**Prdm6 Is Essential for Cardiovascular Development In Vivo**

Andreas Gewies1,2,3,4, Mercedes Castineiras-Vilarino5, Uta Ferch1, Nina Jährling6,7, Katja Heinrich1, Ulrike Hoeckendorf1,12, Gerhard K. H. Przemeck4, Matthias Munding3, Olaf Groß3, Timm Schroeder4, Marion Horsch6, E. Loraine Karran8,10, Aneela Majid9,10, Stefan Antonowicz10, Johannes Beckers8,11, Martin Hrabé de Angelis8,11, Hans-Ulrich Doedt5,6, Christian Peschel12, Irmgard Förster13,14, Martin J. S. Dyer10, Jürgen Ruland1,2,3,4,15*

1 Institut für Klinische Chemie und Pathobiochemie, Klinikum Rechts der Isar, Technische Universität München, Munich, Germany, 2 German Cancer Consortium (DKTK), Heidelberg, Germany, 3 German Cancer Research Center (DKFZ), Heidelberg, Germany, 4 Laboratory of Signaling in the Immune System, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany, 5 Department of Bioelectronics, Institute of Solid State Electronics, Vienna University of Technology, Vienna, Austria, 6 Center for Brain Research, Section of Bioelectronics, Medical University of Vienna, Vienna, Austria, 7 Department of Neurobiology, University of Oldenburg, Oldenburg, Germany, 8 Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany, 9 Helmholtz Zentrum München, German Research Center for Environmental Health, Research Unit Stem Cell Dynamics, Neuherberg, Germany, 10 MRC Toxicology Unit and Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, United Kingdom, 11 Chair of Experimental Genetics, Technische Universität München, Freising-Weihenstephan, Germany, 12 Department of Internal Medicine III, Klinikum Rechts der Isar, Technische Universität München, Munich, Germany, 13 Institute of Medical Microbiology, Immunology and Hygiene, Technische Universität München, Munich, Germany, 14 Immunology and Environment, Life and Medical Sciences (LIMES) Institute, University of Bonn, Bonn, Germany, 15 German Center for Infection Research (DZIF), partner site München, Munich, Germany

**Abstract**

Members of the PRDM protein family have been shown to play important roles during embryonic development. Previous in vitro and in situ analyses indicated a function of Prdm6 in cells of the vascular system. To reveal physiological functions of Prdm6, we generated conditional Prdm6-deficient mice. Complete deletion of Prdm6 results in embryonic lethality due to cardiovascular defects associated with aberrations in vascular patterning. However, smooth muscle cells could be regularly differentiated from Prdm6-deficient embryonic stem cells and vascular smooth muscle cells were present and proliferated normally in Prdm6-deficient embryos. Conditional deletion of Prdm6 in the smooth muscle cell lineage using a SM22-Cre driver line resulted in perinatal lethality due to hemorrhage in the lungs. We thus identified Prdm6 as a factor that is essential for the physiological control of cardiovascular development.

**Introduction**

Prdm6 belongs to the PRDM family of transcriptional repressors which all possess an N-terminal PR domain and C-terminal Krüppel-type zinc finger motifs. While the zinc fingers are responsible for DNA binding, the PR domain is thought to mediate homodimerization and interaction with proteins such as the histone methyl transferase G9a and histone deacetylases [1-3]. Therefore, PRDM proteins are expected to play important roles as histone modifying factors that regulate gene transcription at the chromatin level. The most intensely studied PRDM member PRDM1 (also named BLIMP1) has been shown to mediate methylation of lysine residue 9 of histone 3 [2] and as a transcriptional repressor has been demonstrated to be essential for several physiological processes such as terminal B cell differentiation [4], T cell

*E-mail: jurland@lrz.tum.de*
homeostasis and function [5,6], primordial germ cell formation [7] and regulation of proliferation and differentiation in the sebaceous gland [8]. Other members of the PRDM family were also reported to control developmental processes: Prdm5 regulates collagen gene transcription in developing bone [9]. Prdm9 defines hotspots of genetic recombination during meiosis [10]. Prdm14 was shown to be involved in the maintenance of embryonic stem cells in the mouse [11], and Prdm16 controls the bidirectional switch between skeletal myoblasts and brown fat cells [12]. While PRDM transcription factors control various developmental processes under physiological conditions, aberrant expression of PRDM proteins has been correlated with malignant disease and PRDM genes map to chromosomal regions frequently deleted in tumors [13-16]. Moreover, PRDM proteins can be expressed as PR domain-containing full length proteins or as amino-terminally truncated proteins lacking a functional PR domain by usage of an alternative internal promoter. Loss of PRDM full length expression or a shift in expression towards the truncated shorter form has been implicated in tumorigenesis [2,15,17-20].

Prdm6 was recently characterized as a transcriptional repressor that is expressed and plays a role in the vascular system. Davis and colleagues described Prdm6 as a transcription factor that plays a role in regulating the differentiation and proliferation of smooth muscle cells (SMCs) [3]. Moreover, Prdm6 was described as a factor that controls survival and differentiation of endothelial cells in the vascular system [21]. Furthermore, expression of Prdm6 has been reported in cells of the developing nervous system [22]. Finally, we identified PRDM6 to be transcriptionally deregulated and ectopically expressed from the rare, but recurrent chromosomal translocation t(5;14)(q23;q32) in B cell lymphoma patients (manuscript in preparation). To reveal physiological functions of Prdm6 in vivo, we generated and analyzed conditional Prdm6-deficient mice. We report here that Prdm6 is essential for embryonic development and for vital functions of the cardiovascular system.

Results

Generation of a conditional PRDM6 mutant mouse line

Because the physiological functions of PRDM6 are still largely unknown, we generated a gene-targeted mouse line that allows conditional Prdm6 ablation using Cre-loxP technology [23]. By homologous recombination in murine embryonic stem (ES) cells we flanked exon 3 of Prdm6 with loxP sites (see Materials and Methods and Figure S1 A,B). After injection of ES cells into blastocysts and removal of the neomycin selection cassette via flp-mediated deletion we eventually obtained Prdm6<sup>full<sub>lox</sub></sup> and Prdm6<sup>lox/lox</sup> mice, which were born at expected Mendelian ratios and were phenotypically indistinguishable from their wild type littermates (data not shown). Crossing Prdm6<sup>lox/lox</sup> mice to Cre deleter mice [24] induced the deletion of the loxP-flanked exon 3 sequence in the germ line and resulted in Prdm6<sup>del/del</sup> heterozygous mice. Correct homologous recombination events were confirmed by Southern blot analysis (Figure S1 C). Of note, deletion of Prdm6 exon 3 not only removes the central part of the PR domain but, due to a concomitant frame shift, it also prevents the expression of the complete Prdm6 reading frame downstream of the PR domain so that no functional protein can be expressed.

Prdm6 is essential during embryonic development

Heterozygous Prdm6<sup>del/del</sup> mice were intercrossed to obtain homozygous Prdm6-deficient mutants (Prdm6<sup>del/del</sup>). Prdm6<sup>del/del</sup> and Prdm6<sup>del/del</sup> mice were born at the expected Mendelian ratios. In contrast, we did not observe viable homozygous Prdm6<sup>del/del</sup> offspring, indicating that functional Prdm6 expression is essential for the viability of mice. Thus, we next performed timed pregnancies and analyzed Prdm6<sup>del/del</sup> embryos at different developmental stages. Up to E10.0, we observed Mendelian frequencies of morphologically intact Prdm6<sup>del/del</sup> embryos. However, beyond E10.0 the frequency of viable Prdm6<sup>del/del</sup> embryos declined significantly whereas Prdm6<sup>del/del</sup> and Prdm6<sup>del/del</sup> embryos were present at regular numbers (Figure 1A). RT-PCR analysis confirmed the absence of Prdm6 exon 3 and therefore the deficiency of functional Prdm6 mRNA in Prdm6<sup>del/del</sup> embryos. An alternative exon2– exon4-spliced Prdm6 transcript is produced by the disrupted (del) allele in Prdm6<sup>del/del</sup> and Prdm6<sup>del/del</sup> embryos (Figure S1 D). However, since Prdm6<sup>del/del</sup> heterozygous mice are born at Mendelian ratios and are viable, healthy and fertile, there is no indication for a dominant gain-of-function of the alternatively spliced Prdm6 transcript derived from the knockout (del) allele. The onset of embryonic lethality in the Prdm6<sup>del/del</sup> embryos correlated with the onset of Prdm6 expression at E10.5 in wild type embryos (Figure 1B), a developmental stage at which the cardiovascular system undergoes critical developmental steps [25]. Macroscopic inspection of Prdm6<sup>del/del</sup> embryos revealed that at E12.5 they eventually displayed pale and edematous bodies, implying cardiovascular insufficiency (Figure 1C). Analysis of the cardiac architecture of the Prdm6<sup>del/del</sup> embryos via histological H&E staining revealed a thinning of the myocardial walls, indicating primary or secondary heart failure (Figure 1D).

Prdm6 affects vascular patterning

During the isolation of Prdm6<sup>del/del</sup> embryos from the deciduae, we repeatedly observed vascular malformations exclusively on the yolk sacs of Prdm6<sup>del/del</sup> embryos at stage ±E13.5 (Figure 2A, left panel). Higher magnifications revealed that these malformations were composed of clusters of densely growing and partially dilated blood microvessels (Figure 2A, right panel). However, large vessel vascularization was present in the yolk sacs of Prdm6<sup>del/del</sup> embryos, indicating regular overall vasculogenesis (Figure 2B). To investigate the role of Prdm6 in the development of the small blood vessel architecture in structural detail, we stained vascular endothelial cells with an anti-CD31 (anti-PECAM1) antibody and visualized the yolk sac vascular system via fluorescence microscopy. The yolk sacs of wild type control embryos displayed an organized vascular network with a hierarchy between vessels of higher and lower orders at the developmental stages E10.5 and E11.5 (Figure 2C, upper panels). Also in Prdm6<sup>del/del</sup> yolk sacs a small vessel network was present (Figure 2C, lower panels).
**Figure 1. Prdm6 deficiency results in embryonic lethality.** (A) *Prdm6<sup>wt/del</sup>* mice were intercrossed. Pregnant mice were euthanized and embryos dissected and genotyped at defined developmental stages. The percentages of viable embryos of the respective genotypes at the different stages of embryonic development (dpc = days post coitum) are indicated; wild type *Prdm6<sup>wt/wt</sup>* (wt/wt) and heterozygous *Prdm6<sup>wt/del</sup>* (wt/del) mice are viable, whereas *Prdm6*-deficient *Prdm6<sup>del/del</sup>* (del/del) embryos begin to die after E10.0, with no *Prdm6<sup>del/del</sup>* embryos being found at developmental stages beyond E16.0. (B) Northern blot analysis of *Prdm6* expression using total embryonic RNA from different developmental stages from wild type embryos. *Gapdh* expression analysis served as a loading control. (C) Representative wild type control and *Prdm6*-deficient embryos (del/del) at the indicated developmental stages. White arrows indicate edematous swelling. (D) Transverse heart sections from wild type control and *Prdm6*-deficient embryos were stained with H&E and analyzed by microscopy. The thin myocardium of *Prdm6*-deficient embryos (del/del) is indicated by an arrow. Scale bars correspond to 200 µm.

doi: 10.1371/journal.pone.0081833.g001
However, visual inspection suggested subtle differences in the patterning of the vascular network of Prdm6-deficient yolk sacs compared to wild type control yolk sacs (Figure 2C). Indeed, quantitative analysis revealed that the small vessel network of Prdm6-deficient yolk sacs contains significantly less avascular space and exhibits a significant increase in the mean vessel diameter (Figure 2D and Figure S2). These findings indicate that Prdm6 function is involved in vascular patterning during embryonic development.

**Figure 2. Prdm6 affects angiogenic patterning.** (A) Unusual clusters of densely growing vessel structures on yolk sacs of Prdm6-deficient embryos, as observed under a stereomicroscope. Scale bars correspond to 1 mm (left panel) or 100 µm (right panel). (B) Large vessels in the yolk sacs of E12.5 control and Prdm6-deficient (del/del) embryos under a stereomicroscope. Scale bars correspond to 500 µm. (C) Visualization of E10.5 (left panels) and E11.5 (right panels) yolk sac microvascular systems via immunofluorescent staining with an anti-CD31 primary antibody and a Cy3-conjugated secondary antibody. Scale bars correspond to 200 µm. (D) Quantitative morphometric analysis of the yolk sac vasculature as shown in (C). Avascular space and mean vessel diameters of yolk sacs at E10.5 – E11.5 are shown as mean ± SEM, n=6. More details about this analysis are given in Figure S2.

doi: 10.1371/journal.pone.0081833.g002

**Normal differentiation and proliferation of Prdm6-deficient smooth muscle cells**

It was recently reported that Prdm6 might play a role in SMC function [3]. We therefore next tested whether Prdm6 deficiency affects SMC differentiation and proliferation. To this end, we established Prdm6-deficient ES cell lines from the inner cell mass of early E3.5 Prdm6-deficient embryos and differentiated these ES cells into defined vascular cell lineages under specific culture conditions in vitro. Of note, the Prdm6-deficient ES cell lines differentiated regularly into smooth muscle alpha-actin (SMA)-expressing cells (i.e. pericytes or...
Prdm6 in Embryonic Development

vascular SMCs [26]) at a rate and frequency comparable to wild type ES cell lines (Figure 3A), indicating that Prdm6 is dispensable for SMC-lineage differentiation. Moreover, Prdm6flo/del ES cell lines also regularly differentiated into ECs and cardiomyocytes in vitro (data not shown). Immunohistochemical staining to SMA demonstrated that SMCs were regularly lining arterial vessel walls in Prdm6flo/del embryos, thus indicating that recruitment of SMCs to the vasculature was intact (Figure 3B). To study the proliferation of embryonic vascular SMCs in vivo, we injected bromodeoxyuridine (BrdU) into pregnant mice and subsequently used immunohistology to determine the BrdU content within embryonic SMCs in different vascular regions. We did not observe significant differences in the frequencies of BrdU-positive SMCs between wild type control and Prdm6-deficient embryos, neither in SMCs in the aortic arch arteries nor in the yolk sac (Figure 3 C,D). In conclusion, Prdm6 does not seem to be required for either general SMC differentiation, recruitment to blood vessels or proliferation during embryonic development.

SM22-Cre-induced Prdm6 deletion results in perinatal death associated with pulmonary hemorrhage

We crossed the Prdm6flo/del alleles into the SM22-Cre mouse line [27,28] to generate Prdm6flo/del;SM22-Cre mice in which Prdm6 is selectively disrupted in the SMC lineage . Although SMC-conditional Prdm6 knockout mice were born at expected Mendelian frequency, we did not obtain viable adult SMC-conditional Prdm6 knockout mice (Figure 4A) because all newborn Prdm6flo/del;SM22-Cre pups died within 2 days after birth (Figure 4B). Interestingly, perinatal death induced by SM22-Cre-mediated conditional deletion of Prdm6 was associated with massive hemorrhage in the lungs (Figure 4C).

Prdm6 regulates factors that are involved in angiogenesis

Since Prdm6 acts as a transcription factor [3], we were interested in the identification of target genes that are physiologically controlled by Prdm6. Therefore, we performed genome-wide cDNA microarray analysis and compared gene expression patterns between wild type Prdm6wt/wt and knockout Prdm6del/del embryos. Because we observed an impact of Prdm6 deletion on vascular development, we compared the mRNA expression patterns in the yolk sacs of day E10.5 embryos, which are highly vascularized and easily accessible, allowing high quality RNA isolation. A total of 51 genes were found to be differentially expressed in Prdm6del/del yolk sacs compared to wild-type tissue (Figure S3). Only two genes (Sfrp1 and Mtap1b) were upregulated, while all of the other deregulated genes displayed decreased expression levels in the absence of Prdm6. Several of the differentially regulated genes have been previously implicated in angiogenesis, such as those coding for the Wnt signaling inhibitor Sfrp1, the extracellular matrix protein F-Spondin, and the matrix metalloproteinase MMP2. Quantitative RT-PCR (qPCR) analysis of selected genes confirmed the microarray data (Figure 5), indicating that Prdm6 directly or indirectly controls the expression of a set of genes that are implicated in vascular development, and possibly also in other developmental processes.

Discussion

In this study we reveal an essential role of Prdm6 for the development of the cardiovascular system. Prdm6 total knockout embryos (Prdm6del/del) die during development with an onset of about E10.5. At later stages, Prdm6del/del embryos display signs of cardiac insufficiency, i.e. edema and progressive heart defects. Anti-CD31 staining revealed that a vessel network is present in Prdm6-deficient yolk sacs, indicating that vasculogenesis is intact in Prdm6 deficient yolk sacs. However, the small vessel network of Prdm6-deficient yolk sacs displayed an altered patterning with increased vascular diameters and smaller avascular space when compared to wild type yolk sacs. Prdm6 deficiency therefore apparently affects aspects of angiogenesis. Embryonic lethal phenotypes involving intact vasculogenesis, but impaired angiogenesis have been described for mice that are deficient in a variety of genes, such as Fzd5 [29], Notch1 [30,31], Jagged1 [32], Hey1/Hey2 [33], Smoothened [34], Eph-B4 and Ephrin-B2 [35], Angiopoietin [36], Tie2 [37], Smad5 [38], Quaking [39], HIF2alpha- [40], VE-PTP [41], SCL/Tal-1 [42], and P13K p110-alpha [43]. Inactivation of those key regulators of angiogenesis results in embryonic death latest by E11.0. Compared to that, Prdm6del/del embryos start to die around E10.5 with clearly reduced but countable numbers still alive and without clear morphological defects at E12.5. All Prdm6del/del embryos that can be identified at E12.5 however are edematous, anemic pale and obviously are deceasing. While the above mentioned gene knockouts of angiogenic key regulators arrest angiogenesis in the yolk sac already at the level of the primitive primary plexus with defective development of the large vessel system, Prdm6del/del yolk sacs do possess large vessels and the observed angiogenesis defect of the small vessel network in Prdm6del/del yolk sacs is rather mild. Thus, it is questionable whether the observed subtle changes in vascular patterning can be responsible for the embryonic lethal phenotype of the Prdm6del/del embryos. It appears likely that Prdm6-deficiency might directly induce the observed heart defect that might be the primary cause of embryonic death. Further studies are required to resolve this issue.

Davis et al. proposed that Prdm6 is a transcriptional repressor that suppresses SMC differentiation and promotes SM proliferation [3]. We therefore investigated SMCs in Prdm6del/del total knockout embryos in order to test whether Prdm6 deficiency might have an effect on SMCs that potentially could contribute to the cardiovascular phenotype. However, we could not detect defects in the overall capacity of Prdm6-deficient SMCs to differentiate, proliferate and to be recruited to blood vessels in vivo. Moreover, Prdm6-deficient ES cells were able to differentiate into the pericyte lineage in vitro, to the same extent as wild type ES cells. These findings indicate that Prdm6 function might be required for alternative aspects of SMC function or in additional cell types during vascular development, e.g. the endothelial lineage as has been suggested by Wu et
Future experiments are required to address these questions.

Even though we did not detect SMC defects in Prdm6 del/del total knockout embryos, we crossed our floxed Prdm6 allele to...
a SM22-Cre deleter line which induces Cre-mediated recombination in the SMC lineage [27] but also in other selected cell types such as mesothelial cells in the yolk sac and in cardiomyocytes during early heart development [44-46]. Conditional deletion of Prdm6 by the SM22-Cre driver did not result in embryonic death, which is an additional indication that Prdm6-deficiency in SMCs might not be the main cause for the defect in embryonic development as we observe it in Prdm6[lox/del] total knockout embryos. For the same reason, it is unlikely that Prdm6 plays essential roles in mesothelial cells or cardiomyocytes during early stages of embryonic development. Interestingly however, SM22-Cre driven conditional deletion of Prdm6 resulted in postnatal death associated with lung hemorrhage. In lung, the expression of SM22-Cre has been demonstrated to be confined to vascular smooth muscle cells [27]. Thus, it might be assumed that smooth muscle cells require Prdm6 for maintaining pulmonary vessel integrity. Alternatively, however, it cannot be ruled out that SM22-driven...

Figure 4. Selective disruption of Prdm6 in vascular smooth muscle cells results in perinatal lethality. (A) Prdm6[lox/del];SM22-Cre mice were crossed with Prdm6[lox/lox] mice, and the genotypes of the offspring were analyzed at three weeks of age. The Prdm6[lox/del];SM22-Cre genotype leads to deletion of Prdm6 in the SMC lineage. The frequencies of the resulting genotypes were calculated from a total of 28 offspring animals and compared to the expected Mendelian frequencies. (B) Newborn mice from the same crosses as in (A) were observed at day 1 and day 2 after birth and subsequently were genotyped. (C) Lungs from newborn Prdm6[lox/del] control animals (viable) and SMC-conditional Prdm6[lox/del];SM22-Cre animals (deceasing) were embedded in paraffin, and sections were stained with hematoxylin and eosin. Scale bars correspond to 100 µm.

doi: 10.1371/journal.pone.0081833.g004
Moreover, Wnt/frizzled signaling plays a critical role in distinct transcriptional co-factors [47]. Our microarray data indicate that the related Prdm5 protein was reported to possess the capacity to regulate Wnt4 expression [3], we speculate that Prdm6 might modulate angiogenesis partly through effects on the Wnt/frizzled pathway. Further mechanistic studies are required to test this hypothesis and to understand which target genes are directly or indirectly regulated by Prdm6, thereby also providing hints concerning a potential role of PRDM6 in lymphomagenesis. The present study is a starting point for future investigations of PRDM6 in vivo functions with our conditional knockout mouse model being a valuable tool to further define the role of PRDM6 in the cardiovascular system by its selective deletion in e.g. the endothelial lineage or in cardiomyocytes and to study the possible impact of Prdm6 in other physiological processes, such as neurogenesis with which Prdm6 expression has been reported to be associated [22].

Materials and Methods

Ethics Statement

All animal work was conducted in accordance with German Federal Animal Protection Laws and approved by the Institutional Animal Care and Use Committee at the Technical University of Munich.

Generation of Prdm6 conditional knockout mice and flp/Cre deleter strains

Exon 3 of Prdm6 was flanked by loxP sites via homologous recombination in E14K ES cells according to standard procedures [59]. The embryonic stem (ES) cells containing the correctly recombined (rec) Prdm6 locus (Prdm6<sup>rec</sup>) still also contained the FRT-flanked neomycin resistance selection cassette. Standard ES cell technologies were used to generate...
germline mutant Prdm6<sup>lox/mut</sup> mice. Crossing with fli recombinase deleter mice [60] resulted in deletion of the neomycin resistance cassette and produced Prdm6<sup>lox/mut</sup> mice. The following mouse strains were used: fli deleter mice (Jax human β-actin FLPe deleter strain B6;SjL-Tg(ActFLPe)9205Dym/J), Cre deleter mice (Jax human CMV-Cre deleter strain B6.C-Tg(CMV-cre)1Cgn/J), and SM22-Cre (i.e. SM22alpha-Cre) mice (Jax Tg(Tagln-Cre)1Her/J). The mice were housed in a specific pathogen-free facility according to FELASA recommendations (http://www.felasa.eu). Littermates were used in all experiments.

Genotyping PCR, RT-PCR and qPCR

For genotyping of the Prdm6 wt, del and flox alleles, the following primer combinations were used. wt allele: fwd: 5'-agacagaacatcaagaagggtag-3' plus wt rev: 5'-gcatctgcatctgttaaagctacca-3'). Del allele: fwd: 5'-agacagaacatcaagaagggtag-3' plus del rev: 5'-ccagatttgtgcacccttaagg-3') (570 bp); and flox allele: 5'-agacagaacatcaagaagggtag-3' plus flox rev: 5'-gatatcgctagcgggaagttc-3' (380 bp). RT-PCR and qPCR were performed as described previously [61]. The following primer pairs were used: Prdm6 exon 3 wt allele specific primers (5'-taacagattgtgcacccttaagc-3' plus 5'-ggctgaccaacagtatccatattca-3') and 380 bp. RT-PCR and qPCR were performed as described previously [61]. Briefly, ES cells were co-cultured with OP 9 cells in differentiation medium containing 10% FCS (PAN Biotech) and 10<sup>-4</sup> M beta-mercaptoethanol (Sigma-Aldrich) in alpha-MEM (Gibco/Invitrogen) and then FACS sorted to detect Flk1-positive, Cadherin-negative lateral plate mesodermal cells (Flk1 Ab: clone AVAS12, eBioscience; Cadherin Ab, clone ECCD2). To achieve mural cell differentiation, 2x10<sup>4</sup> sorted mesodermal cells were cultured for 4 days on Collagen IV-coated plates. The cells were fixed in methanol containing 5% DMSO and stained with a monoclonal anti-SMA-Cy3 Ab (Sigma-Aldrich, Clone 1A4).

cDNA microarray analysis

E10.5 yolk sacs from six Prdm6<sup>lox/mut</sup> and six wild type control mice were dissected on ice in DEPC-treated PBS, shock frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated using RNeasy Mini kits (Qiagen), and 400 ng of RNA was amplified according to the instructions of the Target AMP<sup>TM</sup> 1-Round aRNA Amplification Kit 103 (Epicentre Biotechnologies). Genome-wide cDNA microarrays were generated, hybridized and analyzed as described recently [67]. The selection of significantly differentially expressed genes showing reproducible up- or down-regulation included less than 5% false positives (FDR) in combination with fold changes of >1.3. The expression data were submitted to the GEO database (GSE9065), where a full description of our microarray results is also available (GPL4937).

Supporting Information

Figure S1. Generation of a conditional Prdm6 allele. (A) Amino acid sequence of the murine Prdm6 protein according to GenBank accession number NP_001028453. Two methionine start residues are indicated by circles: the first corresponds to the sequence proposed by Wu et al. [21], the second was described by Davis et al. [3]. The PR domain in the central part of the sequence is indicated in bold, whereas the zinc finger region is underlined. Exon-exon borders are marked with dashed vertical lines, and exon numbers are given to indicate by which exons the different parts of the protein are encoded. (B) Targeting strategy for homologous recombination at the Prdm6 locus. The region containing exon 3 of the Prdm6 wt locus, the targeting vector and the distinct recombinant alleles
The vascular networks of a representative wild type control (A) and a Prdm6 knockout (B) yolk sac were analyzed by measuring the avascular space (i.e. intercapillary space) and mean vessel diameters. The white areas in the center panels indicate the avascular spaces as measured by the histogram function of the Photoshop CS6 software. The right panels indicate all points where vessel diameters were measured using the ruler function of Photoshop CS6 software. Vessel diameters were determined in between all branching points. Scale bars correspond to 200 µm.

(PDF)

**Figure S2. Quantitative morphometric analysis of the yolk sac vasculature.** The vascular networks of a representative wild type control (A) and a Prdm6 knockout (B) yolk sac were analyzed by measuring the avascular space (i.e. intercapillary space) and mean vessel diameters. The left panel shows the original image of the anti-CD31 stains of whole mount yolk sacs. The white areas in the center panels indicate the avascular spaces as measured by the histogram function of the Photoshop CS6 software. The right panels indicate all points where vessel diameters were measured using the ruler function of Photoshop CS6 software. Vessel diameters were determined in between all branching points. Scale bars correspond to 200 µm.

(PDF)

**Acknowledgements**

We thank Karl-Ludwig Laugwitz and Andrea Moretti for helpful discussions and support; Konstanze Pechloff and Oliver Gorka for critical reading of the manuscript; Eliö Montanéz for the CD31 staining protocol; Susie Weiss, Lisa Bartnik, Sabrina Krebs, Kristina Brunner, Sandra Geißler, Stephanie Erenoglu, and Andrea Bernshausen for technical assistance. Many thanks to all staff of the animal care facility at the Klinikum Rechts der Isar (ZPF) for excellent support.

**Author Contributions**

Conceived and designed the experiments: JR AG MJSD IF CP TS JB MHDeA HUD. Performed the experiments: AG MCV UF NJ KH UH GKHP MM OG MH ELK AM SA. Analyzed the data: AG JR UF NJ MH MJSD IF. Wrote the manuscript: AG JR MJSD.

**References**

1. Schneider R, Bannister AJ, Kouzarides T (2002) Unsafe SETs: histone lysine methyltransferases and cancer. Trends Biochem Sci 27: 396-402. doi:10.1016/S0968-0004(02)02141-2. PubMed: 12151224.
2. Györny L, Wu J, Fejer G, Seto E, Wright KL (2004) PRDI-BF1 recruits the histone H3 methyltransferase G9a in transcriptional silencing.[see comment]. Nature Immunology 5: 299-308.
3. Davis CA, Haberland M, Arnold MA, Sutherland LB, McDonald OG et al. (2006) PRISM/PRDM6, a transcriptional repressor that promotes the proliferative gene program in smooth muscle cells.[see comment]. Nature Immunology 5: 299-308.
4. Shapiro-Shelef M, Lin KI, McHeyzer-Williams MG, et al. (2003) Bmp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells.[see comment]. Immunity 19: 607-620.
5. Martins GA, Cimmino L, Shapiro-Shelef M, Szaboics M, Herron A, et al. (2006) Transcriptional repressor Bmp-1 regulates T cell homeostasis and function.[see comment]. Nature Immunology 7: 457-465.
6. Kallies A, Hawkins ED, Belz GT, Metcalf D, Hommel M, et al. (2006) Transcriptional repressor Bmp-1 is essential for T cell homeostasis and self-tolerance.[see comment]. Nature Immunology 7: 466-474.
7. Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y et al. (2005) Bmp1 is a critical determinant of the germ cell lineage in mice. Nature 436: 207-213. doi:10.1038/nature03483. PubMed: 15937476.
8. Horsley V, O’Carroll D, Tooze R, Ohinata Y, Salitou M et al. (2006) Bmp1 defines a progenitor population that governs cellular input to the sebaceous gland. Cell 126: 597-609. doi:10.1016/j.cell.2006.06.048. PubMed: 16901790.
9. Galli G, Hornens de Lichtenberg K, Carrara M, Hans W, Wuecling M et al. (2012) Prdm5 regulates collagen gene transcription by association with RNA polymerase II in developing bone. PLoS Genet 8: e1002711. PubMed: 22589746.
10. Brick K, Smagulova F, Kihl P, Camerini-Otero RD, Petukhova GV (2012) Genetic recombination is directed away from functional genomic elements in mice. Nature 485: 642-645. doi:10.1038/nature11089. PubMed: 22660327.
11. Ma Z, Swigut T, Valcove A, Rada-Iglesias A, Wysocka J (2011) Sequence-specific regulator Prdm14 safeguards mouse ESCs from entering extraembryonic endoderm fates. Nature Structural and Molecular Biology 18: 120-127. doi:10.1038/nsmb.2000.
12. Seale P, Bjork B, Yang W, Kajimura S, Chin S et al. (2008) PRDM16 controls a brown fat/skeletal muscle switch. Nature 454: 961-967. doi:10.1038/nature07182. PubMed: 18719582.
13. Mock BA, Liu L, LePaslier D, Huang S (1996) The B-lymphocyte maturation promoting transcription factor BLIMP1/PRDI-BF1 maps to D5S447 on human chromosome 6q21-q22.1 and the syntenic region of mouse chromosome 10. Genomics 37: 24-28. doi:10.1006/geno.1996.0516. PubMed: 8921366.
14. Huang S (1999) The retinoblastoma protein-interacting zinc finger gene RIZ in 1p36-linked cancers. Frontiers in Bioscience 4: D528-D532. doi:10.2741/Huang. PubMed: 10369809.
31. Pasqualucci L, Compagno M, Houldsworth J, Monti S, Grun A et al. (2006) Inactivation of the PRDM1/Blimp1 gene in diffuse large B cell lymphoma. J Exp Med 203: 311-317. doi: 10.1084/jem.20052204. PubMed: 16492805.

32. Le H, Yu JX, Liu L, Busey IM, Wang MS et al. (1998) RIZ1, but not the alternative RIZ2 product of the same gene, is underexpressed in breast cancer, and forced RIZ1 expression causes G2-M cell cycle arrest without apoptosis. Cancer Res 58: 4238-4244. PubMed: 9766644.

33. Jiang GL, Huang S (2000) The yin-yang of PR-domain family genes in tumorigenesis. Histol Histopathol 15: 109-117. PubMed: 10668202.

34. Nishikata I, Sasaki H, Iga M, Tateno Y, Imaoishi S, et al. (2003) A novel EVI1 gene family, MEL1, lacking a PR domain (MEL1S) is expressed mainly in (1;3)(p36;q21)-positive AML and blocks G-CSF-induced myeloid differentiation. Blood 102: 3323-3332.

35. Shing DC, Trubia M, Marchesi F, Radaelli E, Belloni E et al. (2007) Overexpression of sPRDM16 coupled with loss of p53 induces myeloid leukemias in mice. J Clin Invest 117: 3696-3707. PubMed: 18037989.

36. Wu Y, Ferguson JE 3rd, Wang H, Kelley R, Ren R et al. (2008) PRDM6 is enriched in vascular precursors during development and inhibits endothelial cell proliferation, survival, and differentiation. J Mol Cell Cardiol 44: 47-58. PubMed: 17682997.

37. Kinaneri E, Inoue T, Anuga J, Imaoishi I, Kageyama R et al. (2008) Prdm proto-oncogene transcription factor family expression and interaction with the Notch-Hes pathway in mouse neurogenesis. PLOS ONE 3: e3689. 10.1371/journal.pone.0003689. PubMed: 18870550.

38. Schwenk F, Baron U, Rajewsky K (2011) Pre-angiogenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res 39: 5080-5081. doi: 10.1093/nar/gkr695. PubMed: 21033516.

39. Conway SJ, Kruysenaars-Freijag A, Kneer PL, Machnicz M, Koushik SV (2003) What cardiovascular defect does my prenatal mouse mutant have, and why? Genesis 35: 1-21. doi:10.1002/gene.10152. PubMed: 12481294.

40. Avrilik A, Genové G, Betholzé C (2011) Pericyte: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell 21: 193-215. doi:10.1016/j.devcel.2011.07.001. PubMed: 21839917.

41. Holthoer R, Gotthardt M, Shyabbin B, Steinmetz M, Potthast R et al. (2002) Smooth muscle-selective deletion of guanylyl cyclase-C prevents the acute but not chronic effects of ANP on blood pressure. Proc Natl Acad Sci U S A 99: 7142-7147. doi:10.1073/pnas.071605999. PubMed 10947487.

42. Frutkin AD, Shi H, Otsuka G, Levene P, Karlsson S et al. (2005) A critical developmental role for tfgbr2 in myogenic cell lineages is revealed in mice expressing Sm22-Cre, not SMMHC-Cre. Journal of Molecular and Cellular Cardiology 41: 724-731.

43. Terai Y, Abe M, Miyamoto K, Koike M, Yamasaki M et al. (2001) Vascular smooth muscle cell growth-promoting factor-Fspondin inhibits angiogenesis via the blockade of integrin alphavbeta3 on vascular endothelial cells. Journal of Cellular Physiology 183: 378-387. doi:10.1002/jcp.10399. PubMed: 11473366.

44. Adini I, Rabinovitz I, Sun JF, Prendergast GC, Benjamin LE (2003) Raf-1 controls trafficking and stage-specific survival of endothelial cells during vascular development. Genes Dev 17: 2721-2732. doi:10.1101/gad.1134603. PubMed: 14597666.

45. Sabaté C, Malvaux L, Bovy N, Deroanne C, Lambert V et al. (2011) MicroRNA-21 exhibits antiangiogenic function by targeting RhoB expression in endothelial cells. PLOS ONE 6: e16979. doi:10.1371/journal.pone.0016979. PubMed: 21347332.

46. Schnaper HW, Grant DS, Stetler-Stevenson WG, Fridman R, D’Orazi G et al. (1993) Type IV collagenase(s) and TIMPs modulate endothelial cell proliferation in vitro and in vivo. Cardiovasc Res 21: 733-738. doi:10.1093/cr/21.4.733. PubMed: 8797002.

47. Mignatti P, Rifkin DB (1996) Plasminogen activators and matrix metalloproteinases in angiogenesis. Enzyme Protein 49: 117-138. doi:10.1002/epr.570491101. PubMed: 8700002.

48. Dasgupta P, Chellappan SP (2006) Nicotine-mediated cell proliferation and angiogenesis: new twists to an old story. Cell Cycle 5: 2324-2328. doi:10.4161/cc.5.23.3366. PubMed: 17102610.

49. Wang, HG, Watkins G, Douglas-Jones A, Holmgren L, Mansel RE (2006) Angiomotin and angiomotin-like proteins, their expression and correlation with angiogenesis and clinical outcome in human breast cancer. BMC Cancer 6: 16. doi:10.1186/1471-2407-6-16. PubMed: 16430777.

50. Grochown AM, Sullivan KM, D’Amore PA (2006) Cultured endothelial cells display endogenous activation of the canonical Wnt signaling pathway and express multiple ligands, receptors, and secreted modulators of Wnt signaling. Dev Dyn 235: 3110-3120. doi:10.1002/dvdy.20939. PubMed: 17013865.
57. van de Schans VA, Smits JF, Blankesteijn WM (2008) The Wnt/frizzled pathway in cardiovascular development and disease: friend or foe? Eur J Pharmacol 585: 338-345. doi:10.1016/j.ejphar.2008.02.093. PubMed: 18417121.
58. Shu W, Jiang YQ, Lu MM, Morrisey EE (2002) Wnt7b regulates mesenchymal proliferation and vascular development in the lung. Development 129: 4831-4842. PubMed: 12361974.
59. Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science 265: 103-106. doi:10.1126/science.8016642. PubMed: 8016642.
60. Rodríguez CI, Buchholz F, Galloway J, Sequerra R, Kasper J et al. (2000) High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat Genet 25: 139-140. doi:10.1038/75973. PubMed: 10835623.
61. Ferch U, zum Buschenfelde CM, Gewies A, Wegener E, Rauser S et al. (2007) MALT1 directs B cell receptor-induced canonical nuclear factor-kappaB signaling selectively to the c-Rel subunit. Nat Immunol 8: 984-991. doi:10.1038/nimm.2007.143. PubMed: 17660823.
62. Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci U S A 81: 1991-1995. doi:10.1073/pnas.81.7.1991. PubMed: 6326085.
63. Southern E (2006) Southern blotting. Nature Protocols 1: 518-525. doi: 10.1038/nprot.2006.73. PubMed: 17406277.
64. Mo FE, Muntean AG, Chen CC, Stolz DB, Watkins SC et al. (2002) CYR61 (CCN1) is essential for placental development and vascular integrity. Molecular and Cellular Biology 22: 8709-8720.
65. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292: 154-156. doi: 10.1038/292154a0. PubMed: 7242681.
66. Schroeder T, Meier-Stiegen F, Schwanbeck R, Eilken H, Nishikawa S et al. (2006) Activated Notch1 alters differentiation of embryonic stem cells into mesodermal cell lineages at multiple stages of development. Mech Dev 123: 570-579. doi:10.1016/j.mod.2006.05.002. PubMed: 16822655.
67. Horsch M, Schlädier S, Gailus-Durner V, Fuchs H, Meyer H et al. (2008) Systematic gene expression profiling of mouse model series reveals coexpressed genes. Proteomics 8: 1248-1256. doi:10.1002/pmic.200700725. PubMed: 18336826.
Afl II
EcoRV

**A**

**B**

**C**

**D**
Avascular Space: 33.4 %
Mean Vessel Diameter: 16.6 μm

control

Avascular Space: 28.3 %
Mean Vessel Diameter: 21.3 μm

del/del
| Mean ratio | Array TAG ID | Gene symbol | Comment |
|------------|--------------|-------------|---------|
| 1.56       | MG-6-30j4    | Mtap1b      | Microtubule-associated protein 1 B |
| 2.00       | MG-16-171f10 | Sfrp1       | Secreted frizzled-related sequence protein 1 |
| -2.70      | MG-8-117j20  | Man2b2      | Mannosidase 2, alpha B2 |
| -2.17      | MG-8-73p2    | Spon1       | Spondin 1, (f-spondin) extracellular matrix protein |
| -1.77      | MG-4-6h18    | Ifitm3      | Interferon induced transmembrane protein 3 |
| -2.72      | MG-14-64n9   | Mark3       | MAP/microtubule affinity-regulating kinase 3 |
| -2.38      | MG-15-214i1  | Rhob        | Ras homolog gene family, member B |
| -2.40      | MG-3-97p3    | Hemt1       | Hematopoietic cell transcript 1 |
| -1.84      | MG-8-29b14   | Dctn2       | Dynactin 2 |
| -2.22      | MG-4-4e6     | Alas2       | Aminolevulinic acid synthase 2, erythroid |
| -2.36      | MG-8-55p15   | Iqcf4       | IQ motif containing F4 |
| -2.48      | MG-4-146n10  | Pld2        | Phospholipase C-like 2 |
| -2.69      | MG-4-145f7   | Scl43a2     | Solute carrier family 43, member 2 |
| -2.09      | MG-3-16e23   | Slc9a9      | Solute carrier family 9, isoform 9 |
| -2.43      | Fbxo32       | Fbox32      | F-box only protein 32,3 |
| -2.04      | MG-8-71j2    | Nup155      | Nucleoporin 155 |
| -1.80      | MG-4-146i20  | Clptm1      | Cleft lip and palate associated transmembrane protein 1 |
| -1.93      | MG-3-18a15   | CR521370    | |
| -2.61      | MG-4-3b2     | Hba-a2      | Hemoglobin alpha, adult chain 2 |
| -2.23      | MG-4-4k23    | E030041M21Rik | |
| -2.49      | MG-68-143f7  | Hbb-b2      | Hemoglobin, beta adult minor chain |
| -2.67      | MG-4-3k1     | Hba-a1      | Hemoglobin alpha, adult chain 1 |
| -1.71      | MG-8-40g12   | Rad9        | RAD9 homolog |
| -2.71      | MG-47-1h17   | Hbb-bh1     | Hemoglobin Z, beta-like embryonic chain |
| -2.31      | MG-15-3b23   | Col11a1     | Procollagen, type XI, alpha 1 |
| -2.49      | MG-8-42b9    | H3f3a       | H3 histone, family 3A |
| -2.39      | MG-8-11g1    | Slc4a1      | Solute carrier family 4 (anion exchanger), member 1 |
| -2.51      | MG-6-6d16    | Tap1        | Transporter 1, ATP-binding cassette, sub-family B |
| -1.97      | Hoxc8        | Hoxc8       | Homeo box C8 |
| -2.54      | MG-4-4h13    | Grp1        | Glycine/arginine rich protein 1 |
| -1.87      | MG-8-117l6   | Rpl11       | Ribosomal protein L11 |
| -2.63      | MG-4-86f7    | Hb-b2a2     | Hemoglobin alpha, adult chain 2 |
| -2.66      | MG-4-147o3   | Hba-a1      | Hemoglobin alpha, adult chain 1 |
| -2.55      | MG-4-5j21    | Il6st       | Interleukin 6 signal transducer |
| -2.43      | MG-8-86g2    | 1110017116Rik | |
| -2.33      | MG-8-40d4    | Hba-a1      | Hemoglobin alpha, adult chain 1 |
| -1.39      | MG-15-2j1    | Mmp2        | Matrix metalloproteinase 2 |
| -1.48      | MG-4-5e2     | Ugt1a6      | UDP glycosyltransferase 1 family, polypeptide A6 |
| -1.40      | MG-8-13e2    | Xrn1        | 5'-3' exoribonuclease 1 |
| -1.62      | MG-3-10n5    | Nudt4       | Nudix (nucleoside diphosphate linked moiety X)-type motif 4 |
| -2.10      | MG-8-84a9    | Eraf        | Erythroid associated factor |
| -2.80      | MG-4-2e20    | Hba-a1      | Hemoglobin alpha, adult chain 1 |
| -1.42      | MG-16-108a19 | Mbnl1       | Muscleblind-like 1 |
| -2.64      | MG-4-146d10  | Tysnd1      | Tryptsin domain containing 1 |
| -1.42      | MG-8-16n9    | Arrb1       | Arrestin, beta 1 |
| -1.54      | MG-8-96d13   | 2210411K11Rik | |
| -2.64      | MG-4-3k8     | 130001805Rik | |
| -1.49      | MG-4-148g1   | Hist2h3c2   | Histone 2, H3c2 |
| -1.32      | MG-3-3f8     | Amot1       | Angiomotin-like 1 |
| -1.60      | MG-3-218p12  | Car2        | Carbonic anhydrase 2 |
| -2.45      | MG-4-5m17    | Hba-a2      | Hemoglobin alpha, adult chain 2 |