**brca2 and tp53 Collaborate in Tumorigenesis in Zebrafish**

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**Abstract**

Germline mutations in the tumor suppressor genes *BRCA2* and *TP53* significantly influence human cancer risk, and cancers from humans who inherit one mutant allele for *BRCA2* or *TP53* often display loss of the wildtype allele. In addition, *BRCA2*-associated cancers often exhibit mutations in *TP53*. To determine the relationship between germline heterozygous mutation (haploinsufficiency) and somatic loss of heterozygosity (LOH) for *BRCA2* and *TP53* in carcinogenesis, we analyzed zebrafish with heritable mutations in these two genes. Tumor-bearing zebrafish were examined by histology, and normal and neoplastic tissues were collected by laser-capture microdissection for LOH analyses. Zebrafish on a heterozygous *tp53* backgrounds had a high incidence of malignant tumors. The *brca2* mutation status determined both the incidence of LOH and the malignant tumor phenotype. LOH for *tp53* occurred in the majority of malignant tumors from *brca2* wildtype and heterozygous mutant zebrafish, and most of these were malignant peripheral nerve sheath tumors. Malignant tumors in zebrafish with heterozygous mutations in both *brca2* and *tp53* frequently displayed LOH for both genes. In contrast, LOH for *tp53* was uncommon in malignant tumors from *brca2* homozygotes, and these tumors were primarily undifferentiated sarcomas. Thus, carcinogenesis in zebrafish with combined mutations in *tp53* and *brca2* typically requires biallelic mutation or loss of at least one of these genes, and the specific combination of inherited mutations influences the development of LOH and the tumor phenotype. These results provide insight into cancer development associated with heritable *BRCA2* and *TP53* mutations.

**Introduction**

Tumor suppressor genes provide a key barrier to neoplastic transformation by repressing survival and proliferation of abnormal cells. Germline mutations in the tumor suppressor genes *BRCA2* and *TP53* influence tumor susceptibility in many vertebrate species. In humans who inherit one mutated copy of *BRCA2* or *TP53*, tumor development is often associated with loss of the wildtype allele, indicating that somatic loss of heterozygosity (LOH) is important for neoplastic transformation [1–4]. However, tumors can occur in human carriers of *BRCA2* or *TP53* mutations without somatic LOH, suggesting that haploinsufficiency for either gene can lead to tumorigenesis [1,5,6].

*BRCA2* mutation in humans is associated with two distinct cancer susceptibility syndromes. Individuals who inherit one mutant allele for *BRCA2* experience an increased risk for breast and ovarian cancer in adulthood [7,8], while individuals who inherit biallelic *BRCA2* mutations have a high incidence of malignancies during childhood [9]. *TP53* mutation may have a synergistic effect on tumorigenesis in *BRCA2*-associated cancers. Breast and ovarian cancers from *BRCA2*-heterozygous patients often develop *TP53* mutations [10–12], which may precede loss of the wildtype *BRCA2* allele [13], and are not attributable to a generalized or random increase in genetic mutations [10]. Similarly, tumor development is enhanced in *Bra2*-mutant mice and zebrafish with concomitant homozygous *Tp53* mutation or loss [14–17].

Although *TP53* dysfunction is important in *BRCA2*-associated carcinogenesis, the effect of coincident disruptions in these genes on tumorigenesis is not well defined. In this study, we investigated the relationship between inherited mutations in *bra2* and *tp53*, and somatic LOH for these genes, in tumorigenesis in zebrafish [16,18]. Zebrafish with heterozygous *tp53* mutation displayed a high incidence of malignant tumors. LOH for *tp53* was an important factor in malignant tumors from *bra2* wildtype and *bra2* homozygous zebrafish, but was less important in *bra2* homozygous mutant zebrafish. Furthermore, the *bra2* mutation status influenced the age at tumor onset, tumor number, and tumor type. Lastly, malignant peripheral nerve sheath tumors consistently exhibited LOH for *tp53*, while undifferentiated sarcomas more commonly exhibited loss of *bra2* via homozygous mutation. These findings indicate that mutations in *bra2* and *tp53* enhance tumorigenesis in zebrafish, as seen in humans, and that the incidence and type of tumor depends upon the particular combination of mutations.

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Results

Homozygous brca2<sup>Q658X</sup> mutation enhances tumor development in tp53<sup>M214K</sup> heterozygous zebrafish

We previously reported that homozygous tp53<sup>M214K</sup> mutation [18], combined with heterozygous or homozygous brca2<sup>Q658X</sup> mutation, accelerates tumorigenesis in zebrafish [16]. The tp53<sup>M214K</sup> mutation is a missense mutation [18] and the brca2<sup>Q658X</sup> mutation is a nonsense mutation [16] (mutant alleles subsequently designated as ‘m’). Here we describe tumor development in zebrafish that are wildtype (brca2<sup>+</sup>/+), heterozygous (brca2<sup>+</sup>/m), or homozygous (brca2<sup>m/m</sup>) for the brca2<sup>Q658X</sup> mutation, on a heterozygous tp53<sup>M214K</sup> mutant background (tp53<sup>+</sup>/m).

We analyzed 90 tp53<sup>+</sup>/m zebrafish by histology and determined that the overall tumor incidence was 82% in brca2<sup>+</sup>/+;tp53<sup>+</sup>/m zebrafish, 91% in brca2<sup>+</sup>/m;tp53<sup>+</sup>/m, and 100% in brca2<sup>m/m</sup>;tp53<sup>+</sup>/m zebrafish (Table 1). There was no significant difference in tumor development between male and female zebrafish (Table 1). Tumor development occurred between 12.0 and 26.5 months of age (Figure 1A). The mean age at tumor diagnosis was statistically significantly lower in brca2<sup>m/m</sup>;tp53<sup>+</sup>/m zebrafish compared to brca2<sup>+</sup>/+;tp53<sup>+</sup>/m and brca2<sup>+</sup>/m;tp53<sup>+</sup>/m zebrafish (Figure 1A). The overall survival for brca2<sup>m/m</sup>;tp53<sup>+</sup>/m zebrafish declined rapidly compared to brca2<sup>+</sup>/+;tp53<sup>+</sup>/m and brca2<sup>+</sup>/m;tp53<sup>+</sup>/m zebrafish (Figure S1).

To determine if the risk of malignant tumor development correlated with the brca2 genotype in tp53<sup>+</sup>/m zebrafish, the numbers of zebrafish that developed at least one malignant tumor were determined for brca2<sup>+</sup>/+;tp53<sup>+</sup>/m, brca2<sup>+</sup>/m;tp53<sup>+</sup>/m, and brca2<sup>m/m</sup>;tp53<sup>+</sup>/m cohorts (Table 1). Tumors with clear histologic evidence of tissue invasion and destruction were classified as malignant, while tumors that exhibited expansile but noninvasive growth were classified as benign. In all three cohorts, most tumor-bearing zebrafish developed malignant tumors, rather than benign tumors (Table 1 and Table 2). The proportion of zebrafish that developed at least one malignant tumor was not significantly different between any of the three cohorts (Figure 1B and Table 1).

In comparison to the other cohorts, a greater proportion of brca2<sup>m/m</sup>;tp53<sup>+</sup>/m zebrafish developed benign tumors (Figure 1B), due to an increased incidence of benign testicular tumors (Table 2).

Figure 1. Tumor development in tp53<sup>+</sup>/m zebrafish is influenced by brca2<sup></sup> mutation status. (A) Age at tumor diagnosis is significantly lower in brca2<sup>m/m</sup>;tp53<sup>+</sup>/m zebrafish compared to brca2<sup>+</sup>/+;tp53<sup>+</sup>/m and brca2<sup>+</sup>/m;tp53<sup>+</sup>/m zebrafish. (B) The percentage of zebrafish that developed at least one malignant tumor, only benign tumors, or no tumors, in brca2<sup>+</sup>/+;tp53<sup>+</sup>/m, brca2<sup>+</sup>/m;tp53<sup>+</sup>/m, and brca2<sup>m/m</sup>;tp53<sup>+</sup>/m zebrafish. (C) MPNST from a brca2<sup>+</sup>/+;tp53<sup>+</sup>/m zebrafish. (D) Undifferentiated sarcoma from a brca2<sup>m/m</sup>;tp53<sup>+</sup>/m zebrafish. (E) MPNST and nephroblastoma from a brca2<sup>+</sup>/+;tp53<sup>+</sup>/m zebrafish. MPNST, malignant peripheral nerve sheath tumor; NB, nephroblastoma. Scale bars, 20 μm (C,D) and 200 μm (E).

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The most common malignant tumor types observed in this study were malignant peripheral nerve sheath tumors (Figure 1C and Table 2) and undifferentiated sarcomas (Figure 1D and Table 2), the latter of which lacked sufficient histologic differentiation for more specific classification. Some zebrafish developed two or more histologically distinct and anatomically discrete tumors (Figure 1E). Over 50% of brca2+/+;tp53+/m;tp53+/m zebrafish developed more than one tumor; however, less than 20% of brca2+/+;tp53+/m and brca2+/+;m;tp53+/m zebrafish developed multiple tumors (Table 1). When multiple tumors occurred, they were distinguishable by histologic features, and were typically distinctly different tumor types (Figure 1E).

To determine the background level of tumor development over time in zebrafish with brca2 mutation alone, we screened a small group of aged brca2+/+;m;tp53+/m, brca2+/+;m;tp53+/+, and brca2 m/m;tp53+/+ zebrafish for tumor development (Table S1). The overall tumor incidence was higher in the brca2 m/m;tp53+/+ cohort (Table S1 and Figure S2) compared to the other cohorts. This difference was attributable to an increased incidence of testicular tumors in brca2 m/m;tp53+/+ zebrafish, as observed

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**Table 1.** Characteristics of tumor development in brca2+/+;tp53+/m, brca2+/+;m;tp53+/m, and brca2 m/m;tp53+/+ m zebrafish.

| Tumor incidence in tp53+/m zebrafish | brca2+/+ | brca2+/+ | brca2 m/m |
|--------------------------------------|----------|----------|-----------|
| Number of animals analyzed           | 22       | 55       | 13        |
| Animals with tumors                  | 18 (82%) | 50 (91%) | 13 (100%) |
| Male                                 | 6        | 16       | 12        |
| Female                               | 9        | 33       | 0         |
| Sex not determined                   | 3        | 1        | 1         |
| Animals without tumors               | 4 (18%)  | 5 (9%)   | 0 (0%)    |
| Male                                 | 1        | 3        | 0         |
| Female                               | 3        | 2        | 0         |
| Sex not determined                   | 0        | 0        | 0         |

| Proportion of male and female animals with tumors in tp53+/m zebrafish | brca2+/+;tp53+/m males versus brca2+/+;tp53+/m females | p = 1.000 |
|----------------------------------------------------------------------|-------------------------------------------------------|-----------|
| brca2+/+;m;tp53+/+ males versus brca2+/+;m;tp53+/+ females           | p = 0.332                                             |-----------|

| Age at tumor diagnosis in tp53+/m zebrafish | brca2+/+ | brca2+/+ | brca2 m/m |
|---------------------------------------------|----------|----------|-----------|
| Mean age (mo)                               | 18.6     | 19.7     | 15.1      |

| Characteristics of tumor development in tp53+/m zebrafish | brca2+/+ | brca2+/+ | brca2 m/m |
|----------------------------------------------------------|----------|----------|-----------|
| Number of animals analyzed                               | 22       | 55       | 13        |
| Malignant tumor (≥1)                                     | 17 (77%) | 49 (89%) | 10 (77%)  |
| Male                                                     | 5        | 15       | 9         |
| Female                                                   | 9        | 33       | 0         |
| Sex not determined                                       | 3        | 1        | 1         |
| Benign tumor only (≥1)                                   | 1 (5%)   | 1 (2%)   | 3 (23%)   |
| Male                                                     | 1        | 1        | 3         |
| Female                                                   | 0        | 0        | 0         |
| Sex not determined                                       | 0        | 0        | 0         |
| Animals with >1 tumor                                    | 4 (18%)  | 7 (13%)  | 7 (54%)   |
| Male                                                     | 1        | 3        | 6         |
| Female                                                   | 3        | 4        | 0         |
| Sex not determined                                       | 0        | 0        | 1         |

| Proportion of animals with malignant tumors in tp53+/m zebrafish | brca2+/+;tp53+/m versus brca2+/+;m;tp53+/m | p = 0.277 |
|------------------------------------------------------------------|--------------------------------------------|-----------|
| brca2+/+;tp53+/m versus brca2 m/m;tp53+/+                         | p = 1.000                                  |-----------|
| brca2+/+;m;tp53+/+ versus brca2 m/m;tp53+/+                      | p = 0.358                                  |-----------|

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Table 2. Tumor types developed by brca2+/+;tp53+/m, brca2+/m;tp53+/+, and brca2 m/m;tp53+/m zebrafish.

| Tumors developed by tp53+/m zebrafish† | brca2+/+ | brca2+/m | brca2 m/m |
|---------------------------------------|----------|----------|-----------|
| n Age (mo) n Age (mo) n Age (mo) | n Age (mo) n Age (mo) n Age (mo) | n Age (mo) n Age (mo) n Age (mo) |
| Malignant tumor type | | | |
| MPNST | 11 16.0–25.5 | 30 13.5–26.5 | 1 14.5 |
| Undifferentiated sarcoma | 6 13.5–22.0 | 15 13.5–26.5 | 9 12.0–18.0 |
| Nephroblastoma | 0 | 4 16.5–20.5 | 0 | |
| Other malignant tumor | 2 21.5–22.0 | 4 17.5–22.0 | 4 12.5–17.5 |
| Benign tumor type | | | |
| Seminoma | 2 | 12.5–25.5 | 4 | 21.0–25.5 | 4 | 13.5–16.5 |
| Gonadal stromal tumor | 0 | – | 0 | – | 3 | 12.0–16.5 |
| Ultimobranchial adenoma | 2 | 20.0–25.5 | 1 | 25.0 | 1 | 13.5 |
| Total tumors | 23 | 12.5–25.5 | 58 | 13.5–26.5 | 22 | 12.0–18.0 |

Abbreviations: MPNST, malignant peripheral nerve sheath tumor; mo, months.
†The total numbers of each tumor type observed by histologic analysis are reported. Some zebrafish developed more than one tumor (see Table 1).

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previously [16]. The development of multiple tumors in one animal was uncommon in all cohorts (Table S1).

LOH for brca2 and or tp53 is common in malignant zebrafish tumors

To identify LOH in zebrafish samples, we analyzed 31 histologically malignant tumors, four histologically benign tumors, and 28 matched normal tissue specimens from tp53+/m zebrafish that were brca2+/+, brca2+/m, or brca2 m/m (Table S2). Tissue samples were isolated by laser-capture microdissection (LCM) (Figures 2A-C) for DNA extraction, PCR amplification, and sequencing (Methods S1 and Table S3). Multiple tumors in individual zebrafish were isolated as separate samples (Figure 2B).

Sequence analyses revealed that LOH for brca2 and tp53 occurred frequently in malignant tumor specimens, but was not observed in normal tissues (Figures 2D and 2E and Table S2). Interestingly, different tumors analyzed from a single zebrafish did not necessarily exhibit the same LOH profile (Figure 2E and Table S2). Although tp53 LOH always involved loss of the wildtype allele, two tumors from brca2+/m;tp53+/m zebrafish exhibited loss of the mutant brca2 allele (Table S2). Loss of the mutant BRCA2 allele has been reported in human tumors, but the functional significance of this change is unclear [6]. No LOH was detected in three of four histologically benign tumors or in any normal tissue specimens (Table S2).

Malignant zebrafish tumors exhibit distinct LOH profiles that correlate with brca2 genotype

Segregation of malignant tumors by brca2 genotype indicated that LOH status correlated with brca2 mutation status (Figure 3A). In brca2+/+;tp53+/m zebrafish, tp53 LOH occurred in 100% of malignant tumors (8 of 8). In brca2+/m;tp53+/+ m zebrafish, tp53 LOH occurred in 86% of malignant tumors (13 of 15). Interestingly, over half of malignant tumors from brca2+/ m;tp53+/m zebrafish developed LOH for both brca2 and tp53 (8 of 15, 53%). However, LOH for brca2 alone was uncommon in this cohort (1 of 15, 7%). In brca2 m/m;tp53+/m zebrafish, tp53 LOH occurred in only 29% of malignant tumors (2 of 7).

To investigate a second mechanism for brca2 inactivation in malignant tumors from brca2+/m;tp53+/m zebrafish, we analyzed methylation status of the putative promoter region for brca2 in four tumor specimens that retained the brca2 wildtype allele. Significant methylation was not detected in tumor or matched control samples (Table S4).

Malignant tumor type correlates to brca2 genotype and LOH profile

Segregation of malignant tumors by tumor type indicated that brca2 mutation status correlated with both tumor type and LOH profile (Table 3 and Figure 3B). These differences were observed for the two most common malignant tumor types observed in this study, malignant peripheral nerve sheath tumors (MPNST) and undifferentiated sarcoma. The proportion of zebrafish that developed MPNST was not significantly different between brca2+/+;tp53+/+ m and brca2+/m;tp53+/m cohorts (Table 3). However, the proportion of zebrafish that developed MPNST in brca2 m/m;tp53+/+ m zebrafish was significantly lower than both brca2+/+;tp53+/+ m and brca2+/m;tp53+/+ m cohorts (Table 3). The proportion of zebrafish that developed undifferentiated sarcomas in the brca2 m/m;tp53+/+ m cohort was higher than either the brca2+/+;tp53+/+ m or brca2+/m;tp53+/+ m cohorts, but this difference did not reach statistical significance (Table 3).

MPNST and undifferentiated sarcomas were also correlated with different LOH profiles. All MPNST analyzed for LOH had lost the tp53 wildtype allele, regardless of brca2 status (14 of 14) (Figure 3B). Interestingly, this includes the single MPNST analyzed from the brca2 m/m;tp53+/+ m population, although tp53 LOH was otherwise uncommon in tumors from this group. In comparison, 60% of undifferentiated sarcomas (6 of 10) had loss of the wildtype brca2 allele, or occurred in brca2 m/m;tp53+/+ m fish, regardless of the tp53 status (Figure 3B). However, this trend largely reflected the higher incidence of undifferentiated sarcomas in the brca2 m/m;tp53+/+ m cohort (Table 3).

Discussion

BRCA2 and TP53 are well-known tumor suppressor genes that have been linked to defined human cancer syndromes, but the effect of combined genetic disruptions in these genes on carcinogenesis is not well defined. We examined tumor develop-
ment in zebrafish with mutations in \textit{brca2} and \textit{tp53}, and describe the relationship between mutation status, development of somatic LOH, and development of malignant tumors.

In the absence of \textit{tp53} mutation, tumor incidence was increased in \textit{brca2} m/m;\textit{tp53}+/+ zebrafish when compared to \textit{brca2}+/+;\textit{tp53}+/+ and \textit{brca2}+/m;\textit{tp53}+/m zebrafish. However, on a \textit{tp53}+/m background, zebrafish of all three \textit{brca2} genotypes experienced similar tumor incidence. The age at tumor onset was statistically significantly lower, and the proportion of zebrafish with multiple tumors higher, in \textit{brca2} m/m;\textit{tp53}+/- zebrafish when compared to \textit{brca2}+/-;\textit{tp53}+/- and \textit{brca2}+/-;\textit{tp53}+/- zebrafish. These results indicate that: 1) homozygous \textit{brca2} mutation increases tumor development in zebrafish, similar to humans with germline biallelic \textit{BRCA2} mutations, and 2) homozygous \textit{brca2} mutation enhances carcinogenesis in \textit{tp53}+/m zebrafish, supporting previous work that indicates a collaborative relationship between \textit{BRCA2} and \textit{TP53} in human carcinogenesis.

In humans, somatic LOH occurs frequently in tumors from patients who inherit one mutated copy of \textit{BRCA2} or \textit{TP53} [1–4]. To investigate the role for LOH in carcinogenesis in zebrafish with \textit{brca2} and \textit{tp53} mutations, we examined malignant zebrafish tumors and matched normal tissues for evidence of LOH. Loss of the wildtype alleles for \textit{tp53} and \textit{brca2} was common in malignant zebrafish tumors, implicating LOH as an important contributor to carcinogenesis in this species.

Importantly, development of LOH was dependent on \textit{brca2} mutation status. Since all malignant tumors from \textit{brca2}+/-;\textit{tp53}+/- m zebrafish developed \textit{tp53} LOH, we conclude that LOH for \textit{tp53} is necessary for carcinogenesis in \textit{tp53}+/m zebrafish without \textit{brca2} mutation. In contrast, the majority of malignant tumors from \textit{brca2} m/\textit{mtp53}+/m zebrafish did not develop somatic LOH for \textit{tp53}. These findings suggest that on a \textit{tp53}+/m background, the presence or absence of functional \textit{brca2} may influence subsequent genetic alterations required for carcinogenesis.

Figure 2. Malignant zebrafish tumors frequently develop LOH for \textit{brca2} and/or \textit{tp53}. (A–C), Before (upper panels) and after (lower panels) images of LCM-guided sample collection from an MPNST (A), an MPNST and a nephroblastoma (B), and a normal liver (C). Regions of sample collection are outlined in color. (D) MPNST from a \textit{brca2}+/m;\textit{tp53}+/m zebrafish shows loss of the \textit{brca2} and \textit{tp53} wildtype alleles. (E) MPNST and nephroblastoma from a \textit{brca2}+/m;\textit{tp53}+/m zebrafish show disparate LOH profiles. LOH, loss of heterozygosity; MPNST, malignant peripheral nerve sheath tumor; NB, nephroblastoma.

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While most malignant tumors from brca2+/m;tp53+/m zebrafish developed LOH for tp53, LOH for brca2 was comparatively less common. These results suggest that in brca2+/m;tp53+/m zebrafish, biallelic inactivation or loss of brca2 is either not required for tumorigenesis, occurs late in disease, or is achieved by other mechanisms. These possibilities have been previously postulated to explain why some cancers in humans with heterozygous BRCA2 mutation do not develop BRCA2 LOH or promoter methylation [1,6]. In comparison, TP53 mutation is thought to be a relatively early event in the pathogenesis of BRCA2-associated cancer in humans with heterozygous BRCA2 mutation [13]. The high incidence of tp53 LOH in malignant tumors from brca2+/m;tp53+/m zebrafish supports the concept that TP53 dysfunction is a critical and potentially early step in BRCA2-associated carcinogenesis.

In addition to brca2 genotype, LOH status also correlated with malignant tumor type. All MPNST evaluated for LOH had lost the tp53 wildtype allele, regardless of brca2 status. Several previous studies in zebrafish support an association between loss of
functional *tp53* and MPNST [18–21]. In contrast, undifferentiated sarcomas were most commonly associated with homozygous *brca2* mutation. These patterns of gene disruptions may reflect disparate roles for *brca2* and *tp53* in zebrafish tumorigenesis. Interestingly, human cancer syndromes linked to inheritable *BRCA2* mutations are also limited to a small range of tumor types [7–9].

The methods applied in this study represent a minimum estimate for LOH in malignant zebrafish tumors, as screening for LOH was done by sequencing within the exons containing the *brca2* and *tp53* genes. Other mechanisms of gene inactivation may also have contributed to carcinogenesis in this population. When we examined the methylation status of the putative *brca2* promoter in a small set of normal and tumor samples from *brca2+/-;tp53+/m* zebrafish, we did not find evidence of promoter methylation in any sample. While we cannot rule out the possibility that *brca2* promoter methylation contributed to tumorigenesis in this study, *BRCA2* promoter methylation occurs infrequently in tumor specimens from humans with heterozygous *BRCA2* mutations [1].

Human cancers display a remarkable degree of genetic heterogeneity [22,23]. Both inter- and intratumoral heterogeneity are influenced by genetic factors, such as genomic instability, and nongenetic factors, such as tumor microenvironment [23,24]. Two observations from the current study indicate a degree of heterogeneity among malignant zebrafish tumors. First, different tumors from the same animal did not necessarily exhibit the same LOH profile, suggesting that tumor initiation and/or progression could have involved different mechanisms, and may have been influenced by the site of onset and cell of origin. Second, a small number of tumors exhibited partial LOH for *brca2* or *tp53* (Table S2), indicating that a subset of tumor cells retained the wildtype allele for these genes. This suggests the presence of genetically diverse subclones within malignant zebrafish tumors, as has been observed in human cancers [22,23]. Further investigation of genetic diversity in zebrafish tumors will be required to understand tumor heterogeneity in this species.

In this study, we demonstrate that LOH for *brca2* and *tp53* represents a conserved mechanism for carcinogenesis in zebrafish, and suggest that the relative importance of LOH or haploinsufficiency for *brca2* and *tp53* in driving carcinogenesis is dictated by *brca2* genotype (Figure 3C). In zebrafish that are wildtype or heterozygous for *brca2* mutation, *tp53* LOH appears to be a critical step in driving carcinogenesis. In contrast, cancers associated with homozygous *brca2* mutation do not require *tp53* LOH. We have previously reported accelerated carcinogenesis in *brca2+/-;mtp53 m/m* and *brca2 m/m;tp53 m/m* zebrafish compared to *brca2+/-;tp53 m/m* zebrafish [16]. From these two studies, we conclude that carcinogenesis in zebrafish with *tp53* LOH is not required for carcinogenesis in *brca2+/-;mtp53 m/m*. In *brca2+/-;tp53 m/m* zebrafish, *tp53* typically requires biallelic inactivation or loss of at least one of these two genes, and this effect is enhanced by haploinsufficiency or biallelic loss of the other gene.

The collaborative effects of *BRCA2* and *TP53* mutations on carcinogenesis have been previously described in human cancer. Genomic instability is thought to be a significant factor in human carcinogenesis [22,23], and germline *BRCA2* mutation is linked to an increased mutation rate in *BRCA2*-mutant mice [25] and in *BRCA2*-associated human cancer [26,27]. Coincident or subsequent *TP53* pathway disruption in *BRCA2*-deficient cells may permit survival and proliferation of cell populations with significant genetic aberrations, ultimately leading to neoplastic transformation. Further investigation of genetic and/or epigenetic alterations accompanying *brca2* mutation in malignant zebrafish tumors may uncover additional factors that contribute to *BRCA2*-associated cancer in humans.

### Table 3. Incidence of specific malignant tumor types in *tp53+/-* zebrafish.

| Incidence of MPNST in *tp53+/-* zebrafish | brca2+/- | brca2+/- | brca2 m/m |
|-------------------------------------------|---------|---------|-----------|
| Number of animals compared                | 19      | 49      | 10        |
| MPNST                                     | 11 (58%)| 12 (61%)| 1 (10%)   |
| All other malignant tumor types           | 8 (42%) | 19 (39%)| 9 (90%)   |
| Incidence of MPNST in *brca2+/-* versus *brca2+/-* | p = 1.000 |          |           |
| Incidence of MPNST in *brca2+/-* versus *brca2 m/m* | p = 0.019 |          |           |
| Incidence of MPNST in *brca2+/-* versus *brca2 m/m* | p = 0.004 |          |           |

| Incidence of undifferentiated sarcoma in *tp53+/-* zebrafish | brca2+/- | brca2+/- | brca2 m/m |
|----------------------------------------------------------|---------|---------|-----------|
| Number of animals compared                                | 19      | 49      | 10        |
| Undifferentiated sarcoma                                  | 6 (32%) | 14 (29%)| 6 (60%)   |
| All other malignant tumor types                           | 13 (68%)| 35 (71%)| 4 (40%)   |
| Incidence of undifferentiated sarcoma in *brca2+/-* versus *brca2+/-* | p = 1.000 |          |           |
| Incidence of undifferentiated sarcoma in *brca2+/-* versus *brca2 m/m* | p = 0.234 |          |           |
| Incidence of undifferentiated sarcoma in *brca2+/-* versus *brca2 m/m* | p = 0.074 |          |           |

Abbreviations: MPNST, malignant peripheral nerve sheath tumor.

1 One zebrafish in this group developed both an MPNST and an undifferentiated sarcoma, and was counted in both categories for each comparison.

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Materials and Methods

Ethics statement
Zebrafish were monitored for clinical and gross evidence of tumor development and humanely euthanized with 50X Tricaine in system water buffered with Sodium Bicarbonate (0.7 grams/liter). All animal studies were approved by the Intramural Animal Care and Use Committee, National Cancer Institute, National Institutes of Health, Bethesda, MD (Animal Study Protocol #MB-081).

Zebrafish maintenance
Experiments were performed with adult zebrafish from the brca2<sup>Q658X</sup> and <i>tp53<sup>M214K</sup></i> mutant zebrafish lines carrying the <i>brca2</i><sup>Q658X</sup> [16] and <i>tp53<sup>M214K</sup></i> [18] mutations, respectively. All zebrafish evaluated in this study were related. For additional details, see Methods S1.

Histologic analyses
Zebrafish were processed for histology as previously described [16]. Tumors were classified based on histologic features, and the number of histologically and anatomically distinct tumors was determined for each specimen. Histologic diagnoses were made without knowledge of the <i>brca2</i> genotype or loss of heterozygosity status. For additional details, see Methods S1.

LCM and DNA extraction from paraffin-embedded zebrafish tissues
Tissue sections were placed on PEN-membrane glass slides, and tumor and normal tissue specimens were individually collected by laser-capture microdissection. Collected specimens were routinely processed for DNA isolation (See Methods S1).

LOH analyses
LOH analyses were achieved by PCR amplification and sequencing over the <i>brca2</i><sup>Q658X</sup> and <i>tp53<sup>M214K</sup></i> mutation sites. See Methods S1, Figure S3, and Table S3 for details.

CpG island identification and pyrosequencing methylation detection assay
The zebrafish <i>brca2</i> promoter has not been characterized, but a CpG island as determined by NCBI algorithm is predicted to occur 906 base pairs upstream of the 5′ position of the translational start codon for <i>brca2</i> on chromosome 15 (<i>Danio rerio</i> genome version Zv9). DNA isolated from LCM-collected samples was routinely processed for bisulfite conversion, PCR amplification, and Pyrosequencing analysis for methylation status of this CpG island (See Methods S1).

Statistics
Data sets comprised of the proportions of male or female animals that developed tumors were compared by Fisher’s exact test (GraphPad Prism, version 6.0b), and P<0.05 was accepted to indicate statistical significance. Data sets comprised of age at tumor diagnosis were compared by unpaired t-test with Welch’s correction (GraphPad Prism, version 6.0b), and P<0.05 was accepted to indicate statistical significance. Data sets comprised of the proportions of animals with malignant tumors were compared by Fisher’s exact test with Mehta’s modification (GraphPad Prism, version 6.0b), and P<0.05 was accepted to indicate statistical significance. For additional details on statistical comparisons, see Methods S1.

Supporting Information
Figure S1 Overall survival declines rapidly in brca2 m/m;tp53+/-m zebrafish. Kaplan-Meier survival curves for all <i>tp53</i>+/m zebrafish described in this study show that the survival curve for the <i>brca2</i> m/m;tp53+/-m cohort declined rapidly in comparison to <i>brca2</i>+/+;tp53+/-m and <i>brca2</i>+/m;tp53+/-m cohorts. (TIF)

Figure S2 Tumorigenesis is enhanced by tp53 mutation. The percentage of zebrafish that developed tumors (benign or malignant) is higher in the <i>brca2</i> m/m;tp53+/-m cohort than in <i>brca2</i>+/+;tp53+/-m or <i>brca2</i>+/m;tp53+/-m cohorts. (TIF)

Figure S3 Diagram of zebrafish <i>brca2</i> and tp53 genes indicating mutation and SNP positions relevant to LOH analyses. (A) Diagram of zebrafish <i>brca2</i> indicating the locations of the <i>brca2</i><sup>Q658X</sup> mutation, and the locations of single nucleotide polymorphisms (SNPs) used to distinguish wildtype and mutant alleles. (B) Diagram of zebrafish tp53 indicating the locations of the <i>tp53</i><sup>M214K</sup> mutation, and the locations of SNPs used to distinguish wildtype and mutant alleles. Vertical lines indicate PCR primer positions. (TIF)

Table S1 Characteristics of tumor development in <i>brca2</i>+/+;<i>tp53</i>+/-, <i>brca2</i>+/m;<i>tp53</i>+/-, and <i>brca2</i> m/m;tp53+/- zebrafish. (DOC)

Table S2 Summary of LOH analyses performed on tumor specimens and matched normal tissue specimens collected from <i>brca2</i>+/+;<i>tp53</i>+/-m, <i>brca2</i>+/m;<i>tp53</i>+/-m, and <i>brca2</i> m/m;tp53+/-m zebrafish. (PDF)

Table S3 Summary of target and primer sequences used for LOH analyses of normal and tumor specimens. (DOC)

Table S4 Summary of CpG analyses from normal and tumor specimens from <i>brca2</i>+/m;<i>tp53</i>+/-m zebrafish. (DOC)

Methods S1.

Author Contributions
Conceived and designed the experiments: HRS RRW LJE DDH. Performed the experiments: HRS RRW LJE DDH. Analyzed the data: HRS RRW LJE DDH. Contributed reagents/materials/analysis tools: HRS RRW LJE. Wrote the paper: HRS DDH.

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