Prdm1 functions in the mesoderm of the second heart field, where it interacts genetically with Tbx1, during outflow tract morphogenesis in the mouse embryo

Stéphane D. Vincent1, Alicia Mayeuf-Louchart1, Yusuke Watanabe1,∗§, Joseph A. Brzezinski IV2, Sachiko Miyagawa-Tomita3, Robert G. Kelly4 and Margaret Buckingham1

1Department of Developmental & Stem Cell Biology, Institut Pasteur, CNRS URA 2578, Paris, France, 2Department of Structural Biology, University of Washington, Seattle, WA, USA, 3Division of Cardiovascular Development and Differentiation, Department of Pediatric Cardiology, Tokyo Women’s Medical University, Tokyo, Japan and 4Aix-Marseille Université, Developmental Biology Institute of Marseille, CNRS UMR 7288, Campus de Luminy, Marseille, France

Received March 22, 2014; Revised and Accepted May 8, 2014

Congenital heart defects affect at least 0.8% of newborn children and are a major cause of lethality prior to birth. Malformations of the arterial pole are particularly frequent. The myocardium at the base of the pulmonary trunk and aorta and the arterial tree associated with these great arteries are derived from splanchnic mesoderm of the second heart field (SHF), an important source of cardiac progenitor cells. These cells are controlled by a gene regulatory network that includes Fgf8, Fgf10 and Tbx1. Prdm1 encodes a transcriptional repressor that we show is also expressed in the SHF. In mouse embryos, mutation of Prdm1 affects branchial arch development and leads to persistent truncus arteriosus (PTA), indicative of neural crest dysfunction. Using conditional mutants, we show that this is not due to a direct function of Prdm1 in neural crest cells. Mutation of Prdm1 in the SHF does not result in PTA, but leads to arterial pole defects, characterized by mis-alignment or reduction of the aorta and pulmonary trunk, and abnormalities in the arterial tree, defects that are preceded by a reduction in outflow tract size and loss of caudal pharyngeal arch arteries. These defects are associated with a reduction in proliferation of progenitor cells in the SHF. We have investigated genetic interactions with Fgf8 and Tbx1, and show that on a Tbx1 heterozygote background, conditional Prdm1 mutants have more pronounced arterial pole defects, now including PTA. Our results identify PRDM1 as a potential modifier of phenotypic severity in TBX1 haploinsufficient DiGeorge syndrome patients.

**INTRODUCTION**

Congenital heart defects that affect at least 0.8% of newborn children, and cause 30% of spontaneous abortions, represent a major human health problem (1). The heart originates from two main sources of heart progenitors in anterior splanchnic mesoderm; the first and second heart fields (FHF and SHF, respectively) (2). Cell lineage analyses, as well as explant experiments and genetic tracing, have shown that the SHF is the sole source of cells for outflow tract myocardium, as well as contributing to the right ventricle, the atria and the venous pole (2–4). Initially, the cardiac tube grows by the addition of cells from the SHF to its extremities. At the arterial pole, the outflow tract becomes separated to form the future aorta and pulmonary trunk, as a result of...
colonization of cardiac neural crest (CNC) cells derived from
neurectoderm. This is accompanied by the formation of endocardial cushions (5). The outflow tract is initially connected to the
dorsal aorta via the symmetrically positioned pharyngeal arch
arteries (PAAs) that extend from the branchial arches (BAs).

This network is remodelled leading to the formation of a left-
sided aortic arch (2). The mesodermal core of the BAs corresponds
to an extension of the SHF, which gives rise to the myocardium of
the pulmonary trunk and aorta, derived from the outflow tract
(anterior arches—BAs 1,2) and to the associated arterial tree derived
from the PAAs (posterior arches—BAs 3,4,6) (6). This connection
between the heart field and the great vessels is a conserved
feature among vertebrates (7).

An extensive gene regulatory network operates in cardiac
progenitor cells of the SHF (2). Subdomains of this network
can be distinguished. Thus, a number of regulatory genes are spe-
cifically expressed in the anterior part of the SHF that contributes
to the arterial pole of the heart, which is particularly susceptible
to malformations, representing 30% of all neonatal heart defects
in humans. Tbx1, encoding a T-box containing transcriptional
activator, is expressed in the anterior SHF and Tbx1-haploinsuf-
cient DiGeorge syndrome patients are characterized by car-
diac defects at the arterial pole of the heart, such as tetralogy
of Fallot or persistent truncus arteriosus (PTA) (8). Fgf8 is also
expressed in the SHF and Fgf8 hypomorphic mutant embryos
display a similar range of phenotypes (9,10). Both Fgf8 and
Tbx1 are also expressed in the endoderm and endoderm of
the BAs, and conditional deletions of Fgf8 and Tbx1 have shown
tissue-specific requirements for correct heart morphogen-
esis (11–20). Moreover, interaction between Tbx1 and the
FGF signalling pathway has been demonstrated, highlighting
the complex genetic regulation in this region during heart
formation (21).

Another interesting candidate in the context of heart develop-
ment is Prdm1 which is expressed in the BAs region that
includes the SHF, but not in the heart (22,23). It encodes a tran-
scriptional repressor containing a PR/SET domain that binds to
specific DNA targets via its Zinc fingers (24). Prdm1 represses
gene transcription via the recruitment of HDAC1/2, Groucho
family members or histone methyl transferases, and controls
programs of gene expression by direct repression or indirect ac-
tivation, via the repression of repressors, of hundreds of target
genes (24). Recently, it has been shown that Prdm1 also could
act as a direct activator during zebrafish neural crest specifi-
cation (25), but this has not been shown in other models so far.
During mouse embryogenesis, Prdm1 is dynamically expressed
and plays a role in organogenesis and tissue specification.
Cre genetic tracing analysis showed that Prdm1 is expressed
in the progenitors of the right ventricle and the outflow tract
(22). Prdm1 null mutant embryos are characterized by the
lack of development of the BAs posterior to BA2 due to apop-
tosis (23) and by PTA (22), phenotypes similar to those of
Tbx1 or Fgf8 mutants. Fgf8 and Tbx1 expression are down-
regulated in E9.5 Prdm1 null mutant embryos (22,23), suggest-
ing possible interactions.

PTA is caused by the failure of CNC cells that migrate through
the BAs, to divide the outflow tract into the aorta and the pulmo-
rary trunk (26,27). The PTA phenotype may result from a cell au-
tonomous function of Prdm1 in CNC cells since it has been
detected in the neural plate border and neural crest in zebrafish
(28), or from a secondary effect due to loss of Prdm1 in the ecto-
derm, mesoderm and/or endoderm of the BAs region. The apop-
tosis observed in null Prdm1 embryos supports this hypothesis
(23). This may also mask a specific requirement of Prdm1
in the endoderm, ectoderm and/or mesoderm.

In this study, we have refined the expression pattern of Prdm1
and demonstrate that it is expressed in the SHF. Conditional
deletion of Prdm1 in the cardiac mesodermal lineage results
in mutants that display arterial pole malformations but not
PTA, demonstrating a specific requirement for Prdm1 in the
SHF. In its absence, proliferation in the SHF is compromised,
leading to defects in outflow tract rotation and PAA formation.
The expression of a dominant negative form of Prdm1 in the
SHF also leads to arterial pole defects, in keeping with its role
as a repressor. In a Tbx1 heterozygous background, conditional
Prdm1 mutant embryos have a more severe phenotype, with
PTA, demonstrating genetic interaction between these genes.
Tbx1 directly activates Fgf8 in the SHF (29) and we propose
that the more pronounced phenotype in Prdm1 mutants on a
Tbx1+/− heterozygous background is due to the impairment of
the FGF/Tbx1/RA regulatory network that is involved in pha-
yngeal arch (PA) and cardiovascular development (21,30,31).
Our results also identify PRDM1 as a potential modifier of
phenotypic severity in Tbx1 haploinsufficient DiGeorge syn-
drome patients.

RESULTS

**Prdm1 is expressed in the SHF**

Prdm1 is initially expressed in anterior definitive endoderm (32)
and then also in mesoderm in the region of the SHF, showing a
crescent shaped pattern (Fig. 1A and B). This pattern is main-
tained at E8.5 (Fig. 1C) when Prdm1 expression is detected in
the three germ layers, but not in the heart tube (Fig. 1D) (23).
At E9.5 and E10.5, Prdm1 expression is maintained in discrete
regions of ectoderm and endoderm, and is still broadly expressed
in the three germ layers in the region where the caudal BAs are
forming (Supplementary Material, Fig. S1). Immunohistochem-
istry confirms that Prdm1 is present in the SHF, but not in myo-
cardium of the heart tube marked by striated myosin heavy chain
(MF20) expression (Fig. 1E). There is a clear overlap of the
expression of Prdm1 and Isl1 (Ish1), a marker of SHF mesoderm
(33) (Fig. 1F and G). Prdm1 and Isl1 are also co-expressed in
derm and endoderm, where Prdm1 expression is relatively
higher (Fig. 1F and G). In the SHF, Prdm1 is present in a subdo-
minal of Isl1 expression (Fig. 1F). Prdm1 levels are highest close
to the differentiating outflow tract (Fig. 1F), compared with the
more lateral and posterior SHF (Fig. 1G). The Prdm1 expression
domain does not extend to the more posterior Isl1-positive part
of the SHF which gives rise to atrial myocardium (34), consistent
with the Prdm1cre/+R26R genetic tracing that showed a contri-
bution to the arterial pole of the heart, but not to the atria (22).

Although in the zebrafish embryo Prdm1 is expressed in
neural crest cells and is required for their differentiation into
specific neurons (28,35), we did not detect Prdm1 expression in
neural crest cells, and conditional deletion of Prdm1 in these
cells using two Cre lines [Pax3Cre/+ (36) and Tg(HpPAcre)
(37)] did not result in any cardiac defects (data not shown),
demonstrating that Prdm1 is not involved directly in CNC in
the mouse embryo. Neural crest is, however, affected in the Prdm1 null mutants, as a result of apoptosis of the posterior BAs (23), through which these cells normally transit before colonizing the outflow tract. These observations strongly suggest that the PTA phenotype observed in Prdm1 null mutant foetuses (22) is indirect.

Prdm1 is required in the SHF for correct formation of the arterial pole of the heart

In order to delete Prdm1 in mesodermal cardiac progenitors, we used a conditional Prdm1 mutant line (38) crossed onto either Mesp1Cre/+ (39) or a transgenic line Tg(Mef2cCre) (40).

---

**Figure 1.** Prdm1 is dynamically expressed in the SHF in the mouse embryo. (A–C) Whole-mount in situ hybridization for Prdm1 transcripts at E7.5 (A), E7.75 (B), E8.5 (C, transverse section (red dotted line) in D). Prdm1 is expressed in a crescent shaped domain starting at E7.5 (A). Transcripts also accumulate in head mesoderm as development proceeds (B and C) and, as shown in (D), are detected in the endoderm, ectoderm and mesoderm, but not in the heart tube (HT). (E–G) Immunohistochemistry on sections at the levels indicated in the diagrams, with antibodies to striated muscle myosin heavy chain (MF20) that marks myocardium and Prdm1 (E), or the SHF marker Islet1 (Isl1) and Prdm1 (F and G), on transverse (E) or sagittal (F and G) sections of wild-type embryos at E8.5 (E) or E8.25 (F and G). HT, heart tube; the arrow in (D) indicates the SHF; arrowhead in (E) points to the dorsal mesocardial protrusion behind the heart tube where differentiating myocardium is still Isl1-positive; arrows in (F) and (G) point to outflow tract (OFT), Endoderm (En) and Ectoderm (Ec), where both Isl1 and Prdm1 are expressed to varying degrees. The white asterisks in (E) and (G) indicate trapping of the secondary antibody in the foregut pocket.
**Table 1. Abnormal development at the arterial pole of the heart in foetal and postnatal (E16.5-P21) Mef2cCre and Mesp1Cre conditional mutants, and in control and mutant crosses on a Tbx1<sup>+/−</sup> heterozygote genetic background**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of embryos</th>
<th>HEART</th>
<th>AORTIC ARCH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal TGA DORV VSD Stenotic Ao Stenotic PT PTA</td>
<td>Normal IAA-B AOSA High arch Retro SA</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>8 0 0 0 0 0 0 0</td>
<td>8 0 0 0</td>
</tr>
<tr>
<td>Mef2cCre</td>
<td>12</td>
<td>11 0 0 0 0 1&lt;sup&gt;a&lt;/sup&gt; 0 0</td>
<td>1 0 4 10</td>
</tr>
<tr>
<td>Mesp1Cre</td>
<td>13</td>
<td>1+1&lt;sup&gt;b&lt;/sup&gt; 5 6 12 9&lt;sup&gt;c&lt;/sup&gt; 1&lt;sup&gt;d&lt;/sup&gt; 0</td>
<td>0 7 7 10&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>8</td>
<td>7 0 0 0 0 1 0 0</td>
<td>5 0 1 3</td>
</tr>
<tr>
<td>Mef2cCre;Tbx1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>4</td>
<td>1 2&lt;sup&gt;f&lt;/sup&gt; 1&lt;sup&gt;g&lt;/sup&gt; 4 2 0 1</td>
<td>0 1 4 3</td>
</tr>
<tr>
<td>Mesp1Cre;Tbx1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>5</td>
<td>0 1+1&lt;sup&gt;f&lt;/sup&gt; 3&lt;sup&gt;g&lt;/sup&gt; 5 1 1 3</td>
<td>0 0 5 5</td>
</tr>
</tbody>
</table>

TGA, transposition of the great arteries; DORV, double outlet right ventricle; VSD, ventricular septal defect; Ao, aorta; PT, pulmonary trunk; PTA, persistent truncus arteriosus; IAA-B, interruption of aortic arch type B; AOSA, aberrant origin of the subclavian arteries.

<sup>a</sup>Normal semilunar valves.

<sup>b</sup>Stenotic aorta.

<sup>c</sup>Uneven leaflets in seven cases.

<sup>d</sup>Only two leaflets.

<sup>e</sup>Including two cases of a sling connecting the subclavian arteries to the dorsal subclavian arteries.

<sup>f</sup>Not complete TGA; overriding Ao.

<sup>g</sup>PTA facing the RV.

*Mespl* is expressed in all cardiac progenitors (39) and *Mesp1*<sup>Cre</sup> has been successfully used to delete genes such as *Tbx1*, *Fgf8* or *Fgf10* in this population (15,17,20). Therefore, *Mesp1*<sup>Cre+;</sup> *Prdm1<sup>Cre-AACAS</sup> (Mesp1Cre) embryos can be considered as loss of function for *Prdm1* in the SHF. On the other hand, Cre recombination under the control of the *Mef2c* enhancer, Tg(*Mef2cCre*), will be active later, only in the anterior part of the SHF and its derivatives (40). Due to the difference in timing of Cre expression, deletion with the Tg(*Mef2cCre*) in Tg(*Mef2cCre*); *Prdm1<sup>CreaACAS</sup>* (Mef2cCre) embryos occurs after the onset of *Prdm1* expression and can be considered as a late inactivation of *Prdm1* in the SHF. Both deletions did not perturb overall embryonic development but led to highly penetrant cardiovascular phenotypes; however, no PTA was observed (Table 1).

We therefore conclude that the PTA observed in epiblast-specific *Prdm1* mutant embryos (22) is due to a non-cell autonomous effect of Prdm1, mainly from the ectoderm and/or the endoderm, which leads to the loss of neural crest cells. Conditional deletion of *Prdm1* using a *Foxg1*<sup>Cre</sup> allele, which mainly targets pharyngeal epithelia (11), led to complete or partial absence of outflow tract septation and PTA (Supplementary Material, Fig. S2), in keeping with this view. The strong PTA phenotype in the *Prdm1* null embryos probably masked mesoderm-specific defects.

Whereas we did not observe any lethality among the *Mef2cCre* mutant embryos (1.5% compared with an expected 25% Mendelian ratio, n = 204) survive after birth. In the survivors, the heart appeared unaffected but they had an abnormal aortic arch (data not shown). At early stages of cardiogenesis, *Mesp1Cre* mutant hearts were characterized by a shorter outflow tract, as indicated by in situ hybridization for transcripts of *Wnt11* that mark this part of the heart (41), at E9.5 (Fig. 2A and B). This results in defective morphogenesis and abnormal positioning of myocardium at the base of the future ascending aorta, as shown by expression of the *T55* transgene that marks this region of the heart (42) (Fig. 2C and D). In situ hybridization of *Semaphorin 3C* transcripts, which normally accumulate in myocardium at the base of the pulmonary trunk (43), indicated abnormal positioning of this myocardial domain (Fig. 2E and F). Anomalies in outflow tract rotation were confirmed using the *T96-16* transgenic line, also expressed in subpulmonary myocardium (44) (data not shown). Unlike the situation in *Tbx1<sup>+/−</sup>* mutant embryos (43), the identity of pulmonary trunk myocardium is therefore preserved, although malpositioned, in the absence of Prdm1. These abnormalities in outflow tract morphogenesis are associated with arterial pole defects such as transposition of the great arteries, double outlet right ventricle and ventricular septal defects (Fig. 3B compared with 3A, Table 1). In contrast, no defects were detected in the formation of the right ventricle. In some *Mesp1Cre* mutants, stenotic aorta (Fig. 3B) and stenotic pulmonary trunk were also observed (Table 1). Defects were not detected in *Mef2cCre* mutant hearts (Table 1). However, both types of mutants displayed aortic arch artery anomalies, including right aortic arch and right ductus arteriosus (Table 2), although the defects in *Mef2cCre* mutant embryos were milder (Table 1). In both *Mef2cCre* and *Mesp1Cre* mutant embryos, the connections of the subclavian arteries (right or left, in the case of the right or left aortic arch phenotypes, respectively) were abnormal, being either retroesophageal or connected via a high arch, as illustrated for the right subclavian artery (Fig. 3D and E, respectively). These defects do not lead to lethality, as shown by the survival of the *Mef2cCre* mutant animals (data not shown) and were not related to an early defect in the expression of the left–right-specific effector Ptx2 (data not shown).

**Prdm1 is required for the formation of the pharyngeal arch arteries**

The aortic arch defects observed in *Mef2cCre* and *Mesp1Cre* mutant embryos can be attributed to defects in fourth PAA development. In order to see whether the fourth PAAs were absent or degenerating, we performed ink injection in E10.75 embryos (Table 3 and Supplementary Material, Fig. S3). We could not detect fourth PAAs in the *Mesp1Cre* mutants (Fig. 4B) and in *Mef2cCre* mutants, the fourth PAAs were also affected (Fig. 4C), unilaterally (7/17: absence of the...
In order to examine whether the absence of ink in the fourth PAAs of Mesp1Cre embryos was due to a lack of the artery or to a lack of the fourth BA itself, we performed immunohistochemistry using antibodies to Pecam1 that marks endothelial cells and to the SHF marker Isl1. As shown in Figure 4E, there is a hypoplastic fourth BA that does not contain an artery in the Mesp1Cre mutant embryos, seen as Pecam1 labelling of endothelial cells in the control (Fig. 4D). Although we cannot conclude that neural crest cells are not affected in the absence of Prdm1 in the mesoderm, neural crest colonization has taken place, as shown by the presence of AP2α (Fig. 4F and G) and Sox10 transcripts (Fig. 4H and I).

Taken together, these results indicate that Prdm1 is directly required in the SHF for the correct development of the fourth and sixth PAAs.

### Expression of a dominant-negative form of Prdm1 in the anterior SHF and its derivatives affects the development of the heart

In order to interfere with Prdm1 repressive function in vivo, we constructed a conditional transgenic line Tg(pCAG CAT 1b8) expressing a truncated form of Prdm1 (Prdm1β) and GFP, after Cre recombination (45). This truncated form, which lacks a PR/SET domain, displays dramatically reduced repressive activity, but is still able to bind to its DNA targets (46) and therefore interferes with the function of the endogenous full-length Prdm1 (47). By RACE PCR and RT-PCR, we could not detect any such endogenous truncated transcript between E9.5 and E10.5 in control embryos (unpublished data). Mesp1Cre;Tg(CAT 1b8/−) embryos were severely growth retarded and we therefore used the Tg(Mef2cCre+/−) line where Cre activity is restricted to the SHF, to induce the expression of Prdm1β (Mef2cCre1b8). Mef2cCre1b8 transgenic mice died before birth (4 litters analyzed, n = 45). Analysis of the PAAs by ink injection did not reveal defects at E10.75 (Supplementary Material, Fig. S3), but analysis of the heart at the same stage showed hypoplasia of both the outflow tract and the right ventricle (Fig. 5A and B). Hypoplasia of the right ventricle was also evident at E16.5 (Fig. 5C and D compared with 3C), at which stage transgenic hearts lacked septation of the outflow tract, showing PTA (Fig. 5E) and had an abnormal aortic arch (Fig. 5F). This may have resulted from hypoplasia of the aortic sac, although this was not detectable at E10.5 (data not shown). These results show that interfering with Prdm1 repressive function affects the development of SHF derivatives. Aspects of the arterial pole phenotype resemble that of the conditional mutant, although a major decrease in the size of the right ventricle and PTA, seen with the dominant negative transgene, were not observed. Since expression of the transgene is maintained in the heart, Prdm1β may also affect target genes in the right ventricle which derive from cells that activate the Mef2c-Cre transgene in the SHF, although overexpression of the full-length Prdm1 did not have an effect (data not shown). The dominant-negative form of Prdm1 may also interfere with DNA binding of other members of the Prdm family. A recent study demonstrates that Prdm3 (Mds1 and Evi1 complex locus (Mecom)) is expressed very early in the endocardium, which is also derived from the SHF, and that hypomorphic mutants display severe congenital heart defects such as VSD, PTA as...
well as IAA-B (48). Interference with Prdm3 could explain the PTA not seen in the Prdm1 conditional mutant.

**Prdm1** is required for the maintenance of the pool of SHF progenitors

All the observed cardiac phenotypes in the Prdm1 conditional mutants can be explained by a depletion of SHF progenitors that contribute to the arterial pole of the heart. This depletion could be caused by premature differentiation, increased cell death or reduced proliferation.

Prdm1, as a transcriptional repressor, is a good candidate for inhibition of premature differentiation in the SHF. However, we could not detect any expansion of the a-cardiac actin expression domain between E7.75 and E9.5 (Supplementary Material, Fig. S5A–H), ruling out premature differentiation of SHF progenitors. We also analyzed Bmp4 expression, since it is required for the recruitment of progenitors to the differentiating heart tube.

**Table 2.** Asymmetric defects in the development of the aortic arch in foetal (E16.5–E18.5) Me2cCre and Mesp1Cre conditional mutants, and in control and mutant crosses on a Tbx1+/− heterozygote genetic background

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of embryos</th>
<th>DUCTUS ARTERIOSUS</th>
<th>AORTIC ARCH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Me2cCre</td>
<td>9</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Mesp1Cre</td>
<td>13</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Tbx1+/−</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Me2cCre;Tbx1+/−</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Mesp1Cre;Tbx1+/−</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
but could not see any differences (data not shown), further supporting the idea that Prdm1 is not involved in the direct inhibition of the cardiac differentiation program.

In order to analyze cell death, we performed TUNEL staining on serial sections of control and Mesp1Cre mutant embryos at E9.5. There is no difference in the percentage of apoptotic cells between control and mutant embryos in the SHF (Supplementary Material, Fig. S5I), demonstrating that the loss of Prdm1 does not result in increased cell death. A similar result was obtained by staining embryos with Lysotracker Red (data not shown).

To examine an effect on proliferation, we performed phosphorylated-histone 3 (PHH3) and Isl1 co-staining on serial transverse sections of control and Mesp1Cre mutant embryos at E9.5. As shown in Fig. 6A, the percentage of Isl1-positive cells that are PHH3-positive is significantly reduced in Mesp1Cre mutant embryos compared with the controls and there are less Isl1-positive cells (data not shown). This observation was confirmed by counting the number of β-galactosidase-positive cells per section expressing the Mlc1v-nlacZ-24 (1v24) transgene in 1v24/+ and Mesp1Cre;1v24/+ embryos at E9.5 (Fig. 6B). The 1v24 transgene marks the anterior SHF and its contribution to the anterior pole of the heart as a result of insertion into the Fgf10 locus (6). This diminution in the extent of the SHF begins to be detectable on whole mounts of X-gal-stained Mesp1Cre;1v24/+ embryos at E9.25 (Fig. 6C and D). By E10.25 in these embryos, β-galactosidase-positive cells in the mesodermal core of the fourth BAs are reduced (Fig. 6E and F).

### Table 3. Abnormal development of the pharyngeal arch arteries in Mef2cCre and Mesp1Cre conditional mutant Prdm1 embryos at E10.75

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of embryos</th>
<th>2nd PAA</th>
<th>3rd PAA</th>
<th>4th PAA</th>
<th>TOTAL PAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Unit</td>
<td>Absence</td>
<td>Normal</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Mef2cCre</td>
<td>17</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Mesp1Cre</td>
<td>20</td>
<td>17</td>
<td>2</td>
<td>1</td>
<td>17</td>
</tr>
</tbody>
</table>

*Normal left fourth PAA: the right fourth PAA is always affected.
The core mesoderm of the more anterior PAs do not seem to be affected to the same extent. We also observed this depletion of progenitors in Mef2cCre mutant embryos, although at a later stage (Fig. 6H compared with 6G). The shortening of the outflow tract observed in Mesp1Cre embryos compared with controls (Fig. 2A and B) is in keeping with a reduced contribution of the SHF. Reduction in Wnt11 expression, which marks the outflow tract myocardium, is even more striking in the Prdm1BEH/BEH null mutant, where Fgf8 expression throughout the BAs region is reduced (Supplementary Material, Fig. S6A–D), indicative of the effect of Prdm1 in the ectoderm and endoderm which are not affected when only the SHF is targeted (Supplementary Material, Fig. S6E) in Mesp1Cre mutant embryos. The reduction of cells specifically in the SHF and their contribution to the outflow tract are evident in Prdm1BEH/BEH:1v24/+ mutant embryos (Supplementary Material, Fig. S6F–I).
Together, these results demonstrate that Prdm1 is required to maintain the pool of progenitors that contribute to the development of the arterial pole of the heart. Quantification of proliferation in conditional Mesp1Cre mutant embryos demonstrates the importance of its repressive activity in the SHF in this context.

Prdm1 and Tbx1 interact genetically in the SHF during the morphogenesis of the arterial pole of the heart

The different Prdm1 mutant phenotypes at the arterial pole of the heart are reminiscent of those already described when FGF signalling (9,10,12–17) is abrogated, in keeping with up-regulation of inhibitors of tyrosine kinase receptor signalling. Expression of Fgf8 is not strikingly affected in the Mesp1Cre mutant embryos in which ectodermal and endodermal Prdm1 is maintained (Supplementary Material, Fig. S6E). To pursue this further, we looked at possible genetic relationships with components of the FGF pathway that are implicated in the SHF. Expression of Prdm1 in the SHF in conditional Fgf8 mutant embryos or in mutant embryos overexpressing the FGF signalling repressor Sprouty2 in heart progenitors (50) was not affected (Supplementary Material, Fig. S7A–H).

Tbx1 mutants also have cardiac phenotypes that overlap with those of Mesp1Cre+/−;Prdm1CA/CA mutant embryos, and we therefore investigated possible genetic interactions between Prdm1 and Tbx1 which is also transcribed in the anterior SHF. Expression of Tbx1 is not notably affected in Mesp1Cre mutant embryos (Supplementary Material, Fig. S7A–D) and this is the case also for Raldh2, involved in a regulatory loop with Tbx1 (51–54) (Supplementary Material, Fig. S8E and F). We cannot rule out, however, that Tbx1 expression is affected, since the reduction in the number of cells in the SHF in the Mesp1Cre mutant may obscure a potential increase in Tbx1 transcripts in SHF cells when visualized non-quantitatively by in situ hybridization (Supplementary Material, Fig. S8A–D). Conversely, we analysed Prdm1 expression in Tbx1 null embryos. Although Prdm1 expression is affected at E10.5 when development of the caudal BAs region is defective (data not shown), initial expression of Prdm1 is normal in the absence of Tbx1 (Supplementary Material, Fig. S8G and H). These results suggest that there is no direct interaction between Tbx1 or FGF pathway genes and Prdm1 during cardiogenesis.

In order to assess whether an indirect interaction could exist, we analysed the phenotype of Tg(Mef2cCre/+);Prdm1CA/CA and Mesp1Cre+;Prdm1CA/CA mutant embryos in a Tbx1+/− heterozygote background. Analysis of the development of the PAAs by ink injection reveals a clear increase in the severity of the Prdm1 conditional mutant and Tbx1+/− heterozygote phenotypes (Supplementary Material, Fig. S3). Analysis of E17.5 hearts also showed an increase in the severity of the phenotypes both at the level of the aortic arch and of the alignment of the pulmonary trunk and aorta (summarized in Fig. 7F and G). Surprisingly, we also observed PTA in 3/5 Mesp1Cre+; Prdm1CA/CA,Tbx1+/− embryos (Fig. 7C and E). PTA was also observed with the Mef2cCre transgene, ruling out a possible genetic interaction between Tbx1 and Mesp1. In contrast, PTA was never observed in Tbx1+/− heterozygotes (55,56) or Mesp1Cre+;Prdm1CA/CA mutant and Mef2cCre;Prdm1CA/CA mutant embryos (Table 3).

These results demonstrate a genetic interaction between Tbx1 and Prdm1 in the SHF during the morphogenesis of the arterial pole of the heart. The lack of outflow tract septation leading to PTA is the result of a defect in CNC. Tbx1 function is required for the guidance of migrating CNC cells, in the endoderm (11), ectoderm (57) and mesoderm (20). Analysis of neural crest migration did not reveal any obvious disruption, even in the context of the Mesp1Cre−/+;Prdm1CA/CA,Tbx1+/− embryos (Supplementary Material, Fig. S9). We conclude that Prdm1, expressed in the mesodermal progenitors of the SHF that form the outflow tract, via a probable indirect interaction with Tbx1, can contribute to CNC cell function during outflow tract septation, which when compromised results in PTA.

DISCUSSION

In this study, we show that normal cardiogenesis requires the expression of the Prdm1 transcriptional repressor in the SHF. In its absence, SHF progenitors fail to proliferate normally, leading to defects of the distal structures of the arterial pole of the heart. We also show that these defects are enhanced in the absence of one functional copy of Tbx1, of which the human ortholog, TBX1, is involved in DiGeorge syndrome.

Prdm1 is expressed in the SHF from the early cardiac crescent stage (E7.5) to later stages when the heart tube has formed and SHF mesoderm in the posterior BAs is contributing to the formation of the PAAs. It overlaps with Islet1, which is expressed more broadly in posterior and lateral domains, whereas Prdm1 is mainly present in the anterior SHF, at high levels adjacent to the outflow region of the heart, where SHF cells are recruited to form myocardium. Many of the transcription factors required for myocardial differentiation are already present in the SHF, where repression of premature differentiation is an issue. Prdm1, as a transcriptional repressor, is a candidate in this context; however, no premature expression of α-cardiac actin, a myocardial marker, was observed when Prdm1 was mutated in the SHF. Proliferation is reduced however, and this would explain the cardiac phenotypes seen in the mutant. This negative effect on proliferation is in contrast to Prdm1 loss of function in the skin, in progenitors of the sebaceous gland (58) and in the dermal papillae of the vibrissae (22), where proliferation is increased. This is also seen in B lymphocytes (59), consistent with the description of Prdm1 as a tumour suppressor in B cell lymphomas (60,61). However, this function is clearly context dependent since Prdm1 is also expressed in differentiated skeletal muscle cells (23,62) or epidermis (63), where proliferation is not affected in the mutant. In the Sonic hedgehog-positive cells of the forelimb and in ectodermal or endodermal cells of the BAs region, Prdm1 is required for expansion of the progenitor cell pool (22), similar to its role in the SHF, described here. These different tissue-specific effects of Prdm1 underline the importance of the cellular context for its function, probably reflecting the recruitment of cell-specific co-regulators.

Using classical qRT-PCR experiments on the microdissected BAs region, we were not able to identify the molecular nature of the pro-proliferative function of Prdm1 in the SHF, which is probably obscured by the maintenance of Prdm1 function in endoderm and ectoderm (data not shown). We can speculate...
that it operates via repression of inhibitors of WNT and FGF signalling pathways, known to be important for the proliferation of cardiac progenitors (2). FGF signalling, in particular, acts on the anterior part of the SHF where Prdm1 is expressed and the phenotypes of Mesp1Cre/+;Fgf8CA/CA conditional mutants (15) as well as of conditional Tg(SproutyGOF/+);Prdm1CA/CA mutants (50).

In Prdm1 conditional mutants, with the Mesp1Cre allele that targets cardiac progenitors in the primitive streak and thereafter in the SHF, the formation of the early outflow tract is abnormal and this results in later arterial pole defects, such as transposition of the great arteries and partial loss of the PAAs derived from the fourth and sixth BAs. In contrast to Tbx1 mutants, there appears to be no reduction of the myocardial domain normally found at the base of the pulmonary trunk. The right ventricle appears normal, indicating that the absence of Prdm1 function in the SHF mainly affects later contributions of cardiac progenitors (2). Overexpression of the dominant negative version of Prdm1 (Prdm1b), activated by a Mef2c-Cre transgene, results in a reduced outflow tract in keeping with a repressive function of Prdm1 in these progenitors. Unexpectedly, it also leads to a hypoplastic right ventricle that could be explained by the maintenance of expression of the truncated version of Prdm1 in the myocardium where it may interfere with later development of the right ventricle, possibly through effects on the binding of other transcription factors (64,65).

In Prdm1 conditional mutants, with the Mesp1Cre allele that targets cardiac progenitors in the primitive streak and thereafter in the SHF, the formation of the early outflow tract is abnormal and this results in later arterial pole defects, such as transposition of the great arteries and partial loss of the PAAs derived from the fourth and sixth BAs. In contrast to Tbx1 mutants, there appears to be no reduction of the myocardial domain normally found at the base of the pulmonary trunk. The right ventricle appears normal, indicating that the absence of Prdm1 function in the SHF mainly affects later contributions of cardiac progenitors (2). Overexpression of the dominant negative version of Prdm1 (Prdm1b), activated by a Mef2c-Cre transgene, results in a reduced outflow tract in keeping with a repressive function of Prdm1 in these progenitors. Unexpectedly, it also leads to a hypoplastic right ventricle that could be explained by the maintenance of expression of the truncated version of Prdm1 in the myocardium where it may interfere with later development of the right ventricle, possibly through effects on the binding of other transcription factors (64,65).

Prdm1 is expressed in the SHF but also in the ectoderm and endoderm in the region where BAs are forming. However, it is not present in neural crest cells that transit through the anterior BAs before colonizing the outflow tract of the heart. As shown here, conditional deletion of Prdm1 with a Cre targeted to neural crest has no effect on cardiac or arch artery development.
However, in *Prdm1* null mutants, apoptosis of neural crest cells occurs, possibly due to effects on FGF signalling from endoderm and ectoderm, known to be required for neural crest cell survival (9,10), potentially via up-regulation of inhibitors. Mesodermal deletion of *Prdm1* has no effect on neural crest colonization of the BAs. PTA, which results from a neural crest defect, is not observed when *Prdm1* is specifically deleted in the SHF. Surprisingly when the dominant-negative form of *Prdm1* is over-expressed in the SHF, PTA is observed. This may be due to more severe disruption of cardiac progenitors which can indirectly affect neural crest (50) or, as already mentioned, to on-going effects of the transgene in the endocardium or the myocardium which may perturb outflow tract septation. A cell survival effect of *Prdm1* on neural crest cells is not observed in the SHF where there is no increase in apoptosis in the absence of *Prdm1*.

Tbx1 is expressed in the SHF, as well as in the endoderm and ectoderm. Ectodermal deletion of *Tbx1* leads to a reduction of neural crest numbers, associated with the down-regulation of Fgf8 which is a Tbx1 target, with consequent PTA (55,56,57). In *Tbx1* heterozygotes the fourth PAA are affected; however PTA is not observed. We show that when *Tbx1* heterozygotes are crossed with *Mesp1Cre*/*Prdm1* mice, the *Prdm1* mutant phenotype is more severe and, unexpectedly, PTA is observed. This would suggest that in the absence of mesodermal expression of *Prdm1* and with only one functional allele of *Tbx1*, there is an indirect effect on neural crest numbers, potentially mediated by a cooperative effect with Tbx1 on FGF signalling. Haploinsufficiency of *Tbx1* is well established and there are indications that this may be the case for *Prdm1* also, for example in primordial germ cells (23) and indeed hypoplasia of the fourth PAA is observed on a *Prdm1* heterozygous background (data not shown). Furthermore, in *Prdm1* mutants with one null allele and one conditional allele, additional defects appear and phenotypes are more severe, notably now also for the heart in the presence of *Tg(Mef2cCre)* (Supplementary Material, Fig. S10), indicating haploinsufficiency for *Prdm1* in ectoderm and endoderm. In the absence of mesodermal *Prdm1* and with half the dose of *Tbx1*, we can speculate that reduced activation of Fgf8 and Fgf10, which is also a Tbx1 target (67), augments the negative effect of Prdm1 inhibition to produce a more dramatic arterial pole phenotype and now also leads to apoptosis of neural crest in the BAs. *Prdm1*, as well as Tbx1, may affect FGF signalling, potentially via repression of FGF inhibitors, in keeping with the similarity between the phenotype of the conditional *Prdm1* SHF mutant and that seen when the FGF inhibitor, Sprouty2, is overexpressed in the SHF (50). The critical requirement for a threshold level of FGF signalling was demonstrated by *Fgf8/Fgf10* compound mutant analysis for the SHF (17) and by *Spry1/Spry2* compound mutant analysis in a *Tbx1* heterozygous background (68). Our data support a limited additive effect of *Tbx1* haploinsufficiency on the proliferation of SHF progenitors in the context of the mesodermal deletion of *Prdm1*. There is an increased severity in the defects observed between *Mef2cCre* and *Mef2cCre;Tbx1* homozygous embryos (Fig. 7F and G, compare columns 3 and 5) with more rotation defects of the OFT and, to a lesser extent, between the *Mesp1Cre* and the *Mesp1Cre;Tbx1* homozygous mutant foetuses (Fig. 7F and G, compare columns 4 and 6). However, in all the embryos with a mesodermal deletion of *Prdm1*, even in a *Tbx1* heterozygous background, no defects in the most proximal derivative of the SHF, i.e. the right ventricle, were observed. Altogether, these data suggest that when *Prdm1* is deleted in the whole SHF population (*Mesp1Cre*), the strongest effect on the pool of SHF progenitors is observed and that removing one copy of *Tbx1* does not seem to have an additive effect, if one excludes PTA as a secondary consequence. However, when *Prdm1* is deleted later, only in a subset of the SHF cells (*Mef2cCre*), a clear additive effect is observed on the progenitors that will give rise to the arterial pole of the heart. Interestingly, this is probably not only related to a cell autonomous effect within the mesoderm since similar observations were made on *Tg(Mef2cCre/+);Prdm1* mutants, in which one copy of *Prdm1* is null. Indeed, the phenotypes observed in *Tg(Mef2cCre/+);Prdm1* mutants resemble those of *Mesp1Cre* mutants (see Supplementary Material, Fig. S10).

Mutations in the human PRDM1 gene have, so far, only been reported in different myeloid cancers, mainly in diffuse large B cell lymphomas (60,69), but also in primary central nervous system lymphomas (70), anaplastic large T cell lymphomas (71) or NK cell neoplasms (61,72). Most of these mutations lead to the absence of PRDM1 protein. Interestingly, the severity of some of these lymphomas is also associated with an increased expression of the PRDM1β truncated form (73,74). PRDM1 mutations are also associated with increased risk of rheumatoid arthritis (75), systemic lupus erythematosus (76) or Crohn’s disease (77). Due to the crucial function of Prdm1 in placenta formation in the mouse (78), PRDM1 homozygous null mutations in humans are probably embryonic lethal. However, *PRDM1* heterozygotes are probably viable, with only limited defects in the arterial tree and with reduced numbers of primary germ cells. The *Prdm1* null cardiovascular phenotype shows strong similarities with *FGF* or *Tbx1* mutations, both involved in the etiology of DiGeorge syndrome in humans. Whereas in mice, the full range of the DiGeorge syndrome phenotype is only observed on complete loss of Tbx1 function, in humans, DiGeorge syndrome is linked with haploinsufficiency of *Tbx1*. In the mouse, *Tbx1* and *Prdm1* single heterozygotes display only mild cardiovascular phenotypes and *Tbx1* and *Prdm1* double heterozygotes did not display a more severe phenotype (data not shown). Double *Prdm1*/*Tbx1* null mutant embryos display a more severe phenotype at the level of the BAs at E9.5 and E10.5 (hypoplasia of the first BA, data not shown), but the placental defect of *Prdm1* null mutant embryos precludes any further analysis of the cardiovascular phenotype. However our results, demonstrating more severe phenotypes for *Tbx1* heterozygotes on a *Prdm1* mesoderm specific mutant background, point to PRDM1 as a potential candidate gene in modulating the severity of DiGeorge syndrome. It would be of interest to screen severe DiGeorge syndrome patients for potential mutations that affect PRDM1 activity in the SHF or to look for PRDM1 haploinsufficiency which, as for *Tbx1*, may have more functional impact in humans.

**MATERIALS AND METHODS**

**Ethics statement**

All animal experimentation was carried out according to the animal welfare regulations and guidelines, as laid down by the...
French Ministry of Agriculture and practised by the Institut Pasteur animal facility.

Mouse lines and embryo collection

Pax3^{Cre+}, Tg(HtPaCre/+), Tg(Mef2creCre/+), Mesp1^{Cre+}, Foxg1^{Cre+}, Tg(Mlc-lv-nlacZ-24/+), Prdm1^{CA/+}, Prdm1^{BEH+}, Tbx1^{−/−}, Tg(A17-Mylf3-nLacZ-T55/+), Tg(y96-Mylf5-nLacZ-26/+), Fgfl^{CA}, Fgf8^{−/−}, and Tg(Sprü2GOF/+). Lines have already been described (6,23,40,55,79−81). The transgenic Tg(pCAG CAT 1b8/+) line has been recently described (45): briefly, this line expresses, after Cre recombination, the truncated Prdm1β protein that has severely reduced repressive function but is still able to bind to the DNA target sites. For simplicity, Mesp1^{Cre+}, Prdm1^{CA/CA} and Tg(Mef2CreCre/+)/Prdm1^{CA/CA} will be referred to as Mesp1Cre and Mef2cre embryos, respectively, and Tg(Mef2creCre/+);Tg(pCAG CAT 1b8/+ will be referred to Mef2creCre1b8. Stud males carrying the Cre allele and one or two copies of the Prdm1^{CA} alleles were mated with homozygous Prdm1^{CA/CA} females. The day of the vaginal plug was scored as E0.5.

Ink injection

E10.5 embryos were injected with Indian ink (Rotring) diluted in PBS−heparin (20 μg/ml) (6 drops in 3 ml PBS) through the OFT using a fine glass needle and post-fixed in 4% paraformaldehyde (PFA). E11.5 and older embryos were perfused with ink through the placenta and then post-fixed in 4% PFA. Embryos were then clarified in BABB solution (1 volume benzyl alcohol−2 volumes benzyl benzoate). In order to see the vessels, the heart and the head were dissected out. Pictures were taken using a Leica stereomicroscope.

Heart and aortic arch analyses

Fetuses were harvested between E16.5 and E18.5. The jugular veins were sectioned and the embryos were perfused through the placenta with PBS−heparin (20 μg/ml) and then with 4% PFA. Fetuses were stored in 4% PFA until dissection. Pictures were taken at each step using a Leica stereomicroscope.

Whole-mount in situ hybridization, X-gal staining

Whole-mount in situ hybridization was performed according to the standard protocols (82). Prdm1, Fgf8, Wnt11, α-cardiac actin (Actc1) and Sox10 probes have been previously described (17,23,57,83). X-gal staining was performed as previously described (84).

Embryo cryosections

Embryos were dissected at E8.5 or E10.75, and fixed for 1 or 2 h in 4% PFA at 4°C. After three rinses, embryos were transferred into 15% sucrose/PBS at 4°C and embedded in 7% gelatin, 15% sucrose for two times 30 min at 37°C. Embryos were sectioned on a Leica cryostat to give 20 μm thick sections.

LysotrackerRed and TUNEL staining

LysotrackerRed staining was performed as follows: E9.5 dissected embryos were incubated for 30 min at 37°C in 5 μM LysotrackerRed (Molecular Probes). Embryos were then rinsed in PBS and post-fixed in 4% PFA. TUNEL analysis was performed on cryosections using the In Situ Cell Death Detection Kit, Fluorescein (Roche, 11 684 795 001) according to the manufacturer’s instructions.

Immunolocalization on sections

Sections were rehydrated in PBS at room temperature (RT) and permeabilized for 20 min at RT in PBS, 0.05% Triton X-100. Sections were blocked for 20 min at RT in blocking buffer (3% BSA, 1% goat serum, 0.1% Tween 20). The following primary antibodies were used: mouse monoclonal antibodies—anti all striated Myosin (MyHC) (DSHB, MF20), anti Isl1 (DSHB, 39.4D5), anti αS (DSHB, 3B5), anti Pecam1/CD31 (BD Pharmingen, 558738), anti phosphorylated histone 3 (Millipore, 06-570) and anti GFP (Molecular Probes, A-6455), and rat monoclonal—anti Prdm1 (Santa Cruz, 6D3), diluted in the blocking solution overnight at 4°C. After three washes in PBS, sections were incubated with a mix of secondary antibodies diluted in blocking solution, for 2 h at RT. For secondary antibodies, conjugated Alexa 488/546 against the different species (Molecular Probes) were used. After three washes in PBS, sections were mounted in Fluormount (Southern Biotech, 0100-01). Analysis was performed on a Zeiss ApoTome system.

Statistical analysis

Statistical analysis and graphs were performed using Excel, Graphpad Prism and Anastats (http://www.anastats.fr). Data were acquired from serial sections of two embryos for each genotype at the same stage. Data were pooled and then analysed by a Fisher test followed by a Student’s t-test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGMENTS

The authors are grateful to Catherine Bodin and Sabrina Coqueran for technical assistance. We thank Kathryn Calame (Columbia University College of Physicians and Surgeons) for the Prdm1^{CA/CA} mouse line and Tom Reh (University of Washington) for the Foxg1^{CreCre};Prdm1^{CA/CA} embryos. S.D.V. is an INSERM investigator.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Institut Pasteur and the CNRS URA 2578 (National Centre for Scientific Research), with grants from the E.U. Integrated Project ‘Heart Repair’ (LHSM-CT2005-018630) and ‘CardioCell’ (LT2009-223372).
to M.B. S.M.-T. received a fellowship from the Naito foundation.

REFERENCES


embryonic midbrain and cerebellum require different levels of FGF signaling during development. Development, 135, 889–898.


