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PRDM1/BLIMP1 is widely distributed to the nascent fetal-placental interface in the mouse gastrula

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Abstract

Background—PRDM1 is a transcriptional repressor that contributes to primordial germ cell (PGC) development. During early gastrulation, epiblast-derived PRDM1 is thought to be restricted to a lineage-segregated germ line in the allantois. However, given recent findings that PGCs overlap an allantoic progenitor pool that contributes widely to the fetal-umbilical interface, posterior PRDM1 may also contribute to soma.

Results—Within the posterior mouse gastrula (Early Streak – 12-s stages, ~E6.75–9.0), PRDM1 localized to all tissues containing putative PGCs; however, PRDM1 was also found in all three primary germ layers, their derivatives, and two presumptive growth centers, the Allantoic Core Domain and Ventral Ectodermal Ridge. While PRDM1 and STELLA colocalized predominantly within the hindgut, where putative PGCs reside, other colocalizing cells were found in non-PGC sites. Additional PRDM1 and STELLA cells were found independent of each other throughout the posterior region, including the hindgut. The *Prdm1*-Cre-driven reporter supported PRDM1 localization in the majority of sites; however, some *Prdm1* descendants were found in sites independent of PRDM1 protein, including allantoic mesothelium and hindgut endoderm.

Conclusion—Posterior PRDM1 contributes more broadly to the developing fetal-maternal connection than previously recognized, and PRDM1 and STELLA, while overlapping in putative PGCs, also co-localize in several other tissues.

Keywords

Allantoic Core Domain; allantois; hindgut; primordial germ cells (PGCs)

INTRODUCTION

In vertebrates, PRDM1 is a transcriptional repressor that plays essential roles in progenitor cell populations (reviewed in John and Garrett-Sinha, 2009). In some cases, PRDM1 regulates the differentiation of progenitor cells (Turner et al., 1994; Nishikawa et al., 2010; Mould et al., 2012), while in others, PRDM1 is required for stemness (Horsley et al., 2006) and formation of the PGCs (Ohinata et al., 2005; Chuva de Sousa Lopes and Roelen, 2008). During mouse gastrulation, *Prdm1*-null embryos form a reduced population of cells

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exhibiting STELLA protein and alkaline phosphatase (AP) activity, claimed to be the presumptive PGCs, in the base of the allantois (Ohinata et al., 2005; Vincent et al., 2005). In these cells, PRDM1 prevents somatic differentiation by repressing the expression of *Hoxb1* and other somatic genes (Ohinata et al., 2005; Kurimoto et al., 2008; Magnusdottir et al., 2013). PRDM1 also induces expression of *Tcfap2c*, which encodes the AP2 γ protein (Magnusdottir et al., 2013). AP2 γ and PRDM14 promote the expression of *Stella* and thereby contribute, in conjunction with PRDM1, to the establishment and maintenance of the putative PGC population in the nascent posterior region (Kurimoto et al., 2008; Yamaji et al., 2008; Weber et al., 2010; Magnusdottir et al., 2013). However, AP and STELLA are found in a variety of pluripotent stem cells (Bernstine et al., 1973; Bowles et al., 2003; Brambrink et al., 2008), and there is no evidence to show that those presumptive PGCs within the base of the allantois exclusively contribute to the gonadal germ line (reviewed in Mikedis and Downs, 2014).

Thus, while *Prdm1*-expressing cells in the nascent posterior region of the gastrulating mouse embryo have primarily been characterized as PGCs, other observations support a role for PRDM1 in building the somatic tissues of the posterior region. For example, descendants of *Prdm1*-expressing cells contribute to the umbilical vasculature days after the PGCs have left the posterior region (Mould et al., 2012). In addition, STELLA, which was initially characterized as specific to PGCs (Ohinata et al., 2005; Kurimoto et al., 2008), localizes initially to the Allantoic Core Domain (ACD), which is the posterior most terminus of the primitive streak; its descendants build the fetal-umbilical connection (Downs et al., 2009; Mikedis and Downs, 2012). During development, STELLA-positive ACD descendant cells broadly colonize not only putative PGC sites, but the posterior region overall (Mikedis and Downs, 2012). Third, like STELLA, the *Prdm1* population spatiotemporally overlaps the ACD (Ohinata et al., 2005; Downs et al., 2009). Therefore, the posterior *Prdm1* population may not be specific to the so-called PGC lineage but may contribute to many cell types outside of it.

In this study, we examined PRDM1's contribution to the nascent posterior region. First, we systematically localized PRDM1 protein via immunohistochemistry in histological sections to the mouse conceptus from the onset of gastrulation through early hindgut formation (Early streak (ES) – 12-s stages; ~E6.5–9.0). Second, confocal microscopy was used at a subset of these stages to find out whether PRDM1 and STELLA colocalize to a unique population, which might support the presence of a restricted PGC lineage. Finally, we used the same genetic tracing system of Ohinata and colleagues (Ohinata et al., 2005), in which *Prdm1*-expressing cells and their descendants were marked by GFP whether or not they continued to express *Prdm1*, to determine the extent to which posterior *Prdm1*-expressing cells contributed to the embryonic-extraembryonic interface. In particular, we analyzed stages when the allantois has fused with the chorion to initiate placental labyrinth formation and the hindgut is undergoing rapid development (4–9-s; ~E8.25–8.75), neither process of which was assessed in previous studies (Ohinata et al., 2005; Mould et al., 2012).

RESULTS

Localization of PRDM1

Specificity of the PRDM1 antibody—The commercially available rat IgG_{2a} monoclonal antibody to PRDM1 used in these experiments was raised against a glutathione-S-transferase (GST)-fusion protein corresponding to amino acids 255–395 of mouse PRDM1. This antibody was previously used in many studies to detect PRDM1 in mouse tissues (Asimakopoulos and Varmus, 2009; Brzezinski et al., 2010; Muncan et al., 2011). Indeed, immunofluorescent signal from this antibody colocalized with presumptive PGCs that expressed the *Prdm1*-mVENUS reporter (Aramaki et al., 2013). Moreover, specificity of the antibody was previously verified within the villus tips of the intestines from mice in which *Prdm1* had been conditionally deleted (Muncan et al., 2011); further, this antibody specifically detected transfected PRDM1 that was ectopically expressed in HCT116 cells (Muncan et al., 2011). Therefore, strong published evidence supported use of this antibody for localization studies.

Nevertheless, we used the NCBI BLAST tool (Altschul et al., 1990) to identify mouse proteins that share sequence similarity to the synthetic peptide. Other than PRDM1 isoforms, no proteins exhibited similarity to the peptide sequence ($E < 0.01$). More importantly, PRDM1 was not detectable in *Prdm1*-null conceptuses (generous gift of Dr. A. Surani) during a timepoint (7–11-s; ~E8.5–9.0) when the protein was particularly abundant (Fig. 1A–C). Wildtype and mutant conceptuses were immunostained in the same experiment. *Prdm1*^{+/+} specimens (N=4) exhibited PRDM1-positive nuclei in previously reported posterior sites, while no nuclear signal was detected in *Prdm1*^{-/-} specimens (N=4). In addition, controls in which the immunohistochemical reaction (Fig. 1D₁, D₂) was carried out without primary antibody ('minus primary antibody'; Fig. 1E₁, E₂) or with rat IgG_{2a} control isotype (Fig. 1F₁, F₂) at similarly robust stages 5-s (~E8.25) and 8–9-s (~E8.5–8.75) (N=3 specimens for each stage) provided further evidence that anti-PRDM1 specifically identifies PRDM1-positive nuclei in the posterior region. Finally, Western blot analysis of total protein (vEHF-5-s; ~E7.75–8.25) further verified the specificity of anti-PRDM1. Anti-PRDM1 identified one strong and one weak band between the 75 and 100 kDa molecular weight (MW) markers (Fig. 1G, **lane 2**). These bands are consistent with the predicted MWs of mouse PRDM1, whose five known isoforms range in size from 88.2 – 95.8 kDa (Turner et al., 1994; Tunyaplin et al., 2000; Morgan et al., 2009; The UniProt Consortium, 2014). Two additional, relatively weak bands were detected between MW markers 35 and 50 kDa, and a third band was detected between 50 and 75 kDa; these may represent partially degraded PRDM1 protein. All bands were absent in the "minus primary antibody" control (Fig. 1G, **lane 4**) and in the rat IgG_{2a} isotype control (Fig. 1G, **lane 6**). Together, all of these data provide overwhelming evidence for the specificity of anti-PRDM1.

Early Streak (ES) –Late allantoic bud (LB) stages (~E6.5–7.5)—At ES-Late Streak (LS) stages (~E6.5–6.75), PRDM1-positive cells localized to the right side of the proximal epiblast (Fig. 2A) and to the posterior primitive streak that spans the embryonic-extraembryonic junction (data not shown). Subsequently, PRDM1 clustered within the extraembryonic component of the primitive streak (XPS, Downs et al., 2009; Fig. 2B), (No

(allantoic) Bud (OB) – LB stage; ~E7.0–7.5). With the exception of the PRDM1-positive cells in the adjacent posterior epiblast and posterior visceral endoderm, no other cells in this posterior region exhibited PRDM1. By the EB stage, PRDM1-positive cells persisted in the XPS (Fig. 2C) and interface posterior visceral endoderm (Fig. 2D), consistent with a previous report (Ohinata et al., 2005), and extended into the proximal dorsal epiblast, intra-embryonic primitive streak and posterior amniotic ectoderm (~E7.25; Fig. 2C). While amniotic localization was most frequent during neural plate/bud stages, PRDM1 was occasionally found here through 4–5-s (~E8.25) but was rarely detected thereafter. PRDM1 was also observed in the mesoderm of the allantoic bud (Fig. 2D), where expression was not previously reported at this or any other stage (Ohinata et al., 2005). Finally, at all stages examined, PRDM1 was detected in the nuclei of a subset of giant cells (Fig. 2E).

Early Headfold (EHF) to 3-s stages (~E7.75–8.25)—During Early Headfold (EHF) – 3-s stages (~E7.75–8.25), PRDM1 persisted in both proximal and distal components of the Allantoic Core Domain (ACD) (Mikedis and Downs, 2012), distal allantois, intraembryonic posterior primitive streak, interface posterior visceral endoderm, and dorsal epiblast (Fig. 2F, H, I). Distal allantoic PRDM1 was found in cells exhibiting large, round nuclei (Fig. 2G), in endothelializing cells (Fig. 2J), and in cells within which only part of the nucleus was positive (Fig. 2J). Intriguingly, PRDM1 clustered within the ACD and formed an axial file there, the latter of which was previously shown to extend from the ACD (Downs et al., 2009). PRDM1-positive cells were also dispersed throughout the width of the posterior primitive streak (Fig. 2I). Additional PRDM1-positive cells localized to the visceral endoderm of the nook, where the ventral allantois meets the visceral yolk sac, and which is the first site of hematopoiesis in this region (Rhee and Iannaccone, 2012), as well as to the vessel of confluence, which forms directly below the nook (Fig. 2H). The vessel of confluence will ultimately develop into the site where the independently formed vascular systems of the conceptus converge to create a confluence throughout the conceptus (Downs et al., 1998).

4–12-s stages (~E8.25–9.0)—By 4-s (~E8.25), PRDM1-positive cells persisted in the proximal and distal ACD, distal allantois, posterior embryonic primitive streak and its associated mesoderm and epiblast, (Fig. 1D₂). From 4–12-s (~E8.25 – 9.0), PRDM1 was found at varying intensities within the hindgut lip, which is the axial site of hindgut invagination (Fig. 1A, 1D, 2K, 3A–B). As the hindgut formed, PRDM1 localized to both dorsal and ventral components (Fig. 1D₁, 2K), but by 9–10-s, hindgut PRDM1 was restricted to the ventral half (Fig. 3A–B). All PRDM1-positive cells in the hindgut, both dorsal and ventral, were confined to round cells (Fig. 2K, 3A–B) rather than cuboidal cells, thought to signify definitive endoderm.

At 6–8-s (~E8.5), when the ACD is no longer detectable, the allantois completes elongation and fuses with the chorion. PRDM1-positive cells persisted in the allantois, with an occasional PRDM1-positive cell localized to the allantois' proximal ventral wall (Fig. 2K), which overlies the vessel of confluence (Downs et al., 1998; Daane et al., 2011), and which will eventually merge with splanchnopleure that, together with somatopleure, becomes the primary fetal body wall (Brewer and Williams, 2004). In addition, PRDM1 was found in the

vessel of confluence (Fig. 2L), posterior primitive streak, and associated mesoderm and epiblast (data not shown).

At 6–12-s (~E8.5–9.0), PRDM1 was occasionally detected in endothelial cells of the yolk sac blood islands (Fig. 2L). Slightly later, at 9–12-s (~E8.75–9.0) when the distal allantois spreads over the chorionic surface to form with it the placental labyrinth (reviewed in Inman and Downs, 2007), small clusters of PRDM1-positive cells persisted in the proximal allantoic midline (Fig. 3C₁). Distally, allantoic PRDM1 continued to be scattered within large, round nuclei and the nuclei of endothelializing cells while also appearing in only part of the nucleus in other cells (Fig. 3C₂).

Within the posterior body of the embryo, PRDM1 localized to some, but not all, cells of the left and right mesodermal and ectodermal components of the somatopleure (Fig. 3D) as well as to the endodermal component of the splanchnopleure (Fig. 3E). Additional PRDM1-positive cells localized to the posterior mesoderm (Fig. 3F) and occasionally to vessel endothelium in the dorsal aortae and omphalomesenteric artery of the posterior body. Endothelial localization was not unique to the posterior region, as occasional endothelial cells in the yolk sac and anterior embryo also exhibited PRDM1 (Fig. 2L and data not shown). Finally, in specimens at 10–12-s (~E8.75–9.0), a small number of PRDM1-positive cells localized to the Ventral Ectodermal Ridge (VER; Fig. 3F), a presumptive stem cell reservoir for the tailbud formed by the remnant primitive streak at ~E9.0 (Grüneberg, 1956; Goldman et al., 2000).

Together, these results demonstrate that PRDM1 localizes to tissues that span the posterior embryonic-extraembryonic junction, which includes two presumptive growth centers, the ACD and VER, as well as derivatives of all three primary germ layers.

Colocalization of posterior PRDM1 and STELLA

Given that *Prdm1*-expressing cells in the posterior region are thought ultimately to exhibit STELLA (Ohinata et al., 2005), and given the unexpectedly widespread localization patterns of STELLA (Mikedis and Downs, 2012) and PRDM1 (this study), we colocalized PRDM1 and STELLA during the following timeperiods: ~E7.75–8.0 (headfold stages), when the ACD forms and distributes its cells to a variety of posterior tissues (Downs et al., 2009; Mikedis and Downs, 2012); ~E8.25 (4–5-s), when the nascent hindgut invagination appears, and the PGCs are thought to colonize it; and ~E8.75–9.0 (9–12-s), when the Ventral Ectodermal Ridge (VER) is forming (Grüneberg, 1956; Goldman et al., 2000) and chorio-allantoic fusion is completed. Cell numbers were quantified at each stage (Fig. 4A, B). The specificity of anti-PRDM1 and anti-STELLA immunofluorescent signal was verified in “minus primary antibody” controls, in which either anti-PRDM1 (Fig. 4C) or anti-STELLA (Fig. 4D) was omitted from the immunostaining reaction at all three stages (N=2 for each stage interval; see Experimental Procedures). In both control experiments, the nuclei were negative (Fig. 4C, D).

PRDM1/STELLA colocalizing cells—All STELLA sites described in this and the next section had been previously reported (Mikedis and Downs, 2012). However, STELLA had not been colocalized with PRDM1 protein. At all stages, the majority of PRDM1-positive

cells exhibited STELLA (Fig. 4A), and this colocalizing population increased from headfold to 9–12-s stages (Student's t-test, $p=0.035$; Fig. 4A). At all three timeperiods, colocalizing PRDM1 and STELLA appeared in the proximal ACD/allantois, posterior embryonic primitive streak, and posterior visceral endoderm/hindgut (Fig. 4E, F). In addition, colocalizing PRDM1 and STELLA were found in headfold-stage distal ACD (Mikedis and Downs, 2012), and at 9–12-s, in the VER of the tailbud (Fig. 4G), distal allantois (Fig. 4H), and posterior mesoderm (data not shown). The majority of hindgut cells exhibiting PRDM1 were STELLA-positive and vice versa (Fig. 4B).

Independent PRDM1 or STELLA populations—By contrast to the PRDM1/STELLA colocalizing population, which increased over time, the PRDM1-positive/STELLA-negative population was stable during the same timeperiod (Fig. 4A; Student's t-test, $p=0.623$). Furthermore, the STELLA-positive/PRDM1-negative cells, which represented the smallest of the three populations in this analysis, also increased over time (Fig. 4A; Student's t-test, $p=0.026$).

PRDM1-positive cells independent of STELLA were occasionally found in the posterior visceral endoderm and hindgut (Fig. 4F), the embryonic posterior primitive streak, ACD, and distal allantois at 4–5-s, as well as in the tailbud and allantois by 9–12-s (data not shown). While abundant PRDM1 was found in the allantois (Fig. 3C), somatopleure (Fig. 3D), and splanchnopleure (Fig. 3E) by conventional DAB staining, this was not evident in immunofluorescence. Therefore, despite greater amplification of the anti-PRDM1 signal (see Experimental Procedures), anti-PRDM1 immunofluorescent staining is less sensitive than DAB-based chromogenic staining.

At headfold stages, STELLA-positive cells independent of PRDM1 were primarily confined to the posterior visceral endoderm spanning the embryonic-extraembryonic interface. At 4–5-s and 9–12-s stages, they were found in the hindgut (Fig. 4E) and, at the latter stages, within the tailbud (data not shown).

Genetically traced posterior *Prdm1* cells contribute to multiple posterior tissues that do not always coincide with PRDM1

Based on these results, PRDM1, whether alone or colocalizing with STELLA, is found throughout the posterior embryonic-extraembryonic interface. These findings do not accord with previous conclusions that all *Prdm1*-genetically traced cells are limited to the base of the allantois and invariably colocalize with STELLA (Ohinata et al., 2005). To find out the extent to which previous investigators might have interpreted their data based on limited data sets (LB-3-s stages; ~E7.5–8.2; Ohinata et al., 2005), we used the same *Prdm1-Cre* reporter system to clarify the fate of *Prdm1*-expressing cells during an expanded period of time (OB-9-s; ~E7.0–E8.75).

Fertility, litter size, staging, and segregation ratios of the (ROSA26-GFP × *Prdm1-Cre*) reporter strain—Females homozygous for the reporter with a floxed early stop codon, *ROSA26-GFP*, were mated to inbred males hemizygous for the *Prdm1*-driven *Cre* transgene (*ROSA26-GFP* × *Prdm1-Cre*). While litters consisted of a very small number of living conceptuses (2.6 ± 0.2) and several resorptions (3.7 ± 0.3) per litter (Table 1), *Cre*-

positive and *Cre*-negative conceptuses occurred at the expected Mendelian ratio of 1:1 (Pearson's χ^2 test; $p=0.070$). Furthermore, the embryonic day stage of (*ROSA26-GFP* $\times$ *Prdm1-Cre*) conceptuses was neither positively correlated with the number of resorptions nor negatively correlated with the number of *Cre*-positive or -negative conceptuses (Pearson's product-moment correlation analyses; $p>0.05$ for all groups; Table 1), indicating that neither *Cre*-bearing nor *Cre*-negative conceptuses were being resorbed at E7.5–9.0. No gross morphological defects were observed in any specimens. Therefore, (*ROSA26-GFP* $\times$ *Prdm1-Cre*) specimens appeared to exhibit normal development.

The small litters from (*ROSA26-GFP* $\times$ *Prdm1-Cre*) matings appeared to be associated with the *Prdm1-Cre* transgene (Table 2), as the average litter size was statistically similar to that from inbred *Prdm1-Cre* breeding couples (2.8 ± 0.3 pups per litter, Table 2; Student's t-test, $p=0.618$). By contrast, the reporter line breeding couples produced statistically larger litters (6.3 ± 0.6 pups per litter, Table 2; Student's t-test, $p<0.001$). No statistical difference was observed between inbred and outbred *Prdm1-Cre* breeding pairs (2.8 vs. 3.9 pups per litter, respectively; ANOVA with post-hoc Student's t-test, $p=0.185$; Table 2). It is possible that other factors, such as chromosomal abnormalities, might also contribute to the small litter size associated with *Prdm1-Cre*. However, the similarity in size between E7.5–9.0 (*ROSA26-GFP* $\times$ *Prdm1-Cre*) litters and inbred *Prdm1-Cre* litters at birth further supports the conclusion that specimens do not experience embryonic lethality after E7.5. Therefore, despite the small litter size associated with *Prdm1-Cre*, (*ROSA26-GFP* $\times$ *Prdm1-Cre*) specimens collected after E7.5 appear to be useful as models of normal development.

To verify that mouse conceptuses from matings between (*ROSA26-GFP* $\times$ *Prdm1-Cre*) recapitulate the wildtype F2 PRDM1 localization patterns reported above, the latter were compared with *Cre*-negative specimens at both headfold (~E7.75–8.0) and 4–5-s stages (~E8.25; Fig. 5). No significant differences were found in the total number of posterior region PRDM1 cells in the *Cre*-negative and F2 control specimens ($p>0.05$ for each stage range; Table 3). With the exception of the headfold-stage intra-embryonic primitive streak, in which *Cre*-negative specimens contained more PRDM1-positive cells than those of the F2 (24.5 vs. 13.8 cells; $p=0.0005$; Fig. 5C; Table 3), no other differences were observed within any other posterior tissues at these stages ($p>0.05$ for all comparisons; Table 3). Thus, differences within the PRDM1 population specific only to the intra-embryonic primitive streak may be due to genetic background.

Next, because Cre recombinase activity in some transgenic mouse lines can cause DNA damage (reviewed in Schmidt-Supprian and Rajewsky, 2007) and apoptosis (Naiche and Papaioannou, 2007), which can obfuscate results, we compared the frequency of apoptosis in *Cre*-positive and *Cre*-negative control specimens at three developmental intervals: headfold (~E7.75–8.0), 4–5-s (~E8.25) and at 7–9-s (~E8.5–8.75). Compared with controls, *Cre*-positive specimens never exhibited greater numbers of CASPASE-3 (CASP3)-positive fragments in posterior tissues (Table 4). In addition, at 4–5-s stages, the number of PRDM1-positive cells in all tissues of the posterior region of *Cre*-positive specimens was similar to that in *Cre*-negative and F2 control specimens (ANOVA, $p>0.05$ for all comparisons; Table 3). Therefore, Cre recombinase activity does not result in the loss of PRDM1-positive cells in this genetic tracing system.

Collectively, *Prdm1*-expressing cells and their descendants, even if these descendants no longer express *Prdm1*, should therefore be identified in conceptuses derived from (*ROSA26-GFP* × *Prdm1-Cre*) matings, described below.

Prdm1-Cre genetic tracing—In (*ROSA26-GFP* × *Prdm1-Cre*) specimens, the GFP protein product of the transgene required immunohistochemical detection (Ohinata et al., 2005). In all experiments, specificity of the GFP antibody was verified in *Cre*-negative and *Sox17-GFP*^{+/-} control conceptuses (Kim et al., 2007), in which GFP was either absent or strong enough to detect without immunohistochemical amplification. As immunostaining was observed only in the vesicles of the extraembryonic visceral endoderm in *Cre*-negative control specimens (N=15; OB-9-s stages) (Fig. 6A and data not shown), it was disregarded in the *Cre*-positive experimental specimens.

The numbers and distribution of GFP-positive cells in the posterior region are reported in Fig. 5. Overall, GFP-positive cells were significantly lower than the number of PRDM1-positive cells at all stages examined (Fig. 5A, purple versus blue bars).

Not all specimens exhibited GFP in the nascent posterior region: 0/1 specimens at OB stage (~E7.0), 4/6 at EB stage (~E7.25), and 1/1 at LB stage (~E7.5). Thus, while PRDM1 cells were observed at the OB stage (Fig. 2B), GFP-positive cells were detectable by genetic tracing only as early as the EB stage (~E7.25). This is consistent with previous reports that the detection of a *Cre*-activated genetic reporter is delayed relative to the timing of *Cre* transcription (Nagy, 2000). In contrast, the visceral endoderm exhibited a more extreme delay in activation. Even though embryonic and extraembryonic visceral endoderm exhibited widespread *Prdm1* expression as early as E5.5 (Ohinata et al., 2005), by the neural plate stages, this expression was limited to only a few specimens (2/8 specimens, OB-LB stages; 3 cells per specimen; data not shown). Of these, none exhibited GFP in the allantois- and posterior embryonic visceral endoderm while 7.4 ± 2.6 PRDM1-positive cells were found there (Fig. 5B). Also at these stages, only the extraembryonic primitive streak and allantoic bud exhibited GFP-positive cells (Fig. 5B). Therefore, at early stages, this genetic tracing reporter marked far fewer cells than were PRDM1-positive.

By the headfold stage, all tissues that localized PRDM1 also localized GFP (Fig. 6B), though significantly fewer GFP cells were found (Fig. 5C). While an average of 37.3 ± 5.2 posterior GFP-positive cells was observed, similar to the numbers previously reported for late bud to 3-s stages (40.8 ± 5.3 cells; Ohinata et al., 2005), this number was significantly lower than the 81.3 ± 11.0 and 92.5 ± 10.0 PRDM1-positive nuclei observed in the F2 and *Cre*-negative controls (Fig. 5A), respectively. Between 4-s and 9-s stages, most PRDM1-positive tissues also localized GFP (Fig. 6C–E), though again, the latter in significantly fewer numbers (Fig. 5D–E). Moreover, somatopleure, splanchnopleure, embryonic endothelium, and trophoblast giant cells, which localized PRDM1, did not localize GFP. Trophoblast giant cells remained negative because they do not express the *Prdm1-Cre* transgene used in this study (Mould et al., 2012). The other populations are likely negative due to delayed reporter activation, observed with many *Cre* lines (Nagy, 2000), and/or incomplete activation of this specific genetic tracing reporter (Ohinata et al., 2005). Finally, two tissues displayed GFP where PRDM1 had never been seen: the distal mesothelial surface of the allantois (Fig. 6B₂)

and ventral cuboidal hindgut endoderm, which is thought to be definitive endoderm (Fig. 6D₁).

Because *ROSA26-GFP* is not a robust genetic tracing reporter (Ohinata et al., 2005), we further examined the contributions of the *Prdm1*-expressing lineage with another, more robust genetic tracing reporter, *ROSA26-mT/mG*, at a subset of stages (4-s and 11-s). Like the *ROSA26-GFP* reporter, the *ROSA26-mT/mG* reporter exhibited contribution to the allantoic mesothelium, (Fig. 6F), and to the cuboidal endoderm of the hindgut (Fig. 6G), both of which lacked PRDM1-positive cells. *ROSA26-mT/mG* contribution to the hindgut was particularly more abundant than that in the *ROSA26-GFP* reporter. The *ROSA26-mT/mG* reporter also revealed contribution to the splanchnopleure (11-s; Fig. 6G), which was marked by PRDM1 protein but not the *ROSA26-GFP* reporter.

These results suggest that the *Prdm1*-expressing population in the nascent posterior region contributes to putative PGCs and somatic cells, similar to STELLA (Mikedis and Downs, 2012). In *Prdm1*-null embryos, a STELLA-positive population forms, but it is diminished in size (Ohinata et al., 2005). At later stages, putative PGCs (identified via AP or SSEA-1) are not detected in the hindgut (Vincent et al., 2005). We confirmed these findings in the present study. At the EHF stage (~E7.75), *Prdm1*^{+/+} and *Prdm1*^{+/-} specimens exhibited STELLA in all previously reported sites (Mikedis and Downs, 2012), including the allantois, posterior primitive streak, posterior visceral endoderm, epiblast, and amnion (Fig. 7A). Similarly, in the single available *Prdm1*^{-/-} specimen, STELLA was detected in all of these tissues, but in diminished numbers (Fig. 7B, Table 5). No single population had been eliminated. By 8-s, however, STELLA was not detected in any tissue of the posterior region of the *Prdm1*^{-/-} specimen (Fig. 7D), while the *Prdm1*^{+/+} and *Prdm1*^{+/-} specimen exhibited STELLA in the hindgut, allantois, posterior embryonic mesoderm, and surface ectoderm (Fig. 7C), as previously reported (Mikedis and Downs, 2012). These results suggest that *Prdm1* is required in the nascent posterior region for the establishment and persistence of all subpopulations of STELLA and is not uniquely required for the putative PGCs.

PRDM1 colocalizes with *Runx1* in the posterior region

Given that PRDM1 protein and *Prdm1*-genetically traced cells were detected in endothelium of the allantois, posterior embryo, and yolk sac, we asked whether non-endothelialized PRDM1-positive cells near sites of vasculogenesis contributed to vessel endothelium. Therefore, we examined the relationship between the PRDM1 protein and a *Runx1-LacZ* reporter (North et al., 1999; North et al., 2002). RUNX1 is a transcription factor that is found in hemogenic endothelium (Chen et al., 2009) as well as in posterior tissues that are intimately associated with developing hemogenic vessels (Daane and Downs, 2011).

Colocalization of PRDM1 and *Runx1* (N=4; 8–12s stages) occurred at the nascent chorio-allantoic interface (Fig. 8A), where the vascular labyrinth is forming. In addition, some colocalizing cells were found in the splanchnopleure at the site of formation of the omphalomesenteric artery (Fig. 8B). Other colocalizing cells were, surprisingly, associated with the hindgut (Fig. 8C, D). More than half (~60%) of the hindgut's PRDM1-positive population, particularly that most closely associated with the omphalomesenteric artery, colocalized *Runx1* expression (79.0 ± 16.4 nuclei; Fig. 8D); by contrast, less than half

(~40%) of the hindgut's PRDM1 population did not exhibit *Runx1* (49.8 ± 11.8 nuclei; Fig. 8D).

The colocalization of PRDM1 protein and *Runx1* expression indicates that some PRDM1-positive cells in the chorio-allantoic interface may contribute to the vascular labyrinth, while cells in the hindgut and yolk sac may contribute to the omphalomesenteric artery.

DISCUSSION

Using three independent approaches, we have demonstrated that PRDM1 protein and the descendants of *Prdm1*-expressing cells are found in a multitude of somatic cell types within the nascent posterior region, all of which contribute to the fetal-placental interface of the mouse gastrula.

Posterior PRDM1 is not unique to putative PGCs

Prior to this study, *Prdm1* in the nascent posterior region was thought exclusively to identify putative PGCs and their precursors (Ohinata et al., 2005). However, based on results presented here, both PRDM1 protein and *Prdm1* genetically traced cells localized to tissues that are not thought to contain PGCs. In particular, these included the distal ACD and distal allantois, which contribute to the placenta, and not to the embryo (Mikedis and Downs, 2012). Thus, allantoic PRDM1 likely plays roles in placental development. In addition, PRDM1 protein and the *Prdm1* genetic tracing reporter localized to splanchnopleure and/or somatopleure, which build the primary body wall (Brewer and Williams, 2004), and are not thought to contain PGCs.

Many PRDM1-positive cells and *Prdm1*-genetically traced cells exhibited distinctly somatic cellular morphologies rather than the large round morphology associated with PGCs (Clark and Eddy, 1975). For example, PRDM1 protein and/or *Prdm1*-genetically traced cells were detected in squamous vessel endothelium (Fig. 2J, 6F), the squamous cells of allantoic mesothelium (Fig. 6B₂, F), definitive endoderm of the hindgut (both dorsal and ventral components; Fig. 6D₁, G), and splanchnopleure (Fig. 3E, 6G).

Expansive contribution to posterior somatic tissues differs from a previous report (Ohinata et al., 2005), and may be explained in several ways. As the previous study was limited to LB – 3-s (~E7.5–8.25) stages, the investigators likely missed the diversity in PRDM1 contributions to the posterior interface that becomes evident at later stages. In addition, previous *Prdm1* genetic lineage tracing depended on the *ROSA26-GFP* genetic tracer, which is not a robust reporter (Ohinata et al., 2005). Because we wished to standardize our conclusions vis-à-vis those of others, we used this reporter strain. However, future experiments should probably employ the robust *ROSA26-mT/mG* reporter, which demonstrated greater contribution to somatic lineages (Fig 6F–G).

The observation that PRDM1 cells in the nascent posterior region contribute to soma is consistent with previous single cell analyses. While these analyses were used to support a model in which PRDM1 establishes the germ line by repressing the expression of *Hoxb1* and other somatic mesodermal genes and halting somatic differentiation (Ohinata et al.,

2005; Kurimoto et al., 2008), the *Prdm1*-expressing population does not immediately repress *Hoxb1* (Kurimoto et al., 2008). Instead, at the early headfold stage, 61% of cells expressing *Prdm1* also exhibited *Hoxb1* (Kurimoto et al., 2008). Over time, the number of cells coexpressing *Prdm1* and *Hoxb1* decreased, and they made up only ~10% of the *Prdm1*-expressing population by E8.25 (Kurimoto et al., 2008). Because *Prdm1* was thought exclusively to give rise to PGCs, this heterogeneity was interpreted as an asynchronous suppression of *Hoxb1* in *Prdm1*-expressing cells (Kurimoto et al., 2008). However, given the results here, which indicate that the PRDM1 population contributes to soma, it is possible that the *Prdm1* cells exhibiting *Hoxb1* are capable of contributing to soma. In addition, while it was assumed that the loss of the *Prdm1*- and *Hoxb1*-expressing population was due to PRDM1's repression of *Hoxb1* expression (Ohinata et al., 2005; Kurimoto et al., 2008), it is possible that those cells were lost because they down-regulated *Prdm1* and differentiated into soma. Finally, previous analyses were limited to *Prdm1*-expressing cells within the base of the allantois and the hindgut and thus provided no information on the transcriptional characteristics of *Prdm1*-expressing cells outside of these tissues. Therefore, previous single cells analyses of the *Prdm1* population did not exclude the possibility that this population contributed to soma.

It remains obscure if and how PRDM1 functions within the somatic descendants of *Prdm1*-expressing cells in the nascent posterior region. Perhaps some *Prdm1*- and *Hoxb1*-expressing cells stochastically differentiate into soma until PRDM1 suppresses *Hoxb1* expression. Given that the allantois has myeloid potential (Zeigler et al., 2006) and that PRDM1 is a regulator of myeloid differentiation (Chang et al., 2000), PRDM1 may be regulating hematopoietic differentiation in the allantois. This seems particularly likely because PRDM1 localized to distal allantoic cells with large round nuclei, consistent with blood cells, and overlapped *Runx1* expression there (Fig. 8A). In addition, as PRDM1 represses developmental regulators in migrating PGCs (Yamashiro et al., 2016), PRDM1 may similarly repress these genes in somatic cells until additional signals downregulate PRDM1 and allow the somatic cells to respond to differentiation signals. The loss of STELLA-positive populations in all posterior tissues of the *Prdm1* embryo (Fig. 7D) indicates that PRDM1 is required for the persistence of STELLA in somatic cells and putative PGCs. However, the loss of STELLA protein is unlikely to impact development, as *Stella*-null embryos can develop normally (Payer et al., 2003; Bortvin et al., 2004). Regardless, any type of role for PRDM1 in posterior soma will be difficult to determine until the extent to which the PRDM1 population actually contributes to the germ line has been carefully characterized.

PRDM1 cells in the proximal allantois, posterior primitive streak, and overlying endoderm may give rise to putative PGCs, as the streak component of the allantois gives rise to STELLA-positive descendants in the hindgut, while the other two tissues, though not fate-mapped for STELLA contribution, exhibit STELLA (Mikedis and Downs, 2012). It is possible that a PRDM1 subpopulation exclusively contributes to the germ line while a second population contributes to soma. Alternatively, the PRDM1 cells may be a uniform population that contributes to soma and germ line, and only at a later developmental stage does the germ line segregate from soma.

Finally, an intensive review of the literature revealed no formal proof that presumptive allantoic PGCs exclusively contribute to the gonadal germ line (Mikedis and Downs, 2014). Instead, graft-based fate mapping experiments indicate that allantoic STELLA contributes to both soma and putative germ line (Mikedis and Downs, 2012). These data were based, in part, on STELLA immunostaining using a commercially available antibody that closely recapitulated previously reported *Stella* expression and STELLA localization (Saitou et al., 2002; Sato et al., 2002; Ohinata et al., 2005), but the specificity of this antibody, or of any commercially available antibody, has yet to be verified in *Stella*-null embryos. To confirm the results from grafting experiments, genetic tracing of *Stella* within the nascent posterior region using the tamoxifen-inducible *Stella-Cre* system is needed (Hirota et al., 2011). It would also be worthwhile to directly compare the *Stella-Cre* results to those from the *Prdm1-Cre* system, particularly in the hindgut, as results here indicate that PRDM1 does not identify a hindgut population of a single molecular identity. Specifically, PRDM1 colocalized with a subpopulation of STELLA cells and vice versa (Fig. 4A–B). In addition, approximately 50% of hindgut PRDM1 exhibited *Runx1* expression (Fig. 8D). While it is possible that PRDM1 with and without STELLA, as well as PRDM1 with and without *Runx1* expression, is the result of fluctuations of protein expression levels, whether these molecular differences reflect distinct potencies and future cellular contributions needs to be explored in further detail.

PRDM1 in sites of posterior progenitor pools

Both the ACD and VER are progenitor cell pools that distribute descendant cells along the posterior midline. In the case of the ACD, its descendants are found at the fetal-umbilical interface (Mikedis and Downs, 2012), while those of the VER populate the elongating tail (Goldman et al., 2000). A potential role for PRDM1 in the differentiation of the ACD's or VER's progenitor cells is consistent with PRDM1's role in regulating multipotent progenitors of several other tissues, such as the heart and forelimb (Robertson et al., 2007). However, PRDM1 is not a regulator of broader pluripotency, as it is not required for the establishment of naïve pluripotency in mouse embryonic stem cells (ESCs) or primed pluripotency in mouse epiblast stem cells (EpiSCs) (Bao et al., 2012).

PRDM1 cells contribute to the hindgut endoderm

Based on the robust contribution of *Prdm1*-genetically traced cells to the hindgut endoderm (Fig. 5E, 6D, 6G), one would predict that hindgut development would be disrupted in *Prdm1*-null embryos. However, no hindgut defects have been reported in *Prdm1*-null embryos before they died at E10.5 (Vincent et al., 2005). PRDM1-positive cells in the hindgut, particularly those that are STELLA-negative, may contribute to the subsequent development of the gut, in which PRDM1 protein persists from E9.0 through birth (Chang and Calame, 2002). The PRDM1-positive cells detected in the hindgut here exhibited a round morphology that is associated with many tissue-specific stem cells (Wickenhauser et al., 1995; Yu et al., 2006; Grzesiak et al., 2011). While the hindgut's round cells are thought to be the PGCs, they could be the progenitors to the stem cells in the mature gut epithelium, which is replaced every 4–5 days in the mouse (van der Flier and Clevers, 2009). Adult intestinal stem cells do not exhibit PRDM1, but they are derived from an embryonic population that expressed *Prdm1* (Harper et al., 2011; Muncan et al., 2011). Furthermore, in

neonates, *Prdm1* is required to delay precocious formation of the intestinal crypts, which contain both stem and Paneth cells, until the end of the suckling period (Muncan et al., 2011).

Alternatively, these round PRDM1-positive cells could contribute to the pluripotent neural crest cells of the enteric nervous system that complete their rostral-to-caudal colonization of the entire gut around E14.5 (reviewed in Butler Tjaden and Trainor, 2013). Enteric neural crest cells are known to originate from the vagal (somites 1–7) and sacral (caudal to somite 24) regions of the embryonic axis (reviewed in Butler Tjaden and Trainor, 2013), but a hindgut origin for some of these cells has not been explored. Intriguingly, the immature enterocytes of the neonatal intestines are PRDM1-positive and require PRDM1 for normal function, but the adult enterocytes that ultimately replace them do not (Harper et al., 2011; Muncan et al., 2011). Therefore, the PRDM1-positive cells detected in the hindgut here may contribute to later gut development and/or function.

Finally, some of these round PRDM1 cells may be hemogenic or vascular precursors, as they co-express *Runx1* (Fig. 8B–D), and the hemogenic vessel of confluence and omphalomesenteric artery develop in close association with the ventral hindgut (Daane and Downs, 2011). Consistent with this, PRDM1 regulates differentiation of hematopoietic lineages (Turner et al., 1994; Chang et al., 2000). Intriguingly, a subset of AP-positive PGCs in the hindgut exhibited endothelial- and hematopoietic-associated protein PECAM-1 at E9.0 (Wakayama et al., 2003). At later stages (E10.5 and E11.5), a subpopulation of *Oct3/4*-EGFP-positive germ line from the aorta-gonad-mesonephros (AGM) region was found to express hematopoiesis-associated genes (*Brachyury*, *Hoxb4*, *Scf/Tal-1*, and *Gata-2*) and proteins (CD-34, CD-31, and FLK-1) and possess hematopoietic colony forming activity *in vitro* (Scaldaferri et al., 2015). While endothelial cells were previously reported to form normally in *Prdm1*-null embryos, this was based on PECAM staining analyzed in whole mount specimens (Vincent et al., 2005), which cannot provide the resolution needed to assess the extent to which endothelial cells have formed cohesive vessels that properly connect to one another. In support of a role for PRDM1 in endothelial/vascular development, PRDM1 localizes to a subset of endothelial cells throughout the embryo and allantois (here; Vincent et al., 2005; Mould et al., 2012), and *Prdm1*-null mutants exhibit global hemorrhaging, with pooled blood reported in the heart and dorsal aorta and under surface ectoderm (Vincent et al., 2005). Intriguingly, hemorrhaging was not reported in embryos in which *Prdm1* was conditionally deleted in the epiblast via *Sox2-Cre* nor in endothelial cells via *Tie2-Cre* (Robertson et al., 2007). As both of these conditional knockouts survived longer than the *Prdm1*-null embryos, the early lethality in the null embryos was not attributed to endothelial defects (Robertson et al., 2007), but whether the mutant endothelium exhibited subtle defects was not examined. As such, a role for PRDM1 in vascular development, particularly with respect to the posterior vasculature, requires further investigation.

Some of the PRDM1-positive cells in the nascent dorsal hindgut exhibited the round morphology associated with putative PGCs in the ventral hindgut. STELLA and IFITM3 similarly localize to round cells in the nascent dorsal hindgut (Mikedis and Downs, 2012; Mikedis and Downs, 2013), and only after ~E8.75 (9–10-s stage) do these proteins become

confined to round cells of the ventral hindgut. This is surprising, given that PGCs have only been reported to localize exclusively to the ventral hindgut (Lawson and Hage, 1994). Given that these round dorsal hindgut cells exhibit multiple PGC-associated proteins (this study; Mikedis and Downs, 2012; Mikedis and Downs, 2013), they may be part of the putative PGC population. If this is the case, then the putative PGCs are found in both ventral and dorsal components of the nascent hindgut (~E8.25–8.75), which is short in length. Only later, as the hindgut elongates, do putative PGCs exclusively localize to ventral hindgut.

Epiblast- and trophoblast-derived PRDM1 may contribute to labyrinth development

In this study, we found PRDM1 in giant cells, in accord with previous results (Mould et al., 2012). Loss of *Prdm1* disrupted the specification of spiral artery-associated trophoblast giant cells (Mould et al., 2012), which invade the deciduum and remodel the maternal vasculature. In the absence of this trophoblast cell subtype, the formation of the maternal blood sinuses was disrupted and the spongiotrophoblast, from which the spiral artery-associated trophoblast giant cells originate, failed to expand (Mould et al., 2012). These defects secondarily disrupted the expansion of the labyrinth layer underlying the spongiotrophoblast (Mould et al., 2012). Therefore, PRDM1 in giant cells affects labyrinth expansion without directly contributing to the labyrinth.

Epiblast-derived allantoic tissue, where PRDM1 was found (Fig. 3C, 8A), also contributes to the labyrinth. The *Prdm1*-null labyrinth defect is consistent with the contribution of *Prdm1*-expressing cells to embryonic and umbilical endothelium (here; Vincent et al., 2005; Mould et al., 2012) and widespread hemorrhaging reported in *Prdm1*-null mutants (Vincent et al., 2005), which die by E10.5 (Robertson et al., 2007). In embryos in which *Prdm1* was conditionally deleted in epiblast, the labyrinth defect was sufficiently rescued to allow development through E18.5 (Robertson et al., 2007). However, *Prdm1* conditional mutants were not recovered in newborn litters, and the status of the labyrinth was not examined (Robertson et al., 2007). Therefore, it remains unclear whether a conceptus with *Prdm1*-expressing trophoblast and *Prdm1*-null epiblast develops a fully functional labyrinth sufficient to bring the embryo to term. As such, it is possible that the epiblast-derived *Prdm1*-expressing populations facilitate the expansion of the labyrinth, potentially through the contribution of endothelial cells.

PRDM1 exhibits a distinct subcellular localization in some allantoic cells

The observation that PRDM1 localized to only part of, and not throughout, the nucleus in some distal allantoic cells (Fig. 2J, 3C₂) might reflect the internal organization of the nucleus. Individual chromosomes occupy distinct regions of the nuclei during interphase and in non-cycling cells (reviewed in Schneider and Grosschedl, 2007). Because PRDM1 functions as a transcriptional repressor by interacting with a DNA consensus motif via its five Kruppel-type zinc finger motifs (Keller and Maniatis, 1992; Schmidt et al., 2008) and then recruiting chromatin-modifying corepressors to the target locus (Yu et al., 2000; Gyory et al., 2004; Ancelin et al., 2006; Su et al., 2009), the partial nuclear localization of PRDM1 likely reflects its localization to specific chromosomes within the nucleus. Furthermore, this is not the first example of differential nuclear localization of PRDM1; an alternatively spliced PRDM1 isoform that lacks zinc fingers 1–3 due to the absence of exon 7 localizes in

a speckled nuclear pattern when ectopically expressed in HEK 293T cells, in which full-length PRDM1 exhibits broad nuclear localization (Schmidt et al., 2008). This isoform is expressed in mouse B cells (Schmidt et al., 2008), but whether it localizes in this speckled nuclear pattern within these cells remains obscure. However, considering that B cells also express the full-length isoform (Schmidt et al., 2008), it may be difficult to detect a speckled nuclear pattern unless a cell exclusively expresses the alternatively spliced isoform. It is possible that the partial nuclear localization observed here might be the result of cells expressing both full length and alternatively spliced PRDM1.

Conclusions

While PRDM1 in the nascent posterior region was previously thought to be limited to a lineage-restricted germ line, results here overwhelmingly demonstrate vast PRDM1 localization to somatic cells within the nascent posterior region, each population of which is in a nascent state and thus, may require PRDM1 in subsequent differentiation. Along with lack of formal demonstration that presumptive allantoic PGCs exclusively contribute to germ cells in the gonads, the data presented here call into question the extent to which a segregated germ line has, in past studies, been distinguished from the soma within the nascent fetal-umbilical connection of the mouse gastrula.

EXPERIMENTAL PROCEDURES

Mouse strains, animal husbandry, dissection, and staging

All animals were treated in accordance with Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals (Public Law 99–158) as enforced by the University of Wisconsin-Madison. B6CBAF1/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Matings between these mice provided (B6CBAF1/J × B6CBAF1/J) specimens (henceforth referred to as F2 specimens) (Downs, 2006) for the PRDM1 immunohistochemical study and for immunofluorescent localization of PRDM1 and STELLA. Heterozygous *Prdm1^{tm1Masu}* mice were a generous gift from Dr. M. Azim Surani (Ohinata et al., 2005). They were bred with B6CBAF1/J and maintained via interbreeding. Matings of *Prdm1^{+/-}* and *Prdm1^{+/-}* animals provided mutant *Prdm1^{-/-}* and control *Prdm1^{+/+}* and *Prdm1^{+/-}* specimens for PRDM1 and STELLA immunohistochemistry experiments. For genetic tracing experiments, B6.129(Cg)-*Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J* (hereafter referred to as *ROSA26-mT/mG*; Muzumdar et al., 2007) were a gift from Dr. Joan S. Jorgensen. B6.129-*Gt(ROSA)26Sor^{tm2Sho}/J* (hereafter referred to as *ROSA26-GFP*; Mao et al., 2001) and B6.Cg-Tg(*Prdm1-cre*)1Masu/J (hereafter referred to as *Prdm1-Cre*; Ohinata et al., 2005) were obtained from The Jackson Laboratory (Bar Harbor, ME). Some congenic *Prdm1-Cre* animals were maintained via interbreeding while other *Prdm1-Cre* males were outbred to B6CBAF1/J females for one generation and then maintained via interbreeding. As no differences in fertility and Mendelian ratios were observed between congenic and outbred lines (Table 2), both genetic backgrounds were used in this study. However, to maintain consistency within genetic tracing experiments, only B6 congenic *Prdm1-Cre* hemizygous studs were mated to outbred *ROSA26-GFP* homozygous females to produce (*ROSA26-GFP* × *Prdm1-Cre*) specimens, and only outbred *Prdm1-Cre* hemizygous studs

were mated to B6 congenic *ROSA26-mT/mG* homozygous females to produce (*ROSA26-mT/mG* × *Prdm1-Cre*) specimens. B6.Cg-*Sox17^{tm1Sjm} Ptpcrb Thy1a/J* (hereafter referred to as *Sox17-GFP*; Kim et al., 2007) were obtained from The Jackson Laboratory (Bar Harbor, ME). Heterozygous animals were mated to B6CBAF1/J females to obtain GFP-positive control conceptuses for immunohistochemical experiments. For the colocalization analysis of PRDM1 protein and *Runx1* expression, heterozygous *Runx1^{tm2Spe}* (hereafter referred to as *Runx1-LacZ*; North et al., 1999; North et al., 2002) males were mated with non-reporter B6CBAF1/J females to provide *Runx1-LacZ*⁺ specimens (Zeigler et al., 2006).

Animals were maintained under a 12-hr light/dark cycle (lights out at 13:00 or 21:00), and selection of estrous females and mating were as previously summarized (Downs, 2006). Pregnant females were sacrificed by cervical dislocation, conceptuses were dissected from decidua, and Reichert's membrane and associated trophoblast were reflected. Conceptuses were fixed for 2 hours in 4% paraformaldehyde (PFA; made in phosphate-buffered saline, PBS, Sigma) at 4°C, rinsed in PBS, dehydrated in an increasing series of methanol/PBS, and stored indefinitely in absolute methanol at -20°C.

Nominal days postcoitum, abbreviated "E", for "embryonic day", were used to guide morphological staging, as follows (Downs and Davies, 1993): Early, Mid-, and Late Streak (ES, MS, LS; ~E6.5–6.75); Neural Plate/No, Early, and Late (allantoic) Bud (OB, EB, and LB; ~E7.0, 7.25, and 7.5); Early and Late Headfold (EHF, LHF; ~E7.75–8.0); 1–5-s (~E8.0–8.25); 6–8-s (~E8.5); 9–12-s (~E8.75–9.0).

Genotyping

For polymerase chain reaction (PCR) genotyping, genomic DNA was isolated from a small piece of yolk sac or anterior embryonic tissue as previously described (Inman and Downs, 2006). The PCR reaction contained 1× GoTaq buffer (Promega; M791 A; final concentration of 1.5 mM magnesium chloride), 1.0 μM of each primer, 0.2 mM dNTP, and 0.025 U/μL of GoTaq Polymerase (Promega; M3001). For the genotyping of (*ROSA26-GFP* × *Prdm1-Cre*) and (*ROSA26-mT/mG* × *Prdm1-Cre*) conceptuses, the "Generic Cre" standard polymerase chain reaction (PCR) protocol from the Jackson Laboratory was used. An additional 0.5 mM magnesium chloride was added to the reaction for a total concentration of 2.0 mM. Amplification was achieved via 94°C for 3 minutes; followed by 35 cycles of 94°C for 30 seconds, 51.7°C for 1 minute, and 72°C for 1 minute; and completed with a final elongation step at 72°C for 2 minutes. Primers (5'- GCG GTC TGG CAG TAA AAA CTA TC -3') and (5'- GTG AAA CAG CAT TGC TGT CAC TT -3') produced a ~100 bp *Cre* transgene product while primers (5'- CTA GGC CAC AGA ATT GAA AGA TCT -3') and (5'- GTA GGT GGA AAT TCT AGC ATC ATC C -3') served as an internal positive control, producing a 324 bp product. For the genotyping of (*Prdm1^{+/-}* × *Prdm1^{+/-}*) conceptuses, PCR amplification was achieved via 94°C for 1 minute; followed by 37 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds; and completed with a final elongation step at 72°C for 3 minutes. A common forward primer (5'- GCC CAG TGA CTC AAA GCA CT -3') and a reverse primer specific to the wildtype allele (5'- TAT GGT CTT CTC ATG TTG GGG -3') produced a 200 bp product. The same forward primer and a reverse primer specific to the null allele (5'- GGT GTC TGA AGA GCA AAG CTG -3')

produced a 450 bp product. ($F1 \times Sox17-GFP$) conceptuses were genotyped under fluorescence via GFP filter and scored for whether GFP was present ($Sox17-GFP^{+/-}$) or absent ($Sox17-GFP^{-/-}$). $Sox17-GFP$ animals were genotyped via PCR amplification: 94°C for 5 minutes; 38 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute; and 72°C for 2 minutes. A forward primer specific to the wildtype allele (5'- CGA ACA GTA TCT GCC CTT TGT G -3') and a common reverse primer (5'- AAT CTC GTG TAG CCC CTC AAC-3') produced a 320 bp product. A primer specific to the null allele (5'- CTG GAA GGT GCC ACT CCC ACT GT -3') and the common reverse primer produced a 440 bp product.

Antibodies

The following antibodies were used for Western blotting (WB), immunohistochemistry (IHC), and immunofluorescence (IF): AlexaFluor 647-conjugated donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.; 711-605-152; 1.5 mg/mL stock; used at 1/250 dilution for IF); anti-CASPASE 3 (anti-CASP3; rabbit polyclonal; BD Biosciences; 559565; 0.5 mg/mL stock; used at 1/100 for IHC); anti-GFP, unconjugated (goat polyclonal; Abcam; ab6673; 1.0 mg/mL stock; used at 1/150 for IHC and 1/25 for IF); anti-PRDM1 (anti-BLIMP1; rat monoclonal; Santa Cruz Biotechnologies; sc-47732; 0.2 mg/mL stock; used at 1/50 for IHC, 1/25 for IF, and 1/150 for WB); anti-STELLA (rabbit polyclonal; Santa Cruz Biotechnologies; sc-67249; 0.2 mg/mL stock; used at 1/25 for IF); biotinylated donkey anti-goat IgG secondary antibody (Santa Cruz Biotechnologies; sc-2042; 0.4 mg/mL stock; used at 1/500 for IHC); biotinylated donkey anti-rabbit IgG secondary antibody (Santa Cruz Biotechnologies; sc-2089; 0.4 mg/mL stock; used at 1/500 for IHC); biotinylated goat anti-rat IgG secondary antibody, pre-adsorbed against mouse and human IgG (Santa Cruz Biotechnologies; sc-2041; 0.4 mg/mL stock; used at 1/500 for IHC and for IF); DyLight 550-conjugated donkey anti-goat IgG secondary antibody (Abcam; ab96932; 0.5 mg/mL stock; used at 1/250 for IF); DyLight 550-conjugated streptavidin (Abcam; ab134348; 1 mg/mL stock; used at 1/500 for IF); DyLight 650-conjugated donkey anti-rabbit IgG secondary antibody, pre-adsorbed against mouse, rat, and human IgG (Abcam; ab98501; 0.5 mg/mL stock; used at 1/250 for IF); horseradish peroxidase-conjugated goat anti-rat IgG secondary antibody, pre-adsorbed against mouse and human IgG (Santa Cruz Biotechnologies; sc-2065; 0.4 mg/mL stock; used at 1/5000 for WB); normal rat IgG_{2a} (Santa Cruz Biotechnologies; sc-3883; 0.05 mg/mL stock; used at 2/25 for IHC and 2/75 for WB, thereby maintaining final concentrations equivalent to those used for anti-PRDM1).

X-Gal staining and immunohistochemistry (IHC)

Prior to immunostaining, *Runx1-LacZ* specimens were stained overnight with modified X-Gal solution (10mM K₄Fe(CN)₆ · 3H₂O, 2mM MgCl₂, and 1mg/mL X-Gal) as previously described (Daane and Downs, 2011).

PRDM1 and GFP were immunolocalized in whole mount specimens exactly as previously described (Downs, 2008), using the antibody dilutions from the previous section. Four PRDM1^{-/-} mutant and four PRDM1^{+/+} wildtype embryos (7–11-s) were immunostained in the same experiment. PRDM1 immunostaining (anti-PRDM1 with biotinylated goat anti-rat

secondary antibody) used F2 specimens at ES – 12-s stages, with 3 specimens/stage. A total of 4 *Runx1-LacZ*⁺ specimens were immunostained for PRDM1 at 8–12-s stages. Embryos from multiple stages were often stained within each experiment to allow for comparisons between stages. To increase antibody penetration for PRDM1 IHC, the majority of the yolk sac was removed in older specimens (7–12-s; ~E8.5–9.0).

The specificity of the PRDM1 antibody was previously verified in intestinal villi (Muncan et al., 2011). However, when incubated in the absence of the primary antibody, the biotinylated goat anti-rat secondary antibodies (sc-2041, Santa Cruz Biotechnology and BA9401, Vector Laboratories; 0.5 mg/ml stock; used at 1/100 for IHC) produced some extranuclear staining in both F2 specimens and *Prdm1*^{-/-} controls; tissues included the yolk sac, giant cells, hindgut endoderm, and splanchnopleure at the 5-s stage (~E8.25) and/or the 8–9-s stage (~E8.5–8.75) after 10-minute incubation in diaminobenzoate chromagen (DAB) (N=4 for each immunostaining condition at each stage). While extranuclear staining was not detected in “minus primary antibody” controls after 7.5-minutes’ exposure to DAB (Mikedis and Downs, 2009), we chose the longer exposure period, as this optimized the nuclear PRDM1 signal and led to unambiguous identification of positive cells. Extranuclear staining was also evident in isotype control experiments (N=4 at 5-s stage and N=4 at 8–9-s stage) in which the primary antibody was replaced with normal rat IgG_{2a}. Extranuclear staining was likely due to residual cross-reactivity with mouse IgGs that have been well-documented in these sites (Bernard et al., 1977; Rachman et al., 1984) despite pre-adsorption of the secondary antibody against mouse IgG.

Unconjugated anti-GFP with secondary antibody was used for this study, as preliminary experiments with a biotinylated anti-GFP (rabbit polyclonal; Abcam; ab6556; 0.5 mg/mL stock; used at 1/75 for IHC) did not robustly label GFP-positive cells in Cre-positive specimens from (*ROSA26-GFP* × *Prdm1-Cre*) litters compared to the unconjugated anti-GFP paired with the appropriate biotinylated secondary antibody, presumably due to weak GFP reporter signal (Ohinata et al., 2005). GFP immunostaining of (*ROSA26-GFP* × *Prdm1-Cre*) and (*ROSA26-mT/mG* × *Prdm1-Cre*) specimens also used unconjugated anti-GFP and biotinylated donkey anti-goat secondary antibody. As controls to verify anti-GFP specificity, Cre-negative specimens from (*ROSA26-GFP* × *Prdm1-Cre*) litters (N=15; OB-9-s stages) and (*ROSA26-mT/mG* × *Prdm1-Cre*) litters (N=3; EHF-10-s) were used. *Sox17-GFP*^{+/-} conceptuses, in which the GFP is fluorescently detectable without IHC, were used as positive controls for all anti-GFP IHC reactions.

For both antibodies, the antibody complex was visualized with diaminobenzoate chromagen (DAB; DAKO Corporation) at room temperature for 10 minutes, after which specimens were fixed in 4% paraformaldehyde (PFA) for 2 hours or overnight at 4°C. Then, after standard dehydration and clearing, specimens were embedded in wax for transverse or sagittal orientations; a complete series of serials sections of thickness 6 µm was obtained, dewaxed, counterstained with hematoxylin, cover-slipped, and analyzed.

Immunofluorescence (IF)

Immunofluorescent staining was carried out as previously described (Mikedis and Downs, 2013). F2 specimens at headfold (~E7.75–8.0), 4–5-s (~E8.25), and 9–12-s (~E8.75–9.0)

stages were immunofluorescently labeled for PRDM1 and STELLA and counterstained with DNA marker DAPI (Life Technologies; D1306; used at 1.8 ug/mL). Using anti-PRDM1 for IF staining presented two problems, which were addressed by making the following modifications to the original IF protocol (Mikedis and Downs, 2013). First, significant background signal, particularly in the yolk sac, was observed due to nonspecific staining, described above, from the goat anti-rat IgG secondary antibody used to recognize anti-PRDM1. To minimize this background, after standard blocking, specimens were incubated overnight at 4°C with mild agitation with horseradish peroxidase-conjugated goat anti-rat IgG secondary antibody at a concentration of 1/100 stock solution to PBS-ST. Specimens were subsequently washed and incubated in primary antibody as previously described (Mikedis and Downs, 2013). The second problem with anti-PRDM1 IF staining was weak fluorescent signal. To amplify the anti-PRDM1 IF signal, biotinylated goat anti-rat IgG was used in the overnight incubation for secondary antibodies. The following day, after 5 PBS-ST washes of 45 min, specimens were incubated with DyLight 550-conjugated streptavidin in PBS-ST for 3 hours at room temperature and subsequently washed 3 times for 30 min each. Specimens were then stained for DAPI as previously described (Mikedis and Downs, 2013). Throughout this protocol, specimens were costained for STELLA by including the corresponding antibodies in the primary and secondary antibodies incubations. For these experiments, two secondary antibodies were used to recognize anti-STELLA, AlexaFluor 647-conjugated donkey anti-rabbit IgG and DyLight 650-conjugated donkey anti-rabbit IgG secondary antibodies, between which no differences were observed in the signal among stained specimens. “Minus primary antibody” control experiments in which one primary, but not the other, were eliminated from the immunostaining reaction verified the specificity of anti-PRDM1 and anti-STELLA in this IF protocol (for each primary antibody, N=2 per stage interval; N=6 specimens total).

After staining, conceptuses were trimmed to isolate the posterior region using long glass scalpels (Tam and Beddington, 1992). Specimens were mounted on glass slides in Aquamount (Lerner Laboratories; 13800) and coverslipped with no. 1.5 high performance coverslips ($170 \pm 5 \mu\text{m}$; Carl Zeiss Microscopy; 474030-9000). Specimens were analyzed on a Nikon A1R confocal microscope (W.M. Keck Laboratory for Biological Imaging, University of Wisconsin-Madison) using a Plan Apo VC 60× water immersion objective (1.20 numerical aperture). Images were collected on NIS-Elements software (Nikon Instruments Inc.) using the 561 and 638 nm lasers (as well as the 408 nm lasers for DAPI-stained specimens) with a 1.2 AU pinhole size set for the 638 nm laser. Control specimens were imaged with the same settings as experimental specimens. STELLA, PRDM1, and DAPI signals were pseudocolored as red, cyan, and blue, respectively, using NIS-Elements software.

Western blotting (WB)

Total protein was extracted from 3 litters of freshly dissected F2 conceptuses, or approximately 24 conceptuses, (vEHF-5-s; ~E7.75–8.25) divested of trophoblast giant cells, parietal endoderm, and the ectoplacental cone, and flash frozen. Total protein was extracted as previously described (Mysliwiec et al., 2007). Briefly, the conceptuses were homogenized and then subjected to sonication in buffer (0.2% SDS; 11% glycerol; 11mM Tris, pH 8.0; 1.1

mM EDTA, pH 8.0; 1 mM DTT; and a protease solution consisting of 0.02 mM leupeptin, 1.5 μ M aprotinin, and 0.1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)). To detect PRDM1 protein, 20 μ g of total protein extract was subjected to SDS-PAGE under reducing conditions in a 10% polyacrylamide gel, and immunoblotting was performed as previously described (Mikedis and Downs, 2013) using anti-PRDM1 and horseradish peroxidase-conjugated goat anti-rat secondary antibody as the primary and secondary antibodies, respectively. Trail Mix Protein Markers (EMD Millipore; 70980-3; used based on manufacturer's protocol) were detected via chemiluminescence using horseradish peroxidase-conjugated S-protein (EMD Millipore; 69047-3; used at 1/5000) during the secondary antibody incubation. Additional preliminary blots verified that the S-protein did not recognize protein bands in embryonic cell lysate and that neither the anti-PRDM1 primary antibody nor the goat anti-rat secondary antibody recognized protein bands in the Trail Mix Protein Markers. Figure 1G was obtained via a 10 min film exposure. Antibody specificity was verified via elimination of the primary antibody and via a rat IgG_{2a} isotype control.

Cell counts and statistical methods

Cell counts in IHC specimens were obtained as previously described (Mikedis and Downs, 2012). The hindgut lip was defined in transverse orientation as the first section of closed ventral hindgut endoderm plus any subsequent sections of ventral hindgut in which the omphalomesenteric artery had not fully formed between the hindgut and overlying yolk sac endoderm; for those specimens analyzed here, the total number of hindgut lip sections ranged from 1 to 3 sections. So as to not inflate the extent to which (*ROSA26-GFP* $\times$ *Prdm1-Cre*) specimens recapitulated the PRDM1 localization pattern, specimens that did not exhibit positive cells in the posterior region and/or in specific posterior tissues were included as "0" in calculations of the average numbers of PRDM1-positive cells and GFP-positive cells.

Student's t-test (equivariance assumed), calculated in Excel 2008, was used to compare PRDM1- and STELLA-positive populations at headfold and 9–12-s stages; PRDM1-positive/STELLA-negative populations at headfold and 9–12-s stages; PRDM1-negative/STELLA-positive populations at headfold and 9–12-s stages; litter sizes between (*ROSA26-GFP* $\times$ *Prdm1-Cre*) and *Prdm1-Cre*² lines; litter sizes between (*ROSA26-GFP* $\times$ *Prdm1-Cre*) and *ROSA26-GFP*² lines; CASP3-positive populations in Cre-positive and -negative specimens at 4–5-s; CASP3-positive populations in Cre-positive and -negative specimens at 7–9-s; PRDM1-positive populations in F2 specimens and GFP-positive populations in Cre-positive specimens at the EB stage; and PRDM1-positive populations in F2 specimens and GFP-positive populations in Cre-positive specimens at 7–9-s stages.

Pearson's χ^2 analyses, calculated in Excel 2008, was used to test whether Cre-positive and -negative conceptuses from (*ROSA-GFP* $\times$ *Prdm1-Cre*), *Prdm1-Cre*², and *Prdm1-Cre*F1² litters exhibited the expected Mendelian ratio of 1:1.

Pearson's product-moment correlation analyses with associated p-values, calculated via R Project for Statistical Computing version 3.1, were used to analyze the correlation between

dissection (embryonic day) stage and the number of Cre-positive conceptus, Cre-negative conceptuses, and resorptions.

One-way ANOVA with post-hoc Student's t-tests (equivariance assumed), calculated in R Project for Statistical Computing version 3.1, was used to compare litter size in breeding pairs from *Prdm1-Cre*, *Prdm1-CreF1*, and *ROSA26-GFP* lines; number of litters per breeding pair from *Prdm1-Cre*, *Prdm1-CreF1*, and *ROSA26-GFP* lines; PRDM1-positive populations in F2 specimens, PRDM1-positive populations in Cre-negative specimens, and GFP-positive populations in Cre-positive specimens at headfold stages; and PRDM1-positive populations in F2 specimens, PRDM1-positive populations in Cre-negative specimens, PRDM1-positive populations in Cre-positive specimens, and GFP-positive populations in Cre-positive specimens at 4–5-s stages.

All means are expressed as $\pm$ standard error of the mean (sem). Statistical significance, $p < 0.05$.

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Abbreviations

ACD	Allantoic Core Domain (headfold and early somite stages)
AP	alkaline phosphatase
E	embryonic day
EB	Early (allantoic) Bud
EHF	Early Headfold
ES	Early Streak
ESC	embryonic stem cell
IgG	immunoglobulin
IPS	intra-embryonic posterior primitive streak
LB	Late (allantoic) Bud
LS	Late Streak
MS	Mid-Streak

OB	No (allantoic) Bud
PGCs	primordial germ cells
s	somite pairs
vEHF	very Early Headfold
VER	Ventral Ectodermal Ridge
XPS	extraembryonic posterior primitive streak (neural plate stages)

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Fig. 1. Specificity of PRDM1 antibody

Unless otherwise indicated, all sections presented here and in subsequent figures are sagittally oriented with anterior on the left and posterior on the right. PRDM1 immunostaining is dark brown; all sections were counterstained in hematoxylin (bluish-purple color).

(A–C) Anti-PRDM1 (+ αPRDM1) at 7–11-s stage (~E8.5–9.0) in F2 positive control (A), *Prdm1*^{+/+} positive control (B), and *Prdm1*^{-/-} negative control (C). N=4 for each genotype. Transverse orientation: dorsal, left / ventral, right. In positive controls, PRDM1 signal was

detected in nuclei of the hindgut (hg; A, B; outlined in black and magnified within bottom left insets) and splanchnopleure (sp; A, B; outlined in black and magnified within bottom right insets). In *Prdm1*^{-/-} controls, this signal was not detected in the hindgut (C; outlined in gray and magnified within bottom left inset) or splanchnopleure (C; outlined in gray and magnified within bottom right inset), thereby verifying the specificity of the antibody. Extranuclear staining on the apical surface of dorsal hindgut (A–C) and in the vesicles of the yolk sac extraembryonic visceral endoderm (A), was present in both experimental and control (“minus antibody” and isotype) embryos and was therefore interpreted as nonspecific background, as described in Results and Experimental Procedures. These results are representative of all mutant and control specimens. **(D–F)** F2 specimens at 5-s stage (~E8.25) and 8–9-s stage (~E8.5–8.75). Anti-PRDM1 (D), “minus primary antibody” (–1° α) control (E), and rat IgG_{2a} isotype control (F). N=4 for each staining condition at each stage range. Transverse orientation: dorsal, left / ventral, right (D₂, E₂, F₂). Exemplary PRDM1-positive nuclei in the posterior region are highlighted by black arrowheads (D₁, ₂). Nuclear staining was absent in “minus primary antibody” control specimens (E₁, ₂) and in rat IgG_{2a} isotype control specimens (F₁, ₂). As described above, control specimens also exhibited extranuclear staining in hindgut (hg; E₂, F₂) and splanchnopleure (sp; E₂, F₂) that was rarely observed in anti-PRDM1 experimental specimens (D₂). **(G)** PRDM1 Western blot analysis of total protein extracts from F2 mouse gastrulae (vEHF-5-s, ~E7.75–8.25; see Experimental Procedures). Lanes 1, 3, and 5 represent the protein markers as designated to the left of the blots; lanes 2, 4, 6 represent 20 µg embryonic cell lysate. Anti-PRDM1 identified two bands between the 75 and 100 kDa MW markers (lane 2, black asterisks) that are consistent with the predicted MWs of PRDM1 isoforms. Two additional, relatively weak bands were detected between MW markers 35 and 50 kDa, and a third band was detected between 50 and 75 kDa (lane 2, red arrowheads); these may represent partially degraded PRDM1 protein. All bands were absent in the “minus primary antibody” (–1° α) control (lane 4) and in the rat IgG_{2a} isotype control (lane 6). All lanes were run on a single gel; black outlines mark where blots were separated for individual processing. Additional abbreviations: al, allantois; am, amnion; da, dorsal aorta; oa, omphalomesenteric artery. Scale bar in A: 20.3 µm (A–C), 4.1 µm (A–C insets), 25.0 µm (D₁, E₁, F₁), 17.8 µm (D₂, E₂, F₂).

Fig. 2. PRDM1 in posterior tissues at ES-7-s stages

(A) Midstreak (MS) stage (~E6.75). PRDM1-positive cells (outlined in black and magnified within top right inset) localized to the proximal epiblast (epi; black arrowhead, inset) and in cells beginning to move through the primitive streak (red arrowhead, inset), from which mesoderm (m) emerges. In this, and in all subsequent panels where visceral endoderm is present, PRDM1-positive cells are found in both the embryonic and extraembryonic visceral endoderm (eve, xve, respectively; red arrowheads outlined in black).

(B) No (allantoic) Bud/Neural Plate (OB) stage (~E7.0). PRDM1-positive cells localized to the extraembryonic posterior primitive streak (XPS; outlined by dashed lines), over which lies the allantois-associated posterior visceral endoderm (apve).

(C, D) Early (allantoic) Bud/Neural Plate (EB) stage (~E7.25). Examples of PRDM1-positive cells in two EB-stage conceptuses. PRDM1-positive cells persisted in the XPS (C, outlined by dashed lines) and adjacent posterior epiblast (C, black arrow outlined in red). Some positive cells were also detected in the intraembryonic posterior primitive streak (ips; C, black arrowhead), the amniotic ectoderm (C, outlined by black box, enlarged within inset) and the allantoic bud (al; D, red arrowheads).

(E) Early Headfold (EHF) stage (~E7.75). Larger panel is a magnification of region outlined within inset showing PRDM1 localized to the nuclei of trophoblast giant cells (gc; arrowheads).

(F) EHF stage (~E7.75). Within the allantois (al), PRDM1 localized throughout the length of the ACD (~120 μ m (Downs et al., 2009); delineated by brackets), but the majority of cells were found in the proximal ACD (Mikedis and Downs, 2012).

(G) EHF stage (~E7.75). Large panel is an enlargement of region highlighted in black within inset. Some PRDM1-positive cells localized to the distal allantois (arrowhead). In the highlighted exemplary cell, PRDM1 localized throughout most of the nucleus.

(H) 2-s stage (~E8.0). Inset is an enlargement of the nook region, the first site of hematopoiesis in the conceptus (Rhee and Iannaccone, 2012) highlighted in black within larger panel. Asterisk marks vessel of confluence.

(I, J) 3-s stage (~E8.25). Examples of PRDM1-positive cells in two 3-s-stage conceptuses. Within the allantois, the majority of PRDM1-positive cells localized to the proximal ACD (I, asterisk), but PRDM1-positive cells could be found throughout the allantois in more distal regions (I and J, arrowheads). In some cells of the distal allantois, PRDM1 was confined to only part of the nucleus (J, red arrowhead).

(K, L) 7-s stage (~E8.5). Examples of PRDM1-positive cells in two 7-s-stage conceptuses. Larger panel (K) is a magnification of the boxed area within inset. The majority of posterior PRDM1-positive cells localized to the hindgut (hg) invagination, both to the ventral side (K, black arrowheads) and dorsal side (K, red arrowheads). The ventral hindgut population included PRDM1-positive cells in the hindgut lip (K, black arrowheads outlined in red). Outside of the hindgut, additional cells were detected in the allantois, e.g., ventral cuboidal mesothelium (K, black arrow; L, red arrowhead) that overlies the vessel of confluence (K and L left inset, asterisk). Occasional mesodermal cells of the yolk sac were also positive (K, red arrow). PRDM1-positive cells localized to a subset of endothelial cells in the nascent umbilical artery (L, right center inset) and in the blood islands of the yolk sac (L, right top inset). Additional cells localized to putative non-endothelializing allantoic mesoderm (L, right bottom inset). Occasional PRDM1-positive cells localized to the endothelium of the vessel of confluence (L, left inset, black arrowhead).

Additional abbreviations: am, amnion; epc, ectoplacental cone; ipve, intraembryonic posterior visceral endoderm.

Scale bar in A: 44.4 μ m (A), 15.7 μ m (A inset), 50.0 μ m (B, C, H, I, L), 12.5 μ m (C inset, L right middle inset), 29.4 μ m (D), 67.3 μ m (D inset), 37.5 μ m (E), 150.0 μ m (E inset), 56.3 μ m (F), 15.2 μ m (G, J), 158.2 μ m (G inset), 18.3 μ m (H inset), 121.9 μ m (J inset), 15.0 μ m

(K), 123.8 μm (K inset), 12.2 μm (L right upper inset), 16.4 μm (L right lower inset), 39.9 μm (L left inset).

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Fig. 3. Posterior PRDM1 at 8–12-s stages

All panels are in transverse orientation: dorsal, left / ventral, right.

(A) 9-s stage (~E8.75). PRDM1-positive cells localized to the splanchnopleure (outlined and magnified within inset), which will meet in the midline to create the omphalomesenteric artery, ventral hindgut (hg), and hindgut wall (Daane and Downs, 2011).

(B) 9-s stage (~E8.75) shows differing intensities of PRDM1 in the ventral hindgut (hg; boxed areas magnified within insets). B₁ is 24 microns anterior to B₂ in the same specimen.

(C) 9-s stage (~E8.75) shows variable profiles of PRDM1-positive cells within the allantois, including a cluster within the midline of the proximal allantois (al; C₁, boxed region magnified within inset) and endothelial cells which were widely dispersed within the distal allantois (C₂ bottom right inset). While PRDM1 localized throughout the nucleus in many distal allantoic cells (C₂ top and bottom right insets), in many other cells, PRDM1 was confined to a single site within the nucleus (C₂ center right inset). Partial nuclear staining does not appear to be an artifact as adjacent sections were negative.

(D) 11-s stage (~E9.0). Black arrowheads highlight exemplary PRDM1-positive cells in the mesodermal and ectodermal components of the somatopleure (so; bottom inset) and hindgut (top inset).

(E) 11-s stage (~E9.0). Large panel is an enlargement of region highlighted in black within inset. PRDM1 in splanchnopleure endoderm (sp; black arrowheads) overlying the ventral hindgut (hg) and omphalomesenteric artery (oa).

(F) 11-s stage (~E9.0). Large panel is an enlargement of outlined region within inset. PRDM1-positive cells localized to the Ventral Ectodermal Ridge (VER) of the tailbud (outlined by black dashed line; exemplary PRDM1-positive cells highlighted by black arrowheads) as well as to tailbud mesoderm (red arrowheads).

Additional abbreviations: da, dorsal aorta; nt, neural tube; s, somite; xve, extraembryonic visceral endoderm.

Scale bar in A: 50 μm (A, B₁, B₂, C₁, C₂) 20.0 μm (A inset), 9.4 μm (B₁ inset, B₂ inset), 10.1 μm (C₁ inset), 10.5 μm (C₂ insets), 100.0 μm (D), 27.9 μm (D upper inset), 33.5 μm (D lower inset), 23.2 μm (E, F), 129.6 μm (E inset), 104.1 μm (F inset).

Fig. 4. PRDM1 (cyan) and STELLA (red) colocalization within the posterior region

DAPI was used to identify DNA in all specimens.

(A) Posterior PRDM1- and/or STELLA-positive populations at headfold (HF, ~E7.75-8.0; N=6), 4-5-s (~E8.25; N=4), and 9-12-s (~E8.75-9.0; N=6). Standard error of the mean (sem) for PRDM1-positive only; STELLA-positive only; and PRDM1- and STELLA-positive populations at headfold stages (1.4; 0.2; 8.6), 4-5-s stages (4.2; 0.3; 9.9), and 9-12-s stages (8.1; 2.0; 6.3). Both the “STELLA only” and “PRDM1 and STELLA” populations increased between headfold and 9-12-s stages (Student’s t-test; $p=0.026$ and 0.035 , respectively), while the “PRDM1 only” population did not change between these stages ($p=0.623$).

(B) Hindgut PRDM1- and/or STELLA-positive populations at 9-12-s (~E8.75-9.0; N=4). The majority of positive cells colocalized PRDM1 and STELLA, but small populations of “PRDM1 only” and “STELLA only” cells were identified in the hindgut. Error bars represent standard error of the mean (sem).

(C) 4-s stage (~E8.25). Minus primary anti-PRDM1 ($-1^\circ \alpha\text{PRDM1}$) control (C_1) is representative of minus primary control experiments at headfold (~E7.75-8.0; N=2), 4-5-s (~E8.25; N=2), and 9-12-s (~E8.75-9.0; N=2) stages. White arrowheads indicate cells that are positive for STELLA (C_2) and DAPI (C_3); three-way merged image (C_4).

(D) 5-s stage (~E8.25). PRDM1 (D₁) in cells from which anti-STELLA (−1° αSTELLA) was eliminated (D₂) is representative of minus primary control experiments at headfold (~E7.75–8.0; N=2), 4–5-s (~E8.25; N=2), and 9–12-s (~E8.75–9.0; N=2) stages.

(E–H) 9–12-s stages (~E8.75–9.0). Frontal orientations of the posterior region. Regions outlined in white within left panels are enlarged and displayed as single channels (E_{1–3}, E_{5–7}, F_{1–3}, G_{1–3}, H_{1–3}) or as merged images (E₄, E₈, F₄, G₄, H₄) in adjacent right panels. Exemplary cells highlighted by white arrowheads.

(E) Ventral hindgut. Dashed yellow line outlines the bottom edge of the hindgut lip/opening; dashed white lines outline the right and left sides of the hindgut tube in this and panel F. The majority of cells exhibiting STELLA colocalized with PRDM1 (E_{1–4}), but a minority exhibited STELLA alone (E_{5–8}).

(F) Hindgut PRDM1-positive cells without STELLA (F_{1–4}).

(G) Colocalizing PRDM1/STELLA within the Ventral Ectodermal Ridge (ver). Dashed white line outlines the edges of the tailbud; dashed yellow outlines one side of the allantois (al).

(H) Colocalizing PRDM1/STELLA within the allantois.

Scale bar in A₁: 7.3 μm (C_{1–4}, D_{1–4}, F_{1–4}, G_{1–4}), 33.8 μm (E), 6.2 μm (E_{1–4}), 4.6 μm (E_{5–8}), 33.1 μm (F), 3.6 μm (F_{1–4}), 17.8 μm (G), 36.6 (H).

Fig. 5. Comparison of PRDM1- and GFP-positive traced populations within the posterior region PRDM1 and GFP populations were compared within the total posterior region at multiple stages (**A**) as well as within specific posterior tissues at early bud (EB, ~E7.25; **B**), headfold (HF, ~E7.75–8.0; **C**), 4–5-s (~E8.25; **D**), and 7–9-s (~E8.5–8.75; **E**) stages. Specimens at EB and 7–9-s stages were statistically analyzed via two-tailed Student's t-test; headfold and 4–5-s stage specimens were analyzed via one-way ANOVA, which was combined with post-hoc two-tailed Student's t-tests as needed. All p-values are displayed in Table 3. Gray and black asterisks mark PRDM1 and GFP populations, respectively, that are statistically

different ($p < 0.05$). Error bars represent standard error of the mean (sem). For (anti-PRDM1, F2 controls; anti-PRDM1, Cre- controls; anti-PRDM1, Cre+ controls; anti-GFP, Cre+ experimentals), N = (5; nd; nd; 6) at EB, (6; 4; nd; 3) at HF, (4; 4; 3; 5) at 4–5-s, and (6; nd; nd; 6) at 7–9-s.

Additional abbreviations: nd, not done.

Fig. 6. Descendants of *Prdm1*-expressing cells in the posterior region

(A–E) (*ROSA26-GFP* × *Prdm1-Cre*) specimens.

(A) Control, anti-GFP immunostaining, very early headfold (vEHF) stage (~E7.75). A (*ROSA26-GFP* × *Prdm1-Cre*) specimen lacking the *Prdm1-Cre* transgene (Cre⁻), and thereby lacking GFP protein (GFP⁻). No nuclear staining was detected in the conceptus (A₁; the yolk sac above the headfolds (hf) was removed (asterisk) for genotyping), including within the posterior region (A₂), except for spurious nonspecific staining in the nuclei of

some giant cells (gc; A₁) and spots (black arrowheads in A₃) in the vesicles of some extraembryonic visceral endoderm (xve).

(B) LHF stage (~E8.0). GFP-positive cells in the posterior region. GFP-positive cells localized to sites of PRDM1: the intraembryonic posterior primitive streak-associated posterior visceral endoderm (ipve; B₁, bottom inset), intraembryonic posterior primitive streak (ips; B₂, bottom inset), proximal ACD (B₁, center inset; B₂, center inset), and distal allantoic core mesoderm (B₁, top inset). GFP also localized to allantoic mesothelium (B₂, top inset), where PRDM1 was never found. Bracketed dashed line indicates the ACD, whose length averages 120 µm in fixed material at this stage (Downs et al., 2009).

(C) 4-s stage (~E8.25). GFP-positive cells localized to the allantois (top three insets), hindgut (hg; fourth inset from the top), and posterior epiblast (bottom inset), all sites where PRDM1 had been found.

(D) 8-s stage (~E8.5). Some GFP-positive cells in the hindgut exhibited the round morphology (D₁, bottom inset; D₂, insets) previously observed in STELLA-positive PGCs of the hindgut (Mikedis and Downs, 2012), while other GFP-positive cells exhibited a cuboidal epithelial morphology (D₁, top two insets), thought to be associated with somatic hindgut endoderm. PRDM1 was found in round, but not cuboidal, cells.

(E) 8-s stage (~E8.5). GFP-positive cells localized to the distal allantois (al; insets), near the site of fusion between the chorion (ch) and allantois, where PRDM1 had also been found.

(F–G) (*ROSA26-mT/mG* × *Prdm1-Cre*) specimens.

(F) 4-s stage. Some GFP-positive cells localized to presumptive allantoic endothelium (black arrowhead). Others localized to the allantoic mesothelium (red arrowhead) or were closely associated with it (red arrowhead outlined in black). PRDM1 was found in allantoic endothelium but not in allantoic mesothelium.

(G) 11-s stage. GFP localized abundantly to the hindgut (hg), both the round cells (black arrowhead) and cuboidal cells (exemplary cells highlighted by red arrowheads). Additional GFP-positive cells localized to the splanchnopleure (sp; exemplary cells highlighted by red arrowheads outlined in black), where PRDM1 protein was detected.

Other abbreviations: am, amnion; apve, allantois-associated posterior visceral endoderm; da, dorsal aorta; oa, omphalomesenteric artery.

Scale bar in A₁: 100.0 µm (A₁), 25.0 µm (A₂, B–E), 8.7 µm (A₃), 3.8 µm (B_{1,2} insets), 6.3 µm (C insets, D₁ insets), 5.9 µm (D₂ insets), 8.8 µm (E insets), 14.4 µm (F), and 14.9 µm (G).

Figure 7. STALLA in *Prdm1*^{+/-} and *Prdm1*^{-/-} specimens

- (A) EHF stage, *Prdm1*^{+/-} specimen. STALLA localized to the base of the allantois (al), intraembryonic posterior primitive streak (ips), and associated posterior visceral endoderm.
- (B) EHF stage, *Prdm1*^{-/-} specimen. STALLA localized to the base of the allantois, posterior primitive streak, and associated posterior visceral endoderm. However, there were fewer cells than in control specimens (Table 5).
- (C) 8-s stage, *Prdm1*^{+/-} specimen. STALLA localized to the ventral hindgut (hg).
- (D) 8-s stage, *Prdm1*^{-/-} specimen. STALLA was not detected in the hindgut.

Other abbreviations: am, amnion; da, dorsal aorta; oa, omphalomesenteric artery.
Scale bar in A: 50.0 μm (A, B), 65.0 μm (C, D).

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Fig. 8. PRDM1 colocalizes with *Runx1*

(A) 8-s stage (~E8.5). PRDM1 localized to *Runx1-LacZ*-expressing cells (royal blue; exemplary cells highlighted by black arrowheads) at the chorio-allantoic interface. One exemplary cell is outlined in black and enlarged within inset.

(B) 8-s stage. Transverse orientation: ventral, bottom / dorsal, top. PRDM1 colocalized with *Runx1-LacZ* expression in the splanchnopleure near the omphalomesenteric artery that forms alongside the hindgut.

(C) 12-s stage (~E9.0). Transverse orientation: dorsal, left; ventral, right. Some PRDM1-positive cells in the hindgut colocalized with *Runx1-LacZ* expression (black arrowheads); these colocalizing cells tended to be more closely associated with the omphalomesenteric artery (oa). Other PRDM1-positive cells in the hindgut were negative for *Runx1-LacZ* (red arrowhead); these cells tended to be further from the omphalomesenteric artery than colocalizing cells.

(D) Colocalization of PRDM1 and *Runx1-LacZ* expression in the hindgut at 8–12-s (~E8.5–9.0). N=4. Error bars represent standard error of the mean (sem).

Scale bar in A: 50.0 μm (A), 6.6 μm (A inset), 32.2 μm (B), 10.6 μm (B inset), and 22.5 μm (C).

Table 1

Recovery of conceptuses from (*ROSA26-GFP* × *Prdm1-Cre*) litters at E7.5–9.0 stages (46 litters containing 122 conceptuses)

Contents of implantation sites	Average number of conceptuses recovered per litter(sem)	Correlation to dissection stage (Pearson correlation coefficient, ρ)	p-value ^a
Cre+ conceptuses	1.1 (0.1) ^b	0.244	0.953
Cre– conceptuses	1.5 (0.2) ^b	–0.105	0.240
Resorptions ^b	3.7 (0.3)	0.031	0.417

^aPearson's product-moment correlation analysis. For Cre+ and Cre– conceptuses, the alternative hypothesis was that the true correlation of the number of conceptuses recovered to dissection stage was less than 0. For resorptions, the alternative hypothesis was that the true correlation to the number of conceptuses recovered to dissection stage is greater than 0.

^bCre-positive and -negative conceptuses were observed at the expected 1:1 Mendelian ratio ($\chi^2=3.2787$; $p=0.070$).

^c7 additional litters had no conceptuses but an average of 3.3 ± 0.9 resorptions.

Abbreviations: sem, standard error of the mean.

Table 2

Fertility of congenic *Prdm1-Cre*, outbred *Prdm1-Cre*, and *ROSA26-GFP* breeding couples continuously paired for 16 weeks

Transgenic strain	Genetic background	Ratio of successful matings (%)	Total no. litters	Avg. litter size (sem)	Avg. no. litters per pair ^a	Cre- offspring	Cre+ offspring	Offspring not genotyped	p-value for χ^2 analysis of Mendelian inheritance ^b
Congenic <i>Prdm1-Cre</i>	B6.Cg	10/12 (83.3)	18	2.8 (0.3) ^c	1.8 ^d	25	26	1	0.889
Outbred <i>Prdm1-Cre</i>	B6.Cg; B6CBA	6/6 (100.0)	14	3.9 (0.4) ^c	2.3 ^d	25	26	4	0.889
<i>ROSA26-GFP</i>	B6;129	8/10 (80.0)	25	6.3 (0.6) ^c	3.1 ^d	n/a	n/a	n/a	n/a

^a Only includes data from couples that successfully mated at least once.

^b Expected percentage of *Cre+* pups from wildtype by hemizygous matings is 50.0.

^c Average litter size is statistically different among the three transgenic strains (ANOVA; $p=0.000007$). Post-hoc Student's t-test revealed that the *ROSA26-GFP* line had more pups per litter than congenic and outbred *Prdm1-Cre* lines ($p=0.00001$ and 0.003 , respectively); no difference was observed between congenic and outbred *Prdm1-Cre* lines ($p=0.185$).

^d Average number of litters per breeding pair is statistically different among the three transgenic strains (ANOVA; $p=0.008$). Post-hoc Student's t-test revealed that *Prdm1-Cre* pairs had fewer litters than the *ROSA26-GFP* pairs ($p=0.009$); no statistically significant difference was observed between congenic and outbred *Prdm1-Cre* lines ($p=0.300$) nor between outbred *Prdm1-Cre* and *ROSA26-GFP* pairs ($p=0.146$).

Abbreviations: avg, average; no, number; sem, standard error of the mean.

Table 3

Statistical comparisons of GFP-positive populations to PRDM1-positive populations within the posterior region in Fig. 5

	ANOVA (p-value)	Student's t-test (p-value)				
		GFP vs. PRDML, F2 control	GFP vs. PRDML, Cre-control	GFP vs. PRDML, Cre+ control	PRDML, F2 control vs. PRDML, Cre+ control	PRDML, Cre- control vs. PRDML, Cre+ control
Total posterior region (Fig. 5A)						
Early bud (EB)	n/a	0.00005	nd	nd	nd	nd
Headfold (HF)	0.013	0.012	0.005	nd	0.412	nd
4-5-s	0.004	0.004	0.013	0.001	0.531	0.124
7-9-s	n/a	0.003	nd	nd	nd	nd
Posterior tissues at EB stages (Fig. 5B)						
aPVE	n/a	0.068	nd	nd	nd	nd
iPVE		0.014				
XPS		0.00003				
Allantoic bud		0.237				
IPS		0.00005				
Epiblast		0.018				
Amnion		0.109				
Posterior tissues at HF stage (Fig. 5C)						
aPVE	0.625	n/a	nd	n/a	n/a	nd
iPVE	0.752	n/a		n/a	n/a	
Proximal ACD	0.001	0.001		0.001	0.479	
Distal ACD	0.054	n/a		n/a	n/a	
Distal allantois	0.985	n/a		n/a	n/a	
IPS	0.00001	0.0003		0.000003	0.0005	
Epiblast	0.003	0.002	0.002	0.722		
Amnion	0.230	n/a	n/a	n/a	n/a	

	ANOVA (p-value)	Student's t-test (p-value)					
		GFP vs. PRDM1, F2 control	GFP vs. PRDM1, Cre-control	GFP vs. PRDM1, Cre+ control	PRDM1, F2 control vs. PRDM1, Cre- control	PRDM1, F2 control vs. PRDM1, Cre+ control	PRDM1, Cre- control vs. PRDM1, Cre+ control
Posterior tissues at 4–5-s stages (Fig. 5D)							
Hindgut	0.178	n/a	n/a	n/a	n/a	n/a	n/a
Proximal ACD	0.0001	0.0001	0.0003	0.00002	0.614	0.136	0.060
Distal ACD	0.041	0.021	0.033	0.017	0.821	0.754	0.602
Distal allantois	0.684	n/a	n/a	n/a	n/a	n/a	n/a
IPS/posterior mesoderm	0.096	n/a	n/a	n/a	n/a	n/a	n/a
VCM-associated yolk sac	0.231	n/a	n/a	n/a	n/a	n/a	n/a
Posterior epiblast	0.196	n/a	n/a	n/a	n/a	n/a	n/a
Amnion	0.426	n/a	n/a	n/a	n/a	n/a	n/a
Posterior tissues at 7–9-s stages (Fig. 5E)							
Hindgut lip	n/a	0.087	nd	nd	nd	nd	nd
Ventral hindgut (closed)		0.420					
Dorsal hindgut (closed)		0.026					
Allantois		0.005					
Site of chorio-allantoic union		0.426					
Posterior mesoderm		0.044					
Somatopleure		0.006					
Splanchnopleure		0.00002					
Posterior endothelium		0.006					
Amnion		0.401					

Specimens at EB and 7–9-s stages were analyzed via two-tailed Student's t-test. Specimens at headfold and 4–5-s stages were analyzed via one-way ANOVA, which was paired with post-hoc two-tailed Student's t-tests as needed. Boldface font indicates statistically significant p-values ($p < 0.05$).

Abbreviations: ACD, Allantoic Core Domain; aPVE, allantois-associated posterior visceral endoderm; EB, early bud; EHF, early headfold; IPS, intraembryonic primitive streak; iPVE, posterior visceral endoderm associated with the intraembryonic posterior primitive streak; n/a, not applicable; nd, not done; XPS, extraembryonic posterior primitive streak; VCM, visceral cuboidal mesothelium.

Shaded gray regions are for readability.

Table 4

CASP3 in PRDM1-positive posterior tissues in Cre-positive and -negative specimens from (*ROSA26-GFP* × *Prdm1-Cre*) matings

Average number of CASP3+ fragments (sem)			
EHF-LHF (~E7.75–8.0)			
Tissue	Cre+ (N=5)	Cre- (N=5)	p-value
aPVE	0.8 (0.8)	0.4 (0.4)	0.667
Proximal ACD	1.8 (1.1)	1.2 (1.2)	0.723
Distal ACD	2.4 (1.3)	0	0.100
Distal allantois	1.4 (1.4)	0	0.347
Amnion	0	0.4 (0.4)	0.347
Total	6.4 (3.2)	2.0 (1.5)	0.255
4–5-s stage (~E8.25)			
Tissue	Cre+ (N=3)	Cre- (N=3)	p-value
Hindgut	3.0 (1.2)	0.7 (0.7)	0.155
IPS	15.7 (3.0)	11.3 (7.3)	0.612
Posterior epiblast	4.3 (2.3)	5.7 (2.7)	0.726
Allantois	47.7 (4.4)	59.7 (2.0)	0.067
Total	70.7 (6.2)	78.3 (7.7)	0.480
7–9-s stage (~E8.5–8.75)			
Tissue	Cre+ (N=3)	Cre- (N=5)	p-value
Hindgut	14.3 (9.0)	7.0 (2.6)	0.359
Posterior mesoderm	39.3 (7.5)	48.2 (9.4)	0.541
Posterior surface ectoderm	4.0 (2.0)	1.4 (0.9)	0.213
Splanchnopleure	0.7 (0.7)	0.4 (0.4)	0.725
Somatopleure	6.3 (6.3)	5.0 (2.7)	0.827
Allantois	32.7 (5.2)	70.2 (6.4)	0.007 ^a
Total	98.0 (3.6)	135.4 (9.2)	0.025 ^b

Populations were analyzed via two-tailed Student's t-test.

^aThis statistically significant difference indicates that the population of CASP3+ fragments in the allantois is smaller in the Cre+ than Cre– specimens. Therefore, this difference does **not** indicate that more cell death is occurring in Cre+ specimens compared to Cre– specimens, and might be explained by small specimen numbers.

^bStatistical difference between total populations is due to difference in allantoic cell numbers, as when the allantoic population is excluded from the total, no difference is detected (p=0.994).

Abbreviations: ACD, Allantoic Core Domain; aPVE, allantois-associated posterior visceral endoderm; CASP3, Caspase-3; EHF, early headfold; IPS, intraembryonic posterior primitive; LHF, late headfold; sem, standard error of the mean.

Table 5

STELLA-positive cells in ($Prdm1^{+/-} \times Prdm1^{+/-}$) specimens at the EHF stage (~E7.75)

Posterior tissue	<i>Prdm1</i> ^{+/+} (N=1)	<i>Prdm1</i> ^{+/-} (N=1)	<i>Prdm1</i> ^{-/-} (N=1)
Allantois	30	31	16
Epiblast	35	27	1
Posterior visceral endoderm	85	33	4
Posterior primitive streak	11	4	1
Amnion	2	1	1
Total	163	96	23