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FULL LENGTH ARTICLE

Loss of the BRCA1-PALB2 interaction accelerates p53-associated tumor development in mice

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Abstract The BRCA1-PALB2-BRCA2 axis, or the BRCA pathway, plays key roles in genome stability maintenance and suppression of breast and several other cancers. Due to frequent p53 mutations in human *BRCA1* breast cancers and mouse mammary tumors from *Brca1*, *Brca2* and *Palb2* conditional knockout models, it is often thought that p53 inactivation accelerates BRCA1/2 and PALB2-associated tumorigenesis. Here, we studied tumor development in mice with a mutation in *Palb2* that disengages the PALB2-BRCA1 interaction in different *Trp53* backgrounds. Rather than mammary tumors, *Palb2* and *Trp53* compound mutant mice developed, with greatly reduced latencies, lymphomas and sarcomas that are typically associated with germline *Trp53* inactivation. Whole exome sequencing failed to identify any significant differences in genomic features between the same tumor types of *Trp53* single mutant and *Palb2;Trp53* compound mutant mice. These results suggest that loss of the BRCA pathway accelerates p53-associated tumor development, possibly without altering the fundamental tumorigenic processes.

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Introduction

BRCA1 and BRCA2, the two major breast cancer susceptibility proteins, play key roles in the DNA damage response, especially in homologous recombination (HR)-mediated DNA double strand break repair and cell cycle checkpoint control, thereby maintaining genome integrity and suppressing tumor development.[1,2] The two BRCA proteins are physically and functionally linked in HR and checkpoint control by PALB2, which was first identified as a major binding partner of BRCA2.[3–6] Like *BRCA1/2*, monoallelic germline mutations in *PALB2* cause high risk of breast cancer and also increase the risks of ovarian, pancreatic and other cancers.[7,8]

To understand the *in vivo* function of the *BRCA1/2* genes and the mechanisms of their associated tumorigenesis, several different mouse models were created for each gene soon after their cloning.[9] The first studies found that germline, systemic knockout of each gene led to embryonic lethality,[9] underscoring the importance of the genes for fundamental cellular processes and development. Conditional ablation of each gene in the mammary gland indeed led to mammary tumor development; however, the latencies were long, averaging about 1.5 years,[10–13] suggesting the existence of a strong barrier of tumor formation following the loss of the proteins. This barrier is widely believed to be p53, as its mutations were found in most mammary tumors arising from conditional knockout models of both genes.[10,11,13] Co-deletion of *Trp53* with each gene led to much more efficient mammary tumor formation,[14,15] and a *Trp53* heterozygous background also promoted mammary tumorigenesis in *Brca1* CKO mice.[12,13] Similar findings were made with *Palb2* conventional and conditional KO mouse models.[16–19] The above findings suggest that p53 loss of function is required for BRCA- and PALB2-asociated mammary tumorigenesis, at least in mice.

To bypass the embryonic lethality of germline *Palb2* knockout and to study the role of the BRCA1-PALB2 interplay *in vivo*, we previously created a $Palb2^{CC6}$ strain with a mutation in the N-terminal coiled-coil motif of PALB2 that disrupts the binding of BRCA1.[20] Homozygous mutant mice showed increased endogenous DNA damage,[21] a moderate defect in spermatogenesis,[20] and susceptibility to radiation-induced tumor development.[21] In this study, we monitored spontaneous tumor development in the $Palb2^{CC6}$ mice with different *Trp53* backgrounds. Our results showed that combined mutations in the two genes led to accelerated development of tumor types typically associated with loss of *Trp53* rather than *Palb2*.

Materials and methods

Mice

Generation of the $Palb2^{CC6}$ mutant strain was described before.[20] The strain was backcrossed to C57BL/6 background for 6 generations and then crossed with $Trp53^{-/-}$ mice[22] on 129sv background; the progenies were then intercrossed to generate the different cohorts for observation. All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) of the Rutgers Robert Wood Johnson Medical School.

Tumor collection and processing

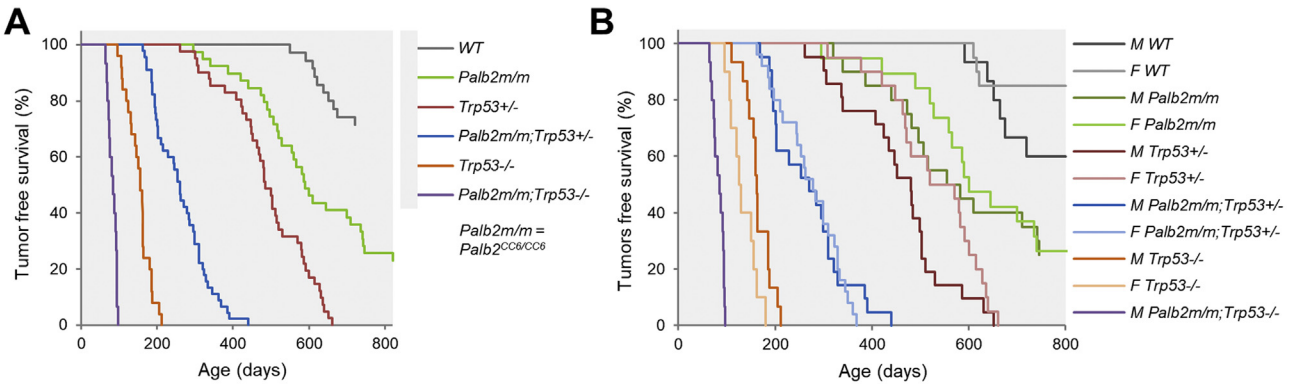
Tumors were collected from mice immediately after euthanization by CO_2 asphyxiation. Half of each tumor was snap frozen and the other half fixed overnight in phosphate-buffered formalin, transferred to 70% ethanol and embedded in paraffin. Paraffin-embedded tumors were sectioned at 5-μm thickness, stained with hematoxylin and eosin (H&E) for histological review.

Whole-exome sequencing analysis

DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen). Tumor and normal DNA samples were subjected to whole-exome sequencing (WES; SureSelect Mouse All Exon Kit, Agilent Technologies) at RUCDR Infinite Biologics (Piscataway, NJ). Paired-end sequencing data were aligned to the reference mouse genome mm10 using the Burrows-Wheeler Aligner (BWA, v0.7.15). Local realignment, duplicate removal and base quality score recalibration was performed using the Genome Analysis Toolkit (GATK, v3.1.1). After pooling the reads from each normal sample and masking repetitive regions using RepeatMasker (v4.0), somatic single nucleotide variants (SNVs) were identified using MuTect (v.1.1.4), and small insertions and deletions (indels) were detected using VarScan2 (v2.3.6) and Strelka (v3.1.1). To identify indels greater than 3 bp, Lancet, Platypus, and Scalpel were employed, and the results were combined to define a consensus call as previously described. SNVs and indels outside the WES capture were filtered out, as were SNVs and indels for which the variant allele fraction (VAF) in the tumor sample was less than 5 times the VAF of the paired normal tissue as previously described. Allele specific copy number aberrations (CNAs), tumor purity and ploidy were obtained from the WES data using FACETS.

Genomic features of HR DNA repair defects

Large-scale state transition (LST) scores were computed from the results of FACETS using the WES data according to Popova et al.[23] A cut-off of $\geq$15 was employed to classify tumors as LST high. The NtAI score, which assesses telomeric allelic imbalance, was defined according to Birkbak et al.[24] The number and length of small deletions in the tumors samples were assessed as previously described.[25]



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Genotype	Sex	Mammary tumor	Ovarian cancer	Thymic lymphoma	Splenic tumor	Other lymphoma	Osteo-sarcoma	Soft tissue sarcoma	Liver tumor	Lung cancer	Other tumors	Total tumors	Total # of mice
WT	M	0	0	0	0	0	1	0	0	0	0	1	16
WT	F	0	0	0	0	0	1	0	0	0	2	3	20
Palb2$^{CC6/CC6}$	M	0	0	1	0	0	0	1	9	1	1	13	21
Palb2$^{CC6/CC6}$	F	2	3	1	3	2	0	0	4	1	1	17	17
Trp53$^{+/-}$	M	0	0	0	2	2	1	5	1	1	4	16	18
Trp53$^{+/-}$	F	0	2	0	1	2	4	3	4	0	2	18	20
Palb2$^{CC6/CC6}$;*Trp53*$^{+/-}$	M	0	0	4	0	0	4	5	1	2	5	21	22
Palb2$^{CC6/CC6}$;*Trp53*$^{+/-}$	F	0	1	4	0	1	13	0	3	1	2	25	25
Trp53$^{-/-}$	M	0	0	9	0	0	1	1	0	1	0	12	12
Trp53$^{-/-}$	F	0	0	7	0	1	0	0	0	0	1	9	11
Palb2$^{CC6/CC6}$;*Trp53*$^{-/-}$	M	0	0	10	0	0	0	0	0	0	0	10	10

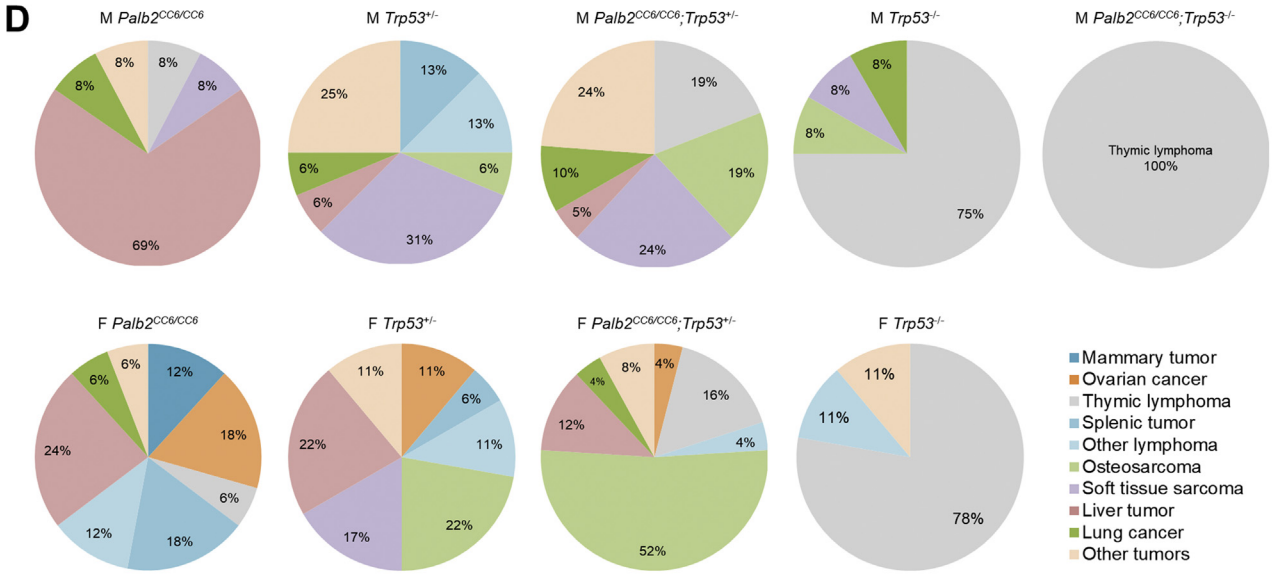

Figure 1 Tumor development in *Palb2*^{CC6} mice with different *Trp53* backgrounds. (**A,B**) Tumor-free survival of mice of indicated genotypes with males and females combined (**A**) or separated (**B**). (**C**) Summary of tumor types and numbers from mice of different genotypes. (**D**) Tumor spectra of mice with different genotypes. The lack of data from *Palb2*$^{CC6/CC6}$;*Trp53*$^{-/-}$ females is due to female-specific embryonic lethality of this genotype.

Statistical analyses

P values were calculated by two-tailed unpaired *t*-test in GraphPad Prism 8. P values <0.05 were considered significant.

Results and discussion

To understand the importance of the BRCA1-PALB2 interaction in spontaneous tumorigenesis and the role of p53 in the process, we crossed the *Palb2*^{CC6} mice (C57BL/6

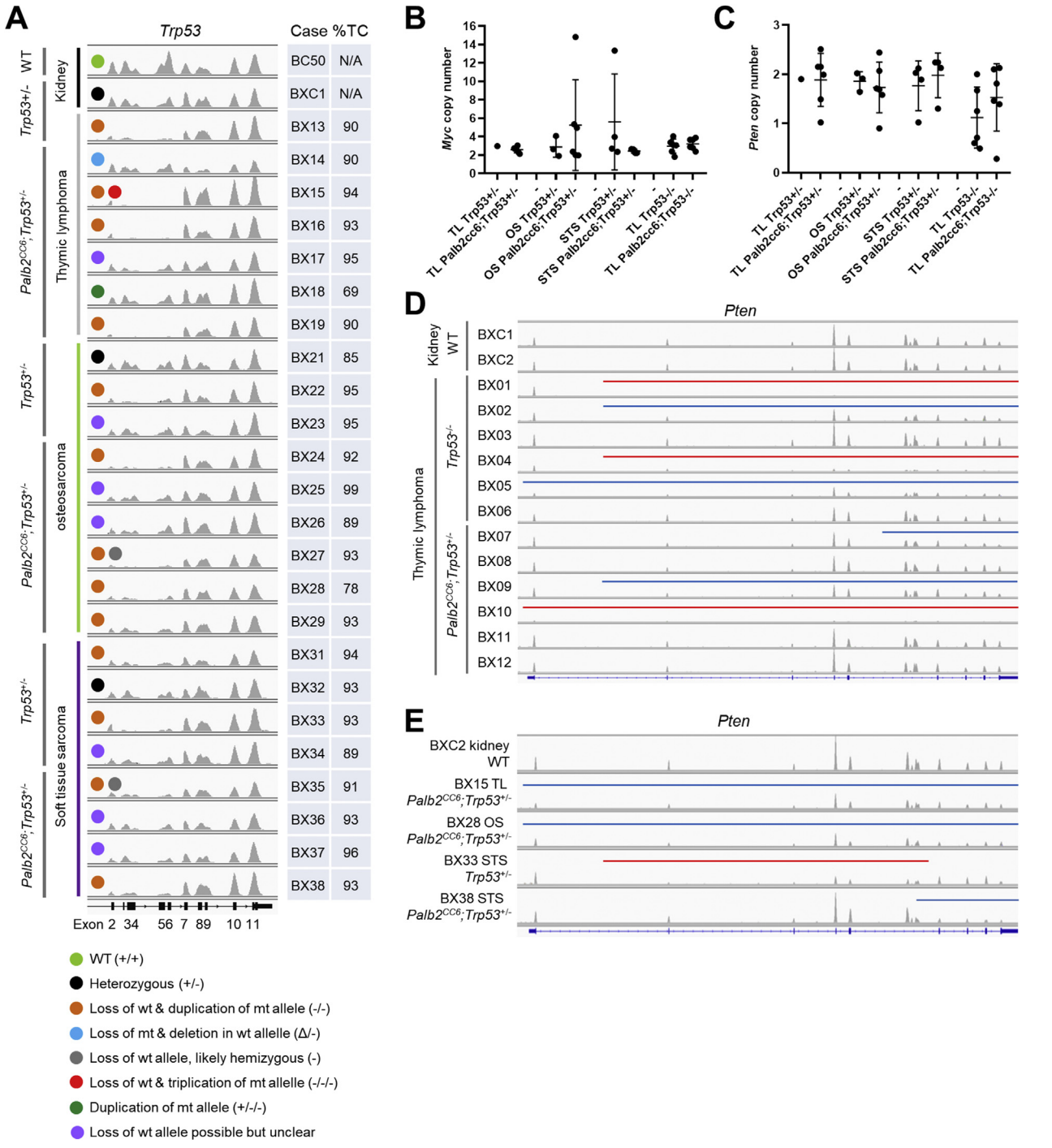

Figure 2 Genomic analyses of tumors from *Palb2*^{CC6} mutant mice in different *Trp53* backgrounds. (**A**) Status of the *Trp53* locus in tumors from *Trp53*$^{+/-}$ and *Palb2*^{CC6};*Trp53*$^{+/-}$ mice. Images were generated with IGV based on WES data. Data range for each track is normalized using the size of the corresponding BAM file, which reflects the number of total reads obtained for the tumor. (**B,C**) Dot plots of computed copy numbers of *Myc* (**B**) and *Pten* (**C**) in all tumors sequenced. (**D,E**) Status of the *Pten* locus in all 12 sequenced tumors from *Trp53*$^{-/-}$ and *Palb2*^{CC6};*Trp53*$^{+/-}$ mice (**D**) and selected tumors from *Palb2*^{CC6};*Trp53*$^{+/-}$ mice (**E**). Data range for each track is normalized as in **A**. Regions of monoallelic and biallelic deletion are indicated by blue and red bars, respectively.

background) with *Trp*53 knockout mice (129sv background), in which exons 2—7 of the gene were replaced by a neomycin drug selection cassette,[22] to produce *Palb2*$^{CC6/CC6}$ mice with wt, +/− and −/− *Trp53* status. These mice were aged along with wt, *Trp*53$^{+/-}$ and *Trp*53$^{-/-}$ mice generated in the same breeding process. Compared with wt mice, *Palb2*$^{CC6/CC6}$ mice showed increased spontaneous tumor development (Fig. 1A).

Overall, males showed earlier tumor development than females (Fig. 1B). Among males, the most common tumor type was liver tumor (69%), followed by lymphoma (including thymic lymphoma, splenic tumors, and other lymphomas) (16%) and soft tissue sarcoma (8%, Fig. 1C,D). Among females, lymphoma was the most common (36%), followed by tumors in the liver (24%), ovary (18%) and mammary gland (12%). The high incidence of liver tumor, especially in males, was unexpected; it could be explained by a possible chronic inflammation caused by increased oxidative stress and/or constitutive activation of NF-κB in these mice, as we reported recently,[21] although this remains to be tested experimentally.

Trp53$^{+/-}$ mice in a C57BL/6 × 129sv mixed background are known to succumb to a variety of tumors mostly between one to two years of age, with lymphomas and sarcomas being the major tumor types.[26] Our observations were similar, with a male/female combined median latency of 482 days and lymphoma, soft tissue sarcoma, osteosarcoma and liver tumor being the top tumor types (Fig. 1A,D). Again, males showed faster tumor development and shorter life span than females (Fig. 1B). Additionally, males were more often affected by soft tissue sarcoma, whereas females showed higher propensity to develop osteosarcoma (Fig. 1D).

Palb2$^{CC6/CC6}$;*Trp53*$^{+/-}$ mice showed greatly accelerated tumor development compared with mice with either mutation alone, with a combined median tumor latency of 253 days (Fig. 1A). Interestingly, although we expected *Trp53* heterozygosity to shift the tumor susceptibility of the *Palb2*$^{CC6/CC6}$ mice towards mammary and perhaps also ovarian and pancreas cancers, the tumor spectrum of the compound mutant mice instead showed a shift to osteosarcoma, a key phenotype of *Trp53*$^{+/-}$ mice,[26] and thymic lymphoma, the signature phenotype of *Trp53*$^{-/-}$ mice[22] (Fig. 1D). In fact, mammary and ovarian tumor incidence was substantially reduced in the compound mutant mice compared with *Palb2*$^{CC6/CC6}$ single mutant mice (4% vs 30%), so was liver tumor incidence in males (5% vs 69%). This suggests that functional loss of the BRCA1-PALB2-BRCA2 pathway accelerates p53-associated tumorigenesis, rather than p53 loss promoting BRCA1/2- and PALB2-associated tumor development.

Consistent with results from earlier studies,[22,26] all *Trp53*$^{-/-}$ mice developed tumors within 7 months of age (Fig. 1A), with thymic lymphoma being the major tumor type, especially in males (Fig. 1D). Remarkably, tumor development was still faster in *Palb2*$^{CC6/CC6}$;*Trp53*$^{-/-}$ mice (Fig. 1A), and thymic lymphoma was the only tumor type in the double mutant mice (Fig. 1D). This finding again demonstrates that disruption of the BRCA1-PALB2-BRCA2 axis accelerates p53-associated tumor development. During the breeding, we noted a female-specific embryonic lethality of *Palb2*$^{CC6/CC6}$;*Trp53*$^{-/-}$ mice (therefore only males were monitored). As *Trp53*$^{-/-}$ mice are known to be prone to female-specific defects in neural tube closure,[27,28] it is possible that mutation of *Palb2* or loss of the BRCA1-PALB2 interaction exacerbated the defect leading to embryonic lethality.

To gain insights into the genetic mechanisms of tumor development in the *Palb2*/*Trp53* mutant mice, we conducted whole exome sequencing (WES) for a total of 36 tumors of 3 different types (thymic lymphoma, osteosarcoma and soft tissue sarcoma) from mice of 6 different genotypes (Table S1). It has been reported that the majority of tumors arising from *Trp53*$^{+/-}$ mice undergo loss of heterozygosity (LOH), losing the wt allele.[22] Therefore, we first examined the *Trp53* locus in tumors from *Trp53*$^{+/-}$ and *Palb2*$^{CC6/CC6}$;*Trp53*$^{+/-}$ mice (Fig. 2A). Among thymic lymphomas, the only one from *Trp53*$^{+/-}$ mice and 3 of 6 from *Palb2*$^{CC6/CC6}$;*Trp53*$^{+/-}$ mice clearly lost the wt allele, as evidenced by a lack of reads in exons 2–7 (Fig. 2A, orange dots). Note that the residual reads in exons 2–7 present in some cases were likely due to impurity of the tumors, i.e. the presence of normal (*Trp53*$^{+/-}$) cells. Interestingly, among the remaining 3 thymic lymphomas from the *Palb2*$^{CC6/CC6}$;*Trp53*$^{+/-}$ mice sequenced, one lost the mutant allele but also contained a deletion of exons 8 and 9 of the wt allele (blue dot), while another appeared to have duplicated the mutant allele while still maintaining the wt allele (dark green dot). Among osteosarcomas, 1 of 3 from *Trp53*$^{+/-}$ mice and 4 of 6 from *Palb2*$^{CC6/CC6}$;*Trp53*$^{+/-}$ mice lost the wt allele. Among soft tissue sarcomas, loss of wt allele occurred in at least 2 of 4 from each group.

For all 3 tumor types, when the wt allele was lost, the mutant *Trp53* allele was often duplicated (copy-neutral LOH); however, in one (thymic lymphoma) case the mutant allele was triplicated (red dot), while a few other cases appeared to be hemizygous (with deletion of the wt allele, grey dots). Therefore, although all tumors from the *Palb2*$^{CC6/CC6}$;*Trp53*$^{+/-}$ mice did not lose the wt *Trp53* allele, overall LOH appeared to be accelerated due to the disruption of the BRCA1-PALB2 interaction, given the faster tumor development. The remaining wt allele could be disrupted by rearrangements, which is not detectable by WES.

We next analyzed the copy number of key cancer genes known to be involved in relevant tumor types studied here. Using computed copy number of 3 as a cutoff, 8/24 (33%) of the tumors arising from *Trp53*$^{+/-}$ and *Palb2*$^{CC6/CC6}$;*Trp53*$^{+/-}$ mice contained a gain or amplification of the *Myc* oncogene. In particular, 3/6 (50%) of osteosarcomas from the *Palb2*$^{CC6/CC6}$;*Trp53*$^{+/-}$ mice contained 5 or more copies of the gene (Fig. 2B). Gain of *Myc* (3–4 copies) was also observed in 4/6 (67%) and 3/6 (50%) of thymic lymphomas from *Trp53*$^{-/-}$ and *Palb2*$^{CC6/CC6}$;*Trp53*$^{-/-}$ mice, respectively. Among tumor suppressor genes, frequent deletions in *Pten* have been reported to occur in thymic lymphomas arising from *Trp53*$^{-/-}$ mice.[29] This was observed in the current study, as 4/6 (67%) of such tumors showed some forms of deletion in the gene (Fig. 2C, D). In comparison, partial or complete loss of *Pten* were observed in 3/6 (50%) of thymic lymphomas from the *Palb2*$^{CC6/CC6}$;*Trp53*$^{-/-}$ mice. Additionally, partial deletions of *Pten* were also seen in a small number of tumors from *Palb2*$^{CC6/CC6}$;*Trp53*$^{+/-}$ mice (Fig. 2E). With respect to the overall copy numbers of the 2 genes, no statistically significant difference was found between the *Palb2* wt and mutant subgroups of each tumor type.

Finally, we compared the number of mutations, large-scale state transition (LST) scores, and the number and length of indels in the different groups of tumors (Fig. S1). A larger number of mutations was found in osteosarcomas from *Palb2*$^{CC6/CC6}$;*Trp53*$^{+/-}$ mice than the same tumor type from *Trp53*$^{+/-}$ mice. Other than that, no significant differences in any parameter were found in other tumor types and groups. Therefore, in most cases, disruption of the BRCA1-PALB2 axis appeared to accelerate rather than alter the genetic changes that lead to tumor development in p53 mutant mice.

In summary, our finding that the *Palb2*^{CC6} mutation accelerates p53-associated tumor development (Fig. 1A, B) suggests that p53 is the more dominant factor that determines tumor spectrum, i.e. tissue specificity. Given the much shorter tumor latency in *Palb2*$^{CC6/CC6}$;*Trp53*$^{+/-}$ than *Trp53*$^{+/-}$ mice and a lack of clear differences in genomic alterations in tumors arising from the 2 groups of mice, our data suggest that loss of the BRCA1-PALB2-BRCA2 axis accelerates p53-associated tumor development without fundamentally altering the tumorigenic process. As for the mechanism, WES of tumors from *Palb2*$^{CC6/CC6}$ mice with a *Trp53* heterozygous background (Fig. 2) suggest that loss of the BRCA1-PALB2 interaction accelerates loss of heterozygosity (LOH) at *Trp53*. In almost all tumors with full *Trp53* inactivation, it occurred by losing, rather than mutating, the wt allele and duplicating the mutant allele, with the one exception being a deletion within the wt allele. Notably, the *Palb2*^{CC6} mutation also accelerated p53-associated tumor formation (mostly thymic lymphomas) in mice with biallelic knockout of *Trp53*, indicating that accelerating *Trp53* LOH is only part of the mechanism and that accelerated alterations in other cancer genes, such as *Myc*, also contributes to accelerated tumor development.

Our findings also imply that at least a subset of *BRCA*- and *PALB2*-associated breast cancers may be, by nature, a malignancy caused by somatic p53 loss of function or dysfunction that is accelerated by a defect in the BRCA1-PALB2-BRCA2 axis. This hypothesis is also supported by the fact that virtually all *BRCA1* mutant human breast cancer harbor *TP53* mutations.[30] However, *TP53* mutations only occur in a minority of *BRCA2* and *PALB2* mutant breast cancers.[31] Therefore, it would be important to determine if p53 or its pathway is inactivated in *BRCA2* and *PALB2* mutant cancers at levels other than primary gene sequence. It should also be noted that as human *BRCA* and *PALB2* mutation carriers are mostly heterozygous, cancer development in humans generally requires somatic, spontaneous inactivation of the wt allele by LOH or mutations. This is not only an extra step compared with biallelic ablations or mutations in mouse models but also a major variable, in terms of timing and frequency, among the 3 genes due to their different genomic loci and chromatin structures. As such, the different requirements of *TP53* inactivation may stem from differences in the timing, frequency, or nature of the inactivation of the wt *BRCA* or *PALB2* alleles in the breast epithelial cells of human mutation carriers.

Conflict of interests

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2020.08.012.

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