Autophagy Opposes p53-Mediated Tumor Barrier to Facilitate Tumorigenesis in a Model of PALB2-Associated Hereditary Breast Cancer

Yanying Huo, Hong Cai, Irina Teplova, Christian Bowman-Colin, Guanghua Chen, Sandy Price, Nicola Barnard, Shridar Ganesan, Vassiliki Karantza, Eileen White, and Bing Xia
INTRODUCTION

About 5% to 10% of breast cancers occur in the form of inherited predisposition in certain high-risk families in which women tend to develop the disease at higher frequencies and at younger ages than the general population. Interestingly, nearly all of the known familial breast cancer genes function, at least in part, in the repair and/or signaling response to DNA damage, particularly double strand breaks (DSB; 1). In addition, several of the susceptibility genes, for example, BRCA1, BRCA2, PALB2, TP53, and ATM, also share a function in reducing cellular levels of reactive oxygen species (ROS), which cause genome damage and promote tumorigenesis (2–6). Thus, a major fraction of hereditary breast cancer seems to result from a common root, namely genome instability.

BRCA1 and BRCA2 carry out key functions in genome stability maintenance by promoting faithful repair of DSBs by homologous recombination (HR) and other relevant processes (7, 8). We discovered PALB2 as a major BRCA2 binding partner that controls its chromatin association and function in HR (9). Subsequent work established PALB2 as a BRCA2-like tumor suppressor gene that is mutated in familial breast and pancreatic cancers as well as in Fanconi anemia (10–16). More recently, PALB2 was shown to directly bind BRCA1 as well, and to link BRCA1 and BRCA2 in HR repair (17, 18). Importantly, multiple patient-derived missense mutations that abrogate PALB2 binding have been identified in both BRCA1 and BRCA2 and shown to disable their HR repair function (9, 18), indicating that the three proteins function together in a BRCA complex/pathway to promote HR and suppress tumor development.

Contrary to an expectation that mice lacking Brca1 or Brca2 may develop breast cancer, complete knockout of either gene was found to result in early embryonic lethality (19). It was then realized that these genes were indispensable for HR, which is essential not only for tumor suppression but also for mammalian development. Consistent with the role of PALB2 as a linker between BRCA1 and BRCA2 in HR, systemic knockout of Palb2 in mice resulted in phenotypes similar to those of Brca1 and Brca2, including early embryonic lethality and induction of the cyclin-dependent kinase inhibitor p21 (20, 21).

The p53 transcriptional program plays essential roles in regulating many critical aspects of cell and tissue physiology that collectively prevent tumorigenesis. Virtually 100% of BRCA1-associated human breast cancers harbor mutations or deletions of the TP53 gene, and BRCA2- and PALB2-associated tumors also frequently contain TP53 mutations (11, 22, 23). Similarly, somatic mutations in Trp53 are frequently found in mammary tumors that develop in Brca1 and Brca2 conditional knockout (CKO) mouse models (24, 25), and Trp53 codeletion or heterozygosity strongly accelerated mammary gland tumor development in all Brca1 and Brca2 models tested (26–30). Moreover, loss of p53 partially rescues the embryonic lethality and developmental defect caused by the knockout of each of the three genes (21, 31). The evidence
indicates that inactivation of the p53 pathway may be a prerequisite for mammary epithelial cells (MEC) to survive DNA damage and escape the resulting cell-cycle checkpoint following BRCA1/2 loss and perhaps also loss of PALB2.

Autophagy is an intracellular waste disposal and recycling process whereby damaged organelles and certain proteins are engulfed in double-membrane vesicles (autophagosomes) and delivered to lysosomes for degradation (32). Through elimination of damaged mitochondria and toxic protein aggregates, and perhaps through other unknown mechanisms, autophagy mitigates oxidative stress and promotes genome stability, thereby suppressing tumorigenesis (33–35). Indeed, monoallelic loss of the essential autophagy gene Beclin 1 (Becn1) in mice leads to increased tumor development at old ages (35–37). Interestingly, autophagy has been shown to be upregulated in RAS-driven cancers, and these cancer cells seem to be “addicted” to and rely on autophagy for survival (38, 39). Thus, autophagy can also facilitate tumor development, presumably by mitigating oxidative stress and promoting tumor cell fitness and nutrient recycling (40, 41).

In this study, we generated and characterized a model of PALB2-associated breast cancer. Moreover, using this model, we explored the role of p53 and autophagy in breast cancer associated with oxidative stress and DNA damage. Our results show that inactivation of p53 is critical for most, if not all, Palb2-associated tumorigenesis; that autophagy facilitates the development of such breast cancer by promoting tumor cell survival; and that the effect of autophagy on mammary tumorigenesis is influenced by p53 status.

RESULTS

Mammary Tumor Development in Palb2 Conditional Knockout Mice

To gain new insights into PALB2-mediated tumor suppression, we targeted the mouse Palb2 gene by inserting loxP sites into introns 1 and 3 (Fig. 1A). Cre-mediated excision of exons 2 and 3 would render exon 4 out of frame and result in a functionally null Palb2 gene (42). To inactivate Palb2 in the mammary gland, Palb2−/− mice were crossed with mice bearing a Cre transgene driven by the mammary gland–specific promoter of whey acidic protein (Wap-cre; ref. 43). The resulting females were mated to undergo two rounds of pregnancy and lactation to induce maximal Cre expression in alveolar MECs, and then monitored for tumor development. As shown in Fig. 1B, 19 of 29 (66%) of mice with MEC-specific knockout of Palb2 developed 20 mammary tumors (T10 = 607 days), directly showing that Palb2 acts as a tumor suppressor in the mammary gland. None of the 18 control animals (with Wap-cre) developed mammary tumors.

Characteristics of Palb2-associated Mammary Tumors

Eighteen of the 20 mammary tumors that developed in the Palb2−/−;Wap-cre mice were analyzed for histology and immunophenotypes. Four characteristic histologic types were observed—solid (poorly differentiated adenocarcinoma), tubular (well-differentiated adenocarcinoma), sarcomatoid [post-epithelial to mesenchymal transition (EMT)], and adenosquamous (adenocarcinoma with squamous differentiation; Fig. 1C). Ten of the 18 tumors (56%) were mostly solid with varying degrees of tubule formation, one was largely tubular, three were mostly sarcomatoid, two had squamous differentiation, and the remaining two were mixtures of solid and sarcomatoid with ongoing EMT (Table 1). Necrosis was a common feature in solid areas but rarely seen in other areas or tumors. Nuclear grades were generally high except in the tubular areas of a few tumors. Although well-defined pushing margins were observed for all of the tumors, at least 15 of them were found to have invasive borders in one or more areas (Fig. 1C and Supplementary Fig. S1). Moreover, 10 of the 18 tumors appeared to have invaded skin or muscle at the time of collection. Additional views of histology are shown in Supplementary Fig. S1.

The status of the estrogen receptor (ER) and progesterone receptor (PR) in the 18 tumors was analyzed by immunohistochemistry (IHC; Fig. 2A). Eight (44%) tumors showed positive ER staining, and four (22%) were PR-positive (Table 1). For ER, the positive tumors generally showed nuclear staining in more than 30% of the cells, but the overall signal strength seemed to be weaker than what is commonly seen in typical human ER+ cancers. Similar findings were made for PR, except that higher background staining was observed in approximately half of the tumors, in which case a “−” status was assigned unless some of the cells showed a strong nuclear signal clearly above the background. Taken together, these results show that somatic deletion of Palb2 driven by Wap-cre can give rise to both ER+ and ER− mammary tumors, a scenario similar to human PALB2-associated breast cancers (10).

Role of p53 in Palb2-associated Mouse Mammary Tumors

The prevalence of TP53 mutations in BRCA- and PALB2-associated human breast tumors led us to sequence the Trp53 gene (cDNA) in tumors that arose from Palb2−/−;Wap-cre mice. Among the 14 tumors analyzed, nine (64%) contained missense mutations or internal deletions, four were wild-type (WT), and the remaining one did not yield cDNA, presumably due to biallelic deletion or extremely low mRNA expression level (Table 1). This finding suggests that the loss of p53 function is important for the development of Palb2-associated mammary tumors. In the four tumors with a wild-type Trp53 transcript, it is still possible that the p53 pathway may be rendered nonfunctional by other mechanisms, such as hyperactivation of MDM2.

To further understand the status of p53 in the tumors, we analyzed its protein levels using IHC (Fig. 2B). Nine (50%) of the 18 tumors were positive, including all of the seven tumors with missense mutations (Table 1). As expected, the two tumors with intragenic deletions/frameshift mutations both showed completely negative staining. Three of the four tumors with WT Trp53 were negative, but one was, surprisingly, strongly positive (##882). Although it is unclear whether the p53 downstream pathway is active in this particular tumor, our findings overall indicate that the loss of normal p53 function is critical for the development of Palb2-associated mammary tumors.

To study the genetic interaction between Palb2 and Trp53, we introduced a floxed Trp53 allele (26) into our model. As shown in Fig. 1B, combined deletion of Palb2 and Trp53 in MECs led to highly efficient tumor development that is much
Autophagy Promotes Breast Cancer Development

Figure 1. Mammary tumor development in mice with tissue-specific ablation of Palb2. A, schematic representation of the generation of the Palb2-floxed and knockout alleles. The full gene structure of Palb2 is shown on top. B, Kaplan-Meier survival curves of mice with mammary gland-specific deletion of Palb2, Trp53, or both genes. C, diverse histology of Palb2-associated mouse mammary tumors. I-IV, the four different types of histology observed; V-VIII, enlarged views of the center regions of I-IV, respectively; IX, a solid tumor with a well-formed pushing margin; X, a solid tumor invading into fat tissue; XI and XII, higher-power views of tumor cell nuclei and mitotic figures.

faster than that caused by Palb2 single deletion. The median tumor latency of the double CKO mice was also slightly shorter than that of the Trp53 single CKO mice (T50 = 246 vs. 289 days), suggesting that the two genes may synergistically suppress breast cancer development. However, the difference did not reach statistical significance (P = 0.0647, log-rank analysis). In addition, we also monitored a small number (n = 9) of Palb2fl/fl;Trp53fl/fl;Wap-cre females, and seven of them developed mammary tumors with latencies from 466 to 736 days, which were in a similar range as tumors arising in Palb2fl/fl;Trp53fl/fl;Wap-cre mice.

DNA Damage in Palb2-Null Tumor Cells and Their Sensitivity to DNA-Damaging Agents

Given the role of PALB2 in DNA repair, we assessed the extent of endogenous DNA damage in tumors by immunohistochemical...
**Figure 2.** Characterization of Palb2-associated mouse mammary tumors by IHC. 

**A,** representative ER and PR staining patterns of the tumors. Because a similar percentage of staining-positive cells was found in most of the positive tumors, assignment of “+” or “++” grades is based purely on the intensity of staining signals. 

**B,** representative staining patterns of p53 in the tumors. Grade assignment is based on the relative staining intensity. 

**C,** different staining patterns of γH2AX top) and 8-oxo-dG (bottom) in the control and Palb2-null tumors.
Table 1. Characteristics of mammary tumors developed in Palb2 CKO mice with Becn1+/+ and Becn1+/− backgrounds

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Becn1</th>
<th>Latency(d)</th>
<th>Tumor</th>
<th>Histology (H&amp;E)</th>
<th>Immunohistochemistry</th>
<th>Trp53 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>914</td>
<td>+/+</td>
<td>383</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>++ + + ++ + ++ + ++ +</td>
<td>GB09A/R270H</td>
</tr>
<tr>
<td>949</td>
<td>+/+</td>
<td>398</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>+ + ++ + ++ + ++ + +</td>
<td>G720A/M240I, G721T/G241W</td>
</tr>
<tr>
<td>741</td>
<td>+/+</td>
<td>422</td>
<td>1</td>
<td>Sarcomatoid + Tubular</td>
<td>++ + ++ + ++ + +</td>
<td>WT</td>
</tr>
<tr>
<td>749</td>
<td>+/+</td>
<td>428</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>+ − SF + ++ + + + +</td>
<td>Δ985-1089</td>
</tr>
<tr>
<td>826</td>
<td>+/+</td>
<td>430</td>
<td>1</td>
<td>Solid → Sarcomatoid</td>
<td>+ − + + ++ + +</td>
<td>N.D.</td>
</tr>
<tr>
<td>824</td>
<td>+/+</td>
<td>431</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>+ − ++ + ++ + + +</td>
<td>T391C/F131L</td>
</tr>
<tr>
<td>808</td>
<td>+/+</td>
<td>450</td>
<td>1</td>
<td>Sarcomatoid + Tubular</td>
<td>− − − + ++ + +</td>
<td>N.D.</td>
</tr>
<tr>
<td>882</td>
<td>+/+</td>
<td>453</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>− + ++ + ++ + + +</td>
<td>WT</td>
</tr>
<tr>
<td>751</td>
<td>+/+</td>
<td>456</td>
<td>1</td>
<td>Tubular + Solid</td>
<td>− − SF − −</td>
<td>WT</td>
</tr>
<tr>
<td>049</td>
<td>+/+</td>
<td>498</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>− − ++ + ++ + +</td>
<td>Δ541-546, Δ664-771</td>
</tr>
<tr>
<td>912</td>
<td>+/+</td>
<td>498</td>
<td>1</td>
<td>Solid → Sarcomatoid</td>
<td>++ − ++ + ++ + +</td>
<td>CB08T/R270C</td>
</tr>
<tr>
<td>915</td>
<td>+/+</td>
<td>529</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>− − + ++ + +++</td>
<td>A632G/H211R</td>
</tr>
<tr>
<td>742</td>
<td>+/+</td>
<td>576</td>
<td>1</td>
<td>Squamous + Tubular</td>
<td>+ ++ − ++ + +</td>
<td>N.D.</td>
</tr>
<tr>
<td>827</td>
<td>+/+</td>
<td>608</td>
<td>T1</td>
<td>Solid + Tubular</td>
<td>− − + ++ + ++ +</td>
<td>GB29A/R277G</td>
</tr>
<tr>
<td>825</td>
<td>+/+</td>
<td>637</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>− − + + ++ +</td>
<td>N.D.</td>
</tr>
<tr>
<td>042</td>
<td>+/+</td>
<td>665</td>
<td>1</td>
<td>Squamous + Tubular</td>
<td>− − − + ++ +</td>
<td>No PCR product</td>
</tr>
<tr>
<td>747</td>
<td>+/+</td>
<td>719</td>
<td>1</td>
<td>Sarcomatoid + Solid</td>
<td>− − ++ + ++ +</td>
<td>T618A/V206D</td>
</tr>
<tr>
<td>907</td>
<td>+/-</td>
<td>520</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>− − SF + −</td>
<td>WT</td>
</tr>
<tr>
<td>163</td>
<td>+/-</td>
<td>522</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>− − ++ + ++ +</td>
<td>WT</td>
</tr>
<tr>
<td>890</td>
<td>+/-</td>
<td>529</td>
<td>T1</td>
<td>Solid + Squamous + Tubular</td>
<td>− − + ++ +</td>
<td>C404T/A135V</td>
</tr>
<tr>
<td>047</td>
<td>+/-</td>
<td>567</td>
<td>1</td>
<td>Squamous + Solid + Tubular</td>
<td>− − − + − +</td>
<td>N.D.</td>
</tr>
<tr>
<td>723</td>
<td>+/-</td>
<td>588</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>± − ++ + ++ +</td>
<td>WT</td>
</tr>
<tr>
<td>119</td>
<td>+/-</td>
<td>626</td>
<td>1</td>
<td>Solid + Tubular</td>
<td>± + + ++ +</td>
<td>WT</td>
</tr>
<tr>
<td>153</td>
<td>+/-</td>
<td>665</td>
<td>1</td>
<td>Tubular + Solid</td>
<td>− − + + ++ +</td>
<td>WT</td>
</tr>
</tbody>
</table>

NOTE: See Fig. 1 for definitions and examples of histology and ER, PR, and p53 immunogrades. “±” indicates weak staining in only some areas of the tumors. For H2AX, “+++”, “++”, and “+” denote tumors in which >30%, 10–30%, and 1–10% of cells, respectively, show ≥3 strongly stained foci or virtually pan-nuclear staining; “SF” denotes tumors in which a single, large focus was observed per cell, which is possibly the inactivated X chromosome. Grade assignment for 8-oxo-dG staining is based on the relative staining intensity, as all positive tumors have more than 60% of cells showing positive staining. Trp53 mutations are shown at both DNA and protein levels, separated by a forward slash. Abbreviations: H&E, hematoxylin and eosin; N.D., Trp53 cDNA sequence not determined due to poor quality of RNA isolated from frozen tissues.
analysis of γH2AX, a marker of DSBs (Fig. 2C). Thirteen (72%) of them showed γH2AX staining regardless of Trp53 status, indicative of the existence of unrepaired DSBs (Table 1). In contrast, little to no staining was detected in tumors arising from Palb2−/−;Trp53−/−;Wap-cre mice, which developed with similar latency (Fig. 2C). Consistent with our recent finding that PALB2 plays a role in the oxidative stress response (4), the Palb2-null tumors were found to have much higher levels of oxidative DNA damage as revealed by immunohistochemical staining of 8-oxo-dG, a marker of such damage, (4), the ing that PALB2 plays a role in the oxidative stress response.

The deletion of the respective proteins in the cell lines was made to generate cell lines from mouse mammary tumors. Several attempts were established from fibroblasts, like BRCA1- and BRCA2-null cells, to retain a WT Palb2 and Trp53. However, to our knowledge, no human PALB2-null breast cancer cells have been established. Thus, to better understand the function and “druggability” of PALB2, we attempted to generate cell lines from the mouse mammary tumors. Several attempts were made to generate cell lines from Palb2-single–null tumors, but only a useful line (PF741) was successfully established that retained a WT Trp53 gene. In contrast, multiple lines were established from Trp53-single–null (Palb2-WT) and Palb2/Trp53-double–null tumors.

The above cells were tested for their ability to repair DNA damage elicited by olaparib, a PARP inhibitor, and MMC. Interestingly, the Palb2-null cells contained more DSBs as revealed by γH2AX immunofluorescence even in the absence of drugs, which was particularly evident in the Palb2/Trp53-double–null cells (Fig. 3A and Supplementary Fig. S2). By 3 hours following treatment, both agents resulted in increased γH2AX staining signal in all three cell types, and distinct RAD51 foci colocalizing with those of γH2AX were observed in Palb2-WT cells, but not in either of the Palb2-null cells. By 8 hours after treatment, γH2AX signals had returned to pretreatment levels in the Palb2-WT cells, whereas the signals persisted in both types of Palb2-null cells. By this time, RAD51 foci had largely disappeared in the Palb2-WT cells, and Palb2-single–null cells showed a diffuse RAD51 staining pattern. Another p53-single–null (control) and two additional Palb2/Trp53-double–null cell lines were tested in parallel and the results were essentially the same.

Next, we conducted neutral comet assays to further assess the levels of DNA breaks in the six cell lines. Compared with the two Palb2-WT cells, all four Palb2-null cells showed substantially higher levels of DNA breaks before drug treatment (Fig. 3B and Supplementary Fig. S2), indicative of a significant defect in the repair of DNA breaks resulting from endogenous factors, such as collapse of replication forks, etc. At 3 hours after drug treatment, increased DNA fragmentation was seen in all cells. By 8 hours after treatment, the levels of DNA breaks were found to have decreased in the Palb2-WT cells but not the Palb2-null cells (Fig. 3B and Supplementary Fig. S2), again indicating an inability of the mutant cells to execute HR-based repair. Consistently, both types of Palb2-deficient cells were hypersensitive to both agents (Fig. 3C and D). The deletion of the respective proteins in the cell lines was confirmed by Western blotting (Fig. 3E). Collectively, these results further underscore the critical role of PALB2 in HR-mediated repair and support the applicability of PARP inhibitors and DNA crosslinkers for PALB2-associated cancers.

Senescence and Apoptosis upon Palb2 Deletion and Rescue by Codelletion of Trp53

To test the immediate consequence of PALB2 loss in primary cells and the role of p53 in this process, we generated mouse embryonic fibroblasts (MEF) from Palb2−/− and Palb2−/−;Trp53−/− mice. After the cells were infected with a Cre-encoding retrovirus to induce gene deletion and subjected to selection, virtually complete loss of the respective proteins was observed (Fig. 4A and B).

As expected, Palb2-null MEFs showed much increased endogenous DSBs, as evidenced by nuclear foci formation of 53BP1 (Fig. 4C). γH2AX foci were not counted in all experiments. However, when we obtained γH2AX with 53BP1, all 53BP1-positive cells were found to be positive for γH2AX, whereas some cells showing multiple but weakly stained γH2AX foci did not display distinct 53BP1 foci (Supplementary Fig. S3). Thus, the actual extent of DNA breaks in the cells should be even greater.

Consistent with our previous finding that PALB2 promotes the nuclear accumulation and function of the antioxidant transcription factor NRF2 (4), the protein was localized mostly in the nucleus in Palb2-WT MEFs but showed a diffuse staining pattern in Palb2-null MEFs (Fig. 4D). Accordingly, Palb2-null MEFs had significantly higher ROS levels throughout the experimental period (Fig. 4E). Together with the fact that Palb2-null tumor cells contained higher levels of oxidative DNA damage (Fig. 2C), these results further underscore the importance of PALB2 in cellular defense against oxidative stress.

Notably, starting from passage 2, large numbers of Palb2-null MEFs appeared flat and enlarged, stained positive for beta-galactosidase, and displayed poor growth (Fig. 4F–H), indicating that the cells were entering senescence. Moreover, an Annexin V assay revealed apoptosis occurring in a substantial fraction of the cells (Fig. 4I). Codelletion of Trp53 completely rescued the slow growth, senescence, and apoptosis phenotypes that resulted from Palb2 deletion. These observations indicate that loss of p53 is able to allow cells to overcome growth arrest or apoptosis induced by DNA damage and oxidative stress after PALB2 loss.

Effect of Becn1 Heterozygosity on Palb2-associated Mammary Tumorigenesis

Autophagy is particularly important for mitigating oxidative stress and suppressing DNA damage response activation during stresses. In addition, recent studies have shown that autophagy can also facilitate cellular senescence (45). Therefore, we suspected that autophagy might play a role in PALB2-associated breast cancer development. To address this, we crossed the Palb2−/−;Wap-cre and Palb2−/−;Trp53−/−;Wap-cre mice to Becn1+/− mice (36). As shown in Fig. 5A, allelic loss of Becn1 significantly delayed mammary tumor formation in Palb2−/−; Wap-cre animals (P = 0.0035, log-rank analysis). Moreover, only seven of the 26 Becn1−/− animals developed mammary tumors. However, allelic loss of Becn1 did not affect tumorigenesis due to combined MEC-specific loss of Palb2 and Trp53, suggesting that the suppression of Palb2-mediated tumorigenesis upon allelic loss of Becn1 is mediated by p53.

The eight tumors that formed in the seven Palb2−/−;Wap-cre; Becn1+/− mice were similar to their Becn1+/− counterparts in terms
Figure 3. DNA repair defect of Palb2-null tumor cells. A, γH2AX and RAD51 foci formation before and after DNA damage induced by olaparib. Tumor cells were treated with 25 μmol/L olaparib for 1 hour and the drug was then removed. Cells were fixed at 3 and 8 hours after drug removal and analyzed by immunofluorescence. B, levels of DNA breaks before and after olaparib treatment. Cells were treated as above, collected at the same time points, and analyzed by neutral comet assay. C and D, sensitivity of the tumor cells to olaparib and MMC. Cells were seeded in 96-well plates, treated with the drugs for 4 days, and analyzed by CellTiterGlo assay. E, Western blots showing PALB2 and p53 protein levels in the tumor cells analyzed in C and D.
Figure 4. Senescence and apoptosis of MEFs following Palb2 loss and the rescue by codeletion of Trp53. A, schematic timeline of the generation and passaging of the MEFs. Two different MEF lines of each genotype were generated and analyzed in parallel. B, Western blots showing loss of PALB2, p53, or both proteins in the MEFs at passage 2. C, 53BP1 nuclear foci formation in the control, Palb2 deletion, and Palb2/Trp53 double deletion MEFs. Top, representative immunofluorescent images of 53BP1 staining during passage 1. Bottom, quantification of foci-positive cells in all three passages. D, NRF2 localization in the MEFs during passage 1, as determined by immunofluorescence. E, cellular levels of ROS in the MEFs in all three passages. F and G, cellular senescence induced by Palb2 inactivation and the rescue by codeletion of Trp53. F, representative images of senescence-associated beta-galactosidase (SA-β-gal) staining of the WT, Palb2-null, and Palb2/Trp53-double-null MEFs, and the quantification is shown in G. H, growth curves of the MEFs showing the growth arrest of the Palb2-null MEFs and the rescue by loss of p53. I, cellular apoptosis following Palb2 inactivation and the rescue by codeletion of Trp53. Apoptotic cells were measured by Annexin V assay. In all above analyses, values shown are the averages of the two independent MEF lines for each genotype, and error bars represent SDs. *P values were determined by two-tailed t-test. **P ≤ 0.005, ***P ≤ 0.01. DCF, 2′,7′-Dichlorofluorescin diacetate.
Autophagy Promotes Breast Cancer Development

RESEARCH ARTICLE

of histology and DNA damage levels (Table 1). Two (25%) of them were marginally positive for ER, showing weak staining signals that were seen in only some areas of the tumors. Moreover, only one of the six tumors (16.7%) sequenced was found to have a Trp53 mutation, as compared with the 64% mutation rate of the Becn1+/−/− tumors. These findings imply that a defect in autophagy may force a different path of tumor evolution following PALB2 loss. Because of the small number of Becn1+/−/− tumors obtained in this work, a larger study may be needed to confirm the results and address the potential mechanisms.

Autophagy and Apoptosis in the Palb2-deficient Mammary Tumors

The finding that allelic loss of Becn1 suppressed Palb2-associated mammary tumorigenesis by a p53-dependent mechanism suggests that autophagy facilitates tumor development. To assess the levels of autophagy activity in the mammary tumors, we analyzed 12 tumor samples (six Becn1+/+ and six Becn1+/−) using electron microscopy. A number of autophagosomes were identified (Fig. 5B), indicating that autophagy indeed occurs in Palb2-associated breast cancer even in the absence of external stress. Notably, autophagosomes were observed in five of the six Becn1+/+ tumors but in only one of the six Becn1+/− tumors, suggesting that allelic loss of Becn1 caused a partial, but appreciable, impairment of autophagy in the setting used. To further confirm the autophagy defect in the Becn1+/− tumors, we compared the levels of p62 (SQSTM1), which is an important substrate for autophagy that accumulates when autophagy is impaired (35). When necrotic areas were excluded, all Becn1+/+ tumors exhibited weak or virtually no staining signal, whereas distinct areas of strong staining were observed in tumors arising from Palb2+/−;Trp53f/f;Wap-cre;Becn1+/− mice (Fig. 5C). In mammary tumors from Palb2+/−;Trp53f/f;Wap-cre;Becn1+/− animals, positive

Figure 5. Role of autophagy in Palb2-associated mammary tumor development. A, Kaplan–Meier survival curves showing mammary tumor development in Palb2-single and Palb2;Trp53-double CKO mice in Becn1+/+ and Becn1+/− backgrounds. B, autophagosomes observed in tumors from mice with indicated genotypes. Note that tumor #827 contains a Trp53 point mutation (Table 1), although it developed in Trp53-WT mice. C, immunohistochemical analysis of autophagy substrate p62 in tumors arising from the four different genetic backgrounds as indicated. Tumors #751 and #163 still retained WT Trp53, whereas #915 and #890 had acquired somatic mutations in Trp53 (Table 1). D, immunohistochemical analysis of cleaved caspase-3, a marker of apoptosis, in the same tumors as in C.
p62 staining was observed, but was markedly weaker than in mammary tumors arising from Palb2<sup>−/−</sup>Wap-cre;Brca2<sup>f/f</sup> mice.

Next, we analyzed the levels of cleaved (activated) caspase-3, which marks apoptotic cells, in the tumor tissues. As in the case of p62, tumors from Palb2<sup>−/−</sup>Wap-cre;Brca2<sup>f/f</sup> mice showed weak or no cleaved caspase-3 staining across nonnecrotic areas, whereas pockets of positive staining were found in tumors that developed in Palb2<sup>−/−</sup>Wap-cre;Brca2<sup>f/f</sup> animals (Fig. 5D). In the Palb2;Trp53 doubly deleted tumors, the staining was all negative regardless of Brca1 status. Thus, combined deficits in DNA repair and autophagy seemed to elevate p53-dependent apoptosis in Palb2<sup>−/−</sup>;Brca1<sup>−/−</sup> mammary tumor cells. To further address the potential correlation between autophagy defect and cell death (apoptosis) in Palb2-associated mammary tumors, we analyzed six tumors with WT Trp53 (3 Palb2<sup>−/−</sup>; Brca2<sup>+/−</sup> and 3 Palb2<sup>−/−</sup>; Brca2<sup>−/−</sup>) by IHC for p62, LC3B (another autophagy substrate), and cleaved caspase-3. As shown in Supplementary Fig. S4, the three Brca2<sup>−/−</sup> tumors stained positive for all three markers, whereas the three Brca1<sup>−/−</sup> tumors showed virtually no staining.

**DISCUSSION**

We showed that ablation of Palb2 in MECs led to mammary tumor development with a median latency of 607 days. The tumors displayed diverse histology but were generally high grade and invasive. With respect to hormone receptors, 44% of tumors analyzed showed positive ER staining and 22% were PR-positive (Table 1). In comparison, human PALB2 cancers are also generally high grade, whereas ER/PR status of the tumors seems to vary significantly depending on mutations and/or populations (10). Overall, approximately 40% of human PALB2 tumors were triple-negative for ER, PR, and HER2, putting the clinical phenotypes of PALB2-driven cancers under both normal and low autophagy conditions.

As noted before, accumulating evidence from both human and mouse studies suggests that the barrier may be mostly enforced by p53. Our finding that the majority of the Palb2-associated tumors analyzed here (9/14) were Trp53-mutated lends further support to the above notion.

The most-prognostic molecular feature shared by Brca1<sup>−/−</sup> and Palb2<sup>−/−</sup> proteins is their role in HR, which is the major mechanism to repair the type of DSBs that inevitably arise during normal DNA replication. Upon loss of any of these proteins, an inability of cells to prevent and repair collapsed replication forks leads to DSB accumulation and a DNA damage response that presumably activates p53. Depending on the circumstances and the extent of p53 activation, cells may undergo G<sub>1</sub>-S arrest, senescence, and/or apoptosis. It still remains to be seen which one(s) is the predominant consequence of Brca1- or Palb2-driven mammary cancer in vivo. This knowledge is important for understanding the developmental path, as well as tissue specificity, of Brca1- and Palb2-associated cancers.

On the basis of existing knowledge and the results obtained in this study, we propose a model of Palb2-associated hereditary breast cancer development as illustrated in Fig. 6. Under normal conditions, PALB2 functions together with BRCA1 and BRCA2 to maintain genome stability and cellular homeostasis to suppress cancer development. When PALB2 is lost, increased DNA damage and ROS cause activation of p53, which induces growth inhibition and perhaps senescence or apoptosis, thereby suppressing tumor formation. Under such adverse conditions, autophagy facilitates cell survival and growth, which allows PALB2-null cells to accumulate further mutations and evolve into cancer cells. When autophagy is defective, increased cell death occurs and the potential for tumor development is reduced. If PALB2 is lost in a cell with already-mutated p53, highly efficient tumor formation occurs under both normal and low autophagy conditions.

The role of p53 in regulating autophagy has been reported by multiple groups and seems to be complex. In particular, one study showed that nuclear p53 promotes autophagy by inducing relevant gene expression, whereas cytoplasmic p53 inhibits autophagy (48). In the present study, allelic loss of Brca1 did not produce any difference in tumor formation in mice with Palb2;Trp53 double deletion in MECs. This finding may have at least two different implications. First, the strong growth advantage conferred by a complete p53 loss may override the reduced fitness elicited by impaired autophagy in Palb2<sup>−/−</sup> MECs or tumor cells. Second, p53 may negatively regulate autophagy in these cells so that loss of p53 leads to a compensation of autophagy function. However, it is important to note that real-world cancers mostly harbor TP53 point mutations combined with loss of heterozygosity instead of biallelic deletions, and it is known that point mutations may possess both loss and gain of functions. Therefore, the actual effect of TP53 mutations on the impact of autophagy on cancer may be variable and again context-dependent.

Our finding is consistent with a recent study that found that allelic loss of Beclin1 delayed tumor development in Atm-deficient mice (3). However, while the above study suggests that Beclin1 heterozygosity leads to a restoration of mitochondrial health damaged by Atm deficiency, no gross difference was noted in the mitochondria of the tumor samples analyzed by electron microscopy in the present study. Still, the Beclin1−/−
Autophagy Promotes Breast Cancer Development

**Figure 6.** A model of the developmental paths of PALB2-associated breast cancer. Under normal conditions, PALB2 functions together with BRCA1 and BRCA2 to maintain genome stability and cellular homeostasis to suppress cancer development. Upon loss of PALB2, p53 is activated, posing a strong barrier to tumor formation, whereas autophagy helps sustain cell viability and proliferation, thereby facilitating tumor cell evolution. However, the impact of autophagy may manifest only when p53 is functional.

Inhibiting autophagy as a potential cancer therapy has gained increasing attention. In this study, Palb2−/−;Becn1−/− tumors had reduced incidence and also seemed to grow more slowly compared with the corresponding Becn1+/− tumors. Consistently, such (Becn1−/−) tumors were found to contain areas undergoing apoptosis. These results suggest that rational autophagy inhibition may selectively kill PALB2-deficient tumor cells. Given the close relationship and functional similarity between PALB2 and BRCA1/2, the same notion may apply to BRCA-deficient tumor cells as well.

**METHODS**

To create a Palb2 CKO mouse model, we targeted the Palb2 locus and generated a strain in which exons 2 and 3 of the gene are flanked by loxP sites (42). The Palb2fl/w or mice were crossed to strains carrying Trp53fl/fl (26), Becn1−/− (36), and Wap-cre (43) alleles to generate all the genotypes in this study. Females of desired genotypes were mated to go through two rounds of pregnancy and lactation to induce Wap expression, and then monitored for tumor development. Tumors were collected when they reached approximately 1.0 cm in diameter. Primary MEFs were generated from E13.5 embryos. All experimental procedures involving animals were carried out in accordance with policies set forth by the Institutional Animal Care and Use Committee of the Robert Wood Johnson Medical School (New Brunswick, NJ) under the protocol numbers I08-073-9 and I11-029-5. To delete the Palb2 and Trp53 genes in MEFs, freshly generated cells with floxed alleles were infected with a Cre-encoding retrovirus and selected with puromycin. Mammary tumor cells were generated from tumor specimens dissociated with collagenase. Olaparib and MMC sensitivities were determined by the CellTiter Glo cell proliferation assay (Promega). Levels of ROS were measured using the 2′,7′-Dichlorofluorescin diacetate assay. Cellular senescence and apoptosis were determined using the senescence-associated β-galactosidase assay and Annexin V assay, respectively. Western blotting and immunofluorescence staining were conducted using standard protocols. Neutral comet assay was conducted using the CometAssay kit from Trevigen following the manufacturer’s protocol. For details, see Methods in the Supplementary Information.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: Y. Huo, H. Cai, V. Karantza, E. White, B. Xia Development of methodology: Y. Huo, H. Cai, C. Bowman-Colin, B. Xia Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Huo, H. Cai, I. Teplova, S. Price, N. Barnard, B. Xia Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Huo, I. Teplova, S. Ganesan, B. Xia Writing, review, and/or revision of the manuscript: Y. Huo, H. Cai, S. Ganesan, V. Karantza, E. White, B. Xia Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Huo, H. Cai, I. Teplova, G. Chen, S. Price, N. Barnard, B. Xia Study supervision: B. Xia
Acknowledgments

The authors thank Drs. David Livingston and Chrysi Kanellopoulou for supporting the initial stage of the work and for their critical comments on the manuscript. The authors also thank Dr. Shoreh Miller for valuable assistance in mouse breeding.

Grant Support

This work was supported by the National Cancer Institute (ROI1CA138804; to B. Xia), the American Cancer Society (RSG #TBG-119822; to B. Xia), and The Cancer Institute of New Jersey (to B. Xia).

Received January 7, 2013; revised April 25, 2013; accepted April 25, 2013; published OnlineFirst May 6, 2013.

REFERENCES

Autophagy Promotes Breast Cancer Development
