Inactivation of *Palb2* gene leads to mesoderm differentiation defect and early embryonic lethality in mice

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Mutations of the *PALB2* tumor suppressor gene in humans are associated with hereditary predisposition to breast and also some other cancers. In the present study, we have characterized mice deficient in *Palb2*. The data show that the *Palb2*¹⁄² mice are normal and fertile, and lack macroscopic tumors when followed up till the age of 8 months. Homozygous (HO) *Palb2*²⁄² mice present with embryonic lethality and die at E9.5 at the latest. The mutant embryos are smaller in size, developmentally retarded and display defective mesoderm differentiation after gastrulation. In *Palb2*²⁄² embryos, the expression of cyclin-dependent kinase inhibitor p21 is increased, and *Palb2*²⁄² blastocysts show a growth defect *in vitro*. Hence, the phenotype of the *Palb2*²⁄² mice in many regards resembles those previously reported for *Brca1* and *Brca2* knockout mice. The similarity in the phenotypes between *Palb2*, *Brca1* and *Brca2* knockout mice further supports the functional relationship shown *in vitro* for these three proteins. Accordingly, our data in vivo suggest that a key function for PALB2 is to interact with and to build up appropriate communication between BRCA1 and BRCA2, thereby licensing the successful performance of the physiological tasks mediated by these two proteins, particularly in homologous recombination and in proper DNA damage response signaling.

INTRODUCTION

Tumor suppressor genes encode proteins that are negative regulators of cell proliferation by being involved in cell division and apoptosis, and are also often essential for the activation of repair after DNA damage. Loss of function mutations in tumor suppressors release the cells from normal growth restraint and cause uncontrolled cell proliferation, promoting the development of malignancies (1–3). Germline mutations in tumor suppressors such as *BRCA1* (breast cancer gene 1) and *BRCA2* (breast cancer gene 2) are associated with an inherited predisposition to breast cancer, and inactivation mutations of these genes in mice cause abnormalities in cellular proliferation and differentiation, and in embryonic development (1,3).

PALB2 (partner and localizer of BRCA2) was originally identified as a BRCA2-interacting protein and it plays an important role in the regulation of the activity and intracellular localization of BRCA2. In response to DNA damage, PALB2 localizes to chromatin at the site of the lesion where it assembles as oligomers, which then serve as an anchor for BRCA2. The PALB2–BRCA2 interaction is vital for the proper function of BRCA2 in the homologous recombination (HR)-based DNA double-strand break repair and in the intra-S phase checkpoint control functions of BRCA2. Accordingly, disruption of PALB2–BRCA2 interaction results in defective HR, and increases cellular sensitivity to DNA damaging agents (4,5). Recently, an interaction between PALB2 and BRCA1 was also reported. Importantly, PALB2 mediates the communication between BRCA1 and
BRCA2 by directly associating with BRCA1, and the PALB2–BRCA1 interplay is also critical for HR (6,7).

Similarly to BRCA1, defects in the PALB2 tumor suppressor gene are associated with hereditary predisposition to breast cancer. Studies on the role of PALB2 mutations in breast cancer susceptibility have been conducted in several Caucasian and Asian populations, and the results have indicated a 2–6-fold increased risk for the mutation carriers (8–18). In our previous study, we identified a relatively common Finnish PALB2 1592delT founder mutation, the carriers of which are estimated to be at a 40% risk for developing breast cancer by the age of 70 years (15). In addition, PALB2 has been suggested to be involved in some other cancer types as well (10,19).

Analogous to BRCA2, PALB2 is a Fanconi anemia (FA) associated gene also known as FANCN (8,20,21). FA is a rare autosomal or an X-linked recessive disease characterized by developmental abnormalities, progressive bone marrow failure and cancer susceptibility (22). Biallelic inactivating mutations in PALB2 cause the disease subtype FA-N. Most of the mutations observed in this type of FA are frameshifts leading to a truncated protein product and are thought to be either functionally null (8), although the hypomorphic nature of these alleles cannot be totally excluded. FA-N resembles the FA-D1 phenotype caused by biallelic mutations in BRCA2, where the affected children display typical features of FA and cellular hypersensitivity to DNA cross-linking agents. Interestingly, FA caused by biallelic mutations in BRCA2 or PALB2 has a more severe clinical phenotype than other types of FA, with higher risks of leukemia and brain tumors (8,21,23).

The biological and physiological consequences of BRCA1 and BRCA2 defects in humans have been extensively assessed by modeling in mouse. Brca1- and Brca2-deficient mice display very similar phenotypes, including early embryonic lethality and decreased cell proliferation. Thus, studies in vivo have shown that both Brca1 and Brca2 are necessary for normal embryogenesis in mice (1,3). In the present study, we have generated a mouse model lacking a functional Palb2 gene. Mice heterozygous (HE) for the insertion of a gene trap construct inactivating Palb2 (Palb2<sup>−/+</sup>) were viable, phenotypically normal and fertile, whereas HO inactivation of the gene (Palb2<sup>−/−</sup>) results in an embryonic lethal phenotype. Thus, the current analysis of Palb2 HO knockout embryos in vivo and in vitro demonstrates that Palb2 plays a critical role in early embryonal development, similarly to Brca1 and Brca2.

RESULTS

Analysis of Palb2 expression during early fetal development in mice

An embryonic stem (ES) cell line [CG0691, Sanger Institute Gene Trap Resource (SIGTR) database (http://www.sanger.ac.uk)] containing a gene trap vector inserted into the Palb2 gene on mouse chromosome 7 (Fig. 1A) was used to produce mice deficient in Palb2 expression. The insertion of the gene trap vector leads to a transcript consisting of the first exon of the Palb2 fused with a Lac-Z reporter gene (Fig. 1B). The 5′-RACE analysis of the fusion transcript further confirmed that the gene trap vector had integrated in the first intron of Palb2. The absence of Palb2 expression in the Palb2<sup>−/−</sup> mice was confirmed by using quantitative RT–PCR (Fig. 2).

The expression of Palb2 in HO knockout mice (harboring only the mutant allele construct Palb2 Ex1-bgeo) was visualized by using X-Gal staining. The data indicated that Palb2 was broadly expressed in the mouse embryo, and high levels of expression were detected as early as at embryonic day 7.5 (E7.5) in the primitive streak (Fig. 1C, upper panel). At E8.5, Palb2 expression was observed in the open neural folds both at the anterior and posterior regions of the body, and the expression was ubiquitous (Fig. 1C, lower panel).

Palb2 mutation results in embryonic lethality

Mice HE for the Palb2 gene trap insertion (Palb2<sup>+/-</sup>) were viable, phenotypically normal and fertile. To address whether the loss of one Palb2 allele would lead to increased frequency of spontaneous tumors, HE mice were followed up till the age of 8 months, but no macroscopic tumors were observed in any of the organs analyzed (mammary, kidney, heart, adrenals, lungs, liver, intestinal, spleen, brain, ovary, testis, epididymis and prostate). However, we cannot, at this point, completely rule out the slight possibility that the HE mice may still develop some tumors at a more advanced age. For this reason, HE mice will be followed up until they are at least 2 years old. No viable Palb2<sup>−/-</sup> mice were identified among the 200 pups genotyped at the age of 2 weeks from HE matings. This indicated that in mice the HO inactivation of Palb2 results in embryonic lethality. To assess the consequences of the loss of Palb2 on embryonic development, embryos from HE intercrosses were analyzed at different stages of gestation. At E7.5, Palb2<sup>−/-</sup> embryos were detected at the expected Mendelian frequency. However, resorptions and empty conceptus were observed from this stage onward. No viable Palb2<sup>−/-</sup> mouse embryos were observed beyond E9.5. This is also evident from the observed significant deviation from the expected Hardy–Weinberg genotype distribution analyzed at birth (P = 0.006 for E9.5 and E11.5 embryos and P = 9.41 e−14 for postnatal mice, Table 1). In addition, several attempts were made to generate Palb2<sup>−/-</sup> ES cell lines, but all of them proved unsuccessful (data not shown).

Palb2 mutation results in morphological and histological abnormalities

The morphology of the Palb2<sup>−/-</sup> embryos was already distinguishable from the wild-type (WT) and Palb2<sup>+/+</sup> littermates at E6.5 (data not shown) and the histological analysis revealed that the Palb2<sup>−/-</sup> null embryos were markedly smaller in size than their WT littermates (Fig. 3A–C), even though the two-layer structure of ectodermal and endodermal cells enclosing the proamniotic cavity was readily identified in some of the knockout embryos (Fig. 3B and C). By E7.5, the difference in size between WT and Palb2<sup>−/-</sup> embryos was even more obvious. In whole mount analysis of the null embryos, only a poorly defined boundary between the embryonic and extra embryonic regions was observed, in contrast to the proper ectoplacental cone, primitive streak and head fold present in the WT embryos (Fig. 4A and B). Histological
analysis of WT embryos revealed a well-defined anterior–posterior axis with a proper formation of the epiblast and the typical structures for the mesodermal layer, ectoplacental cone, chorion and amnion (Fig. 3D) and the embryos had initiated gastrulation, indicated by a formation of the primitive streak. However, the embryonic ectoderm of the \textit{Palb2}(2\textsuperscript{+/2}) embryos appeared with an increased mass of cells and the embryonic cavities were not properly formed. In addition, the amnion of the \textit{Palb2}(2\textsuperscript{+/2}) embryos was undetectable (Fig. 3E and F). At E8.5, the \textit{Palb2}(2\textsuperscript{+/2}) embryos showed normal extra embryonic structures (Fig. 4C and D), while the difference in size between the WT and \textit{Palb2}(2\textsuperscript{+/2}) embryos was further increased (Fig. 4E and F). The \textit{Palb2}(2\textsuperscript{+/2}) embryos had developed a head fold and a neural groove, the mesoderm formation and patterning was initiated, as indicated by \textit{Lim1} expression, being a marker for intermediate mesoderm, primitive streak-derived tissues and extra embryonic tissues for head formation. However, the mesoderm failed to differentiate properly as the somite structures never formed (Fig. 5). Analyzing \textit{sonic hedgehog (Shh)} expression revealed a notochord with diffuse and discontinuous morphology (Fig. 5). Furthermore, lack of proper \textit{Brachyury} expression (data not shown) revealed that the \textit{Palb2}(2\textsuperscript{+/2}) embryos lacked proper tail-bud structures, and the notochord did not progress caudally. At E9.5, the \textit{Palb2}(2\textsuperscript{+/2}) embryos did not show proper organogenesis (Fig. 4I and J). In addition, most of the mutant embryos were reabsorbed, or were undergoing degeneration within the yolk sac (Table 1).

**Disruption of \textit{Palb2} in mouse decreases embryonic growth in vitro and in vivo**

The \textit{Palb2}(2\textsuperscript{+/2}) blastocysts showed a normal appearance, indicating that the lack of functional \textit{Palb2} protein does not affect the pre-implantation stage of development (Fig. 6A and B). However, 85\% of the mutant blastocysts presented with
impaired outgrowth of the inner cell mass (ICM) in vitro, while only 25% of ICM of the WT and HE blastocysts failed to grow (Fig. 6C–F). This was in contrast to the trophoblastoid giant cells that developed normally in the cultured Palb2\(^{−/−}\) embryos (Fig. 6D and F). These results are consistent with the growth retardation observed in the mutant embryos in vivo.

Expression of p21 is increased in Palb2 mutant embryos

Quantitative RT–PCR analysis was used to confirm the lack of Palb2 mRNA in the Palb2\(^{−/−}\) embryos (Fig. 2), and the level of Brca1, Brca2, Rad51, p53, p27 and p21 expression was also determined. No difference was observed between the Palb2\(^{−/−}\) and WT embryos in their expression of Brca1, Brca2 or Rad51 (data not shown). However, the reduced growth of the Palb2\(^{−/−}\) embryos were associated with a 6-fold increased expression of p21 (\(P = 0.002\)), while the levels of p53 (\(P = 0.435\)) and p27 (\(P = 1.000\)) mRNAs were not altered in Palb2\(^{−/−}\) embryos when compared with the WT littermates (Fig. 2).

DISCUSSION

Studies have indicated that PALB2 interacts with both BRCA1 and BRCA2 in vitro, and has a crucial role on their DNA damage response functions (5–7). Previous studies have also shown that BRCA1 and BRCA2 have indispensable roles in early mouse embryogenesis, and Brca1 and Brca2 deficiency has been showed to cause embryonic lethality in mice (1,3,24,25). Identically, in the present study, the Palb2 deficient mouse embryos were found to be abnormal as early as at E6.5, and by E7.5 all embryos were developmentally retarded, although they had initiated gastrulation before dying at around E9.5. Similarly to Palb2\(^{−/−}\) mice, embryos lacking Brca1 or Brca2 show very severe growth retardation at E6.5, and Brca1 deficient embryos die before E7.5, and Brca2 deficient embryos before E9.5 (1,3). The phenotypic similarity between Brca1, Brca2 and Palb2 deficient embryos is an agreement with the data indicating that PALB2 acts in the same cellular processes as BRCA1 and BRCA2. Furthermore, similarly to the results from conventional Brca1 and Brca2 genetic defect modeling in mouse (26), the Palb2 heterozygotes studied here carrying one defective allele together with one WT copy did not, at least to this date of follow up, display an increased propensity for tumor development. However, it is not yet fully understood to what extent the disease modeling for BRCA1, BRCA2 and PALB2 in mouse corresponds to the situation in humans.

Our inability to generate ES cells deficient in Palb2, together with the abundant Palb2 expression in the primitive streak already evident at E7.5, suggest that Palb2 is essential for cell proliferation during embryogenesis in rapidly dividing cells. In addition, at E8.5, Palb2 was found to be broadly expressed in the entire embryo, but was particularly high in the open neural folds at both the anterior and posterior regions of the embryo, in accordance with an increased cell proliferation at these regions. The essential role for Palb2 in cell proliferation is also supported by our studies in vitro showing that at E3.5 the Palb2\(^{−/−}\) blastocysts appeared normal, but their ICM did not proliferate normally. Similarly to that shown for Brca2 mutant blastocysts, a growth defect was observed in Palb2\(^{−/−}\) blastocyst culturing. Thus, it appears that neither Palb2, Brca2, nor Brca1 deficient embryos are capable of growing in vitro.

Gastrulation in the mouse embryo takes place at around E6.5, upon which the mesoderm is formed from the epiblast. In this process, the volume of the embryonic tissue increases up to 40-fold, largely due to the expansion of the tissue giving rise to the extra embryonic ectoderm. Accordingly, between E5.5 and E7.5, the cell number in the embryo increases by over 100-fold (27,28). Palb2\(^{−/−}\) mouse embryos initiate gastrulation at E7.5, but the proper formation of embryonic cavities is disrupted and the amnion is missing. These defects are also observed in Brca1 and Brca2 mutant embryos (1,3) and a reduced size is the shared feature of Brca1, Brca2 and Palb2 deficient embryos. However, while the differentiation of Brca1 and Brca2 stops at gastrulation, Palb2\(^{−/−}\) mutant embryos show neural plate and neural groove development. Furthermore, by E8.5, also neural folds were formed in the Palb2\(^{−/−}\) embryos, but the more posterior structures never form a proper neural tube. Thus the Palb2 deficient embryos develop to a slightly more advanced stage when compared with the Brca1 and Brca2 deficient mice. Accordingly, shh expression revealed the formation of notochordal process in the Palb2\(^{−/−}\) embryos, but the
notochordal mesoderm remained diffuse and showed interrupted pattern and failure to form a proper notochord from the pre-chordal plate more posteriorly. A similar discontinuous notochordal structure has been observed in \textit{Palb2} \textsuperscript{-/-} embryos, the development of which, however, progresses somewhat further but results in severe posterior dysgenesis (29). In \textit{Palb2} \textsuperscript{-/-} embryos, the most cranial structures are formed but the paraxial mesoderm remains undifferentiated as indicated by the lack of somite formation in the growth retarded embryos, but thereafter the differentiation is either deceased or severely delayed. We could not detect any epithelialization of the paraxial mesoderm, nor was heart

**Table 1.** Genotype distribution of the mice from heterozygote breeding

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of mice</th>
<th>+/-</th>
<th>++/-</th>
<th>--/-</th>
<th>Absorbed</th>
<th>Deviation from HWE (P-value)</th>
</tr>
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<tbody>
<tr>
<td>E7.5</td>
<td>20</td>
<td>5 (26%)</td>
<td>11 (54%)</td>
<td>4 (20%)</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>E8.5</td>
<td>35</td>
<td>10 (29%)</td>
<td>18 (51%)</td>
<td>4 (12%)</td>
<td>3 (8%)</td>
<td>0.35</td>
</tr>
<tr>
<td>E9.5</td>
<td>157</td>
<td>49 (31%)</td>
<td>72 (46%)</td>
<td>8 (5%)</td>
<td>28 (18%)</td>
<td>0.006</td>
</tr>
<tr>
<td>E11.5</td>
<td>39</td>
<td>10 (26%)</td>
<td>21 (54%)</td>
<td>0</td>
<td>8 (20%)</td>
<td>0.006</td>
</tr>
<tr>
<td>Postnatal, 1d</td>
<td>200</td>
<td>62 (31%)</td>
<td>138 (69%)</td>
<td>0</td>
<td>–</td>
<td>9.41 ( e^{-14} )</td>
</tr>
</tbody>
</table>

The embryos were collected on days 7.5, 8.5, 9.5 and 11.5 of pregnancy from \textit{Palb2} heterozygous intercrosses. The genotypes were determined by PCR using DNA extracted from the yolk sac of E7.5–E11.5 embryos, or earmarks of postnatal mice. Resorptions were not subjected to genotypic analysis and were therefore not accounted for in the HWE analysis.

E, embryonic day; HWE, Hardy–Weinberg equilibrium.

**Figure 3.** Histological analysis of \textit{Palb2} \textsuperscript{-/-} embryos at E6.5–E7.5. (A) Hematoxylin-eosin staining of a WT embryo at E6.5. (B and C) \textit{Palb2} \textsuperscript{-/-} embryos at E6.5. (D) WT embryo at E7.5. (E and F) Abnormal formations of embryonic cavities are observed in \textit{Palb2} \textsuperscript{-/-} embryos at E7.5. Only the ectoplacental cavity was detectable, and the cell mass was increased in the amniotic cavity region (arrow in E). (F) The histological appearance of the \textit{Palb2} \textsuperscript{-/-} embryos at E7.5 was variable, while some of the embryos lacked almost all normal histological structures. ac, amniotic cavity; am, amnion; ex, exocoelomic cavity; ch, chorion; epc, ectoplacental cavity; ep, ectoplacental cone; cc, ectoderm; me, mesoderm; cn, endoderm; ven, visceral endoderm. Bars 100 \( \mu \)m.
formation observed in the mutant embryos which speaks further for a failure in mesodermal organogenesis. Therefore, the phenotype observed in $\text{Palb2}^{2/-}$ embryos cannot be explained solely based on lack of the proliferation boost after gastrulation, but there is also a failure in the differentiation of

Figure 4. Morphology of $\text{Palb2}^{2/-}$ embryos. (A and B) At E7.5, the $\text{Palb2}^{2/-}$ embryos were smaller than their WT littermates, and presented with a poorly defined boundary between the embryonic and extra embryonic region (arrow). (C and D) A WT and $\text{Palb2}^{2/-}$ embryo at E8.5 with a normal yolk sac. (E and F) The $\text{Palb2}^{2/-}$ embryos were notably smaller and developmentally retarded when compared with the WT embryos. (G)

Figure 5. Expression of mesodermal markers in E8.5 $\text{Palb2}^{2/-}$ embryos. Whole mount RNA in situ analysis of $\text{Lim1}$ in WT (A) and in $\text{Palb2}^{2/-}$ embryos (B). Shh expression of WT (C and E) and mutant embryos (D–F). Dorsal view of same embryos shows discontinuous (square) notochord of $\text{Palb2}^{2/-}$ embryos (D). A higher magnification of such an interruption of the Shh pattern of the notochord in $\text{Palb2}^{2/-}$ (arrowheads in F). Shh expression in notochord of WT embryos (C and E). Histological serial vibratome sections of E8.5 WT (G) and $\text{Palb2}^{2/-}$ embryos (H) with Shh in situ hybridization counterstained with eosin, showing complete absence of somites. nt, neural tube; n, notochord; ps, primitive streak; lpm, lateral plate mesoderm; im, intermediate mesoderm; pm, paraxial mesoderm. Scale bar, 200 μm.

E9.5 WT embryos with a normal appearance of the yolk sac and its vascularization. (I) In $\text{Palb2}^{2/-}$ embryos at E9.5, the yolk sac was smaller and paler than in WT embryos. (J) A WT embryo at E9.5. (K) $\text{Palb2}^{2/-}$ embryos at E9.5 were encased in a spherical structure formed by the yolk sac and the amnion, and completely voided of normal morphology.
that the PALB2 protein is essential for cell proliferation. We have also provided evidence supporting the requirement of Palb2 for cell cycle progression in early embryonic cells. Indeed, Palb2 seems to be indispensable for early embryonic development in mice. Defective Palb2 function during early embryonic development results in insufficient cell proliferation, which might originate from the accumulation of a lethal amount of DNA damage, and in a mesoderm differentiation failure. Together these problems occurring at such an early embryonal developmental stage are eventually causing the death of the Palb2<sup>0/0</sup> mouse embryos. Further, establishment of conditional Palb2 mutant mice will help define the precise role and mode of action of Palb2 during organogenesis and tumorigenesis.

**MATERIALS AND METHODS**

**Generation of Palb2 gene trap mice**

The gene trap clone CG0691 (Sanger Institute Gene Trap Resource) was injected into C57BL/6N mouse blastocysts to generate chimeric mice. Breeding of the chimeric mice with C57BL/6N mice produced HE (Palb2<sup>+/−</sup>) offspring carrying the trapped allele. In order to locate the exact integration site of the gene trap insertion, overlapping PCR amplicons compatible with a gene trap-specific 5′ reverse primer were designed to cover intron 1 of the Palb2 gene. Primers used for the PCR reaction are indicated in Supplementary Material, Table S1. Direct sequencing revealed the exact integration site of the gene trap (data not shown). All the studies were carried out in a mixed genetic background (129/C57BL/6N, 1:1). The C57BL/6N mice used as blastocyst donors were obtained from Charles River Laboratories (Willmington, MA, USA). The mice were fed with a Soya-free diet, and they were maintained in a specific pathogen-free stage at the Central Animal Laboratory of the University of Turku. All studies carried out with the mice were approved by the Finnish ethical committee for experimental animals (2008-00142), complying with international guidelines on the care and use of laboratory animals.

**Genotype analysis**

Genotyping of the mice was performed on DNA, extracted from yolk sacs of E7.5–E9.5 embryos, or earmarks of 2-week-old mice. Primers (WTs) 5′-CTTTTGGGGGA TAGCACTCA-3′ and (WTa) 5′-TTGTTGCTGCTGAGT TCCTG-3′, specific for the targeted intronic sequence, and a primer from the gene trap (Gta) 5′-AGTATCGGCCTCA GGAAGATCG-3′ were used to detect the WT and the mutant allele, respectively. Primer pair WTs/WTa amplified a 720 bp fragment in both heterozygote and WT DNA samples, whereas the primer pair WTs/GTa amplified a 510 bp fragment in both Palb2<sup>0/0</sup> and Palb2<sup>0/0</sup> DNA. The mutant allele was also identified by primers 5′-TTA TCG AGC GTG GTG GTG ATT C-3′ and 5′-GGC GCT ACA TCG GGC AAA TAA TAT C-3′ that amplified a 680 bp internal segment of the Lac-Z reporter gene.
β-Galactosidase staining

E7.5–E9.5 embryos were used to visualize the expression of the fusion gene resulted from the gene trap insertion. The embryos were dissected, washed in PBS and fixed in PBS (pH 7.3) containing 0.2% glutaraldehyde, 2 mM MgCl₂ and 5 mM EDTA for 30 min. Thereafter, the embryos were washed overnight in PBS containing 0.02% NP-40, 2 mM MgCl₂ and 0.01% Na-deoxycholate, and incubated overnight at 37°C with β-galactosidase substrate (X-Gal; 1 mg/ml; Fermentas, MD, USA) in washing buffer, supplemented with 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide. The stained embryos were further fixed in 4% paraformaldehyde at room temperature for 20 min, washed with PBS and photographed.

Histology

E6.5–E8.5 embryos were isolated in ice-cold PBS, fixed overnight in 4% paraformaldehyde at 4°C, dehydrated and embedded in paraffin. Four micrometer thick sections were cut and stained with hematoxylin and eosin. embedded in paraffin. Four micrometer thick sections were cut and stained with hematoxylin and eosin.

In situ hybridization

Whole-amount in situ hybridization was performed using DIG-labeled cRNA probes for Lim1, Shh, Brachyury as described by Wilkinson and Green (1990; 34) using InSituPro automate (Intavis, Germany). Samples were photographed with a Leica stereomicroscope equipped with a DC300 camera and the IM1000 software. For histology, fixed whole-amount embryos were embedded in 5% agarose and 10 μm vibratome sections were cut (Leica vibratome), counterstained with eosin, analyzed and photographed with Zeiss Axiophot II microscope with Axioscam and AxioVision 3.1 software.

Blastocyst culture in vitro

Palb2(+/-) males and females were mated and E3.5 embryos were collected by flushing the uterus of the pregnant females with PBS. Blastocysts were individually cultured in ES cell medium without leukemia inhibitory factor on gelatin of feeder cells (Gibco, Invitrogen, Carlsbad, CA, USA) in 5% CO₂ at 37°C. Cultured embryos were followed every 24 h for 7 days and the morphology of the embryos was determined, and the embryos were genotyped by PCR.

Analysis of mRNA expression

Total RNA was isolated from the E8.5 mouse embryos with the RNeasy Mini Kit (Qiagen, Hilden, Germany), and 1 μg of total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using reverse transcriptase (Finnzymes Oy, Espoo, Finland), and quantitative RT–PCR was performed using the primers for mouse Brca1, Brca2, Rad51, p21, p53, p27 and Palb2. All primers used in qRT–PCR are described in Supplementary Material, Table S2. Quantitative RT–PCR reactions were carried out using DNA Engine Opticon system™ (MJ Research, MA, USA), and the QuantiTect SYBR Green RT–PCR Kit (Qiagen, CA, USA) with continuous fluorescence detection. All qRT–PCR data were normalized to the expression of mouse Ppia gene.

Statistical methods

The genotypic distribution of the embryos at different age (E7.5–E11.5 and 1d) was analyzed for concordance to the Hardy–Weinberg equilibrium, by using the Pearson’s χ² or Fisher’s exact tests. The online Hardy–Weinberg equilibrium test (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to conduct this assessment. All P-values were two sided. The results of qRT–PCR were analyzed by t-test or Mann–Whitney rank-sum test. Significance was set as P < 0.05 and the values were presented as mean ± SD.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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