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Critical Function of AP-2gamma/TCFAP2C in Mouse Embryonic Germ Cell Maintenance[1]

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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, suggested 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.

AP-2γ, developmental biology, gamete biology, gene regulation, HOXB1, PRDM1, primordial germ cells, somatic differentiation, TCam-2, TCFAP2C

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.

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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 α-naphtylphosphate (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 Involving Animals as promulgated by 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*+/–) were crossed with *cre*-expressing mice (*cre*+). The resulting *Tcfap2c* heterozygous *cre* double transgenic animals (*Tcfap2c*+/–; *cre*+) were crossed with $Tcfap2c^{flox/flox}$ mice to receive the null mutants. The day on which a copulation plug was found was defined as E 0.5.

Laser Microdissection

P.A.L.M. membrane slides (Carl-Zeiss MicroImaging, Göttingen, Germany) were irradiated with ultraviolet light for 30 min. Paraffin-embedded tissue was sectioned to 10 μm thickness and stained with haematoxylin and eosin using standard procedures. Seminiferous tubules were dissected using P.A.L.M. Microlaser Technology (Carl-Zeiss MicroImaging). Dissected tissue was collected in DNA lysis buffer (1 mM EDTA pH 8, 10 mM Tris-HCl pH 8, 0.5% Tween20, and 100 μg/ml proteinase K) and incubated at 37°C for 24 h, heated to 95°C for 12 min the next day, and used as a template for PCR.

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.biolreprod.org). Thirty-five cycles (30 in the case of β-actin) were run using a standard protocol.

Quantitative RT-PCR

High-quality total RNA was extracted from the cell lines using an RNAqueous-4PCR kit (Ambion/Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) according to the manufacturer's instructions. Samples were treated with DNase and checked for residual DNA contamination by PCR using a primer set for *SOX2*, which is a single exon gene (see Supplemental Table S1). Complementary DNA was generated as described previously [17]. Quantitative PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). For the detection of expression of a set of genes related to various differentiation lineages, a number of primer sets were used. Primer sequences are given in Supplemental Table S1. Target mRNA was quantified relative to HPRT transcripts as follows: target mRNA value $= 2^{(\text{mean Ct HPRT} - \text{Ct target})}$ [17].

Whole Mount In Situ Hybridization

Whole mount in situ hybridizations were performed as described previously [18]. *Tcfap2c*-full length cDNA subcloned in pBS vector was used for generation of the probe. Embryos were photographed using a binocular (Leica) and a Zeiss Axiocam digital camera system (Carl Zeiss). Files were processed using Adobe Photoshop Elements and Illustrator software (Adobe).

Histology and Imunohistochemistry

Embryos or organs were fixed in 4% neutral buffered formalin at 4°C overnight and embedded in paraffin. Sections of 1–3 μm thickness were stained with hematoxilin and eosin using standard procedures. For immunohistochemistry, sections were incubated using the following primary antibodies: YBX2 (MSY2) (1:2000, kindly provided by Dr. R.M. Schultz, Department of Biology, University of Pennsylvania, Philadelphia, PA), GATA1 (1:200, Santa Cruz, Heidelberg, Germany), GCNA (kindly provided by Dr. G. Enders, University of Kansas, Kansas City, KS), NOS1 (nNOS) (1:200, BD Biosciences, Heidelberg, Germany), TCFAP2C (Clone 6E4, 1:250, kindly provided by Dr. H. Hurst, Cancer Research UK, London, UK), for 1 h at 37°C except YBX2 (4°C, overnight). The secondary antibodies were anti-rabbit (1:500; DAKO, Hamburg, Germany) and anti-rat (1:200; DAKO) for 30 min at room temperature. Signals were visualized using Vectastain ABC Kit (Vector Laboratories, Axxora Deutchland, Lörrach, 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 (Prdm-1 3H2-E8, 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-ApoTome (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 (DPBS, Invitrogen, Germany) and dissociated with 0.05% trypsin/EDTA solution (PAN-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 2×10^5 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-

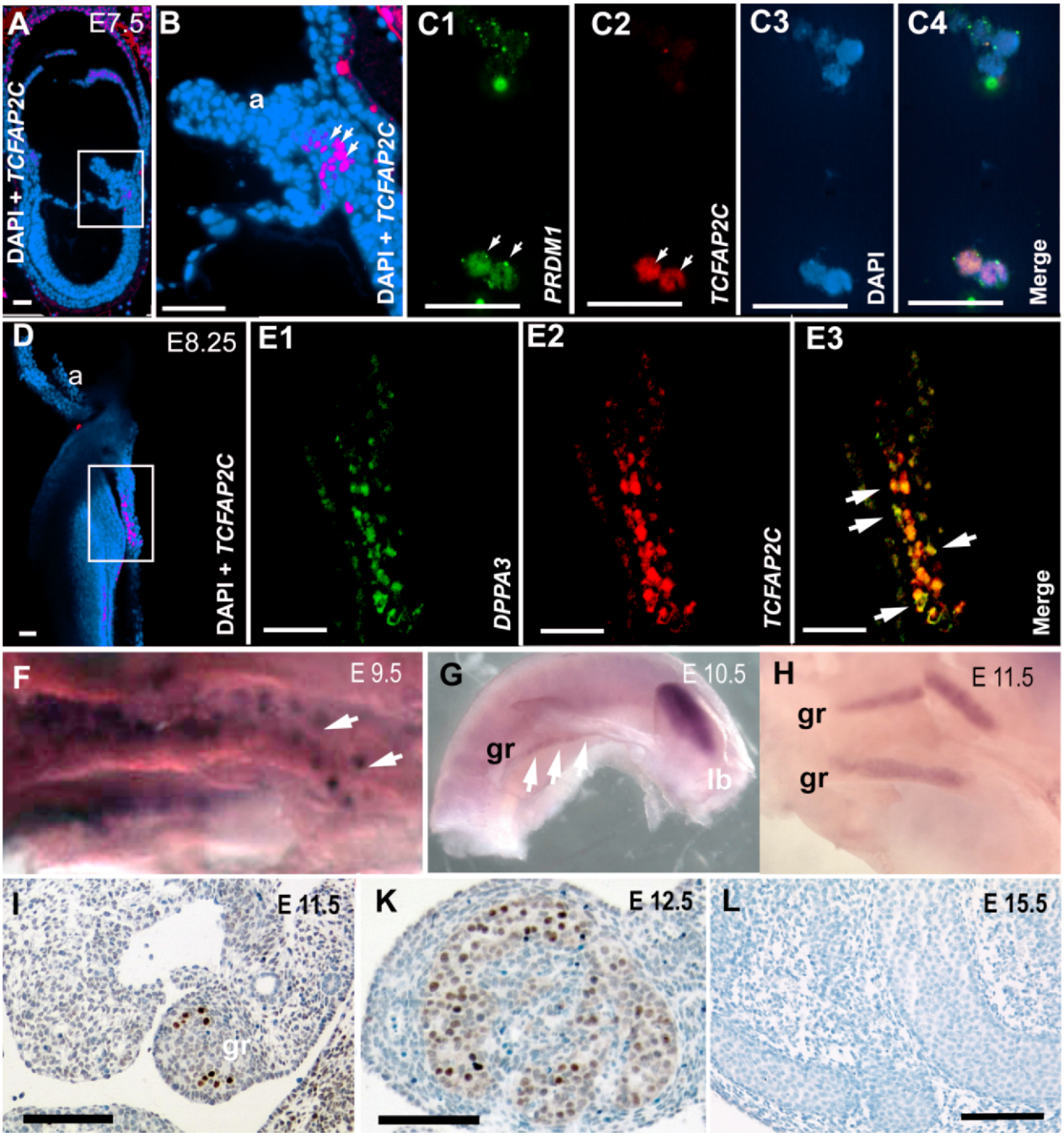


FIG. 1. *Tcfap2c* is expressed in primordial germ cells and gonocytes. Immunoflourescent detection of TCFAP2C at (**A**–**C**) E 7.25 and (**D** and **E**) E 8.25. Boxed area in **A** is enlarged in **B**. **C1**–**C4**) Double labeling of cell suspension generated by dissecting boxed area from **A** using (**C1**) PRDM1 and (**C2**) TCFAP2C antibodies; (**C3**) counterstaining with DAPI; and (**C4**) merge of **C1**–**C3**. **D**) Detection of TCFAP2C expression at E 8.25 along the forming hindgut. Boxed area in **D** is enlarged in **E1** and **E2** and shows the fluorescent signal for DPPA3 and TCFAP2C, respectively. **E3**) Merge of **E1** and **E2**, demonstrating the coexpression of both markers. Messenger RNA in situ hybridization of *Tcfap2c* at (**F**) E 9.5, (**G**) E 10.5, and (**H**) E 11.5. Arrows in **B**, **C1**, **C2**, **E3**, **F**, and **G** indicate primordial germ cells. **I**–**L**) Antibody detecting TCFAP2C in sections at (**I**) E 11.5, (**K**) E 12.5, and (**L**) E 15.5. a, allantois; gr, genital ridge; lb, limb bud. Bar = 50 μm.

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 *Tfap2c* alleles [21]. These *Tcfap2c*$^{flox/flox}$ 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*$^{flox/flox}$:*Mox2-cre* and *Tcfap2c*$^{flox/flox}$:*Alpl-cre* 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*$^{flox/flox}$ 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 *Tfap2c*$^{flox/flox}$:*Sox2-cre* 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, E 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*$^{flox/flox}$: *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*$^{flox/flox}$:*Sox2-cre* 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 head-fold 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 LysoTracker positive cells in wild type (11 $\pm$ 2.55) compared to *Tcfap2c*-deficient (11.74 $\pm$ 2.73) embryos were not significantlt 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 cells obtained from EB cultures represent candidate PGCs [20]. *Tcfap2c*-deficient ES cells were generated from *Tcfap2c*-deficient blastocysts derived from a *Tcfap2c* +/– 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*-deficient ES cells. We used

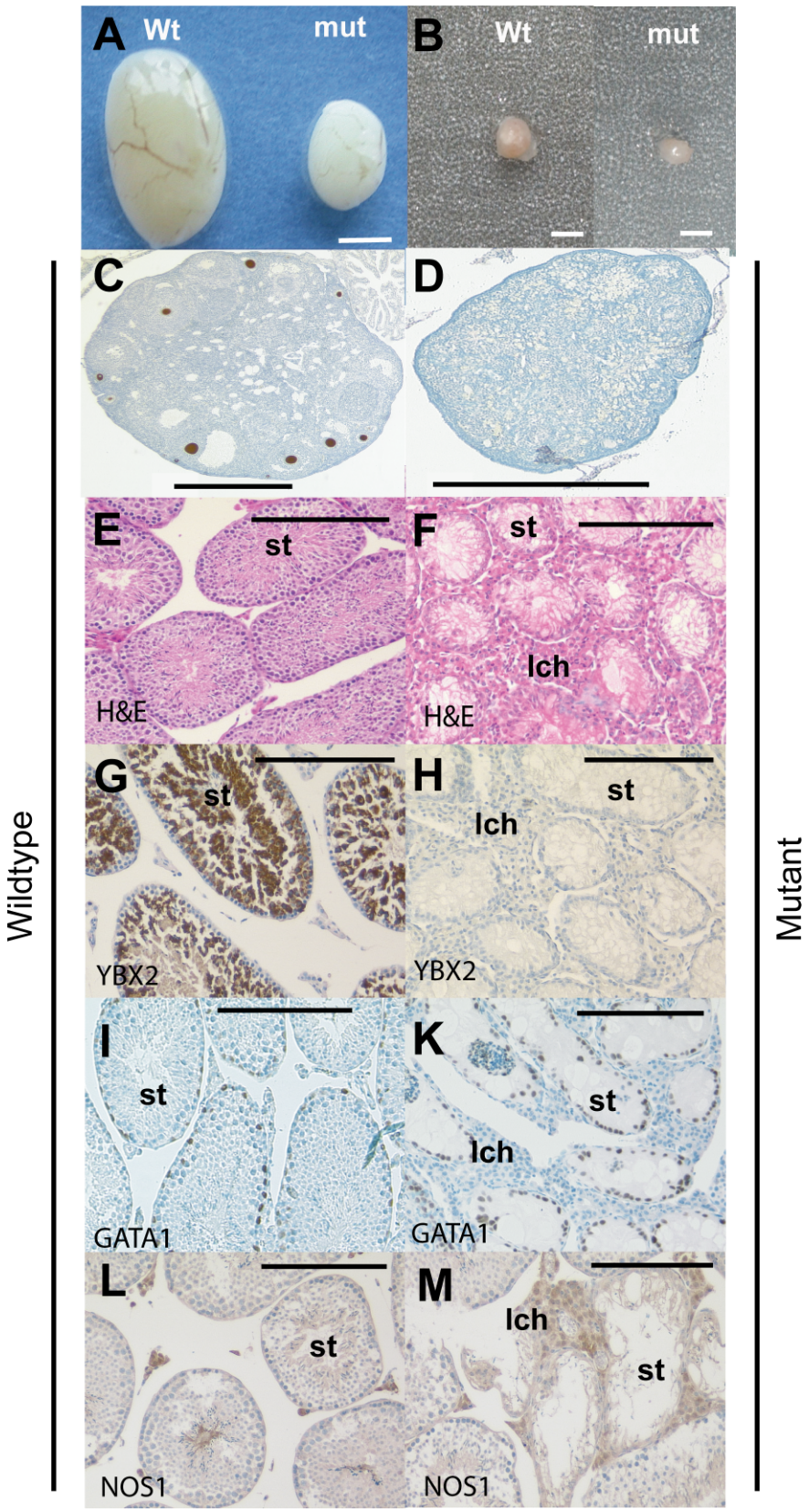


FIG. 2. Loss of *Tcfap2c* leads to lack of germ cells. **A**) Testes and (**B**) ovaries of control (Wt) and *Tcfap2c*$^{flox/flox}$:*Mox2-cre* (mut) mice. Bar = 2 mm. MSY-2 immunohistochemistry on ovarian tissue of (**C**) control and (**D**) *Tcfap2c*$^{flox/flox}$:*Mox2-cre*. Sections of (**C**, **E**, **G**, **I**, **L**) control and (**D**, **F**, **H**, **K**, **M**) *Tcfap2c*$^{flox/flox}$:*Alpl-cre* mutants stained with hematoxylin and eosin (H&E) or the antibody indicated. Specimen and sections were prepared from 3-mo-old animals. st, seminiferous tubules; lch, Leydig cell hyperplasia. Bar = 200 μm (**E**–**M**) and 500 μm (**C** and **D**).

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

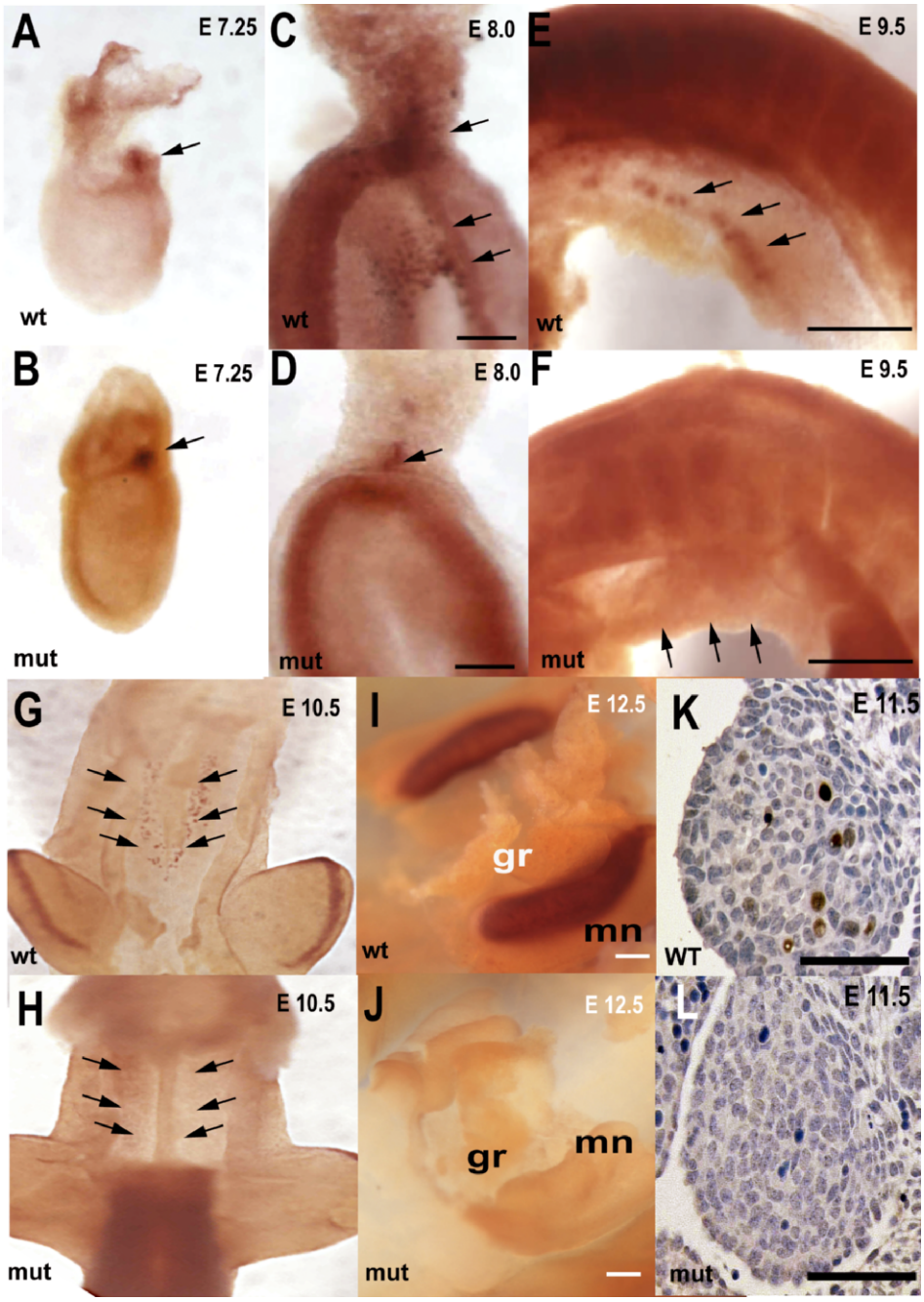


FIG. 3. *Tcfap2c* is required for germ cells. Alkaline phosphatase (AP) whole mount staining of control (wt) and mutant (*Tcfap2c*$^{flox/flox}$:*Sox2-cre*, mut) (**A** and **B**) whole embryos, (**C** and **H**) trunk, and (**I** and **J**) genital ridge at the embryonic age indicated. The arrows indicate the position of AP-positive germ cells. **K**, **L**) Antibody to GCNA detection of germ cells in sections of genital ridges of E 11.5 control (WT) and mutant (mut). gr, genital ridge; kd, kidney; mn, mesonephros. Bar =100 μm (**C**–**J**) and 25 μm (**K** and **L**).

for mature PGCs (*Nanos3*, *Dazl*, and *Mutvh*), which are also expressed in ES cells, decreased significantly in SSEA1-positive cells of *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 *Tcfap2c*-deficient EB cultures (*Hoxb1*) or were induced stronger and earlier in this assay (*Hoxa1*, *T*, and *Snai1*) (Fig. 5A, lower). Taken together, these data suggest, that in *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 *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 *Tcfap2c* in the genetic program orchestrated by *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 *PRDM1* and *PRMT5* [32], and *TCFAP2C*, *SOX17*, *KIT*, *DPPA3*, *NANOG*, and *POU5F1* [33–35]. In addition, TCam-2 cells exhibit symmetric dimethylation of arginine-3 on histones H2A and/or H4 tails (H2A/H4R3me2s) [32]. This modification has been demonstrated to be specific for PGCs and is directed by the interaction of *Prdm1* and the arginine-specific histone methyltransferase *Prmt5* [36].

First, we tested whether TCam-2 cells could serve as an in vitro model for *PRDM1*-mediated repression of the somatic program on the genetic and epigenetic level. Here, short

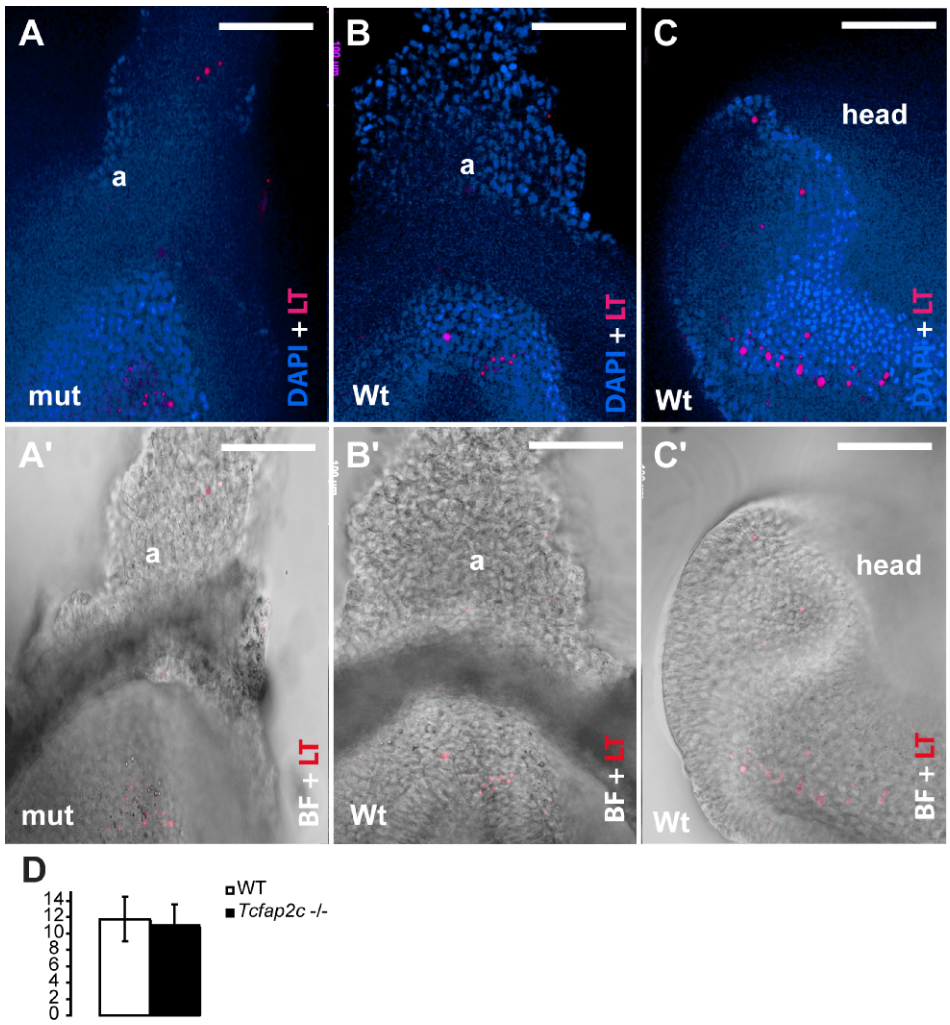


FIG. 4. Cell death is not enhanced in mutant PGCs. Photomicrography of LysoTracker analysis (red signal, LT) of (**A** and **A′**) mutant (mut) and (**B** and **B′**) wild type (Wt) animals at E 8.0. The allantois (a) and posterior end of embryo is shown. **C**, **C′**) Apoptosis in the head mesenchyme 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 *Tcfap2c* mutants (11.74 ± 2.73).

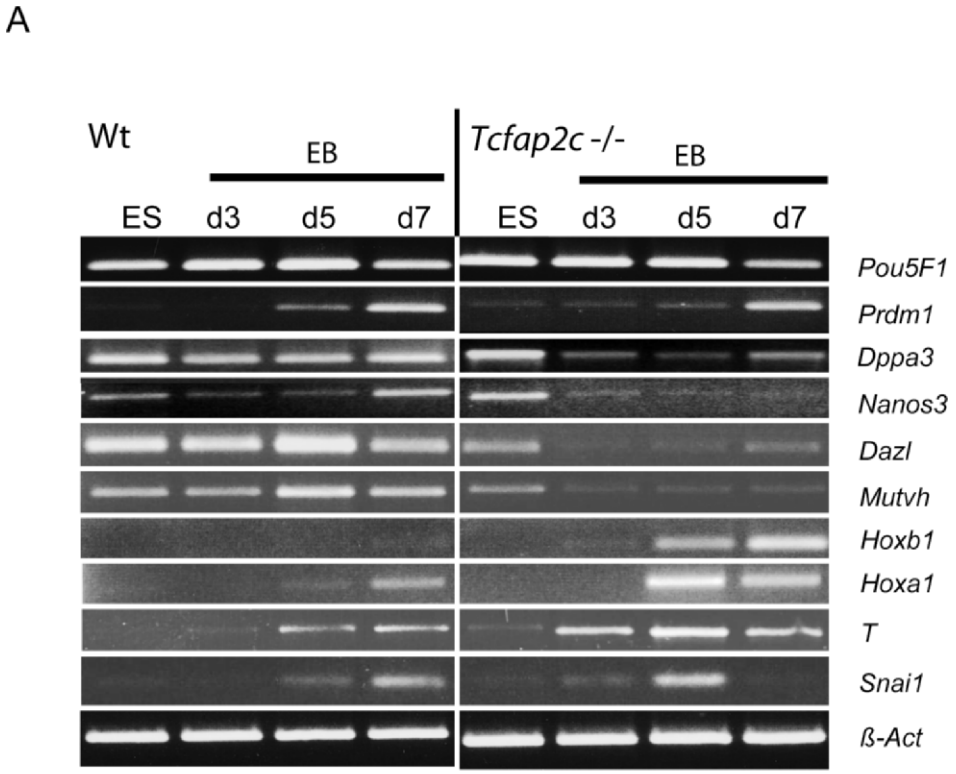
interfering RNA (siRNA)-mediated knockdown of *PRDM1* led to a reduction of *TCFAP2C* expression levels and a concomitant upregulation of *HOXB1*. In addition, expression of the *PRDM1* target gene *MYC* was induced while the expression of *NANOG* remained unaffected (Supplemental Fig. S3A). Western 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 *REXO1*), 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 *HOXA1* 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 *Snai1* 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*



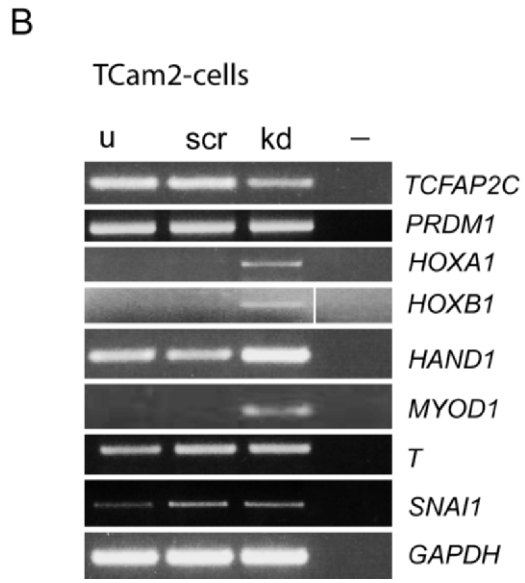


FIG. 5. Lack of *Tcfap2c* results in upregulation of somatic markers. **A**) Wild-type (Wt) and *Tcfap2c*-deficient (*Tcfap2c* –/–) ES cells were subjected to hanging drop cultures to induce embryoid body formation (EB) for 3 (d3), 5 (d5), and 7 (d7) days. SSEA1-positive cells were isolated after trypsinization, and RT-PCR analyses of expression of the genes indicated was performed. Primer sequences are given in Supplemental Table S1. The experiments were repeated 10 times. **B**) TCam-2 cells were treated with siRNA mixture to repress TCFAP2C (kd) and analyzed with respect to untreated cells (u) or cells treated with unspecific siRNA (scr). RT-PCR was used to detect the expression of the genes indicated; (–) indicates negative control without RT. Primer sequences are given in Supplemental Table S1. The experiments were repeated three times.

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* knockdown 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 cell cycle progression, and the repression of the DNA-methyltransferase machinery [9]. Using an EB-based differentiation protocol, we demonstrate induction of *Prdm1* 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 PGCs 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 IGCNU/CIS (intratubular 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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