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Critical Function of AP-2gamma/TCFAP2C in Mouse Embryonic Germ Cell Maintenance

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ABSTRACT

Formation of the germ cell lineage involves multiple processes, including repression of somatic differentiation and reacquisition of pluripotency as well as a unique epigenetic constitution. The transcriptional regulator Prdm1 has been identified as a main coordinator of this process, controlling epigenetic modification and gene expression. Here we report on the expression pattern of the transcription factor Tcfap2c, a putative downstream target of Prdm1, during normal mouse embryogenesis and the consequences of its specific loss in primordial germ cells (PGCs) and their derivatives. Tcfap2c is expressed in PGCs from Embryonic Day 7.25 (E 7.25) up to E 12.5, and targeted disruption resulted in sterile animals, both male and female. In the mutant animals, PGCs were specified but were lost around E 8.0. PGCs generated in vitro from embryonic stem cells lacking Tcfap2c displayed induction of Prdm1 and Dppa3. Upregulation of Hoxa1, Hoxb1, and T together with lack of expression of germ cell markers such Nanos3, Dazl, and Mutyh suggested that the somatic gene program is induced in Tcfap2c-deficient PGCs. Repression of Tcfap2c in Tcam-2, a human PGC-resembling seminoma cell line, resulted in specific upregulation of HOXA1, HOXB1, MYOD1, and HAND1, indicative of mesodermal differentiation. Expression of genes indicative of ectodermal, endodermal, or extraembryonic differentiation, as well as the finding of no change to epigenetic modifications, supported control by other factors. Our results implicate Tcfap2c as an important effector of Prdm1 activity that is required for PGC maintenance, most likely mediating Prdm1-induced suppression of mesodermal differentiation.

INTRODUCTION

Primordial germ cells (PGCs) are the progenitors of gametes and are specified within the proximal epiblast around Embryonic Day 6.5 (E 6.5) in mice through signals from extraembryonic tissues [1, 2]. At E 7.25, PGCs can be identified due to their alkaline phosphatase reactivity as a cluster of approximately 45 cells at the base of the developing allantois [3, 4]. PGCs migrate into the embryo proper along the hindgut to colonize the developing genital ridges that will differentiate to become testes or ovaries [5, 6]. Compared to their neighboring somatic cells, PGCs are characterized by high expression of Ifitm3 and Dppa3 (previously known as “fragilis” and “stella,” respectively). In contrast, genes indicative of somatic differentiation, such as Hoxb1 and Hoxa1, are repressed. It has been proposed that repression of somatic differentiation is one mechanism by which the PGCs escape from the somatic fate and retain pluripotency. Recently, the transcriptional repressor BLIMP1 (B-lymphocyte-induced maturation protein-1, official symbol PRDM1) [7] has been identified as a key factor controlling the specification of PGCs [8]. Upon loss of Prdm1, PGC specification is blocked at an early stage, resulting in lack of activation of PGC-specific genes and derepression of HOX genes leading to induction of somatic differentiation [8]. Global gene expression profiling of the initial 48 h of germ cell development was used to decipher the regulatory network governed by PRDM1 to specify PGCs [9]. Genes found highly upregulated in early PGCs following PRDM1 induction comprise several transcriptional regulators, among them Tcfap2c, Prdm14, Sox3, Sox17, Elf3, Elk1, Isl2, Mycn, Klf2, 4932441K18Rik (Fiat), Sp8, and Smad3 [9].

The precise roles these PRDM1 targets play in the suppression of somatic differentiation have so far remained unknown. Here, we focused on one transcriptional regulator identified in this study, Tcfap2c (also termed AP-2γ), and analyzed the consequences of a loss of function mutation in vivo and in vitro.

Transcription factor Tcfap2c belongs to a family of five closely related genes that are expressed during embryogenesis [10, 11]. Disruption of Tcfap2c leads to midgestation lethality due to a defect in the trophectoderm cells [12, 13]. Within the embryo proper, Tcfap2c is expressed in a variety of tissues, including PGCs [14]. In humans, Tcfap2c is expressed in fetal germ cells from Week 12 through Week 37 of pregnancy, while it is downregulated and subsequently absent in healthy adult testes [15, 16]. In spite of this highly regulated temporal and spatial expression pattern, nothing is known about the function of Tcfap2c in germ cell development. Therefore, using both in vitro and in vivo models, we have investigated the role of TCFAP2C during PGC development.
**MATERIALS AND METHODS**

**Genotyping**

DNA was prepared from tail biopsies or embryonic tissues using standard procedures. Genotyping of Tcfap2c alleles was done as described previously [12]. Primers used to detect the cre alleles were 5'-CCA CGA CCA AGT GAC AGC AAT G-3' and 5'-CAG AGA CGG AAA TCC ATC GCT C-3', both of which amplify a 373bp DNA fragment. Cycle conditions were 94°C for 45 sec, 56°C for 30 sec, and 72°C for 45 sec for 35 cycles.

**Whole Mount Alkaline Phosphatase Staining of Embryos**

Embryos were dissected in PBS, fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 10 min at room temperature, washed twice in PBS, and then incubated in freshly prepared alkaline phosphatase (AP)-staining solution, i.e., 250 mM Tris-maleic acid, pH 9, 0.4 mg/ml 2-naphthylphosphate (Sigma-Aldrich, Taufkirchen, Germany), and 1 mg/ml Fast Red (Sigma-Aldrich), for 30 min in the dark. Embryos were cleared in 40% glycerol for 1 h and 80% glycerol overnight at 4°C. Embryos were photographed using a binocular (Leica, Bensheim, Germany) and a Zeiss AxioCam digital camera system (Carl Zeiss, Jena, Germany). Files were processed using Adobe Photoshop Elements and Illustrator software (Adobe, San Jose, CA).

**Animal Care and Husbandry**

All animals were housed at 25°C with a 12L:12D photoperiod and were given water and a standard rodent diet ad libitum. All animal experiments were approved by the Landesamt fuer Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (#8.87–50.10.31.08). The experiments were conducted in accordance with the International Guiding Principles for Biomedical Research. All experiments were performed in accordance with the Society for the Study of Reproduction. To obtain Tcfap2c-deficient mice, we carried out a multistep mating scheme where mice heterozygous for the Tcfap2c allele (Tcfap2c<sup>+/−</sup>) were crossed with cre-expressing mice (cre<sup>+</sup>). The resulting Tcfap2c heterozygous cre transgenic animals (Tcfap2c<sup>+/−</sup>; cre<sup>+</sup>) were crossed with Tcfap2c<sup>Flx/Flx</sup> mice to receive the null mutants. The day on which a copulation plug was found was defined as E 0.5.

**RT-PCR**

Total RNA was extracted using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany). 100 ng was used for cDNA synthesis (SuperScriptIII; Invitrogen, Karlsruhe, Germany). PCR was performed with the primers listed in Supplemental Table S1 (all the Supplemental Data are available online at www.bioreprod.org). Thirty-five cycles (30 in the case of target) were used. Primer sequences are given in Supplemental Table S1. Five microliters anti-SSEA-1 antibody (0.1 mg/ml; R&D Systems, Minneapolis, USA) were added to the reaction mixture and incubated for 1 h at room temperature. Signals were visualized using Vectastain ABC Kit (Vector Laboratories, Axxora Deutschland, Lorrach, Germany). Sections were photographed using Diskus software (Diskus, Hilden, Germany). Files were processed using Adobe Photoshop Elements 5 and Illustrator software (Adobe).

**Isolation and Immunostaining of Primordial Germ Cells**

Embryos at E 7.5 were collected in Dulbecco modified Eagle medium (DMEM) (Gibco, Karlsruhe, Germany) supplemented with 0.5% BSA. Embryonic fragments from the base of the allantois were isolated using a microscalpel and incubated in 0.05% trypsin and 0.5 mM EDTA for 7 min, followed by dissociation into single cells by trituration with a mouth pipette. Dissociated single cells were pipetted onto poly-L-lysine-coated glass slides and fixed in 2% PFA. Cells were permeabilized in 0.1% Triton-X-100/PBS, and primary antibody incubation was carried out overnight at 4°C. The following antibodies and dilutions were used: anti-Tcfap2c (H-77, 1:100; Santa Cruz Biotechnology, Inc.), anti-Blimp-1 (Pdm-1 H2-E, 1:20; Affinity Bioreagents, Golden, CO), Alexa-488 (1:500; Invitrogen, Karlsruhe, Germany), and Alexa-594 (1:500; Invitrogen).

**Detection of Cell Death**

LysoTracker (Invitrogen, Germany) staining was performed as previously described [19]. Acidic compartments within apoptotic cells and regular cells engulfing debris from apoptotic cells are labeled. For these analyses, E 8.0 mouse embryos were prepared, incubated in LysoTracker solution, i.e., 5 μl LysoTracker, 1 ml DMEM (without phenol-red), and 2 mg/ml BSA (Sigma Aldrich, Munich, Germany), at 37°C for 40 min, washed twice in DMEM/BSA, twice in PBS, and fixed with 4% PFA in PBS for 2 h at 4°C. Whole mounts were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich), and the staining was analyzed using a Zeiss-Apochromat (Carl Zeiss) with a fluorescence module and appropriate filter set.

**Differentiation and Selection of PGC from Embryoid Body Cultures**

Essentially, the procedure was performed as described previously [20]. For embryoid body (EB) formation, ES cells were washed twice in calcium- and magnesium-free Dulbecco phosphate solution (DBPBS, Invitrogen, Germany) and dissociated with 0.05% trypsin/EDTA solution (PN-Biotech, Aidenbach, Germany). Trypsin was neutralized with DMEM (Invitrogen) containing 15% fetal bovine serum (HyClone, Bonn, Germany). To remove feeder cells, the ES cells were plated twice on a 10-cm culture dish (TPP, Trasadingen, Switzerland) with 0.2% gelatin. After 45 min, nonadherent ES cells were collected, and hanging drop culture (750 ES cells per 25 μL) on lids of dishes filled with PBS was initiated. After 3 days in hanging drop culture, the resulting EBs were transferred to bacterial dishes (10–15 EB per dish) to prevent attachment. Every second day, one-third of the medium was replaced. SSEA-1 positive cell fractions were obtained using MACS-Beads and MACS-LS columns (Miltenyi Biotec, Cologne, Germany) according to the manufacturer’s protocol. Five microliters anti-SSEA-1 antibody (0.1 mg/ml; R&D Systems, Heidelberg, Germany) per 2 × 10<sup>4</sup> total cells were used.

**RESULTS**

Tcfap2c Is Expressed in Primordial Germ Cells and Gonocytes

First the expression of Tcfap2c in PGCs and gonocytes during embryogenesis was analyzed. Using immunohistochem-
istry, we detected Tcfap2c-positive cells starting from the early bud stage at E 7.25 (the earliest stage analyzed) at the base of the allantois, the region where PGCs are located at this developmental stage (Fig. 1, A and B, and Supplemental Fig. S1A). To confirm that the cells expressing Tcfap2c were germ cells, the base of the allantois (box in Fig. 1A) was dissected, trypsinized, and labeled with antibodies detecting the PGC markers Prdm1 and Tcfap2c (Fig. 1, C1 and C2). Prdm1 and Tcfap2c were coexpressed in these cells (Fig.1, C3 and C4), suggesting that Tcfap2c is expressed in PGCs. Next, we extracted RNA from the dissected allantoises at E 7.25. RT-PCR analyses showed that the markers for PGCs Kit and Dnd1 (previous symbol Ter) were coexpressed with Tcfap2c (Supplemental Fig. S1B). These results are in agreement with data obtained from transgenic mice expressing PRDM1-mVenus, where transgene expression was detected in Tcfap2c-positive cells at the base of the allantois in E 7.25 embryos [9]. Taken together, these data demonstrate that
Tcfap2c is expressed in PGCs shortly after specification. At E 8.25, PGCs have begun to migrate into the embryo along the hindgut and express the marker Dppa3. At this stage, Tcfap2c expression (Fig. 1D) colocalizes with the signal obtained for Dppa3 (Fig. 1, E1–E3). Using in situ hybridization, we could detect Tcfap2c transcripts in the region of the hindgut at E 9.5. The observed pepperlike pattern is typical for migrating PGCs (Fig. 1F). At E 10.5 and E 11.5, we detected Tcfap2c transcripts in postmigratory PGCs in the genital ridges (Fig. 1, G and H). Staining of paraffin sections with a TCFAP2C-specific antibody confirmed this pattern from E 11.5 to E 12.5 (Fig. 1, I and K). TCFAP2C protein or RNA could neither be detected in germ cells in genital ridges after E 12.5 nor in adult testes or ovaries (Fig. 1L and Supplemental Fig. S1, C–E). These results are in agreement with data obtained from humans where expression of Tcfap2c was observed in primordial germ cells and gonocytes, but not in prespermatogonia and spermatogonia [15, 16]. These results prompted us to investigate the role of Tcfap2c in germ cell development.

Tcfap2c Deficient Mice Fail to Reproduce

A complete loss of Tcfap2c is lethal to the embryo around E 6.5 due to a placenta defect and thus precludes the analysis of PGc development [12, 13]. To overcome this difficulty, we took advantage of transgenic mice bearing conditionally mutated Tcfap2c alleles [21]. These Tcfap2c<sup>flox/flox</sup> mice [21] were bred to either Mox2-cre mice [22] expressing the Cre recombinase from E 6 on in the embryo proper or to Alpl-cre mice [23] expressing the Cre protein in PGCs. Both, Tcfap2c<sup>flox/flox,Mox2-cre</sup> and Tcfap2c<sup>flox/flox,Alpl-cre</sup> animals developed normally but failed to reproduce and displayed a similar phenotype with respect to germ cell development. Testes and ovaries of the mutant animals were dramatically reduced in size compared to wild-type controls (Fig. 2, A and B). In female animals, lack of germ cells in ovaries was demonstrated using YBX2 as marker for oocytes (Fig. 2, D vs. C). In male animals, atrophy of the seminiferous tubules was apparent (Fig. 2, F vs. E), and immunohistochemistry demonstrated that mutant testes did not contain any germ cells as judged by the marker YBX2 (Fig. 2, H vs. G) [24]. In contrast, GATA1-positive Sertoli cells were present (Fig. 2, K vs. I) [25] as well as Leydig cells that could be visualized with anti-NOS1 (Fig. 2, M vs. L) [26]. Of note, Leydig cell hyperplasia (Fig. 2, lch) was obvious in the mutant testis, which most likely is a secondary effect due to lack of germ cells. In some animals, the germ cell atrophy was incomplete. PCR analysis of laser microdissected tissue revealed that the Cre-mediated loss of Tcfap2c had not occurred in such testicular areas (Supplemental Fig. S2), suggesting that the incomplete atrophy was due to an incomplete Cre-mediated excision. Indeed, the recombination frequency of the Alpl-cre allele has been reported to reach 72% [27]. These results demonstrate that loss of Tcfap2c leads to sterility.

Tcfap2c Deficient Germ Cells Are Lost Shortly after Specification

Because the temporal expression of Tcfap2c in the germ cell lineage is restricted to PGCs and gonocytes, we tried to determine at which stage during early germ cell development might Tcfap2c be essential. To address this question, we crossed the Tcfap2c<sup>flox/flox</sup> mice with Sox2-cre transgenic mice. The Sox2-cre transgene is expressed from E 5.0 in all cells of the epiblast [28]. Therefore, Sox2-cre transgenic mice could be used in a manner similar to the Mox2-cre-transgenic mice to overcome the placenta defect caused by complete loss of Tcfap2c. However, Cre-mediated recombination is more efficient compared to Mox2-cre or Alpl-cre, thus, precluding a mosaic recombination within a tissue (unpublished data and [28]). Germ cell development of Tcfap2c<sup>flox/flox, Sox2-cre</sup> or control embryos was monitored using AP staining. PGCs could be detected at the primitive streak stage (E 7.25) at the base of the allantois both in the mutants and in the littermate controls (Fig. 3, A and B, arrows). The total number appeared not to be altered. However, at E 8.0, the numbers of PGCs in mutant mice were drastically reduced compared to control mice. In mutant animals, only a few PGCs were detectable near the base of the allantois, and none had initiated migration in contrast to the wild types (Fig. 3, D vs. C, arrows). At E 9.5, no migrating PGCs could be detected along the hindgut in mutant animals in contrast to controls (Fig. 3, É vs. F, arrows). Moreover, at E 10.5 the genital ridges of mutant animals lacked AP staining indicative of PGCs (Fig. 3, H vs. G, arrows.). At E 12.5, wild-type genital ridges contained large amounts of germ cells as indicated by the intense staining. In contrast, the genital ridges of Tcfap2c<sup>flox/flox</sup>; Sox2-cre mice were completely devoid of the AP signal (Fig. 3, J vs. I). Immunohistochemical staining using the germ cell marker GCNA at E 11.5 confirmed that mutant animals lacked germ cells (Fig. 3, K and L). These results demonstrate that the loss of Tcfap2c in PGCs interfered with early germ cell development from E 8.0 onward.

No Enhanced Apoptosis in Tcfap2c-Deficient Germ Cells

To investigate a possible loss of PGCs due to apoptosis, as observed in Pou5f1-deficient mice [29], LysoTracker analysis [19] of E 8.0 Tcfap2c<sup>flox/flox; Sox2-cre</sup> embryos was performed. LysoTracker signal in the region between the allantois and the developing hindgut, where PGCs are localized at this point of embryonic development, was not enhanced in the mutants (Fig. 4A) compared to wild types (Fig. 4B). Apoptosis in E 8.0 headfold mesenchyme (Fig. 4C) was easily detected and served as positive control. The bright-field images were merged with the signal obtained by the LysoTracker to show the embryo and allantois (Fig. 4, A’–C’). Statistical analysis of this experiment showed that the differences between the numbers of Lyso-Tracker positive cells in wild type (11.74 ± 2.73) embryos were not significant different (Fig. 4D; P = 0.722, Student t-test). In addition, no difference in apoptosis could be detected using whole mount TUNEL-assays (data not shown) [30]. Therefore, cell loss by apoptosis is unlikely to be the mechanism responsible for the lack of germ cells in Tcfap2c mutant animals.

PGCs Derived from Tcfap2c-Deficient Embryoid Body Cultures Upregulate Somatic Markers

The defects observed in PGCs of Tcfap2c mutants are a phenocopy of the defects observed in Prdm1-deficient mice. Loss of Prdm1 leads to induction of somatic differentiation as demonstrated by the upregulation of Hoxb1 [8]. We used an in vitro model for PGC differentiation to test whether lack of Tcfap2c would likewise result in Hoxb1 gene activation. PGCs are known to be present in EB cultures derived from murine embryonic stem cells. Stage-specific embryonic antigen (SSEA1)-positive candidate PGCs [20]. Tcfap2c<sup>-deficient</sup> ES cells were generated from Tcfap2c<sup>-deficient</sup> blastocysts derived from a Tcfap2c<sup>+/−</sup> line that was bred into the 129Sv background (our unpublished results). EB cultures were initiated from wild-type ES cells as well as from Tcfap2c<sup>-deficient</sup> ES cells. We used
two different lines of Tcfap2c-deficient ES cells, yielding similar results. After 3, 5, and 7 days, the EBs were trypsinized, and SSEA1-positive cells (the nascent PGCs) were isolated using immunomagnetic bead sorting. As published [20], expression of Pou5f1 remained constant in SSEA1-positive cells while Pou5f1 levels declined in the SSEA1-negative population (data not shown). The markers for early PGCs, Prdm1 and Dppa3, could be detected in both wild-type and Tcfap2c-deficient cultures (Fig. 5A, top), indicating that PGCs had been specified in both genotypes. However, the markers
for mature PGCs \((\text{Nanos3}, \text{Dazl}, \text{and} \, \text{Mutvh})\), which are also expressed in ES cells, decreased significantly in SSEA1-positive cells of \(\text{Tcfap2c}\)-deficient EB cultures compared to wild-type controls over the time observed (Fig. 5A, middle). Notably, the markers for somatic differentiation could either be detected only in cells from \(\text{Tcfap2c}\)-deficient EB cultures \((\text{Hoxb1})\) or were induced stronger and earlier in this assay \((\text{Hoxa1}, \text{Snai1})\) (Fig. 5A, lower). Taken together, these data suggest, that in \(\text{Tcfap2c}\)-deficient EB cultures, PGCs are specified but further germ cell-specific differentiation is impaired and somatic differentiation is initiated.

**Loss of TCFAP2C Leads to Upregulation of Mesoderm Markers**

Kurimoto et al. [9] demonstrated that \(\text{Prdm1}\) regulates germ cell specification by the inhibition of somatic differentiation, specifically by repressing HOX cluster activation, epithelial-mesenchymal transition, cell cycle progression, and the DNA-methyltransferase machinery. Therefore, we analyzed the role of \(\text{Tcfap2c}\) in the genetic program orchestrated by \(\text{Prdm1}\). As an in vitro model, we used TCam-2 [31], a cell line derived from a human seminoma that displays significant characteristics of PGC. Indeed, TCam-2 cells express several PGC markers such as \(\text{PRDM1}\) and \(\text{PRMT5}\) [32], \(\text{TCFAP2C}\), \(\text{SOX17}\), \(\text{KIT}\), \(\text{DPPA3}\), \(\text{NANOG}\), and \(\text{POU5F1}\) [33–35]. In addition, TCam-2 cells exhibit symmetric dimethylation of arginine-3 on histones H2A and/or H4 tails \((\text{H2A/H4R3me2s})\) [32]. This modification has been demonstrated to be specific for PGCs and is directed by the interaction of \(\text{Prdm1}\) and the arginine-specific histone methyltransferase \(\text{Prmt5}\) [36].

First, we tested whether TCam-2 cells could serve as an in vitro model for \(\text{PRDM1}\)-mediated repression of the somatic program on the genetic and epigenetic level. Here, short
interfering RNA (siRNA)-mediated knockdown of PRDM1 led to a reduction of TCFAP2C expression levels and a concomitant upregulation of HOXB1. In addition, expression of the PRDM1 target gene MYC was induced while the expression of NANOG remained unaffected (Supplemental Fig. S3A). Western blot analyses demonstrated that the symmetric dimethylation on histone H2A/H4 was lost after PRDM1 knockdown (Supplemental Fig. S3B). These data indicated that TCam-2 seminoma cells can serve as a model to study PRDM1-mediated gene programs in PGCs and further corroborated that TCFAP2C represents a PRDM1 target gene.

Next, we established a siRNA-mediated knockdown of TCFAP2C in TCam-2 cells to analyze TCFAP2C-dependent genetic programs in more detail. We compared the knockdown efficiency of three single siRNAs to their mixture (as suggested by the supplier) and detected the strongest inhibition of TCFAP2C RNA and protein when using the mixture (Supplemental Figure S3C). Next, we performed quantitative RT analyses of 31 markers (see Supplemental Table S1). As depicted in Supplemental Fig. 3D, expression of pluripotency genes (NANOG, POU5F1, SOX17, SOX2, UTF1, and REX1), genes involved in DNA de novo methylation (DNMT3A, DNMT3B, and DNMT3L), signaling (SMAD4, SMAD7, and STAT5A), and various markers for ectoderm and endoderm differentiation remained unaffected after downregulation of TCFAP2C. However, the genes indicative of mesoderm differentiation, MYOD1 and HAND1, showed strong upregulation. Also GATA2, a marker found in mesoderm-derived hematopoietic progenitors [37], was upregulated. Using conventional RT-PCR, induction of MYOD1 and HAND1 as well as HOXAI and HOXB1 could be detected in TCam-2. Induction of T was only weak compared to the results of the EB cultures while the levels of Snail were not altered in this context. Of note, levels of PRDM1 remained unaffected by TCFAP2C knockdown (Fig. 5B). Taken together, we demonstrated that repression of TCFAP2C leads to upregulation of HOXB1 and the mesoderm markers MYOD1 and HAND1 while markers of other germ layers, cell cycle progression, signaling, and DNA methyltransferases remain unaffected. These results therefore suggest that TCFAP2C is involved in the repression of a set of genes inducing mesodermal differentiation.

In summary we propose a model, wherein Tcfap2c is an effector of PRDM1-mediated repression of somatic differentiation, in particular differentiation into mesoderm. Pluripotency-associated markers and genes involved in epigenetic reprogramming are not affected.

DISCUSSION

In this study, we show that Tcfap2c is specifically expressed in PGCs and gonocytes from E 7.25 to E 12.5 of murine development, suggesting a role in germ cell biology. Using a conditional deletion approach for Tcfap2c, we show that PGCs are lost shortly after specification that results in sterile animals, independent of sex. In addition, we show that repression of Tcfap2c leads to upregulation of Hoxb1, Hand1, and Myod

![Cell death is not enhanced in mutant PGCs. Photomicrography of LysoTracker analysis (red signal, LT) of (A and A') mutant (mut) and (B and B') wild type (Wt) animals at E 8.0. The allantois (a) and posterior end of embryo is shown. A–C) Apoptosis in the head mesenchyme of wild-type animal serves as positive control. A–C) Counterstaining with DAPI. A–C) Merge of bright field (BF) and lysotracker (red, LT) signal. Bar = 100 μm. D) Graph showing the number and standard deviation of cells positive in the posterior end of embryos (n = 8) in the LysoTracker study for wild type (WT; 11 ± 2.55) and Tcfa2c mutants (11.74 ± 2.73).]
expression, which is indicative of mesoderm differentiation. The expression level of the master coordinator of germ cell development, Prdm1, remains unaffected, supporting the idea that Tcfap2c acts as a downstream mediator in this pathway.

In PGCs, Tcfap2c expression is induced shortly after their specification. Secretory bone morphogenetic proteins (Bmp) Bmp 4, Bmp8b, and Bmp2 and the downstream signaling molecules of the SMAD pathway are involved in the specification of PGCs [38]. The exact molecular mechanism, however, has not been elucidated. Interestingly, in Xenopus epidermis and rat neural crest, Bmp signaling has been reported to induce Tcfap2c expression, suggesting a Bmp-mediated induction of Tcfap2c in nascent PGCs as a possible mechanism [39–42].

Does the lack of TCFAP2C interfere with PGC specification? Experimental data argue against this scenario. If Tcfap2c were required to mediate Bmp and Smad signaling, PGC specification would not take place. However, AP-positive PGCs were detected at E 7.0 in Tcfap2c-mutant mice. Also, SSEA1-positive cells from Tcfap2c-deficient EB cultures showed induction of Prdm1 and Dppa3, early markers for PGCs. In addition, expression levels of SMAD4 and SMAD7, the downstream effectors of BMP signaling, remained unaffected in TCam-2 cells after siRNA mediated knockdown.
of TCFAP2C. However, at E 8.0 PGCs in the mutants appeared reduced in number and had not initiated migration into the embryo suggesting an impaired proliferation of the founding population and/or an altered migratory behavior.

Loss of germ cells in the Tcfap2c mutants may be accomplished by two mechanisms: cell death by apoptosis or differentiation into somatic cells. PGCs harboring mutations in Pou5f1 or Kit undergo apoptosis; hence, these factors are regarded as survival factors for germ cells during migration and proliferation [29, 43]. We showed that levels of Pou5f1 and Kit were not affected by lack of Tcfap2c both in EB differentiation assays and in Tcfap2c knockout experiments. In addition, the number of apoptotic cells is not enhanced in Tcfap2c-deficient embryos. Therefore, we conclude that Tcfap2c and Pou5f1 or Kit act in different pathways during PGC development and migration.

However, parallels to the phenotype caused by loss of Prdm1 are apparent [8]. Prdm1-deficient PGCs are specified but fail to migrate, and they are prone to uncontrolled somatic differentiation [8]. Indeed, in PGCs, Tcfap2c expression has been shown to depend on and correlate with Prdm1 expression [9]. In PGCs, Prdm1 is required for the repression of developmental programs such as the epithelial-mesenchymal transition, repression of HOX cluster activation, repression of developmental programs such as the epithelial-mesenchymal transition, repression of HOX cluster activation, repression of DNA methyltransferases machinery [9]. Using an EB-based differentiation protocol, we demonstrate induction of Dppa3 and Dppa3 in candidate PGCs. In the literature, established PGCs have been characterized as Dppa3-positive and Hoxb1-negative cells [44, 45]. Lack of Tcfap2c in EB cultures, however, leads to induction of Hoxb1 in DPPA3 (and PRDM1)-positive cells. It has been shown, that embryos of the late primitive streak stage (E7.75) coexpress Prdm1 and Hoxb1 during specification [9]. Genetic lineage-tracing experiments revealed that all Prdm1-expressing cells at early stages contribute almost invariably to DPPA3-positive PGCs, which eventually repress Hoxb1 [8]. The fact that the SSEA1-positive cells of the EB cultures coexpress Dppa3 and Prdm1 indicates that these PGCs have been correctly specified. Reexpression of Hoxb1 after 3 to 5 days in the Tcfap2c-deficient EB cultures even in the presence of PRDM1 protein suggests that Tcfap2c acts downstream of Prdm1, and Prdm1-induced repression of Hoxb1 might be mediated by Tcfap2c. Results from siRNA-mediated knockdown in Tcam-2 cells further corroborate these conclusions. Here, TCFAP2C knockdown lead to reexpression of Hoxb1 in the presence of PRDM1 protein as well. In particular, Tcfap2c seems to specifically suppress mesoderm differentiation, as demonstrated by upregulation of HAND1 and MYOD following TCFAP2C downregulation. In contrast, the expression levels of markers for ectoderm, endoderm, or extraembryonic differentiation were not affected by TCFAP2C, suggesting that these differentiation pathways might be controlled by other downstream regulators of PRDM1. Also the repression of DNA methyltransferases DNMT3A, B, and L in PGCs, which results in a genome-wide DNA demethylation [46], is not affected by downregulation of TCFAP2C. Recent results suggest that Prdm14 controlled epigenetic reprogramming and reacquisition of pluripotency in PGCs represents a Prdm1-independent pathway [47]. Based on our data, we propose that Tcfap2c is acting downstream of Prdm1 and represses one aspect of somatic (i.e., mesoderm) differentiation in PGCs.

The expression pattern described for Tcfap2c in mice is in accordance with the expression of TCFAP2C in human fetal germ cells [15, 16], suggesting that this protein has conserved functions in both species. Moreover, TCFAP2C has been shown to be expressed in undifferentiated germ cell tumors, namely in IGCLN/CIS (intrafetal germ cell neoplasia unclassified, carcinoma in situ) and seminomas [15, 16]. The absence of TCFAP2C in differentiated germ cell tumors (e.g., embryonal carcinoma and teratocarcinoma) provokes speculation as to whether the loss of TCFAP2C expression in germ cell tumors may enable the activation of a somatic developmental program, leading to differentiation into embryonal carcinoma and teratocarcinoma.

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