the amplification with gene-specific primers (Supplementary Table 1) and SYBR Green PCR Master Mix (Thermo Scientific). The fold enrichment was calculated according to manufacturer’s instructions.

Human carotid lesion samples. Human atherosclerotic lesions were collected during carotid endarterectomy. One part was fixed in 4% paraformaldehyde while another part was immediately stored in RNAlater for RNA isolation and qPCR. The study protocol for the collection of human atherosclerotic lesions was first approved from Ethics Committee of the Medical Faculty of RWTH Aachen University. The written informed consent was obtained from all participating patients. Background information regarding the patients were reported in the Supplementary Table 2. Classification of the type of the lesion was made according to the nomenclature of Serruys with a threshold of 30%46. Correlation of Kaplan-Meier’s survival analysis was used to investigate the effect of athero-progression on huh-1ncWDR59, SOX17, and CDRNA2A expression.
Statistical analysis. All analyses were performed in a blinded way. Power calculation with StatMate (GraphPad Software) was performed to calculate the number of replicates needed for each experiment. The data represent the mean ± SEM and were compared using unpaired t-test, one-way analysis of variance (ANOVA), or two-way ANOVA (Prism, GraphPad Software), two-sided, unless stated otherwise. A P value of <0.05 was considered significant.

Additional methods are available in the Supplementary Information File (see Supplementary Methods section).

Data availability. All relevant data are included in the manuscript and available from the authors upon reasonable request. MiRNA expression profile from microarray is available online (https://doi.org/10.1038/ncomms10521) and in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE53435, GSE53435, and GSE114805.

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Author contributions

L.N. and A.S. designed the study and wrote the manuscript. L.N. carried out all experiments in vivo and in vitro and analyzed the data. C.G. helped in performing qPCR. C.G. analyzed the RNA-seq data. Y.W. performed the analyses of human atherosclerotic lesions and the LysCre-Dicer array for IncRNAs expression. M.Z. performed monocyte isolation and helped with mice experiments. A.D.F. helped with
micronuclei analysis. P.H. contributed to EC-Dicer mice experiments. R.Z. contributed to the RNA-seq analysis. All authors discussed the results and commented on the manuscript.

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