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**Palb2** synergizes with **Trp53** to suppress mammary tumor formation in a model of inherited breast cancer

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Germ-line mutations in **PALB2** lead to a familial predisposition to breast and pancreatic cancer or to Fanconi Anemia subtype N. **PALB2** performs its tumor suppressor role, at least in part, by supporting homologous recombination-type double strand break repair (HR-DSBR) through physical interactions with **BRCA1**, **BRCA2**, and **RAD51**. To further understand the mechanisms underlying **PALB2**-mediated DNA repair and tumor suppression functions, we targeted **Palb2** in the mouse. **Palb2**-deficient murine E5 cells recapitulated DNA damage defects caused by **PALB2** depletion in human cells, and germ-line deletion of **Palb2** led to early embryonic lethality. Somatic deletion of **Palb2** driven by **K14-Cre** led to mammary tumor formation with long latency. Codelivery of both **Palb2** and Tumor protein 53 (Trp53) accelerated mammary tumor formation. Like **BRCA1** and **BRCA2** mutant breast cancers, these tumors were defective in **RAD51** focus formation, reflecting a defect in **Palb2** HR-DSBR function, a strongly suspected contributor to **Brca1**, **Brca2**, and **Palb2** mammary tumor development. However, unlike the case of **Brca1**-mutant cells, Trp53bp1 deletion failed to rescue the genomic instability of **Palb2**- or **Brca2**-mutant primary lymphocytes. Therefore, **Palb2**-deficient DNA damage control is, in part, distinct from that executed by **Brca1** and more similar to that of **Brca2**. The mechanisms underlying **Palb2** mammary tumor suppression functions can now be explored genetically in vivo.


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the resulting transcript is also a candidate for degradation via nonsense-mediated decay.

Targeting of the Palb2 locus and integration of both loxP recombination sites was confirmed by Southern blot analysis (Fig. S1C). Heterozygous ES cells (Palb2fl/fl) were injected into blastocysts, and the resulting chimeras from two individual clones were bred to either Fip-deleter mice (to eliminate the Frt-flanked neoycin resistance cassette and generate a conditional allele) or Cre-deleter mice (to generate a conventional Palb2 KO allele). Germ-line transmission of the Palb2fl/fl allele occurred from nearly all chimeras, and mice were successfully genotyped for the Fip- and the Cre-recombined alleles (Palb2fl/fl or Palb2+/−, respectively).

We attempted to derive ES cells from the Palb2−/− allele. Three, independent Palb2−/− ES cell lines were derived from a single, heterozygous Palb2fl/fl cross. Expression analysis of Palb2 mRNA by quantitative real-time RT-PCR (qRT-PCR) confirmed that one of these ES lines was Palb2fl/fl and the other two were Palb2+/− (Fig. S1D). The loss of full-length Palb2 expression from these lines was further confirmed by Western blotting, using a polyclonal anti-mouse PALB2 antibody raised against the N-terminal 200 residues of the mouse PALB2 protein (Fig. 1B). Therefore, Palb2 loss did not prevent ES cell derivation and subsequent survival. The three ES cell lines we derived were morphologically comparable, proliferated at the same rate as wild-type (WT) ES cells and were capable of differentiation into embryoid bodies.

ES cells that are deficient for Brca1 or Brca2 have been notoriously difficult to isolate and are severely compromised in their proliferation (17, 18). In keeping with these findings, Palb2−/− ES cells could not be derived from embryos carrying a conventional PALB2 gene-trap allele (19). Because PALB2 operates immediately upstream of BRCA2 and is required for BRCA2 localization at DNA double strand breaks, it is possible that the viability and robustness of our Palb2−/− ES cells were due to residual expression of a truncated PALB2 species. Such a polypeptide could, in theory, result from translation initiation downstream of the engineered Palb2 genomic deletion. No such truncated protein was detected with our polyclonal antibody (Fig. 1B).

To test whether the conditional gene targeting approach that was used had generated an allele that would be rendered null after Cre action, the response of Palb2fl/fl and Palb2−/− ES cells to DNA damaging agents that cause double strand breaks was analyzed. Normally, exposure of PALB2-proficient cells to ionizing radiation (IR) leads to the formation of phosphorylated histone H2A.X (γH2AX) nuclear foci and subsequent recruitment of BRCA1, BRCA2, and RAD51 to these structures. As expected, after exposure to IR, γH2AX and BRCA1 IRIF formation was unaffected in Palb2−/− cells (Fig. 1C, Fig. S2A). However, the recruitment of RAD51 was severely compromised (Fig. 1C). This defect was also evident at the biochemical level because no increase in chromatin loading of RAD51 after IR could be detected in nuclear extracts of IR-treated Palb2−/− cells (Fig. 1D).

Because biallelic PALB2 mutations in humans cause Fanconi anemia, a hallmark of which is increased sensitivity to DNA cross-linking agents such as mitomycin C (MMC), the sensitivity of Palb2−/− ES cells to MMC as well other DNA damaging agents was assayed. Both Palb2−/− ES lines displayed increased sensitivity to MMC, IR, and the radiomimetic drug, neocarzinostatin (Fig. 1E, Fig. S2 B and C). These findings further imply that these Palb2−/− cells are functional KOs for Palb2 because they are compromised in multiple, known Palb2-associated functions. Thus, upon Cre-mediated recombination in vivo, the aforementioned conditional Palb2 allele appears to be converted to a Palb2-null allele.

**Loss of Palb2 in the Germ Line Results in Early Embryonic Lethality.** Germ-line deletion of Brca1 or Brca2 results in early embryonic lethality (17, 20, 21). Although Palb2−/− ES cells displayed no apparent growth defects compared with Palb2fl/fl controls, Palb2 loss could still be deleterious in differentiated progeny cells, and thereby negatively affect mouse development. Indeed, we were unable to obtain Palb2−/− mice from heterozygous crosses (Fig. S3A), consistent with previous reports (19, 22). Dissection of embryos from timed pregnancies revealed that Palb2-null embryos could be recovered only up to E12.5, but even then at sub-Mendelian ratios. These embryos repeatedly exhibited severe malformations. At earlier time points, morphological aberrations of Palb2−/− embryos were less obvious. However, these mutant embryos were clearly smaller than WT or heterozygous littermate embryos (Fig. S3F), and some displayed exencephaly as well as malformations of the placental labyrinth and yolk sac-associated blood islets (Fig. S3 C–F). The fact that Palb2 nulligosity resulted in embryonic lethality detectable at E8.5–E10.5 is consistent with earlier reports showing that homozygous Palb2-deficient mice also die during embryogenesis at ~E8.5 (19, 22).

Embryonic lethality due to loss of Brca1 or Brca2 can be delayed by concomitant loss of P53 (encoded by Trp53) or the CDK inhibitor p21 (encoded by Cdkn1a gene) (23, 24). Trp53 loss also delayed the lethality of Palb2 KO embryos, which otherwise exhibited increased p21 abundance (22). We therefore tested whether loss of p21 expression affects Palb2−/− embryonic lethality by generating Palb2; Cdkn1a−/− embryos. As expected, loss of p21 expression did delay embryonic lethality of Palb2 KO embryos by 2–3 d (Fig. S3A). However, all Palb2/Cdkn1a double KO embryos still displayed multiple malformations and impaired growth compared with Palb2 heterozygous or WT littermates.
and were eventually resorbed. Therefore, these Palb2<sup>fl/fl</sup> embryonic rescue effects were incomplete and failed to suppress embryonic lethality.

Because the establishment of the placenta and onset of embryonic hematopoiesis are critical steps in development that take place around the time of lethality of Palb2 embryos, we asked whether the lethality of Palb2 KO embryos could be bypassed by a WT placenta. To this end, we used the Palb2<sup>+/-</sup> and Palb2<sup>fl/fl</sup> ES cells we had generated to perform tetraploid complementation assays. In this assay, diploid KO ES cells are aggregated to tetraploid WT blastocysts to generate KO embryos that are supported by a WT placenta because the tetraploid WT blastomeres are still capable of forming a placenta but cannot contribute to the embryo.

We found that embryos derived from the Palb2<sup>fl/fl</sup> ES cells were already underdeveloped and malformed at E9.5 compared with their Palb2<sup>+/-</sup> counterparts (Fig. S3 I and J). At E12.5, embryos derived from Palb2<sup>fl/fl</sup> ES cells appeared normal whereas embryos from Palb2<sup>fl/fl</sup> ES cells had been resorbed (Fig. S3 K and L). Likewise, breeding of the Palb2<sup>fl/fl</sup> conditional allele to Meox2-Cre knock-in (K1) mice (in which Cre is expressed from the endogenous Meox2 locus only in the embryo proper and not in the placenta or extraembryonic tissues) (25) only yielded viable mice in which the Palb2 deletion was incomplete. Collectively, these findings indicate that Palb2 is an essential gene during development, and its deficiency in the embryo proper is incompatible with life. These findings are analogous to previous results showing that the lethality of Brca1<sup>+/−</sup> embryos could not be rescued by tetraploid complementation assay (20).

**Palb2 is a Breast Tumor Suppressor in Mice.** To assess the effect of Palb2 loss-of-function on mammary tumorigenesis, we crossed Palb2<sup>fl/fl</sup> mice with keratin 14-promoter-driven Cre (K14-Cre) transgenic mice (26). K14-Cre transgenic animals preferentially express Cre recombinase in the basal epithelium of the mammary ducts, as well as in skin and oral mucosa. K14-Cre has previously been used to model murine Brca1 and Brca2 mammary tumorigenesis (27).

PALB2, like BRCA1 and -2, appears to be a breast cancer suppressor in humans (9, 10, 28). Therefore, in an effort to develop a tractable system for studying how Palb2 operates in this regard, we set out to develop a Palb2 mouse breast cancer model. Mammary tumor formation initiated by BRCA1 or BRCA2 loss requires concomitant loss of functional p53 (encoded by Trp53 in mice) (27, 29). This observation was considered in efforts to establish a Palb2 model.

We first generated Palb2<sup>−/−</sup>/Trp53<sup>−/−</sup> double conditional mice by crossing Palb2<sup>fl/fl</sup>, K14-Cre transgenic mice with Trp53 conditional normal mice. All mice that harbored the conditional alleles for Palb2 and/or Trp53 in the absence of K14-Cre were phenotypically normal, fertile, and capable of nursing their litters. During the period of tumor monitoring (up to 600 d after birth), Trp53<sup>−/−</sup>; K14-Cre female mice developed spontaneous mammary tumors with a frequency of ~80% and a mean tumor-free interval (T<sub>1/2</sub>) of 320 d. By contrast, Palb2<sup>fl/fl</sup>; Trp53<sup>−/−</sup>; K14-Cre double conditional mice developed tumors much faster (T<sub>1/2</sub> = 192 d, P = 2.4 × 10<sup>−5</sup>), indicating that Palb2 loss accelerates tumor formation on a Trp53<sup>−/−</sup> background (Fig. 2A). These latencies are comparable with Brca2<sup>−/−</sup>; K14-Cre and Brca1<sup>−/−</sup>; K14-Cre mice (T<sub>1/2</sub> = 181 and 213 d, respectively) (27, 30).

Somatic loss of one Trp53 allele displayed, as expected, a haploinsufficient tumor suppressor phenotype (27, 30), given that Palb2<sup>−/−</sup>; Trp53<sup>−/+</sup>; K14-Cre mice developed tumors significantly faster than Palb2<sup>−/−</sup>; Trp53<sup>−/−</sup>; K14-Cre mice (T<sub>1/2</sub> = 225 d vs. 420 d, respectively, P = 2.5 × 10<sup>−12</sup>; Fig. S4C). Palb2 loss of function also accelerated tumor formation on a Trp53<sup>−/−</sup> heterozygous (H+/−) background, again reflecting the genetic interaction of these two genes (Fig. 2B).

K14-Cre-mediated loss of Palb2 and Trp53 led, predominantly, to tumor formation in breast, skin, and oral mucosa (Fig. 2E), as previously reported for K14-Cre-driven Brca1 and Brca2 cancers (27, 30–32). All of the mammary cancers were estrogen and progesterone receptor (ER/PR)-negative, basal-like (Fig. S4 D–F), much like the Brca1<sup>−/−</sup> and -2 tumors generated by K14-Cre (30, 32). Although most of the tumors found in Palb2<sup>−/−</sup>; Trp53<sup>−/−</sup>; K14-Cre mice were breast carcinomas, Palb2<sup>−/−</sup>/Trp53<sup>−/−</sup> compound KO mice displayed an expanded spectrum of tissues affected by tumors (Fig. 2E), suggesting that combined loss of PALB2 and P53, possibly due to some expression of Cre in other tissues, also results in tumor formation that is not restricted to the mammary gland.

Mice harboring conditional alleles for Palb2 and Trp53, but no K14-Cre transgene, and Palb2<sup>−/−</sup>; Trp53<sup>−/−</sup>; K14-Cre mice did not display overt tumor formation during the observation period (Fig. 2C), despite the intrinsic mutagenic activity of Cre in mammalian cells (31, 33). Therefore, tumor formation in the above-noted experiments is a product of targeted gene deletion.

All tumors from Palb2<sup>−/−</sup>; Trp53<sup>−/−</sup>; K14-Cre mice (n = 12) had lost both copies of Palb2 and Trp53 (Fig. 2D). Similarly, in all tumors from Palb2<sup>−/−</sup>; Trp53<sup>−/−</sup>; K14-Cre mice (n = 15), the conditional Palb2 and Trp53 alleles were recombined. The WT copy of Trp53 was also lost in most tumors, probably through LOH.

Early reports describing a lack of PALB2 LOH in clinical tumors samples from heterozygous patients suggested that PALB2 could be a haploinsufficient tumor suppressor in humans whereas other reports showed that multiple PALB2 tumors revealed PALB2 LOH, implying that the PALB2 tumor formation process is not uniform (9, 34, 35). In our experimental setting, no haploinsufficiency for tumor suppression was observed for Palb2, as indicated by the comparable latency in Palb2<sup>−/−</sup>; Trp53<sup>−/−</sup>; K14-Cre and Palb2<sup>−/−</sup>; Trp53<sup>−/−</sup>; K14-Cre tumor development (P = 0.46, Fig. S4A). Similarly, when compared on a Trp53<sup>−/−</sup>; K14-Cre background, cohorts of Palb2<sup>−/−</sup> and Palb2<sup>fl/fl</sup> mice developed tumors with similar latency and frequency (P =
implying that heterozygous Palb2 loss of function did not contribute to tumor formation.

Moreover, Palb2 heterozygous mouse breast tumor lines displayed proper RAD51 localization at IRIF, consistent with preserved HR function (see Fig. 4C). By contrast, Palb2−/− breast tumor cell lines displayed the same defect in RAD51 accumulation observed in Palb2-null primary cells (see Fig. 4C). These findings suggest a role for HR deficiency in the genesis of Palb2 tumors, a state that is not compatible with retention of a functional copy of the gene. Moreover, an analysis of tumors that arose in Palb2+/−; Trp53fl/+; K14-Cre mice implies that a significant fraction of these tumors retained at least one copy of PALB2, suggesting that loss of one copy of the gene did not contribute to tumor formation in this model (Fig. 2D).

Although we observed long latency tumors only in Palb2−/−; K14-Cre mice on a WT Trp53 background (T1/2 = 420 d), tumor formation was nonetheless highly significant compared with Palb2+/−; Trp53fl/+; K14-Cre controls (P = 5.4 × 10−36, Fig. 2C). The majority of these tumors were small lesions in the head and neck, and a few were mammary tumors. All of these mammary tumors were Palb2−/−, and all displayed either mutations in or loss of Trp53 by LOH.

The finding that loss of Palb2 alone is sufficient to induce long latency tumor formation contrasts with most Brca1 and Brca2 mouse models in which significant numbers of these tumors could not be detected, unless Trp53 was codelleted (27, 30, 36, 37). One explanation for this finding is that somatic Palb2 loss might be better tolerated than somatic Brca1/2 loss on a Trp53 WT background. This hypothesis fits with the finding that Palb2 nullizygosity gives rise to a less severe phenotype in ES cells than biallelic Brca1 or Brca2 loss (17, 18, 27, 38).

Genomic Features of Palb2/Trp53-Deficient Mammary Tumors. Genomic instability is a hallmark of human cancer, and it promotes tumor initiation and progression. Experimental mouse tumor models have recapitulated this aspect of human tumorigenesis (39). To gain insight into the genomic structures of the tumors that arose due to the loss of Palb2, we performed high-resolution comparative genomic hybridization (CGH) (40) analysis of Palb2/Trp53, Brca1/Trp53, Brca2/Trp53, and Trp53-only-deficient mammary tumors that arose in K14-Cre mice (Fig. 3.A–C).

Segmentation analysis of the CGH data was performed for each tumor to assess the number of genomic segments with deviating copy number changes (aka genomic segmentation), as a readout of genomic instability (41). Palb2/Trp53 and Brca1/Trp53 tumors displayed apparently greater genomic instability (genomic segmentation) compared with Brca2/Trp53 and Trp53-only tumors, but the difference was not statistically significant. However, when the relative dose of amplified segments (log2 dose ≥ 0.5) was analyzed, Palb2/Trp53 mammary tumors (n = 8) displayed a significantly higher average dose of amplified segments than either Brca1/Trp53 (n = 4; P < 0.0001, Fig. 3C) or Brca2/Trp53 tumors (n = 5; P = 0.0014, Fig. 3C). The low numbers of deletions (log2 dose ≤ −0.5) detected in Brca2/Trp53 and Trp53-only tumors precluded further analysis of this aspect of genomic instability (Fig. 3B).

No significant difference in focal genome amplification appeared when Palb2/Trp53 tumors were compared with Trp53-only tumors, indicating that Palb2/Trp53 tumors share a similar amplification-prone genomic profile with Trp53-only tumors, despite their marked difference in tumor formation kinetics. The inability of Palb2 loss to suppress focal genomic amplifications (unlike what was observed in Brca1/Trp53 and Brca2/Trp53 tumors) could be accounted for by three, alternative explanations.

First, these differences could be due to residual activity of the conditional Palb2 allele we generated, and other Palb2 loss of function mutations might trigger the formation of true Brca2 tumor phenocopies. Second, our allele is a functional null, as our studies suggest, but complete loss of Palb2 is similar to a BRCA2 hypomorphic phenotype rather than a complete loss of BRCA2 function. Alternatively, there are PALB2 functions that are, at least in part, nonoverlapping with the tumor suppressing functions of its BRCA2 partner protein. New experiments with additional Palb2 and Brca2 mutant mouse strains would be required to distinguish between these possibilities.

Finally, tumor heterogeneity likely affected the CGH profiles (Fig. S5) in ways that made it difficult to identify regions of chromosomal imbalances that were unique to Palb2/Trp53 tumors. Conceivably, a more comprehensive analysis with a much larger collection of tumor samples would reveal such regions.

Loss of 53BP1 Fails to Rescue the HR Defect Caused by PALB2 Deficiency. Loss of 53BP1 can rescue the HR defect and lethality observed in either Brca1Δ11/Δ11 or Brca1-null cells and mice (42–45). Similarly, decreased expression of the P53 binding protein 1 (53BP1) was detected in triple negative breast cancers as well as human BRCA1 tumors (45). Therefore, we asked whether Trp53bpl (which encodes mouse 53BP1) expression is reduced in Palb2/Trp53 KO tumors, and whether its absence rescues the HR defect associated with Palb2 loss. Quantitative RT-PCR analysis of Trp53bpl mRNA in freshly isolated Palb2 breast tumor samples
tumor suppression was not complemented by 53BP1 loss. In keeping with existing evidence, PALB2 and BRCA2 may be de facto HR effectors that cannot be replaced or bypassed, except by artificially forcing the loading of RAD51 onto chromatin at/near DSB, which 53BP1 loss has not yet been shown to promote (45, 47–49). These observations, along with earlier results (4), also suggest that PARP inhibition might be a potential therapeutic regimen in PALB2-deficient tumors, as it is in BRCA1- and BRCA2-associated tumors (46).

**Conclusions**

In summary, we have shown that *Palb2* is a breast tumor suppressor gene in mice as it is in humans and that it synergizes with *Trp53* to suppress tumor formation. The outcome of dual *Palb2*/*Trp53* nullizygosity in the mouse mammary gland is highly penetrant breast cancer. In keeping with the fact that *Palb2* is also a breast tumor suppressor in humans, *Palb2* might be viewed as a *BRCA3*-like allele. Moreover, tumorigenesis driven by *Palb2* loss in the mouse is not entirely suppressed on a *Trp53* WT germline background, unlike most *Brcal* and *Brcar2* mouse models of breast tumorigenesis (50).

Despite many similarities to *Brcar2/Trp53* and *Brcal/Trp53* breast tumors, *Palb2* tumors displayed certain divergent genomic features that might be viewed as separating them from BRCA1- and -2 cancers. Specifically, we observed patterns of genomic aberrations that were different in *Palb2/Trp53*-derived tumors from those detected in *Brcal/Trp53*- or *Brcar2/Trp53*-derived tumors. These data are consistent with the hypothesis that *Palb2* possesses biological functions that extend beyond those of its major interactors, BRCA1 and BRCA2. Alternatively, the effect of *Palb2* deletion may mimic a phenotype akin to partial loss of BRCA2, resulting in a less dramatic genomic instability profile in the relevant tumor cells.

Although the genomic instability of *Brcal*-deficient cells can be rescued by loss of *Trp53bp1*, deletion of the latter had, if anything, an adverse effect in *Palb2* KO cells. In that context, *Palb2* is more similar to *Brcar2*, the absence of which leads to an HR defect that also cannot be rescued by *Trp53bp1* deletion.

Haploinsufficiency for *Palb2* tumor suppression was not detected in this model although one cannot rule out the possibility that it would be manifest in a different model system and/or with enlarged cohorts of experimental mice. For example, the tumors in this mouse model driven by *K14-Cre* were uniformly of the triple negative phenotype. This characteristic might well contribute to the absence of haploinsufficiency in our system, in the same way that BRCA1 mammary tumors derived from distinct cell populations display preferential patterns of consecutive LOH events along the tumorigenesis pathway (32, 51).

We believe that this mouse model will be useful in understanding how *Palb2* serves its breast cancer suppression function.
Mice harboring tumors were humanely killed when the tumor diameter reached 2.0 cm in its greatest dimension. Mice that were otherwise severely diseased/ distressed were also killed according to institutional guidelines. Menter-Cox logrank test was applied for comparison of tumor-free survival of mouse cohorts.

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Tumorigenesis Studies. Mouse cohorts were monitored for tumor formation biweekly. Mammary tumor formation was scored when a palpable tumor of 1.0 cm in its greatest diameter could be detected, as previously described (27, 30).

Materials and Methods

ES Cell Derivation, Embryo Harvesting, and Tetraploid Complementation Assay. Generation of the conditional allele for Palb2 and additional experimental details are described in SI Material and Methods. Oligo sequences used are described in Table S1. Palb2 KO derivation was performed according to standard protocols (52). Pregnant mice from timed matings were killed at indicated time points by CO<sub>2</sub> asphyxiation following institutional guidelines. Uterine horns and embryos were dissected under the microscope, and isolated embryos were directly used for digestion, DNA extraction, and genotyping. Negative selection of Palb2 KO embryos was analyzed according to the Hardy-Weinberg equilibrium model, using an online tool (http://hgg.dbc.gcontinued...barcoding/17/10:1242-1252.


