Supporting Information

Bowman-Colin et al. 10.1073/pnas.1305362110

SI Materials and Methods

Generation of a Conditional *Palb2* Knockout Mouse. A targeting vector was constructed by cloning a ~8-kb DNA fragment encompassing intron 1 through intron 4 of the mouse partner and localizer of BRCA2 (*Palb2*) locus (Fig. S1). A *loxP* site was inserted in intron 1 and another *loxP* site was inserted in intron 4. After sequence verification of the final targeting construct, 30 μg of the ClaI-linearized vector were electroporated into V6.5 mouse ES cells following standard protocols (1). Approximately 200 neo-resistant ES cell clones were screened by Southern blotting for correct cointegration of both *loxP* recombination sites. For verification of correct targeting of the 5′ end, gDNA was digested with BamHI and hybridized with an exon 1 probe. Correct recombination at the 3′ end of the construct was verified by hybridization of blots of BsaBI-digested genomic DNA with a 3′ probe. Two different clones that displayed correct cointegration of both *loxP* sites were used to generate chimeric mice. Chimeric mice were crossed with C57Bl6 (WT) mice, and germline transmission of the conditional $Palb2^{neo}$ allele was confirmed by PCR genotyping of the resulting offspring. *Palb2* conditional mice were then crossed with FLP deleter mice [129S4/SvJaeSor-Gt(ROSA)26Sor$^{tm1(FLP1)Dym}$/J; stock no. 003946; The Jackson Laboratory] to achieve removal of the *neo* cassette and generation of a $Palb2^{fl}$ allele. For generation of a $Palb2^{\Delta}$ (null) allele, $Palb2^{fl/+}$ mice were crossed with Cre deleter mice (B6.C-Tg$^{(CMV\text{-}cre)1}$*Cgn*/J; stock no. 006054; The Jackson Laboratory). Cohorts of mice for tumorigenesis studies were generated by interbreeding $Palb2^{fl}$ mice, keratin 14 promoter-driven Cre transgenic mice (*K14-Cre*) [Tg(KRT14-cre)1Amc/J; stock no. 004782; The Jackson Laboratory] and Tumor protein 53 ($Trp53^{F2\text{-}10}$) mice (2).

Embryo lethality rescue experiments were performed by crossing either $Palb2^{\Delta}$ mice with *Cdkn1a* KO mice (3) [B6;129S2-$Cdkn1a^{tm1Tyj}$/J, stock no. 003263; The Jackson Laboratory] or $Palb2^{fl}$ mice with *Meox2-Cre* mice (3) [B6.129S4-$Meox2^{tm1(cre)Sor}$/J, stock no. 003755; The Jackson Laboratory]. For the homologous recombination (HR) rescue experiments, mice carrying the conditional exon 11 allele for *Brca1* ($Brca1^{F11}$) (4) and the conditional exon 11 allele for *Brca2* ($Brca2^{F11}$) (5) were obtained from the National Cancer Institute mouse repository. $Trp53bp1^{\Delta/\Delta}$ mice (6) were a gift from Dr. Junjie Chen and *CD19-Cre* mice [Cg-$^{Cd19tm1(cre)Cgn}$*Ighb*/J] (7) were purchased from The Jackson Laboratory (stock no. 004126).

Genotyping. Mouse tail genomic DNA was prepared using standard protocols. Routine genotyping PCR to detect the 5′ *loxP* site, using primers A1F and A1R, yields a 355-bp for the $Palb2^{WT}$ allele and a 432-bp band for the $Palb2^{fl}$ allele. PCR genotyping of the 3′ *loxP* site with primers A2F and A2R yielded a 189-bp product for the $Palb2^{WT}$ allele, a 347-bp band for the $Palb2^{flox}$ allele, and a 1,783-bp PCR product for the $Palb2^{neo}$ allele. PCR with primers A1F and A2R yields bands of 1.48 kb for the $Palb2^{flox}$ allele, 1.25 kb for the $Palb2^{WT}$ allele, and a 459-bp for the $Palb2^{\Delta}$ allele. The *K14-Cre* transgene was genotyped with primers K14F1 and CreR1, yielding an ~500-bp band in *K14Cre*-transgenic animals.

Genotyping of the Trp53 conditional allele with primers p53-1F/p53-1R (5′ *loxP* site) yields a 288-bp product for the WT allele and 370-bp for the conditional allele whereas genotyping with primers p53-10F/p53-10R yielded bands of 419 bp for the WT allele and 572 bp for the conditional *Trp53* allele. Genotyping of *Cdkn1a* KO mice was performed using primers P21KO2R, P21wt2R, and P21commonF, simultaneously. This primer combination yielded bands of 217 bp and 355 bp for the WT and KO alleles, respectively.

PCR was carried out in 10-μL reactions containing 2.5 μL of 1:10 diluted tail digests and 1μM primers, in 1× quantitative PCR (qPCR) mix (iQ SYBR Green Supermix; Bio-Rad Laboratories). Cycling parameters were as follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, then 72 °C for 2 min (2-step) PCR, followed by 72 °C for 7 min as a final extension. Genotyping of the other alleles not specified herein was carried out as described in the corresponding referenced publication or using the protocol available at The Jackson Laboratory website (www.jax.org), for the respective mouse stock number. Embryo genotyping followed the same protocol used for mouse tail genotyping.

Cell Culture, ES Cell Derivation, and Dose–Response Curves. For ES cell derivation (8), uterine horns from mated mice were collected at 3.5 days post-coitum (*dpc*) and flushed with ES cell medium. Blastocysts were plated in individual wells containing feeder layers and fed with fresh medium every 3 d. The MEK1 kinase inhibitor PD98059 (Cell Signaling Technologies) was added at 50 μM. Cells were trypsinized 1–2 times a week, until cell growth from the inner cell mass was evident. Breast tumor cell lines were established from dissected mouse tumors, as previously described (9). For dose–response curves, 10^3 cells were plated in 96-well plates, in 200 μL of medium. Cell viability was assayed 3 d after drug/ ionizing radiation (IR) treament using Cell Titer Glo reagent (Promega), according to the manufacturer's instructions.

Immunofluorescence, Antibodies, Western Blotting, and Immunohistochemistry. Immunofluorescence was performed following cell fixation with paraformaldehyde/sucrose as described (8). Commercial antibodies used for IF were as follows: γH2A.X (JBW301, 1:2,000; Millipore), RAD51 (H-92, 1:150; Santa Cruz). Antiserum against mouse BRCA1 was used at 1:2,000 dilution (10). Chromatin-associated protein extracts were generated and blotted as described (11). Blots were probed for 2 h at room temperature with antibodies developed against the recombinase RAD51 (H-92; Santa Cruz) and histone H3 (9715; Cell Signaling). They were diluted at 1:500 and 1:2,000, respectively. Anti-mouse PALB2 rabbit antiserum was generated after immunization with a recombinant GST fusion protein containing the N-terminal 200 residues of mouse full-length PALB2 purified with a Melon IgG Gel Purification Kit (ThermoScientific). The purified antiserum was used for probing blotted whole cell extracts of ES cells at 1:2,000 dilution. Immunohistochemistry staining of formalin-fixed mouse tumor samples was performed as described (29).

Quantitative Real Time-PCR and qPCR for Gene Dosage Analysis. Quantitative real-time RT-PCR (qRT-PCR) and qPCR for gene dosage were performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer's guidelines. The Comparative CT method was used to calculate relative expression/dose levels. Genomic DNA and total RNA were extracted using DNeasy and RNeasy Mini Kits (Qiagen), according to the manufacturer's instructions. For cDNA synthesis, 5 μg of total RNA was reverse transcribed with SuperScript III (Invitrogen) according to the manufacturer's instructions, and 20 ng of this cDNA was used in each qPCR reaction. The mouse homolog of the human gene encoding the Ribosomal Protein, Large P0-RPLP0 (ARBP), was used as an internal control for

qRT-PCR assays. For gene dosage qPCR analyses, 25 ng of genomic DNA were used as template for determination of *Palb2* and *Trp53* copy numbers, and the average signals from the *Brca1* and *Il2* loci were used for normalization. The primer pairs used for gene copy number quantization were chosen based on optimal efficiency matching and a readout of 100% dose (2N copy number) on genomic DNA from normal tissues from both WT and *Palb2*$^{fl/fl}$; *Trp53*$^{fl/fl}$ mice in which no Cre transgenes were present. All primers were added at 1 μM final concentration, and relevant sequences are provided in Table S1.

HR Rescue Experiments. Metaphase spreads from splenic B lymphocytes were prepared, stained 48 h after activation with LPS (25 μg/mL; Sigma) and IL-4 (5 ng/mL; Sigma), and analyzed as described (12).

Array CGH Analyses. For aCGH experiments, Mouse Nimblegen CGH 3 × 720K Microarrays were used according to the manufacturer's instructions (Roche Nimblegen). Comparative genomic hybridization (CGH) analysis was performed as described (13). Briefly, CGH raw data were smoothed using the CBS package in programming language R with standard parameters, with the exception of the addition of the "sdundo" parameter applied to segment splits that are >3 segment SDs apart (14). Resulting segments were extracted and counted to assess the number of amplified segments and homozygously deleted segments. Amplified and deleted segments were defined as $\log_2 > 0.5$ and < -0.5, respectively. To remove small outlier segments, we only considered segments with 10 or more probes. The pairwise Wilcoxon rank sum test was used to test for differences in the relative doses of amplified segments between the tumor genotypes.

1. Pasparakis M (2005) Making gene-modified mice. *Ernst Schering Res Found Workshop* 50:1–26.
2. Liu X, et al. (2007) Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. *Proc Natl Acad Sci USA* 104(29):12111–12116.
3. Brugarolas J, et al. (1995) Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* 377(6549):552–557.
4. Xu X, et al. (1999) Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nat Genet* 22(1):37–43.
5. Jonkers J, et al. (2001) Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. *Nat Genet* 29(4):418–425.
6. Ward IM, et al. (2005) 53BP1 cooperates with p53 and functions as a haploinsufficient tumor suppressor in mice. *Mol Cell Biol* 25(22):10079–10086.
7. Rickert RC, Roes J, Rajewsky K (1997) B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Res* 25(6):1317–1318.
8. Kanellopoulou C, et al. (2009) X chromosome inactivation in the absence of Dicer. *Proc Natl Acad Sci USA* 106(4):1122–1127.
9. Evers B, et al. (2008) Selective inhibition of BRCA2-deficient mammary tumor cell growth by AZD2281 and cisplatin. *Clin Cancer Res* 14(12):3916–3925.
10. Bouwman P, et al. (2010) 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. *Nat Struct Mol Biol* 17(6):688–695.
11. Xia B, et al. (2006) Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol Cell* 22(6):719–729.
12. Callén E, et al. (2007) ATM prevents the persistence and propagation of chromosome breaks in lymphocytes. *Cell* 130(1):63–75.
13. Bouwman P, et al. (2011) Loss of p53 partially rescues embryonic development of Palb2 knockout mice but does not foster haploinsufficiency of Palb2 in tumour suppression. *J Pathol* 224(1):10–21.
14. Venkatraman ES, Olshen AB (2007) A faster circular binary segmentation algorithm for the analysis of array CGH data. *Bioinformatics* 23(6):657–663.

Fig. S1. (*A*) Alignment of known PALB2 domains with their corresponding coding exons. The yellow shadow indicates the predicted frameshifted ORF encoded by the null allele resulting from recombination of the inserted LoxP sites. The pink shadow indicates exons 2 and 3 which are conditionally targeted by insertion of LoxP sites. (*B*) Schematic representation of the targeting strategy. The wild-type allele (WT), targeting construct, and targeted allele are depicted. Dashed lines indicate regions of homology. Black triangles represent *loxP* sites, and small arrows above and below exons 2 and 3 represent the primers used for RT-PCR. Green bars represent the 5′ and 3′ probes used in the Southern blot in *C*. (*C*) Southern blot of genomic DNA from targeted ES cell clones digested with indicated restriction enzyme (BamHI or BsaBI) and hybridized with 5′ (*Upper*) and 3′ (*Lower*) probe. Note that only some of the targeted clones (3′ probe) that show cointegration of the second *loxP* site with the 5′ probe were used. (*D*) Real-time RT-PCR analysis of *Palb2* mRNA levels in three ES cell lines of the indicated genotypes derived from heterozygous *Palb2*$^{fl/-}$ crosses.

PNAS PNAS PNAS PNAS

Fig. S2. Response of *Palb2*$^{-/-}$ cells to DNA damage. (*A*) Recruitment of BRCA1 to double strand breaks marked by phosphorylated histone H2A.X (γH2AX) foci 2 h after exposure to 5 Gy of ionizing radiation both in *Palb2*$^{fl/fl}$ and *Palb2*$^{-/-}$ cells. (*B* and *C*) *Palb2*$^{fl/fl}$ and *Palb2*$^{-/-}$ ES cell survival after Mitomycin C exposure (*B*) and after IR exposure (*C*).

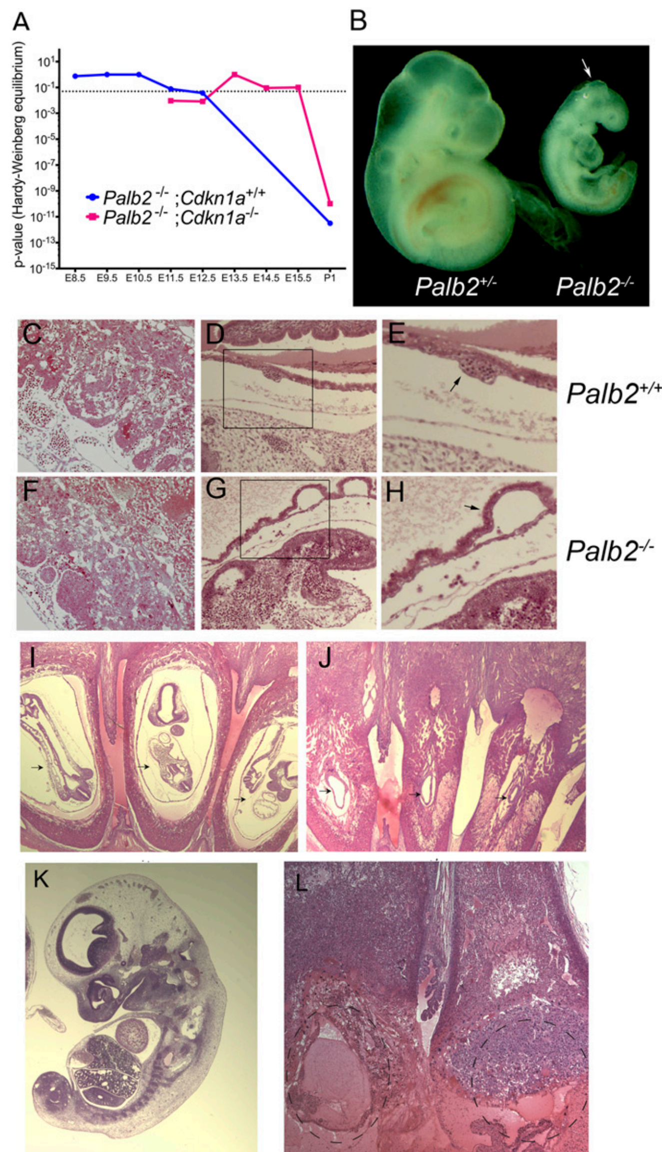

Fig. S3. Early lethality of *Palb2*$^{-/-}$ embryos is not rescued by loss of p21 or by *Palb2* WT placentas. (*A*) Graph indicating the *P* values of Hardy–Weinberg disequilibrium (negative selection) for *Palb2* KO embryos retrieved from *Palb2*$^{+/-}$ matings at various embryonic ages. The *Cdkn1a* (*p21*)-deficient background delays the lethality of *Palb2*-deficient embryos and the time point at which the negative selection of *Palb2* KO embryos become significant ($P \leq 0.05$). (*B*) Bright-field images of *Palb2*$^{+/-}$ and *Palb2*$^{-/-}$ embryos at E10.5. Arrow indicates an embryo marked by exencephaly. (*C–H*) Hematoxylin-eosin staining of placenta (*C* and *F*) and yolk sac blood islets of WT (*C–E*) or *Palb2*$^{-/-}$ embryos (*F–H*). Frames *E* and *H* display higher magnification of the areas delimited on *D* and *G*. (*I* and *J*) Hematoxylin-eosin staining of in utero embryo sections analyzed in a tetraploid complementation assay at E9.5, using *Palb2*$^{fl/fl}$ ES cells (*I*) or *Palb2*$^{-/-}$ ES cells (*J*). (*K* and *L*) Hematoxylin-eosin staining of in utero embryo sections analyzed in a tetraploid complementation assay at E12.5, using either *Palb2*$^{fl/fl}$ ES cells (*K*) or *Palb2*$^{-/-}$ ES cells (*L*). By this stage, all *Palb2*$^{-/-}$ embryos have been reabsorbed, leaving empty decidual cavities (dotted circles on *L*).

Fig. S4. Effects of *Palb2* and *Trp53* heterozygosity in *K14-Cre* mice. Kaplan–Meier curves of tumor-free survival indicating that *Palb2* is not haploinsufficient for tumor formation either on a *Trp53* conditional null (*A*) or conditional heterozygous background (*B*). On the other hand, *Trp53* displays a strong haploinsufficient tumor phenotype on a $Palb2^{fl/fl}$ background (*C*). (*D–F*) Mammary tumors arising in *Palb2/Trp53* double conditional mice are triple-negative. Representative IHC stainings for the estrogen receptor (ER, Fig. S4*C*), progesterone receptor (PR, Fig. S4*D*) and ErbB2 (HER2, Fig. S4*E*). The arrows on the top left corner of *D* and *E* indicate normal ductal carcinoma structures that display typical nuclear positivity for ER and PR, respectively.

Brca2$^{fl/fl}$; *Trp53*$^{fl/fl}$; *K14-Cre*

Brca1$^{fl/fl}$; *Trp53*$^{fl/fl}$; *K14-Cre*

Trp53$^{fl/fl}$; *K14-Cre*

Palb2$^{fl/fl}$; *Trp53*$^{fl/fl}$; *K14-Cre*

Log_2 mean dose

Chromosome #

Fig. S5. CGH profiles from all of the tumors analyzed in this work. Representative profiles displayed on Fig. 4*A* were chosen from this collection of CGH profiles. As in Fig. 4*A*, each dot on the graphs represents the average signal from a segment spanning 10 or more consecutive probes, and the negative/positive log_2 mean ratio from each data point indicates the extent of loss/gain in each segment, respectively.

Fig. S6. Attempted rescue of PARPi-induced acute chromosomal damage in *Brca1 and Brca2*-deficient B lymphocytes by *Trp53bp1* deletion. *Trp53bp1* deletion did not rescue the HR deficiency-associated abnormalities in chromosome spreads from PARPi-treated *Brca2*-deficient B lymphocytes (*A*) but did rescue them in *Brca1*-deficient B lymphocytes (*Brca1*$^{fl/fl}$*;CD19-Cre*) (*B*).

Table S1. Oligonucleotide sequences

Oligo use	Gene	Oligo ID	Sequence (5′– 3′)
Genotyping	*Cdkn1a* (p21)	P21wtR2	AGTGCCCTGGACTTTGGGATACTACACATAAACTGTATAGCATTAGTGCTGCT
		P21KOR2	TTGGATATCATAATTTAAACAAGCAAAACCAAATTAAGGGCCAGCTCAT
		p21CommonF	GTCACCCAGCAAAGCCTTGATTCTGATCTGGGCAGTCTAGCT
	Trp53	cond-p53-int1-F	ACAAAAAACAGGTTAAACCCAGCTTGACCAAGTGCCATTGGTCCATGGAT
		cond-p53-int1-R	AGCACATAGGAGGCAGAGACAGTTGGAGGCCAGCCTGGTCTACAGA
		cond-p53-int10-F	AAGGGGTATGAGGGACAAGGTATGGTGTCATGCTCCTATAATCTCAGCAGTA
		cond-p53-int10-R	GGGGAGGGATGAAGTGATGGGAGCTAGCAGTTTGGGCTTTCCTCCTTGATCA
	Palb2	GENOPALB2-A2R	CTGGCAATCCAATTGAAGGCACTGGGTATTGCTTGAATTGTATAACATGA
		GENOPALB2-A2F	AGCCAGAAGCTTCCTCACAGCTAAGCCACTCTGGTA
		GENOPALB2-A1R	AACTATGATTCACTCACCTGAAGTCGAGCAAGTGTC
		GENOPALB2-A1F	AGAAGTCCATCCTAGGGAAGTAATCCTGCAAACTGA
	K14-Cre	CRER1	GGATCCGCCGCATAACCAGTGAAACAGCATTGCTGTCACTT
		hK14F1	GATGAGGCGGATGAGAGGAGGGACCTGGCTGGGAGT
Gene dosage	*Palb2*	qPCRdose-mPALB2-5F	TGGTTTTGGTTTGAAACAGGGCCTCACTA
		qPCRdose-mPALB2-5R	CTATCTCACCAGCCCCTGAGCCATCT
	Trp53	qPCRdose-mP53-4F	GAAGGAAAGGTCCCAGTCCTCTCTTTGCT
		qPCRdose-mP53-4R	TGGGGAACTAATGAAACACATTGTCC
	Brca1	qPCRdose-mBRCA1-5F	AAGCTGGAGATGAAGGCAAGCTGCAC
		qPCRdose-mBRCA1-5R	GGCATCACTCCACTGCCCCTTAGTGT
	Il2	qPCRdose-mIL2-7F	GCTCTTAGAACTCCAGCAAGGGTAGA
		qPCRdose-mIL2-7R	ATTTACTTGGGACAAGCTCTTTCTAGGACCA
qRT-PCR	*Trp53bp1*	m53BP1F	GTTACCTCAGCCAAACAGGACAAGCA
		m53BP1F	CCCTTCCTTCTCCTCCTCTAACTC
	Palb2	mPalb2mRNA.ND2-F	CACTTGCTCGACTTCAGCGTGCCAA
		mPalb2mRNA.ND2-R	CTGGCTGGGGCACACCATCTTCTATG
	Arbp	mARBPF	CCAACTACTTCCTCAAGATCATCCAACTT
		mARBPR	CATCTGCTTGGAGCCCACGTTGT