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

Citation for published version:
Matrone, G, Xia, B, Chen, K, Denvir, MA, Baker, AH & Cooke, JP 2021, 'FLI1+ cells transcriptional analysis reveals LMO2-PRDM16 axis in angiogenesis', *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.2008559118

Digital Object Identifier (DOI):
10.1073/pnas.2008559118

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Proceedings of the National Academy of Sciences

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Download date: 27. Sep. 2022

1 **Title**: FLI1$^+$ cells transcriptional analysis reveals LMO2-PRDM16 axis in angiogenesis

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

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28 **Key words**: Endothelial cells, differentiation, FLI1, LMO2, PRDM16, zebrafish.

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36 **Significance statement**
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38 Understanding molecular signals driving the development of vascular endothelium is
39 important in seeking novel therapies for human disease. Focusing on the zebrafish model, we
40 herein report new mechanistic insights into development of the vascular endothelium, as well
41 as angiogenesis. This work highlights the novel role of an epigenetic modifier and known
42 oncogene in developmental angiogenesis in zebrafish. In particular, we show that the
43 expression of the histone methyltransferase PRDM16 is high in endothelial cells and
44 PRDM16 is necessary for the endothelial differentiation and migration *in vivo* and *in vitro* in
45 iPSC-derived endothelial cells. Moreover, PRDM16 expression is mediated by LMO2, a
46 well-known oncogene implicated in angiogenesis and leukemogenesis. We hence unveil a
47 novel role of PRDM16 in endothelial development and angiogenesis and therefore propose
48 that PRDM16 could be a novel target for therapeutic modulation of angiogenesis.
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74 **Abstract**
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76 A network of molecular factors drives the development, differentiation and maintenance of
77 endothelial cells. Friend leukemia integration 1 transcription factor (FLI1) is a *bona fide*
78 marker of endothelial cells during early development. In zebrafish *Tg(Fli1:EGFP)^{y1}* we
79 identified two endothelial cell populations, high-FLI1$^+$ and low-FLI1$^+$, by the intensity of
80 green fluorescent protein signal. By comparing RNA-Seq analysis of non-FLI1 expressing
81 cells (FLI1$^-$) with these two (FLI1$^+$) cell populations we identified several novel upregulated
82 genes, not previously recognised as important, during endothelial development.
83 Compared to FLI1 negative (FLI1$^-$) and low-FLI1$^+$ cells, high-FLI1$^+$ cells showed
84 upregulated expression of the zinc finger transcription factor PRDI-BF1 and RIZ homology
85 domain containing 16 (PRDM16). PRDM16 knockdown (KD) by morpholino in the
86 zebrafish larva was associated with impaired angiogenesis and increased number of low-
87 FLI1$^+$ cells at the expense of high-FLI1$^+$ cells. In addition, PRDM16 KD in endothelial cells
88 derived from human induced Pluripotent Stem Cells (iPSC) impaired their differentiation and
89 migration *in vitro*. Moreover, zebrafish mutants with loss-of-function for the oncogene LIM
90 domain only 2 (LMO2-mut) also showed reduced PRDM16 gene expression combined with
91 impaired angiogenesis. PRDM16 expression was reduced further in endothelial (CD31$^+$) cells
92 compared to CD31$^-$ cells isolated from LMO2-mut embryos. ChIP-PCR demonstrated that
93 LMO2 binds to the promoter and directly regulates the transcription of PRDM16. This work
94 unveils a novel mechanism by which PRDM16 expression is activated in endothelial cells by
95 LMO2 and highlights a possible new therapeutic pathway by which to modulate endothelial
96 cell growth and repair.
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103 **Main text**
104
105 The endothelium is the inner lining of blood and lymphatic vessels, made of a continuous
106 monolayer of endothelial cells (EC) interconnected by specialized junctions. It is present in all
107 vertebrates [1] and is highly specialized to adapt to the need of the different tissues and organs [2].
108 It regulates not only the transit of nutrients and oxygen from the blood into tissues and the
109 removal of metabolic waste products, but also blood fluidity, platelet aggregation, vascular
110 tone, inflammation and angiogenesis. In fact, endothelial dysfunction is a fundamental
111 pathophysiology associated with many human diseases and conditions including air pollution
112 [3], smoking [4], heart failure [5], hypertension [6], atherosclerosis [7] and cancer [8]. Therefore, a
113 detailed understanding of the underlying mechanisms of endothelial cell proliferation and
114 differentiation are crucial to the aim of seeking novel therapeutic tools to promote restoration
115 of dysfunctional or damaged endothelium.
116 Zebrafish have a closed circulatory system and the anatomical processes and the molecular
117 mechanisms underlying vascular development are very similar to humans [9]. During
118 embryogenesis, endothelial and blood progenitor cells share a common ancestor, the
119 hemangioblast, derived from the lateral plate of the mesoderm [10], that gives rise to
120 hematopoietic stem cells and endothelial precursors. Early blood vessels form *de novo* from
121 these endothelial progenitors, the angioblasts, in a process named vasculogenesis [11]. This
122 primary vasculature expands and remodels through sprouting into a mature vascular network,
123 also known as developmental angiogenesis [12]. The differentiation of endothelial cells (EC)
124 requires an orchestrated network of different transcription factors, yet to be fully defined [13].
125 One of these, FLI1, a member of the E26 transformation-specific (ETS) family of
126 transcription factors, can be detected at low levels as early as the three-somite stage within the
127 posterior lateral mesoderm in zebrafish [14]. FLI1 is required for hemangioblast formation, as
128 shown in *Xenopus* and zebrafish (*Danio rerio*) [10]. Hence, FLI1 is one of the earliest known

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

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

154 were sorted and cultured on fibronectin-coated petri dish with endothelial cell growth medium
155 (**Fig. 1E**). Fibronectin is an extracellular matrix protein that serves as adhesive support and
156 regulates the spreading, migration, and contractility of EC during vascular development [17]. At
157 24 h after seeding, only 29 % of low-FLI1$^+$ cells were attached to the fibronectin compared to
158 the 89 % of high-FLI1$^+$ cells (**Fig. 1E-F**). In addition, from 24 h to 48 h after seeding the
159 increase in high-FLI1$^+$ cells was significantly greater than low-FLI1$^+$ cells (45 % vs 5 %)
160 (**Fig. 1E-F**).

161 To investigate the gene expression profile, we performed RNA-Seq for FLI1$^-$, low-FLI1$^+$ and
162 high-FLI1$^+$ cell populations. We compared the level of genes in each pair of cell populations
163 and defined differential genes by requiring a fold change larger than 2 and a false discovery
164 rate (FDR) <0.05 **(Fig. 2)**. We were particularly interested in genes encoding novel epigenetic
165 factors that were previously unrecognised in endothelial differentiation.
166 RNA-Seq analysis revealed a set of 441 genes up-regulated and 1024 genes down-regulated
167 in low-FLI1$^+$ compared to FLI1$^-$ cells **(Fig. 2A),** a set of 660 genes up-regulated and 1333
168 genes down-regulated in high-FLI1$^+$ compared to FLI1$^-$ cells **(Fig. 2B)** and a set of 751 genes
169 up-regulated and 793 genes down-regulated in high-FLI1$^+$ compared to low-FLI1$^+$ cells **(Fig.
170 2C)**. Mesodermal markers showed an increased level of FPKM (Fragments Per Kilobase of
171 transcript per Million mapped reads) from FLI1$^-$ to low-FLI1$^+$ to high-FLI1$^+$ cells (**Fig. S1A)**,
172 whereas opposite patterns were observed for ectodermal (**Fig. S1B)** and endodermal markers
173 (**Fig. S1C)**. Pathway enrichment analysis showed clear differences between the groups **(Figs.
174 2D-E, Fig. S2** and **Dataset S1**). Compared to FLI1$^-$, and as expected, Low-FLI1$^+$ and high-
175 FLI1$^+$ groups showed enrichment of blood vessel development pathways, including vascular
176 development, angiogenesis, lymphangiogenesis, and TGF-β signalling pathway **(Fig. S2A)**.
177 with the highest enrichment of these pathways observed in the high-FLI1$^+$ cells **(Fig. 2D)**.
178 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].

202 To assess the role of PRDM16 in zebrafish vascular development, we conducted PRDM16
203 knockdown (KD) studies by injection of two different, non-overlapping PRDM16 translation-
204 blocking morpholinos (Mo) in *Tg(Fli1:EGFP)^{y1}* once-cell stage embryos, following previous
205 guidelines [27]. We injected each Mo separately, at the same dose (0.5 ng per egg), and then we
206 co-injected each morpholino at 1/2 dose of each (0.25 ng per egg). While the phenotype was
207 just slightly apparent with ½ dose of each Mo when injected alone, co-injection of two
208 different Mo produced a stronger effect, similar to that produced by a single morpholino at the
209 dose of 0.5 ng per egg. This additive action strongly suggests that the MO effects are specific
210 for PRDM16. The effective KD was verified by western blotting (**Fig. S5A)**. The survival rate
211 of PRDM16-KD embryos at 120 hpf was approximately 70 % compared to 92 % in controls
212 (**Fig. 3D)**. The main features of the phenotype in PRDM16-KD embryos assessed by
213 brightfield microscopy included body bending and reduced skin pigmentation (**Fig. S5B-C)**.
214 We did not observe an increase in tP53 gene expression, that could cause off-target effects, at
215 the dose of Mo used in this study and up to 4ng, therefore we decided not to co-inject tP53
216 Mo. Using fluorescence microscopy, we observed that the development of intersegmental
217 vessels (ISV), which sprout from dorsal aorta via angiogenesis [28], was significantly reduced in
218 PRDM16-KD embryos compared to control (**Fig. 3E-F**), with a phenotype penetrance of
219 >70%. Indeed, PRDM16 Mo injected embryos with the ISV phenotype showed a significant
220 reduction of PRDM16 compared to control embryos or those embryos not showing the ISV
221 phenotype, as showed by WB analysis (**Fig. S5D**). This suggests that the ISV deficiency is a
222 specific feature of the PRDM16 depletion. In fact, at 30 hpf ISVs bridged fully ventro-
223 dorsally in control embryos, whereas they sprouted only halfway across their dorsal trajectory
224 in PRDM16 morphants (data are reported graphically as average ISVs length, **Fig. 3E-F**).
225 These observations were confirmed in the *Tg(kdrl:mCherry)^{y171}* zebrafish, where the red
226 fluorochrome mCherry is expressed under control of Kdrl gene, an endothelial marker. The

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

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

253 To assess whether the role of PRDM16 is conserved during the differentiation of human EC,
254 we differentiated human induced pluripotent stem cells (iPSC). iPSCs at passage between 21-
255 23 were differentiated to endothelial lineage using our standardized protocol [29] (**Fig. 4A**).
256 Cells were treated with SiRNA, Ctrl or targeting PRDM16, for 6 hours until the end of the
257 mesodermal differentiation. At the end of the protocol, endothelial cells showed the typical
258 EC cobblestone-like shape in the two groups (**Fig. 4B**). Real time PCR showed a reduction of
259 PRDM16 expression of ≥ 70% in SiRNA PRDM16 treated cells compared to control, as also
260 evidenced by western blotting (**Fig. 4C**). Furthermore, real time PCR showed a significant
261 reduction in endothelial markers following PRDM16 KD (**Fig. 4D**). Accordingly, at this stage
262 we sorted double positive cells for the endothelial markers CD31 (PECAM1 gene) and
263 CD144 (CDH5 gene). We observed a reduced percentage of $CD31^{+}$-$CD144^{+}$ cells generated
264 from iPSCs treated with SiRNA PRDM16 compared to control (51.7% vs 6.4%; $p \leq 0.01$; **Fig.**
265 **4E-F**). We purified cells that were double positive for CD31 and CD144 and confirmed the
266 reduced expression of endothelial markers in EC after PRDM16 KD (**Fig. 4G**). We observed
267 no difference in the proliferation rate of iPSC-derived EC in the two groups, as shown by
268 EdU assay (**Fig. S8A**). TUNEL assay in cells from 9 to 15 days of the differentiation
269 protocol, that encompass differentiation and maturation stages of EC, shows that cell death is
270 not increased following PRDM16 KD (**Fig. S8B**). Furthermore, in the endothelial cell
271 migration assay, we observed that iPSC-derived EC treated with SiRNA for PRDM16
272 migrated significantly less, as shown by a larger residual gap between the edge of the wound
273 monolayer, compared to cells treated with SiRNA Ctrl (**Figure 4H-I**).

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

281 LIM-domain-only (LMO)2 is a transcription factor essential to both hematopoietic [31] and
282 endothelial pathways [32], and it is normally expressed in mature vascular endothelium [33].
283 LMO2 is a proto-oncogene implicated in leukemogenesis [34, 35] and has been suggested as a
284 potential therapeutic target in various clinical indications [36]. By virtue of its LIM-domain
285 zinc-finger-like structures, LMO2's canonical function is to act as a bridging molecule [37] to
286 assemble a DNA-binding complex which includes the TAL1, E47, GATA1 and Ldb1 proteins
287 in erythroid cells [38] and in EC [39]. We have previously shown that LMO2 is necessary for EC
288 proliferation during tissue regeneration in adult zebrafish [40], and for the regulation of EC
289 migration mediated by SPHK1 in zebrafish embryos [41]. In the present study, we have
290 knocked-out LMO2 in zebrafish by CRISPR/Cas9 (**Fig. 5)** to further investigate the role of
291 this transcription factor in embryogenesis and we have explored its possible interaction with
292 PRDM16. The gRNA targeted the sequence between intron/exon two of the LMO2 gene and
293 produced an insertion of four nucleotides (AGAT) resulting in a frameshift and a stop codon a
294 few nucleotides downstream (**Fig. 5A**). Western blotting analysis confirmed LMO2 KO (**Fig.
295 5B**). The survival of LMO2-mut embryos was approximately 75 % at 120 hpf compared to
296 94% in controls (**Fig. 5C**). The gross phenotype of LMO2-mut embryos under brightfield
297 microscopy appeared normal and exhibits mild features of body bending and reduced skin
298 pigmentation (**Fig. S9A-B**). These features are similar to those reported previously [42]. As

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

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

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

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

339

340

341 **Methods**
342
343 *Ethics statement*

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

348 *Zebrafish aquaculture and husbandry*

349 Zebrafish *Wik, Tg(fli1:eGFP)*^{y1} and *Tg(kdrl:mCherry)*^{y171} strains were maintained according

350 to standard procedures. Fish were kept at 28℃ under a 14/10 h light/dark cycle and fed with

351 dry meal (Gemma Micro, Westbrook, ME) twice per day. Embryos were obtained by natural

352 mating and kept in E3 embryo medium at 28.5℃. All the experimental procedures were

353 performed under anaesthesia with Tricaine 0.02 mg/ml.

354 *Maintenance of Human iPSC*

355 The human iPSC lines were obtained from Coriell Cell Repositories (Camden, New Jersey)

356 and were maintained on Matrigel (BD Biosciences, cat.n. 354277)-coated plates (Corning) in

357 mTeSR1 medium (STEMCELL Technologies cat.n. 85850) according to their protocol. The

358 iPSCs were passaged approximately every four days, RELSR (STEMCELL Technologies cat.

359 05873) dissociation reagent was used to detach colonies. Cells were maintained in humidified

360 incubators at 37°C and 5% CO2. Pluripotency of iPSC was periodically characterized by

361 morphology and immunostaining of pluripotency markers.

362 *Differentiation of iPSC to endothelial cells*

363 Endothelial cells (EC) differentiation was carried out following an established protocol [46],

364 with some modifications. In brief, the iPSCs at passage between 21-23 were grown to 80%

365 confluence, and placed in differentiation medium Advanced DMEM/F12 (ThermoFisher

366 cat.n. 11320033), supplemented with Wnt agonist CHIR 99021 5 μM (Selleck, cat.n. S2924),

367 bone morphogenetic protein-4 (BMP4, 25 ng/m) (Peprotech cat.n. 120-05), B27 supplement

368 (ThermoFisher cat.n. 17504044), and N2 supplement (ThermoFisher cat.n. 17502048)

369 (*Mesodermal differentiation*). After 3 days, cells were dissociated with HyQtase (GE

370 Healthcare cat.n. SV30030.01) and plated in StemPro media (ThermoFisher cat.n. 10639011),

371 supplemented with forskolin 5 μM (LC Laboratories cat.n. F-9929), vascular endothelial

372 growth factor (VEGF) 50ng/mL (Peprotech cat.n. 100-20), and polyvinyl alcohol 2 mg/mL

373 (Sigma-Aldrich cat.n. 360627) (*Endothelial differentiation*). After 4 days, cells were washed
374 twice with PBS and cultured in endothelial growth media (EGM-2MV, Lonza cat.n. CC-
375 3202), supplemented with additional VEGF (100 ng/ml) for 4 more days (*Endothelial*
376 *maturation*). Cells were passaged once they reached 80-90% confluence. During the whole
377 differentiation protocol, cells were maintained at 37°C and 5% CO_2 in a humidified
378 incubator.

379 *PRDM16 knockdown in differentiating endothelial cells*

380 At day 3 of the mesodermal differentiation stage, 6h before replacing the medium, cells were
381 transfected with 5 nM siRNA targeting PRDM16 or ctrl (Silencer Select siRNA,
382 ThermoFisher) using Lipofectamine RNAiMax (Life Technologies, cat.n. 13778-075 in Opti-
383 MEM (Life Technologies, cat.n. 31985-062). After 6h, mesodermal medium was aspirated
384 and replaced with endothelial differentiation medium. Effective knockdown was analysed by
385 real-time PCR and wester blotting (anti-PRDM16 antibody, human polyclonal, cat.n. PA5-
386 20872 Thermo Fisher Scientific).

387 *Flow cytometry characterization and purification of iPSC-derived EC*

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

395 *Proliferation assay*

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

398 *Apoptosis assay*

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

410 *Cell migration assay*

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

415 *Enzymatic isolation of cells from zebrafish embryos*

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

420 replaced with 1 ml solution of 1X PBS containing trypsin (0.25%, Gibco), 50 µg collagenase
421 P (Roche) and 1 mM EDTA. Embryos were disaggregated using a 200 µl pipette tip and
422 incubated for 30 min at 28.5 °C with further pipetting every 5 min. Digestion was quenched
423 with stop solution (200 µl; 1X PBS containing 30% (v/v) calf serum and 10 mM CaCl2) and
424 samples were centrifuged at 400g for 5 min at 4 °C. Supernatants were discarded, cell pellets
425 washed twice with chilled solution of DMEM containing 1% (v/v) calf serum, 0.8 mM CaCl2,
426 50 U ml−1 penicillin/streptomycin, centrifuged and resuspended in the same medium. Cell
427 suspensions were filtered through a 40 µm cell strainer (BD Biosciences) into FACS tubes.

428 *Flow cytometry characterization and purification of Fli1^{+} cells from zebrafish*

429 Cell suspensions were analysed using a BD FACS Aria (BD Biosciences). DAPI was used to
430 identify viable single cells, whereas FSC-H and FSC-A were used to select cell singlets.
431 Wild-type (*Wik*) zebrafish was used to set the gate between GFP^{-} (*i.e.* FLI1^{-}) and GFP^{+} (low-
432 FLI1^{+}) cell populations whereas the gate between low- and high-FLI1^{+} populations was
433 placed at the onset of the high-FLI1^{+} cells. At least 50,000 of FLI1^{-}, low and high-FLI1^{+} cells
434 (Ex: 488 nm; Em: 530 nm) were sorted from groups of n=50 *Tg(Fli1:eGFP)*^{y1} embryos into a
435 15 ml falcon tube containing chilled PBS and 10 % FBS. The number of viable cells was
436 confirmed under fluorescence stereomicroscope (Leica M205) by using a Neubauer chamber.

437 *Culture of zebrafish cells*

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

441 *Dil-Ac-LDL uptake assay*

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

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

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

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

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

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

469 *Imaging*

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

476 *Preparation of RNA-seq libraries and sequencing*

477 $FLI1^-$, low-$FLI1^+$and hig-$FLI1^+$ from at least n=50 *Tg(Fli1:EGFP)*^{y1} embryos per group were
478 harvested by FACS at 72 hour post-fertilization and prepared for analysis by RNA-seq. Total
479 RNA from cells was isolated, fragmented, reverse transcribed to cDNA, ligated to adapters,
480 and subject to brief PCR amplification in preparation of the Illumina library. The integrity and
481 quality of RNA and complementary DNA were monitored using an Agilent Bioanalyzer
482 2100. The samples were run on an Illumina Hi-Seq 2500 system with 100 base paired-end
483 sequencing (50 million reads per sample). Samples were run in duplicate.

484 *Bioinformatic analysis*

485 RNA-Seq reads were aligned to the zebrafish genome danRer10. We use the full set of known
486 Gene downloaded from the UCSC Genome browser as reference genes. RNA-Seq read counts
487 for each gene in each sample was calculated using Cuffdiff function in Cufflinks version
488 2.2.1. The Cuffdiff also calculates fragment per kilobase per million reads (FPKM) for each
489 gene. We further subject the reads counts to EdgeR version 3.12.0 for differential expression
490 analysis, and define differential genes based on false discovery rate (FDR) cutoff 1e-5. We

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

495 *Chromatin immunoprecipitation (ChIP)-PCR assay*

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

509 *Generation of zebrafish mutants using CRISPR/Cas9*

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

516 temperature for 5 minutes prior to injecting into the cytoplasm of WT AB zebrafish embryos
517 at 1-cell stage. For detecting the zebrafish mutants, genomic DNA was extracted from
518 individual zebrafish larva using the Quick-DNA isolation kit (Zymogen), and a short genomic
519 region (200–400 bp) flanking the target site was amplified by polymerase chain reaction
520 (PCR). For LMO2, the primers were: (F) 5'-GCACATGTTTGCCTGTATTTGT-3', (R) 5'-
521 CAGAGGTCACAGCTCAGACAGT-3'. Purified PCR products (200 ng) were denatured,
522 reannealed, and then digested with EnGen Mutation Detection kit (New England Biolabs) that
523 uses T7 Endonuclease I. The samples were run out on agarose gel 2% to distinguish mutant
524 from wildtype embryos. PCR products of positive mutants were subcloned into pGEM-T
525 vectors (Promega) that were then used to transform competent cells. After overnight culture at
526 37°C, a single colony was selected for sequencing.

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

536 *In vitro transcription of* PRDM16 mRNA
537 PRDM16 mRNA, with 7-methyl guanosine cap structure at the 5´end and Poli(A) tail at the
538 3´end, were transcribed using the HiScribe T7 ARCA mRNA Kit (New England Biolabs, cat.
539 E2060) following manufacturers' instructions.

540 *Rescue experiments by injection of PRDM16 mRNA*

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

550 *Defining the zebrafish embryo phenotype*

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

558 *Kaplan-Meier analysis of survival*

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

561 *RNA extraction and quantitative PCR*

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

564 RNaseZap (Life Technologies Ltd) to deactivate environmental RNase. Efficient disruption
565 and homogenization of tissue was done using sterile RNase-free disposable pestles (Fisher
566 science, cat. 12-141-368) mounted on a cordless motor for 30 s and then passing the lysate 5-
567 10 times through the needle (18-21 gauge) amounted on a RNAse free syringe. RNA integrity
568 was assessed on basis of 18S and 28S ribosomal RNA (rRNA) bands. mRNA was reverse
569 transcribed into cDNA using qScript cDNA Synthesis Kit (Quanta Bio, cat. 95047.) Primers
570 (IDT Technologies) targeting all genes of interest (see **Table S1** for the full list of primers)
571 and SYBR Green PCR kit (Invitrogen, Carlsbad, CA) were used for real-time qPCR, that was
572 performed with the QuantStudio 12 k Flex system (Applied Biosystems, Foster City, CA)
573 following the manufacturer's instructions. Genes expression was expressed as relative fold
574 changes using the ΔCt method of analysis and normalized to α-actin.

575 *Extraction of proteins*

576 Zebrafish embryos were euthanised with an overdose of tricaine, then washed three times in
577 PBS and homogenized with a pestle (Sigma, cat. Z359971) in 100 μL RIPA buffer (25
578 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1%
579 SDS) supplemented with protease/phosphatase inhibitors cocktail. The lysate was kept on ice
580 for 40 min. Then, the tube was centrifuged at 3000g for 5 min and the supernatant transferred
581 into a clean pre-chilled tube. Bicinchoninic acid (BCA) protein Assay (Thermo Scientific, cat.
582 23225) was used to measure protein concentration.

583 *Western blotting*

584 Samples were loaded on polyacrylamide gel electrophoresis (4-15% gradient) for 2 h and
585 transferred on PVDF membranes for 2h. Membranes were blocked with non-fat milk 5% in
586 PBST (PBS+0.1% Tween) for 1 hour at room temperature and probed with primary antibody
587 overnight at 4°C. Antibody used were: anti-PRDM16 rabbit polyclonal (ProSci, cat. 5555,
588 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.

Acknowledgements

This work was supported by the European Commission (MSCA-IF-EF-ST n. 797304) (GM), the Cullen Trust for Health Care (JPC and GM), National Institutes of Health R01 grants HL 148338 (JPC and KC), GM125632 (KC), HL133254 (JPC and KC) and HL148338 (JPC). No potential conflict of interest was reported by the authors.

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

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

616 **Supplementary Materials**

617 Fig S1 – S10; Table S1; Dataset S1

618

619 **Legends**

620

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

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

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

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

663 markers CD31 and CD144 in iPSC-EC showing a significantly reduced population of $CD31^{+}$-
664 $CD144^{+}$ in PRDM16 KD cells compared to controls, as shown in the bar graph in **F**. In
665 negative samples, no 1st antibodies were added**. G.** Immunofluorescence staining for
666 endothelial markers CD31 and CD144 (VE-Cadherin). iPSC-EC with no 1st antibodies added
667 were used as negative control. **H.** Migration assay in iPSC-EC showed that PRDM16 KD
668 reduces cell migration, as shown by a larger residual gap between the edges of the wounded
669 monolayer, quantified in **I**. N=3 experiments, Student t-test, ***$p \leq 0.01$ compared to controls.

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

687

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