FLI1+ cells transcriptional analysis reveals LMO2-PRDM16 axis in angiogenesis

Title: FLI1^{+} cells transcriptional analysis reveals LMO2-PRDM16 axis in angiogenesis

Running title: Novel role of LMO2-PRDM16 axis in angiogenesis

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Significance statement

Understanding molecular signals driving the development of vascular endothelium is important in seeking novel therapies for human disease. Focusing on the zebrafish model, we herein report new mechanistic insights into development of the vascular endothelium, as well as angiogenesis. This work highlights the novel role of an epigenetic modifier and known oncogene in developmental angiogenesis in zebrafish. In particular, we show that the expression of the histone methyltransferase PRDM16 is high in endothelial cells and PRDM16 is necessary for the endothelial differentiation and migration *in vivo* and *in vitro* in iPSC-derived endothelial cells. Moreover, PRDM16 expression is mediated by LMO2, a well-known oncogene implicated in angiogenesis and leukemogenesis. We hence unveil a novel role of PRDM16 in endothelial development and angiogenesis and therefore propose that PRDM16 could be a novel target for therapeutic modulation of angiogenesis.

Abstract

A network of molecular factors drives the development, differentiation and maintenance of endothelial cells. Friend leukemia integration 1 transcription factor (FLI1) is a *bona fide* marker of endothelial cells during early development. In zebrafish *Tg(Fli1:EGFP)^{y1}* we identified two endothelial cell populations, high-FLI1^{+} and low-FLI1^{+}, by the intensity of green fluorescent protein signal. By comparing RNA-Seq analysis of non-FLI1 expressing cells (FLI1^{-}) with these two (FLI1^{+}) cell populations we identified several novel upregulated genes, not previously recognised as important, during endothelial development. Compared to FLI1 negative (FLI1^{-}) and low-FLI1^{+} cells, high-FLI1^{+} cells showed upregulated expression of the zinc finger transcription factor PRDI-BF1 and RIZ homology domain containing 16 (PRDM16). PRDM16 knockdown (KD) by morpholino in the zebrafish larva was associated with impaired angiogenesis and increased number of low-FLI1^{+} cells at the expense of high-FLI1^{+} cells. In addition, PRDM16 KD in endothelial cells derived from human induced Pluripotent Stem Cells (iPSC) impaired their differentiation and migration *in vitro*. Moreover, zebrafish mutants with loss-of-function for the oncogene LIM domain only 2 (LMO2-mut) also showed reduced PRDM16 gene expression combined with impaired angiogenesis. PRDM16 expression was reduced further in endothelial (CD31^{+}) cells compared to CD31^{-} cells isolated from LMO2-mut embryos. ChIP-PCR demonstrated that LMO2 binds to the promoter and directly regulates the transcription of PRDM16. This work unveils a novel mechanism by which PRDM16 expression is activated in endothelial cells by LMO2 and highlights a possible new therapeutic pathway by which to modulate endothelial cell growth and repair.

Main text

The endothelium is the inner lining of blood and lymphatic vessels, made of a continuous monolayer of endothelial cells (EC) interconnected by specialized junctions. It is present in all vertebrates [1] and is highly specialized to adapt to the need of the different tissues and organs [2]. It regulates not only the transit of nutrients and oxygen from the blood into tissues and the removal of metabolic waste products, but also blood fluidity, platelet aggregation, vascular tone, inflammation and angiogenesis. In fact, endothelial dysfunction is a fundamental pathophysiology associated with many human diseases and conditions including air pollution [3], smoking [4], heart failure [5], hypertension [6], atherosclerosis [7] and cancer [8]. Therefore, a detailed understanding of the underlying mechanisms of endothelial cell proliferation and differentiation are crucial to the aim of seeking novel therapeutic tools to promote restoration of dysfunctional or damaged endothelium.

Zebrafish have a closed circulatory system and the anatomical processes and the molecular mechanisms underlying vascular development are very similar to humans [9]. During embryogenesis, endothelial and blood progenitor cells share a common ancestor, the hemangioblast, derived from the lateral plate of the mesoderm [10], that gives rise to hematopoietic stem cells and endothelial precursors. Early blood vessels form *de novo* from these endothelial progenitors, the angioblasts, in a process named vasculogenesis [11]. This primary vasculature expands and remodels through sprouting into a mature vascular network, also known as developmental angiogenesis [12]. The differentiation of endothelial cells (EC) requires an orchestrated network of different transcription factors, yet to be fully defined [13]. One of these, FLI1, a member of the E26 transformation-specific (ETS) family of transcription factors, can be detected at low levels as early as the three-somite stage within the posterior lateral mesoderm in zebrafish [14]. FLI1 is required for hemangioblast formation, as shown in *Xenopus* and zebrafish (*Danio rerio*) [10]. Hence, FLI1 is one of the earliest known

endothelial markers, acting downstream to *npas4l (cloche)* [15] [16] and upstream of transcription factors such as Scl/Tal1, Lmo2, Gata2, Etsrp and Flk1. As such, FLI1 helps to initiate and maintain endothelial cell (EC) fate [10].

The zebrafish transgenic line *Tg(Fli1:EGFP)^{y1}* has enhanced green fluorescent protein signal (EGFP) driven by the FLI1 promoter. This model has been extensively used to elucidate endothelial and blood vessel development [14]. FLI1 is highly specific for EC although low levels of GFP signal can also be detected in some other tissue, including mesenchyme and developing cartilage of the jaw [14]. In the current study, we observed that FLI1 gene expression increased rapidly during zebrafish embryogenesis between 6 h and 72 hours post fertilisation (hpf) (**Fig. 1A**). Accordingly, we FACS-purified three populations of cells from *Tg(Fli1:EGFP)^{y1}* embryos on the basis of their differential expression of FLI1 (**Fig. 1B**), termed FLI1$^-$, low-FLI1$^+$ and high-FLI1$^+$cells. We compared these subpopulations to discover novel genes involved in early endothelial development. We reasoned that if a gene is specific for the differentiation of EC it will be expressed more in the high-FLI1$^+$ population compared to the low-FLI1$^+$. Hence, separating the high-FLI1$^+$ from the low-FLI1$^+$ will achieve two key aims: 1) reveal candidates genes that are highly expressed only in high-FLI1$^+$ cells, the level of which would be diluted or masked if observed in a population containing both high-FLI1$^+$ and low-FLI1$^+$ cells; 2) discriminate those genes whose expression is significantly higher in low-FLI1$^+$ compared to high-FLI1$^+$. For this study, we used embryos at 72 hpf since we observed that FLI1 gene expression and the percentage of GFP$^+$ cells were close to maximal at this timepoint (**Fig. 1C-D**) and a high-FLI1$^+$ population was clearly distinguishable at this early developmental stage (**Fig. 1B**). A GFP$^-$ (Fli1$^-$) population was defined using zebrafish control *Wik* line, whereas the gate between the two GFP$^+$ populations (low-FLI1$^+$ and high-FLI1$^+$) was placed at the onset/edge of the high-FLI1$^+$ population (**Fig. 1B**). To confirm that cells expressing the highest GFP signal were in fact *bona fide* EC, low and high-FLI1$^+$ cells

were sorted and cultured on fibronectin-coated petri dish with endothelial cell growth medium (**Fig. 1E**). Fibronectin is an extracellular matrix protein that serves as adhesive support and regulates the spreading, migration, and contractility of EC during vascular development [17]. At 24 h after seeding, only 29 % of low-FLI1$^+$ cells were attached to the fibronectin compared to the 89 % of high-FLI1$^+$ cells (**Fig. 1E-F**). In addition, from 24 h to 48 h after seeding the increase in high-FLI1$^+$ cells was significantly greater than low-FLI1$^+$ cells (45 % vs 5 %) (**Fig. 1E-F**).

To investigate the gene expression profile, we performed RNA-Seq for FLI1$^-$, low-FLI1$^+$ and high-FLI1$^+$ cell populations. We compared the level of genes in each pair of cell populations and defined differential genes by requiring a fold change larger than 2 and a false discovery rate (FDR) <0.05 **(Fig. 2)**. We were particularly interested in genes encoding novel epigenetic factors that were previously unrecognised in endothelial differentiation.

RNA-Seq analysis revealed a set of 441 genes up-regulated and 1024 genes down-regulated in low-FLI1$^+$ compared to FLI1$^-$ cells **(Fig. 2A),** a set of 660 genes up-regulated and 1333 genes down-regulated in high-FLI1$^+$ compared to FLI1$^-$ cells **(Fig. 2B)** and a set of 751 genes up-regulated and 793 genes down-regulated in high-FLI1$^+$ compared to low-FLI1$^+$ cells **(Fig. 2C)**. Mesodermal markers showed an increased level of FPKM (Fragments Per Kilobase of transcript per Million mapped reads) from FLI1$^-$ to low-FLI1$^+$ to high-FLI1$^+$ cells (**Fig. S1A)**, whereas opposite patterns were observed for ectodermal (**Fig. S1B)** and endodermal markers (**Fig. S1C)**. Pathway enrichment analysis showed clear differences between the groups **(Figs. 2D-E, Fig. S2** and **Dataset S1)**. Compared to FLI1$^-$, and as expected, Low-FLI1$^+$ and high-FLI1$^+$ groups showed enrichment of blood vessel development pathways, including vascular development, angiogenesis, lymphangiogenesis, and TGF-β signalling pathway **(Fig. S2A)**. with the highest enrichment of these pathways observed in the high-FLI1$^+$ cells **(Fig. 2D)**. This was also true for other endothelial markers, such as CHD5, KDR, PECAM, TIE1 and

FLI1 (**Fig. S3A-B)**, as expected, and as confirmed by Q-PCR (**Fig. S3C)**. These data suggest that a high-FLI1^{+} population is composed of ECs, whereas the low-FLI1^{+} population could be composed of a variety of cell types, including ECs at an early maturation stage (**Figs. 2D, S2A and S3)**.

While several transcription factors are known to play a role in endothelial development and maturation [13], the role of other epigenetic factors in these processes is not fully understood. These are enzymes (methyltransferases, demethylases, deacetylases, acetyltransferases and chromatin remodelers [18]) that modify the epigenome directly through DNA methylation or modifications of histones. Accordingly, we identified 510 epigenetic factors from our RNA-Seq dataset that were differentially expressed in our three defined cell populations (**Fig. S4)**. We were particularly interested to screen epigenetic factors which had low or null expression in FLI1^{-} and high expression in the other two cell groups.

In a shortlist of 15 candidate genes, we found three members of the PRDI-BF1 and RIZ homology domain containing (PRDM) gene family: PRDM5, 11 and 16 (**Fig. 3A)**. Of these, PRDM16 showed the highest level of expression in the high-FLI1^{+} cells (**Fig. 3B)**, confirmed by Q-PCR (**Fig. 3C)**, and so this was selected for further study. PRDM16 is a 140 kDa zinc finger protein, the chromatin modifying activity of which is structurally defined by the presence of a conserved N-terminal histone methyltransferase [19]. PRDM16 is quite ubiquitously expressed and is known to be involved in hematopoiesis [20], palatogenesis [21], brown fat determination and differentiation [22] and in neurovascular network formation during brain development [23]. In zebrafish, PRDM16 plays a role in craniofacial development [24]. It has also been implicated in several human conditions, including cardiomyopathy [25] and leukemogenesis [26].

To assess the role of PRDM16 in zebrafish vascular development, we conducted PRDM16 knockdown (KD) studies by injection of two different, non-overlapping PRDM16 translation-blocking morpholinos (Mo) in *Tg(Fli1:EGFP)*^{y1} once-cell stage embryos, following previous guidelines [27]. We injected each Mo separately, at the same dose (0.5 ng per egg), and then we co-injected each morpholino at 1/2 dose of each (0.25 ng per egg). While the phenotype was just slightly apparent with ½ dose of each Mo when injected alone, co-injection of two different Mo produced a stronger effect, similar to that produced by a single morpholino at the dose of 0.5 ng per egg. This additive action strongly suggests that the MO effects are specific for PRDM16. The effective KD was verified by western blotting (**Fig. S5A)**. The survival rate of PRDM16-KD embryos at 120 hpf was approximately 70 % compared to 92 % in controls (**Fig. 3D)**. The main features of the phenotype in PRDM16-KD embryos assessed by brightfield microscopy included body bending and reduced skin pigmentation (**Fig. S5B-C)**. We did not observe an increase in tP53 gene expression, that could cause off-target effects, at the dose of Mo used in this study and up to 4ng, therefore we decided not to co-inject tP53 Mo. Using fluorescence microscopy, we observed that the development of intersegmental vessels (ISV), which sprout from dorsal aorta via angiogenesis [28], was significantly reduced in PRDM16-KD embryos compared to control (**Fig. 3E-F**), with a phenotype penetrance of >70%. Indeed, PRDM16 Mo injected embryos with the ISV phenotype showed a significant reduction of PRDM16 compared to control embryos or those embryos not showing the ISV phenotype, as showed by WB analysis (**Fig. S5D**). This suggests that the ISV deficiency is a specific feature of the PRDM16 depletion. In fact, at 30 hpf ISVs bridged fully ventro-dorsally in control embryos, whereas they sprouted only halfway across their dorsal trajectory in PRDM16 morphants (data are reported graphically as average ISVs length, **Fig. 3E-F**). These observations were confirmed in the *Tg(kdrl:mCherry)*^{y171} zebrafish, where the red fluorochrome mCherry is expressed under control of Kdrl gene, an endothelial marker. The

phenotype of PRDM16 morphants was significantly rescued by co-injecting PRDM16 Mo and PRDM16 mRNA indicating that the observed effects were specific for PRDM16 KD (**Figs. 3E-F & S5A)**. As a negative control of the rescue experiment [27], co-injection of PRDM16 Mo with a mutant RNA that does not encodes proteins failed to rescue the morpholino induced defects. In contrast, vasculogenesis was not affected in PRDM16-KD embryos, as shown by the normal development of dorsal aorta and cardinal vein (**Fig. 3E**). We performed cell proliferation and apoptosis assays in *Tg(Fli1:EGFP)*^{y1} zebrafish whole embryos, at 22 hpf (end of segmentation stage) and at 30, 38 and 46 hpf (pharyngula period), to assess if these processes were involved in the reduced angiogenesis observed in PRDM16-KD embryos (**Fig. S6**). EdU proliferation assay (**Fig. S6A**) showed that the number of GFP+-EdU+ cells, marking proliferating endothelial cells, were not different in PRDM16-KD embryos compared to controls. Interestingly, GFP+-EdU+ cells in PRDM16-KD embryos are more localised in the dorsal aorta, compared to control where GFP+-EdU+ cells are localised also in the ISV. Similarly, TUNEL assay (**Fig. S6B**) showed that the number of GFP+-TUNEL+ cells, labelling apoptotic endothelial cells, were not different in PRDM16-KD embryos compared to controls. Overall, these data suggest that PRDM16 is required for developmental angiogenesis. FACS analysis of cells isolated from PRDM16-KD *Tg*(*Fli1:EGFP)*^{y1} embryos revealed that the percentage of high-FLI1^{+} cells was significantly reduced compared to control (1.5% vs 7%, respectively), whereas the low-FLI1^{+} population was significantly increased (7.9% vs 3.2% in control) (**Fig. 3G-H)**. The expression of endothelial markers was significant lower in FLI1^{+} from PRDM16-KD compared to control (**Fig. S7**). *In vitro*, total FLI1^{+} cells, plated on fibronectin-coated dishes, isolated from PRDM16-KD exhibited a lower GFP signal compared to control. In addition, they showed a non-homogeneous morphology, a reduced LDL uptake and nitric oxide production compared

to control (**Fig. 3I-K)**. Taken together, these data suggest that PRDM16 is essential for angiogenesis *in vivo* and that PRDM16 regulates the functionality of EC.

To assess whether the role of PRDM16 is conserved during the differentiation of human EC, we differentiated human induced pluripotent stem cells (iPSC). iPSCs at passage between 21-23 were differentiated to endothelial lineage using our standardized protocol [29] (**Fig. 4A**). Cells were treated with SiRNA, Ctrl or targeting PRDM16, for 6 hours until the end of the mesodermal differentiation. At the end of the protocol, endothelial cells showed the typical EC cobblestone-like shape in the two groups (**Fig. 4B**). Real time PCR showed a reduction of PRDM16 expression of ≥ 70% in SiRNA PRDM16 treated cells compared to control, as also evidenced by western blotting (**Fig. 4C**). Furthermore, real time PCR showed a significant reduction in endothelial markers following PRDM16 KD (**Fig. 4D**). Accordingly, at this stage we sorted double positive cells for the endothelial markers CD31 (PECAM1 gene) and CD144 (CDH5 gene). We observed a reduced percentage of $CD31^{+}$-$CD144^{+}$ cells generated from iPSCs treated with SiRNA PRDM16 compared to control (51.7% vs 6.4%; $p \leq 0.01$; **Fig. 4E-F**). We purified cells that were double positive for CD31 and CD144 and confirmed the reduced expression of endothelial markers in EC after PRDM16 KD (**Fig. 4G**). We observed no difference in the proliferation rate of iPSC-derived EC in the two groups, as shown by EdU assay (**Fig. S8A**). TUNEL assay in cells from 9 to 15 days of the differentiation protocol, that encompass differentiation and maturation stages of EC, shows that cell death is not increased following PRDM16 KD (**Fig. S8B**). Furthermore, in the endothelial cell migration assay, we observed that iPSC-derived EC treated with SiRNA for PRDM16 migrated significantly less, as shown by a larger residual gap between the edge of the wound monolayer, compared to cells treated with SiRNA Ctrl (**Figure 4H-I**).

Horn *et al.* [30] showed that PRDM16 is expressed in mouse embryos by E14.5 in a broad range of tissues including brain, lung, kidney, and gastrointestinal tract. The mechanisms by which a ubiquitous factor such as PRDM16 is modified by tissue or organ context to provide such a variety of functions is mostly unknown. There are several possible mechanisms acting in isolation or in combination such as protein modifications, specific epigenetic landscapes or interactions with various cell or tissue-specific factors that make PRDM16 both multifunctional and tissue-specific at the same time.

LIM-domain-only (LMO)2 is a transcription factor essential to both hematopoietic [31] and endothelial pathways [32], and it is normally expressed in mature vascular endothelium [33]. LMO2 is a proto-oncogene implicated in leukemogenesis [34, 35] and has been suggested as a potential therapeutic target in various clinical indications [36]. By virtue of its LIM-domain zinc-finger-like structures, LMO2's canonical function is to act as a bridging molecule [37] to assemble a DNA-binding complex which includes the TAL1, E47, GATA1 and Ldb1 proteins in erythroid cells [38] and in EC [39]. We have previously shown that LMO2 is necessary for EC proliferation during tissue regeneration in adult zebrafish [40], and for the regulation of EC migration mediated by SPHK1 in zebrafish embryos [41]. In the present study, we have knocked-out LMO2 in zebrafish by CRISPR/Cas9 (**Fig. 5)** to further investigate the role of this transcription factor in embryogenesis and we have explored its possible interaction with PRDM16. The gRNA targeted the sequence between intron/exon two of the LMO2 gene and produced an insertion of four nucleotides (AGAT) resulting in a frameshift and a stop codon a few nucleotides downstream (**Fig. 5A)**. Western blotting analysis confirmed LMO2 KO (**Fig. 5B)**. The survival of LMO2-mut embryos was approximately 75 % at 120 hpf compared to 94% in controls (**Fig. 5C)**. The gross phenotype of LMO2-mut embryos under brightfield microscopy appeared normal and exhibits mild features of body bending and reduced skin pigmentation (**Fig. S9A-B)**. These features are similar to those reported previously [42]. As

previously reported, we also found that LMO2 mutants had fewer circulating red blood cells, consistent with reduced Gata1 gene expression [43] and impaired hematopoiesis [44], mild pericardial edema [44] and a mild cephalomegaly [43]. Immunostaining for FLI1 and KDR showed a significantly reduced length of ISV in LMO2-mut embryos compared to control (**Fig. 5D-E)**, confirming the role of LMO2 in developmental angiogenesis. In contrast, vasculogenesis was only slightly affected, consistent with a previous study in mouse [32].

As LMO2 and PRDM16 similarly affect angiogenesis, we investigated the relation between the two genes. To test the hypothesis that LMO2 regulates PRDM16, we first assessed PRDM16 gene expression in LMO2-mut embryos. We observed that PRDM16 expression was significantly reduced from 48 to 120 hours post-fertilization compared to control (**Fig. S10).** Interestingly, there is a gradual increase of PRDM16 gene expression in LMO2-mut embryos, suggesting that other factors could be implicated in the regulation of PRDM16. Therefore, we tested the ability of PRDM16 to rescue the vascular defects of LMO2-mut. We showed that injection of PRDM16 mRNA in LMO2-mut resulted in ISV of similar length to controls (**Fig. 5D-E)**. Embryos control injected with PRDM-16 mRNA alone did not show any difference in ISV phenotype compared to control, showing that the rescue of the ISV phenotype in LMO2-mut embryos was a clean rescue experiment (**Fig. 5D-E**). To test whether the reduced expression of PRDM16 in LMO2-mut was global or more specific to endothelial cells, we performed Q-PCR for PRDM16 in $CD31^+$ and $CD31^-$ cells FACS-purified from LMO2-mut and control. We found that the reduction of PRDM16 gene expression was more significant in $CD31^+$ cells compared to the reduction observed in $CD31^-$ cells, demonstrating endothelial cell-specific effects of LMO2 for PRDM16 (**Fig. 5F)**. To further investigate whether LMO2 associates with the promoter region of the PRDM16 gene, a chromatin immunoprecipitation (ChIP) assay followed by PCR (ChIP-PCR) was performed in zebrafish embryos using a LMO2-specific antibody. PCR identified a DNA fragment from

the PRDM16 promoter region, which was co-precipitated by the LMO2 antibody but not by pre-immune serum **(Fig. 5G)**, indicating association of LMO2 with the PRDM16 promoter. As a control, a region located 1 kb downstream of the LMO2–binding site could not be efficiently co-precipitated. Taken together, these data demonstrate that LMO2 associates with, and activates transcription of, PRDM16.

In this report, we took advantage of the *Tg(Fli1:EGFP)*^{y1} zebrafish to discover novel epigenetic factors implicated in endothelial development and differentiation **(Fig. 5H)**. Among many candidates, we focussed on PRDM16. We demonstrated that PRDM16 is essential for angiogenesis, and that its expression is mediated by LMO2 specifically in endothelial cells by LMO2 association with the PRDM16 promoter region. We showed that PRDM16 is involved in the process of differentiation and maturation of EC as shown *in vivo* in the zebrafish and *in vitro* during differentiation of iPSC-derived EC. This work highlights a novel mechanism by which PRDM16 could promote endothelial lineage by LMO2-mediated regulation during endothelial development, and points towards a potentially novel therapeutic targets for endothelial dysfunction in a wide range of vascular disease settings.

Methods

Ethics statement

All experiments with zebrafish were performed in accordance with the recommendations of the Institutional Animal Care and Use Committee at the Houston Methodist Research Institute, and with the United Kingdom Animals (Scientific Procedures) Act 1986 at the Queens Medical Research Institute [45].

Zebrafish aquaculture and husbandry

Zebrafish *Wik, Tg(fli1:eGFP)^{y1}* and *Tg(kdrl:mCherry)^{y171}* strains were maintained according to standard procedures. Fish were kept at 28℃ under a 14/10 h light/dark cycle and fed with dry meal (Gemma Micro, Westbrook, ME) twice per day. Embryos were obtained by natural mating and kept in E3 embryo medium at 28.5℃. All the experimental procedures were performed under anaesthesia with Tricaine 0.02 mg/ml.

Maintenance of Human iPSC

The human iPSC lines were obtained from Coriell Cell Repositories (Camden, New Jersey) and were maintained on Matrigel (BD Biosciences, cat.n. 354277)-coated plates (Corning) in mTeSR1 medium (STEMCELL Technologies cat.n. 85850) according to their protocol. The iPSCs were passaged approximately every four days, RELSR (STEMCELL Technologies cat. 05873) dissociation reagent was used to detach colonies. Cells were maintained in humidified incubators at 37°C and 5% CO2. Pluripotency of iPSC was periodically characterized by morphology and immunostaining of pluripotency markers.

Differentiation of iPSC to endothelial cells

Endothelial cells (EC) differentiation was carried out following an established protocol [46], with some modifications. In brief, the iPSCs at passage between 21-23 were grown to 80% confluence, and placed in differentiation medium Advanced DMEM/F12 (ThermoFisher cat.n. 11320033), supplemented with Wnt agonist CHIR 99021 5 µM (Selleck, cat.n. S2924), bone morphogenetic protein-4 (BMP4, 25 ng/m) (Peprotech cat.n. 120-05), B27 supplement (ThermoFisher cat.n. 17504044), and N2 supplement (ThermoFisher cat.n. 17502048) (*Mesodermal differentiation*). After 3 days, cells were dissociated with HyQtase (GE Healthcare cat.n. SV30030.01) and plated in StemPro media (ThermoFisher cat.n. 10639011), supplemented with forskolin 5 µM (LC Laboratories cat.n. F-9929), vascular endothelial growth factor (VEGF) 50ng/mL (Peprotech cat.n. 100-20), and polyvinyl alcohol 2 mg/mL

(Sigma-Aldrich cat.n. 360627) (*Endothelial differentiation*). After 4 days, cells were washed twice with PBS and cultured in endothelial growth media (EGM-2MV, Lonza cat.n. CC-3202), supplemented with additional VEGF (100 ng/ml) for 4 more days (*Endothelial maturation*). Cells were passaged once they reached 80-90% confluence. During the whole differentiation protocol, cells were maintained at 37°C and 5% CO2 in a humidified incubator.

PRDM16 knockdown in differentiating endothelial cells

At day 3 of the mesodermal differentiation stage, 6h before replacing the medium, cells were transfected with 5 nM siRNA targeting PRDM16 or ctrl (Silencer Select siRNA, ThermoFisher) using Lipofectamine RNAiMax (Life Technologies, cat.n. 13778-075 in Opti-MEM (Life Technologies, cat.n. 31985-062). After 6h, mesodermal medium was aspirated and replaced with endothelial differentiation medium. Effective knockdown was analysed by real-time PCR and wester blotting (anti-PRDM16 antibody, human polyclonal, cat.n. PA5-20872 Thermo Fisher Scientific).

Flow cytometry characterization and purification of iPSC-derived EC

At the end of the protocol (see figure 4A), cells were dissociated into single cells with HyQtase for 5 minutes at 37°C, washed 5 min with PBS containing 5% BSA and then passed through a 40-μm cell strainer. Cells were then incubated with either CD31 mAb (PE mouse anti-human, BD Pharmingen, cat. 555446) or CD144 mAb (FITC mouse anti-human, BD Pharmingen, cat. 560411) for 30 min. Isotype-matched antibody served as negative control. Cells and purified using FACSAria (BD, Franklin Lakes, New Jersey) flow cytometer and data analysed by Flowjo software.

Proliferation assay

Click-IT EdU kit (Thermo Fisher Scientific) was used, following manufacturer's instructions, to assess proliferation in iPSC-derived endothelial cells, followed by FACS analysis.

Apoptosis assay

Apoptosis was detected by TUNEL assay using the ApopTag rhodamine In Situ Apoptosis Detection kit (Chemicon,Temecula, CA), following manufacturers' instructions.
For experiments in whole-mount zebrafish, embryos were fixed in 4% paraformaldehyde (PFA) at 4 °C, washed in PBS twice for 5 min, permeabilized with proteinase K (10 µg/ml) for 20 min at room temperature, followed by two washes in PBS. Then they were placed in prechilled ethanol:acetic acid (2:1) at - 20 °C for 10 min, washed in PBS-T (PBS 1X, 0.1% Tween-20) twice before incubation in equilibration buffer and further steps as described in the manufacturer's protocol. TUNEL assay staining was quantified by counting positive staining puncta (TUNEL+ nuclei) in the vessel of *Tg(Fli1:EGFP)*^{y1} in z-stack confocal images using ImageJ. For *in vitro* experiments, cultured iPSC cells during the endothelial differentiation and maturation stages were treated as above for TUNEL and then immunostained for CD144.

Cell migration assay

Confluent endothelial cells were cultured on 12-wells dishes. Cells were wounded by scratching with a micropipette tip, rinsed with PBS, and then incubated for 16 h. Wound closure was monitored through the use of digital photography and measured using the ImageJ program. Cell migration was expressed as the migrated distance.

Enzymatic isolation of cells from zebrafish embryos

Cells were isolated according to Shestopalov *et al.* [47]. In brief, *Tg(Fli1:EGFP)*^{y1} embryos at the appropriate developmental stage were dechorionated, euthanised and washed three times with sterile PBS. Embryos were placed in a 1.5 ml tube, washed with calcium-free Ringer solution (200 µl for 30 embryos; 116 mM NaCl, 2.6 mM KCl, 5 mM HEPES, pH 7.0) and

replaced with 1 ml solution of 1X PBS containing trypsin (0.25%, Gibco), 50 µg collagenase P (Roche) and 1 mM EDTA. Embryos were disaggregated using a 200 µl pipette tip and incubated for 30 min at 28.5 °C with further pipetting every 5 min. Digestion was quenched with stop solution (200 µl; 1X PBS containing 30% (v/v) calf serum and 10 mM CaCl2) and samples were centrifuged at 400g for 5 min at 4 °C. Supernatants were discarded, cell pellets washed twice with chilled solution of DMEM containing 1% (v/v) calf serum, 0.8 mM CaCl2, 50 U ml−1 penicillin/streptomycin, centrifuged and resuspended in the same medium. Cell suspensions were filtered through a 40 µm cell strainer (BD Biosciences) into FACS tubes.

Flow cytometry characterization and purification of Fli1$^+$ cells from zebrafish

Cell suspensions were analysed using a BD FACS Aria (BD Biosciences). DAPI was used to identify viable single cells, whereas FSC-H and FSC-A were used to select cell singlets. Wild-type (*Wik*) zebrafish was used to set the gate between GFP$^-$ (*i.e.* FLI1$^-$) and GFP$^+$ (low-FLI1$^+$) cell populations whereas the gate between low- and high-FLI1$^+$ populations was placed at the onset of the high-FLI1$^+$ cells. At least 50,000 of FLI1$^-$, low and high-FLI1$^+$ cells (Ex: 488 nm; Em: 530 nm) were sorted from groups of n=50 *Tg(Fli1:eGFP)^{y1}* embryos into a 15 ml falcon tube containing chilled PBS and 10 % FBS. The number of viable cells was confirmed under fluorescence stereomicroscope (Leica M205) by using a Neubauer chamber.

Culture of zebrafish cells

Low and high-FLI1$^+$cells were cultured on petri dishes coated with fibronectin at a concentration of 2 µg/cm^2 in endothelial basal medium supplemented with EGM2 bullet kits (Lonza). Medium was replaced every two day.

Dil-Ac-LDL uptake assay

Uptake of Ac-LDL was assessed by incubating cells with ac-LDL-594 (Thermo Fisher Scientific) at 1:200 dilutions for 3 h. Then, cells were washed with PBS and the fluorescence

measured in at least n = 5 high power fields using imageJ and plotted as mean of the fluorescent signal (integrated density) in each cell.

Nitric oxide assay

Nitric oxide (NO) metabolites nitrite and nitrate were detected in the culture medium as an indirect measurement of cellular NO (Cayman Chemical, Nitrate/Nitrite Assay Kit). In brief, cell culture medium was first added with nitrate reductase that converts nitrate to nitrite. Then, Griess reagents added to the sample convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance (540 nm wavelength) derived from this azo-chromophore accurately determines nitrite concentration. Cellular nitrate/nitrite production is quantitated by subtracting the level of nitrate/nitrite present in the media alone from the total nitrate/nitrite level present in the medium during cell growth.

Immunocytochemistry

Embryos were euthanized in tricaine and enzymatically dissociated as above, fixed in 2% PFA for 10 min, washed 2X PBS, blocked in PBS-TritonX 0.1% for 30 min and stained with anti-CD31 (PECAM-1) Monoclonal Antibody FITC-conjugated (Thermo Fisher Scientific, cat. 11-0311-82, rat 1:200) for 1 h, washed 2X PBS and FACS analysed.

Immunohistochemistry

Zebrafish embryos were euthanized in Tricaine 1mM and fixed in 4% paraformaldehyde (PFA, Sigma) at 4°C overnight. Embryos were permeabilized using proteinase K (10 μg/ml), fixed again in PFA 4% for 30 minutes, washed three times in PBS-Triton-X100 (0.1%) and blocked in Bovine Serum Albumin 5% in PBS for 3 h. Then, embryos were incubated with anti-FLI1 antibody (Sigma, cat.n. SAB2100822, rabbit, dilution 1:100) or anti-Kdrl antibody, followed by incubation with anti-rabbit antibody (Alexa fluor 555, Cell Signalling, 1:500).

Then, specimens were washed in PBS and mounted in glycerol 100%. Blood vessels formation and endothelial cells were assessed in whole-mount embryos.

Imaging

Fluorescence images of zebrafish were acquired using a Leica M205FA stereomicroscope equipped with a mercury lamp with filter sets (Ex: 470 nm, Em: 525 nm for GFP; Ex: 555 nm; Em: 565 nm for Alexa fluor 555) equipped with a Leica DFC500 digital camera, and a confocal microscope (Leica SP5) to capture high resolution z-stack images. The same microscope was also used to capture brightfield images of embryos. Images of isolated cells were acquired using an EVOS FL imaging systems AMF4300 (Thermo Fisher Scientific).

Preparation of RNA-seq libraries and sequencing

FLI1^{-}, low-FLI1^{+}and hig-FLI1^{+} from at least n=50 *Tg(Fli1:EGFP)^{y1}* embryos per group were harvested by FACS at 72 hour post-fertilization and prepared for analysis by RNA-seq. Total RNA from cells was isolated, fragmented, reverse transcribed to cDNA, ligated to adapters, and subject to brief PCR amplification in preparation of the Illumina library. The integrity and quality of RNA and complementary DNA were monitored using an Agilent Bioanalyzer 2100. The samples were run on an Illumina Hi-Seq 2500 system with 100 base paired-end sequencing (50 million reads per sample). Samples were run in duplicate.

Bioinformatic analysis

RNA-Seq reads were aligned to the zebrafish genome danRer10. We use the full set of known Gene downloaded from the UCSC Genome browser as reference genes. RNA-Seq read counts for each gene in each sample was calculated using Cuffdiff function in Cufflinks version 2.2.1. The Cuffdiff also calculates fragment per kilobase per million reads (FPKM) for each gene. We further subject the reads counts to EdgeR version 3.12.0 for differential expression analysis, and define differential genes based on false discovery rate (FDR) cutoff 1e-5. We

subject interesting gene groups to the DAVID website (https://david.ncifcrf.gov) for functional enrichment analysis. Enriched functional terms were defined based on Benjamini adjusted p value cut-off 0.05. Genomic tracks were generated by UCSC Genome browser. Epifactors gene list was downloaded from (https://epifactors.autosome.ru/).

Chromatin immunoprecipitation (ChIP)-PCR assay

ChIP assay was performed following the manufacturer's instructions (Cell Signalling Technology, Beverly, MA). Briefly, 50-70 embryos per group were disaggregated in single cells as described above. DNA and protein were crosslinked by 1% formaldehyde. Chromatin was isolated and digested with Micrococcal Nuclease. Then, the DNA-protein complex was precipitated with control IgG or antibody against LMO2 (rabbit polyclonal, ChIP grade, Abcam, AB72841) overnight at 4°C and protein A/G conjugated magnetic beads for 1 hr. Cross-links were reversed. The extracted DNA was used as template for PCR amplification of the targeted promoter region. The extracted DNA from unprecipitated DNA-protein complex was used as input. The promoter region of PRDM16 (NCBI Reference Sequence: XM_021478491.1) was identified with FINDM software. Primers sequences used were: (F) 5'-GCAGAGTGCGACGGTAAA-3', (R) 5'-CGTCCAGACAGAACTTCACAT-3' to detect PRDM16 promoter and (F) 5'-CACTTCTCAAGAGCCCACTTAAT-3', (R) 5'-CTGCTGAGACTACTCCCTATGT-3' for control sequence.

Generation of zebrafish mutants using CRISPR/Cas9

Zebrafish mutant lines for LMO2 were generated via CRISPR/Cas9-based mutagenesis as previously described [48]. In brief, guide RNAs (gRNA) specific for target sites on LMO2 gene sequence were identified using CHOPCHOP (https://chopchop.cbu.uib.no). gRNA sequence was 5'-GCTGATCTGCAGGGAGCCGG-3' and was prepared as previously described [49]. gRNAs were then co-injected with 600 ng/ul of Cas9 Protein (PNA bio) and 200 mM KCl. Cas9/sgRNA complexes were formed by incubating Cas9 protein with gRNA at room

temperature for 5 minutes prior to injecting into the cytoplasm of WT AB zebrafish embryos at 1-cell stage. For detecting the zebrafish mutants, genomic DNA was extracted from individual zebrafish larva using the Quick-DNA isolation kit (Zymogen), and a short genomic region (200–400 bp) flanking the target site was amplified by polymerase chain reaction (PCR). For LMO2, the primers were: (F) 5'-GCACATGTTTGCCTGTATTTGT-3', (R) 5'-CAGAGGTCACAGCTCAGACAGT-3'. Purified PCR products (200 ng) were denatured, reannealed, and then digested with EnGen Mutation Detection kit (New England Biolabs) that uses T7 Endonuclease I. The samples were run out on agarose gel 2% to distinguish mutant from wildtype embryos. PCR products of positive mutants were subcloned into pGEM-T vectors (Promega) that were then used to transform competent cells. After overnight culture at 37°C, a single colony was selected for sequencing.

PRDM16 knockdown

Suppression (knockdown, KD) of PRDM16 gene (NCBI: DQ851827.1) in zebrafish embryos was achieved by injection of two antisense non-overlapping morpholino (Mo) (Gene Tools, Oregon) targeting the translational start site: 5'-CCTCGCCTTGGATCTCATCTTGTC-3' and 5'-TTGTAGATTCCTCGCGTCCTCCTTG-3'. A mismatch was used as control: 5'-CgTCcCCTTcGATCTCATgTTcTC-3'. Using a standard microinjector (IM300 Microinjector; Narishige, Tokyo, Japan), an optimized dose of 0.5 ng (0.5 nL bolus) of morpholino placed in a pulled glass capillary was injected in each embryo at 1–2 cell stage, just beneath the blastoderm.

In vitro transcription of PRDM16 mRNA

PRDM16 mRNA, with 7-methyl guanosine cap structure at the 5´end and Poli(A) tail at the 3´end, were transcribed using the HiScribe T7 ARCA mRNA Kit (New England Biolabs, cat. E2060) following manufacturers' instructions.

Rescue experiments by injection of PRDM16 mRNA

To determine whether the effects of the PRDM16-targeted morpholino were specifically due to loss of the target gene, we performed rescue experiments by co-injecting PRDM16-Mo with PRDM16 mmRNA wild-type. A bolus of 1 nl of solution containing 0.5 ng of PRDM16-Mo a and 0.5 ng of PRDM16 RNA wild-type was injected into each egg. As negative control for the rescue experiment, 0.5 ng of mutant RNA (120 base-pair; Ultramer RNA, IDT technologies, Coralville, Iowa) lacking the 5'-UTR region and that does not encode any functional protein was co-injected with the Mo. Furthermore, a bolus of 1 nl of solution containing 0.5 ng of PRDM16-mRNA was also used to rescue the phenotype in LMO2-mutants.

Defining the zebrafish embryo phenotype

Whole embryo phenotype in LMO2-mut embryos and following PRDM16 morpholino and rescue experiments were described on the basis of morphologic features observed under bright-field microscopy: reduced body length, curved body, reduced swimming, chorionated larvae, edema. The phenotype was assessed using a simple six points scoring system, according to the severity of that feature and where one point was normal. At least four different clutches of larvae were assessed per experimental group. The percentage of embryos showing the phenotype (penetrance) was recorded.

Kaplan-Meier analysis of survival

Kaplan-Meier analysis was used to measure the survival of adult zebrafish or larvae following each defined treatment, using PRISM 7 software.

RNA extraction and quantitative PCR

mRNA was extracted from embryos using column purification (RNeasy Mini Kit, Qiagen, cat. 74104) according to the manufacturer's instructions. Working surfaces were cleaned with

RNaseZap (Life Technologies Ltd) to deactivate environmental RNase. Efficient disruption and homogenization of tissue was done using sterile RNase-free disposable pestles (Fisher science, cat. 12-141-368) mounted on a cordless motor for 30 s and then passing the lysate 5-10 times through the needle (18-21 gauge) amounted on a RNAse free syringe. RNA integrity was assessed on basis of 18S and 28S ribosomal RNA (rRNA) bands. mRNA was reverse transcribed into cDNA using qScript cDNA Synthesis Kit (Quanta Bio, cat. 95047.) Primers (IDT Technologies) targeting all genes of interest (see **Table S1** for the full list of primers) and SYBR Green PCR kit (Invitrogen, Carlsbad, CA) were used for real-time qPCR, that was performed with the QuantStudio 12 k Flex system (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Genes expression was expressed as relative fold changes using the ΔCt method of analysis and normalized to α-actin.

Extraction of proteins

Zebrafish embryos were euthanised with an overdose of tricaine, then washed three times in PBS and homogenized with a pestle (Sigma, cat. Z359971) in 100 µL RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with protease/phosphatase inhibitors cocktail. The lysate was kept on ice for 40 min. Then, the tube was centrifuged at 3000g for 5 min and the supernatant transferred into a clean pre-chilled tube. Bicinchoninic acid (BCA) protein Assay (Thermo Scientific, cat. 23225) was used to measure protein concentration.

Western blotting

Samples were loaded on polyacrylamide gel electrophoresis (4-15% gradient) for 2 h and transferred on PVDF membranes for 2h. Membranes were blocked with non-fat milk 5% in PBST (PBS+0.1% Tween) for 1 hour at room temperature and probed with primary antibody overnight at 4°C. Antibody used were: anti-PRDM16 rabbit polyclonal (ProSci, cat. 5555, Poway, CA) anti-LMO2 rabbit polyclonal (Abcam, cat. AB72841) and anti-β-tubulin (loading

control) rabbit polyclonal (Abcam, cat. Ab6046). Membranes were washed 3X (5 min per wash) with PBS and incubated with HRP-conjugated goat anti-mouse or rabbit antibodies for 1 hour at RT. Membranes were washed 3X with PBS for 5 minutes. Antigen antibody complexes were then detected by exposure for 5 min to the enhanced chemiluminescence solution (ECL, Amersham). Then, the membrane was placed down on a film layer and exposed to photographic film (BioMax XAR Film Kodak, Sigma-Aldrich). The film was developed and immunoreactivity (band density) was quantified by using densitometry (source: http://rsbweb.nih.gov/ij/docs/user-guide.pdf) using ImageJ.

Data access

All RNA-Seq data have been deposited to the GEO database (accession n. GSE149152).

Statistical analysis

Results were expressed as the mean ± SEM. Each experiment was performed 3 times. The Shapiro-Wilk test was used to confirm the null hypothesis that the data follow a normal distribution. Statistical comparisons between two groups or multiple groups were then performed, respectively, via Student t-test or ANOVA test using PRISM 7 software followed by Bonferroni post-hoc test. A P value <0.05 was considered significant.

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Author contributions

G.M. is the senior author of this work and is primarily responsible for the conception, design and experimental investigation, data collection and analysis, resources, and original and

revised drafts; B.X. and K.C contributed to bioinformatic analysis; M.D. and A.H.B advised on experimental design and contributed to manuscript editing and discussions; J.P.C. contributed to conceptualization, resources and critical review of the manuscript.

Supplementary Materials

Fig S1 – S10; Table S1; Dataset S1

Legends

Figure 1 – Characterization of low and high-FLI1$^+$ cells. A. Q-PCR analysis of FLI1 gene expression during zebrafish development (from 1 h to 120 hours post-fertilization). **B.** Following enzymatic disaggregation of *Tg(Fli1:EGFP)^{y1}* zebrafish embryos at 72 hpf, two populations of low and high-FLI1$^+$ cells were identified. *Wik* zebrafish was used to set the gate for FLI1$^-$ cells. **C-D.** Line graphs showing the percentage of low and high-FLI1$^+$ cells at different developmental stages (C) and relative FLI1 gene expression (D). **E.** Fluorescent images of low and high-FLI1$^+$ cells 24 h after seeded on fibronectin-coated dishes with endothelial growth medium. **F.** Table of cell characterization (% attached cells and cell number increase). N=3 experiments, ANOVA test followed by Bonferroni post-hoc was used to compared means. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$.

Figure 2 - Bioinformatic analysis. **A-C.** Heat maps displaying the expression level of genes up- or down-regulated in the negative, low and high-FLI1 cells, with each two populations compared separately. Genes which fold change was bigger than 2 and false discovery rate (FDR) less than 0.05 were included in the heat maps. **D-E**. Bar plots showing enrichment Q-values of functional terms in genes upregulated (D) or downregulated (E) in high-FLI1$^+$ compared to low-FLI1$^+$. Upregulated or downregulated genes were defined based on EdgeR FDR cutoff 1e-5.

Figure 3 – Assessment of PRDM16 function in *Tg(Fli1:EGFP)^{y1}* zebrafish. A. PRDM16 was selected from a list of epigenetic modifiers. **B**. RNA-Seq read mapping of PRDM16 for all 3 groups of FLI1$^-$, low- and high-FLI1$^+$, in duplicate. **C**. Q-PCR analysis confirmed RNA-Seq data showing the higher expression of PRDM16 in FLI1$^+$ cells. **D.** Kaplan-Meyer survival curve of zebrafish embryos following PRDM16-targeted morpholino (Mo) injection and control (mismatch). **E**. Brightfield images (upper panels) of whole embryos showing the gross phenotype following injection of PRDM16 Mo. Fluorescent images of the trunk regions in *Tg(fli1:eGFP)* (middle panels) and *Tg(kdrl:mCherry)* (lower panels) embryos, showing changes in the intersegmental vessels (ISVs) following PRDM16 KD. These effects were rescued when the PRDM16 Mo was co-injected with PRDM16 mRNA. The average length of ISVs is graphically reported in **F**. **G-H**. FACS graph and data analysis showing changes in the percentage of low and high-FLI1$^+$ population following PRDM16 KD. **I-J-K**. Analysis of total FLI1$^+$ cells isolated from PRDM16-KD and control embryos, and cultured on fibronectin-coated dishes. Merged brightfield and fluorescent images (**I**), fluorescence images showing the uptake of acetylated low-density lipoprotein (**J**), Nitrate/Nitrite assay as measure of nitric oxide (**K**). N = 3 experiments, ANOVA test followed by Bonferroni post-hoc was used to compared means. ***$p \leq 0.001$, *$p \leq 0.05$.

Figure 4 – Assessment of PRDM16 function in iPSC-EC. A. Differentiation protocol for generation of iPSC-derived endothelial cells (see details in material and method section). At day 7, cells were incubated with siRNA Ctrl or targeting PRDM16. **B.** Brightfield images of an iPSC colony and of the iPSC-derived endothelial cells, showing a different phenotype following treatment with SiRNA for PRDM16. **C.** Real time PCR bar graph and western blot showing the reduced expression of PRDM16 mRNA and protein in cells following PRDM16 siRNA treatment. **D.** Real time PCR data showing the reduced expression of endothelial markers in iPSC-derived EC following PRDM16 KD. **E**. FACS analysis for the endothelial

markers CD31 and CD144 in iPSC-EC showing a significantly reduced population of $CD31^{+}$-$CD144^{+}$ in PRDM16 KD cells compared to controls, as shown in the bar graph in **F**. In negative samples, no 1^{st} antibodies were added**. G.** Immunofluorescence staining for endothelial markers CD31 and CD144 (VE-Cadherin). iPSC-EC with no 1^{st} antibodies added were used as negative control. **H.** Migration assay in iPSC-EC showed that PRDM16 KD reduces cell migration, as shown by a larger residual gap between the edges of the wounded monolayer, quantified in **I**. N=3 experiments, Student t-test, ***$p \leq 0.01$ compared to controls.

Figure 5 - LMO2 impacts zebrafish angiogenesis by regulating PRDM16. **A.** LMO2-mut in zebrafish by CRISPR/Cas9. gRNAs produced a 4 nucleotides (nt) insertion in the exon 2 and a stop codon downstream. **B**. Western blotting using an anti-LMO2 antibody confirmed the absence of LMO2. β-Tubulin was used as loading control. **C.** Kaplan-Meyer survival curve of LMO2-mut zebrafish. **D.** Brightfield images (upper) showing the LMO2-mut embryo gross phenotype. Fluorescent images of the trunk region following immunostaining for Fli1 (middle) and Kdrl (lower) showing the reduced average length of ISVs in LMO2 mut and the rescue of vascular defects when PRDM16 mRNA was injected. These data are graphically shown in **E**. **F.** Q-PCR analysis showing the expression of PRDM16 in $CD31^{+}$ and $CD31^{-}$ cell isolated from LMO2-mut embryos. **G.** ChIP-PCR showing the association of LMO2 to the PRDM16 gene. Gel electrophoresis of PCR products (G). The PRDM16 promoter region, but not a control region ~1-kb downstream, was precipitated by the anti-LMO2 antibody. Sheared chromatin before immunoprecipitation and immunoprecipitation using spermin served as positive and negative controls, respectively. **H.** Graphical abstract showing the relationship LMO2-PRDM16 and the role of PRDM16 on angiogenesis. N = 3 experiments, ANOVA test followed by Bonferroni post-hoc was used to compared means. ***$p \leq 0.001$; *$p \leq 0.05$.

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