Supporting Information

Shive et al. 10.1073/pnas.1011630107

SI Materials and Methods

Histological Staging of Zebrafish Specimens. Staging of zebrafish embryos, ovaries, and testes was performed as described previously (1–3).

WISH of Zebrafish Embryos. The *brca2* probe contained a 3.2-kb portion of the 3′ end of the cDNA, and the *vasa* probe contained the 3′ 549 bases of the cDNA. WISH experiments were performed as described previously (4, 5), with modifications (see below). Embryos used for genotyping after WISH were prepared as described previously (6), with minor modifications. Approximately 50 embryos were analyzed per developmental stage from three independent experiments. For *vasa* expression, 25–35 embryos from two independent experiments were imaged individually and then genotyped for the *brca2*^{Q658X} mutation.

Modifications to the WISH Protocol. The following modifications were made to the WISH protocol: (*i*) Tween 20 was eliminated from prehybridization washes; (*ii*) the hybridization buffer included 0.1% CHAPS and 0.5 mM EDTA; (*iii*) hybridization reactions were incubated at 60 °C or 65 °C; (*iv*) posthybridization washes included 0.3% CHAPS in 2× SSC; and (*v*) proteinase K digestion, acetic anhydride treatment, and RNase treatment were eliminated.

In Situ Hybridization of Adult Zebrafish Gonads. The *brca2* probe contained the 3′ 330 bases of the cDNA. Unstained sections were processed for in situ hybridization as described previously (7), with the following modifications: (*i*) Zebrafish were fixed in 4% paraformaldehyde at 4 °C for 24 h, transferred to 70% ethanol, and embedded in paraffin, and 5-μm unstained sections were prepared; (*ii*) sections were treated with proteinase K (5 μg/mL or 10 μg/mL) at 37 °C; (*iii*) the hybridization buffer contained 0.2 mg/mL Sonicated Salmon Sperm DNA (Stratagene); (*iv*) hybridization reactions were conducted at 50 °C or 60 °C; (v) 5× In Situ Hybridization Blocking Solution (Vector Laboratories) was used for blocking; (*vi*) the BCIP/NBT Alkaline Phosphate Substrate Kit IV (Vector Laboratories) was used for detection; and (*vii*) slides were briefly dehydrated in ethanol grades before coverslipping.

Genotyping. Tail amputation and DNA extraction for genotyping were performed as described previously (5), with minor modifications. Tissue was digested in 100 μL of DNA extraction buffer [10 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.2% Triton X-100] with 0.2 mg/mL of proteinase K (Fermentas) at 50–55 °C for a minimum of 2 h, followed by 10 min at 95 °C. A *brca2* fragment was amplified by PCR with primers designed to create an *Alu*I restriction site based on the presence of the *brca2*^{Q658X} point mutation. PCR products were incubated with *Alu*I digestion buffer (0.07 U/μL *Alu*I in 1× Tango buffer; Fermentas) at 37 °C for a minimum of 2 h.

Histological Analysis of Gonadal Development. Juvenile zebrafish were collected at 21, 31, 41, and 51 dpf and euthanized, genotyped, and individually fixed as described above. Specimens were grouped in tissue cassettes by genotype. The 21-dpf juveniles were embedded in 2% agarose before being placed in tissue cassettes. Ten sections were screened per group; specimens were assessed for the presence of gonadal tissue, presence of identifiable perinucleolar oocytes in gonads (signifying juvenile ovary formation), presence of spermatogonia in spermatogonial cysts (signifying testicular differentiation), and proliferation and differentiation of oocytes (signifying ovarian differentiation).

Histological Analyses of Meiosis in Spermatocytes. Testes from 10 adult males (age 5.5 mo) of each genotype were dissected and grouped by genotype for fixation as described above. To determine the total percentage of spermatocysts containing cells in meiosis, five tubules were randomly selected from each of 10 testis sections, for a total of 50 tubules. Tubules in which the tubular lumen and margins could not be reasonably ascertained were disregarded, and sections without at least five well-defined tubules in a cross-section were not included. For each tubule, the total number of spermatocysts was counted, and the number of spermatocysts containing spermatocytes with meiotic figures was recorded (Table S2). The numbers of spermatocysts per tubule from testes of each *brca2* genotype were compared using an unpaired *t* test (Table S2). The numbers of meiotic spermatocysts per tubule from testes of each *brca2* genotype were compared using an unpaired *t* test with Welch's correction (Table S2).

TUNEL Assay on Zebrafish Testes. TUNEL labeling was performed on 5-μm unstained sections with a commercially available kit (In Situ Cell Death Detection Kit, AP; Roche Diagnostics). After TUNEL labeling, sections were counterstained with Nuclear Fast Red Solution (Sigma-Aldrich) and mounted with Clear-Mount (Electron Microscopy Services). TUNEL assays were performed with each sample set in three independent experiments.

Immunohistochemistry for Cleaved Caspase-3. Sections were deparaffinized in xylene, rehydrated through a series of ethanol grades, and incubated in 3% H_2O_2/70% methanol to block endogenous peroxidase activity. For antigen retrieval, sections were incubated in 1× Target Retrieval Solution (DAKO) with steam. Protein block (DAKO) and normal goat serum were used for blocking, and the Vector Avidin/Biotin Blocking Kit (Vector Laboratories) was used to block endogenous biotin. Detection of cleaved caspase-3 was done using a rabbit polyclonal anti–cleaved caspase-3 antibody (1:200 dilution; Cell Signaling) with a goat anti-rabbit biotinylated secondary antibody (1:400 dilution; DAKO). Detection was achieved by incubating sections with a preformed avidin–biotinylated enzyme complex (Vectastain ABC Kit; Vector Laboratories), followed by application of 3,3′-diaminobenzidine tetrahydrochloride (DAB-Plus Substrate Kit; Invitrogen). After detection, sections were counterstained with Mayer's hematoxylin solution (Sigma-Aldrich), dehydrated, and coverslipped.

Evaluation of Tumor Incidence in Adult Zebrafish. Adult zebrafish were regularly monitored for evidence of morbidity or overt tumor development. Tumor development in the *tp53*^{M214K} homozygous zebrafish parent line was defined by observation of macroscopically identifiable tumors, and a subset was also screened by histology to confirm diagnosis. Progeny from an incross of *brca2*^{Q658X} heterozygous;*tp53*^{M214K} heterozygous zebrafish were evaluated for macroscopic tumor development and by histology. The ages at tumor onset in each genotype of zebrafish were compared using an unpaired *t* test (Table S3).

Image Acquisition. Embryos were imaged with a Leica MZ16 stereomicroscope and Leica DFC420 camera with Leica Imaging Suite. Histological sections were imaged with an Olympus BX41

and Diagnostic Instruments 14.2 color mosaic camera with SPOT version 4.6.4.3 imaging software (SPOT Imaging Solutions).

Primer Sequences for *brca2*^{Q658X} Genotyping by PCR and Restriction Digest.

Forward primer (mismatched bases in italics): TCCTGCAC-CAAGACCCCCTGTA*A*GC
Reverse primer: CTGTCAAAGTGCCATTTTCTTCAAG

Primer Sequences for *brca2*^{Q658X} Genotyping by Sequencing.

Forward primer: TGAGGCTATAGTAAAGGCAAAGGC
Reverse primer: GTCTTGGAAGCATCACTAACACTCAC

Primer Sequences for *tp53*^{M214K} Genotyping by Sequencing.

Forward primer: CATATTCACAGACCACCAGCCC
Reverse primer: TCACACTAAAAGCATACCTCGCC

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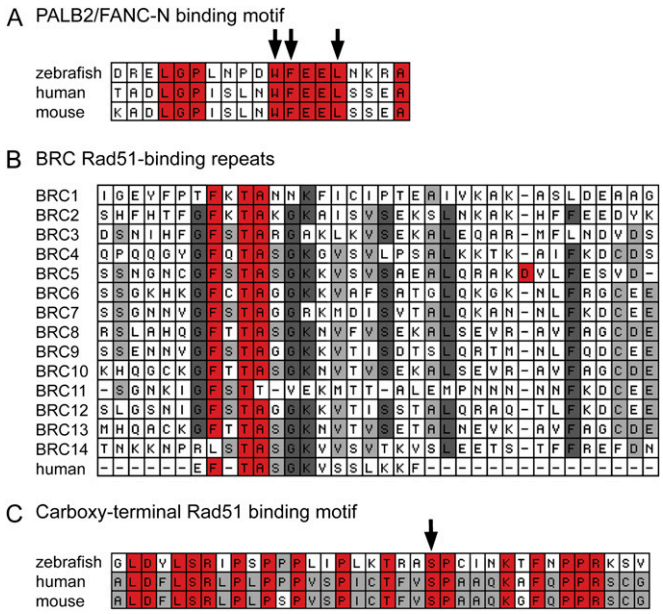

Fig. S1. Amino acid sequence motifs found in zebrafish brca2. The zebrafish (accession no. NP_001103864), mouse (accession no. NP_001074470), and human BRCA2 (accession no. NP_000050.2) were compared using MacVector (MacVector), and sequence motifs were identified using InterProScan (8). The amino acid positions for each motif are given in parentheses. (*A*) Alignment of the 10-residue minimal PALB2-binding motif from human (21–39), mouse (21–39), and zebrafish (10–28) BRCA2. Identical residues are boxed in red. The arrows indicate the core interacting residues Trp31, Phe32, and Leu35 (9). (*B*) Alignment of the zebrafish brca2 BRC Rad51-binding repeat motifs to a consensus sequence from the eight human BRCA2 BRC repeats (10). The positions of the zebrafish BRC repeats are as follows: 1, 586–620; 2, 896–928; 3, 1003–1037; 4, 1129–1163; 5, 1238–1272; 6, 1295–1329; 7, 1361–1395; 8, 1401–1435; 9, 1445–1479; 10, 1488–1522; 11, 1533–1564; 12, 1573–1607; 13, 1617–1651; 14, 1685–1719. Residues found in >90% of the sequences are boxed in red, those found in >70% are boxed in dark gray, and those found in >50% are boxed in light gray. The dashes indicate gaps in the alignment or no defined consensus. (*C*) Alignment of the BRCA2 carboxyl-terminal Rad51-binding domain (11) from zebrafish (2796–2831), human (3270–3305), and mouse (3193–3228). Identical residues are boxed in red, and residues common in two sequences are boxed in gray. The arrow indicates the conserved CDK phosphorylation site (12).

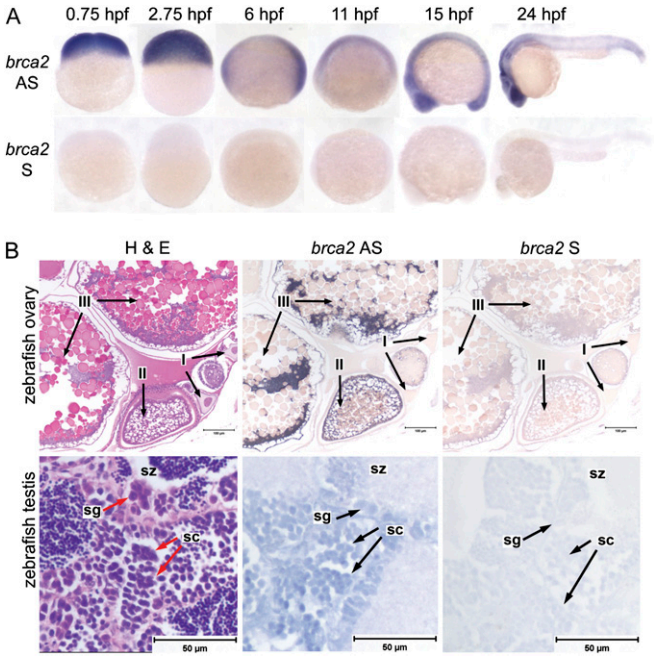

Fig. S2. *brca2* expression in WT embryonic and adult zebrafish. (*A*) WISH for *brca2* expression in zebrafish embryos with antisense (AS; *Upper*) and sense (S; *Lower*) probes at the indicated stages demonstrates expression of *brca2* throughout embryogenesis. (*B*) In situ hybridization for *brca2* expression in sections of adult zebrafish ovary and testis with AS (*Middle*) and S probes (*Right*) indicates that *brca2* is expressed in oocytes, spermatogonia, and spermatocytes (arrows). H&E-stained sections of ovary (*Left*, *Upper*) and testis (*Left*, *Lower*) are provided for anatomic reference. I, stage I oocyte; II, stage II oocyte; III, stage III oocyte; sg, spermatogonia; sc, spermatocytes; sz, spermatozoa. (Scale bars: 100 µm for ovary; 50 µm for testis.)

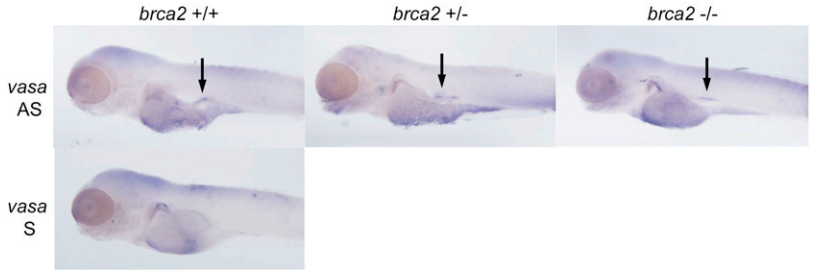

Fig. S3. Germ cell specification and localization at 4 dpf. WISH for the germ cell marker *vasa* indicates similar numbers and localization of PGCs at 4 dpf in zebrafish embryos of each *brca2* genotype. Arrows indicate *vasa*-expressing PGCs. AS, antisense RNA probe; S, sense RNA probe.

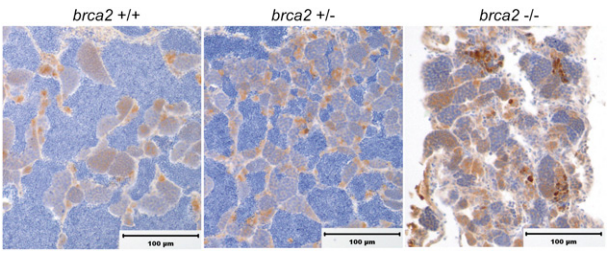

Fig. S4. Immunohistochemistry for cleaved caspase-3 in testes from WT, *brca2*^{Q658X} heterozygous, and *brca2*^{Q658X} homozygous zebrafish. Multiple clusters of apoptotic spermatocytes are present in testes from *brca2*^{Q658X} homozygotes, whereas few individual apoptotic cells are present in testes from WT and *brca2*^{Q658X} heterozygotes. (Scale bars: 100 µm.)

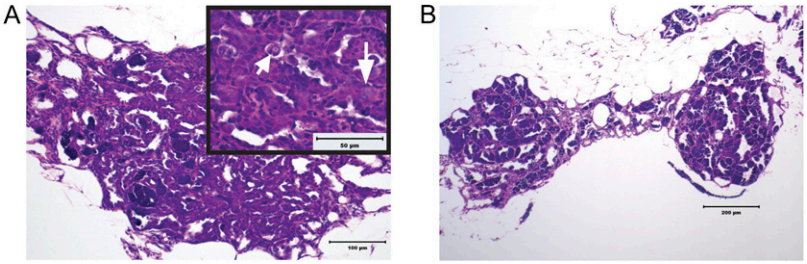

Fig. S5. Hyperplastic, dysplastic, and degenerative changes in H&E-stained sections of testes from adult *brca2*^{Q658X} homozygous zebrafish. (*A*) Testicular tubules are lined by numerous germ cells that form multicellular layers (*Inset*, long arrow). Multifocally, dysplastic germ cells with large nuclei, vacuolated chromatin, and prominent nucleoli are seen (*Inset*, short arrow). (*B*) Testes from *brca2*^{Q658X} homozygotes show segmental degeneration, with loss of spermatocytes in testicular tubules. (Scale bars: 100 μm in *A*; 200 μm in *B*; 50 μm in the *Inset* in *A*.)

Table S1. Genotypes of progeny from incrosses of *brca2*^{Q658X} heterozygotes conform to Mendelian ratios

	WT	*brca2*^{Q658X} heterozygotes	*brca2*^{Q658X} homozygotes
n	221	402	213
Actual	26.4%	48.1%	25.5%
Expected	25%	50%	25%

Zebrafish were genotyped as juveniles or adults, and data were collected on zebrafish derived from 12 different incrosses of *brca2*^{Q658X} heterozygous zebrafish. The percentages of actual genotypes were not statistically significantly different from expected genotypes (χ^2 = 1.378; *P* = 0.504).

Table S2. Quantitative analysis of meiotic spermatocysts in zebrafish testes

Brca2 genotype	Spermatocysts per 50 tubules	Meiotic spermatocysts per 50 tubules	% of meiotic spermatocysts
WT zebrafish	338	15	4%
Brca2^{Q658X} heterozygous zebrafish	364	14	4%
Brca2^{Q658X} homozygous zebrafish	351	38	11%
Comparisons	*P* value	*P* value	
WT and *brca2*^{Q658X} heterozygous zebrafish	0.2867	0.8422	
WT and *brca2*^{Q658X} homozygous zebrafish	0.5074	0.0026	
Brca2^{Q658X} heterozygous and *brca2*^{Q658X}homozygous zebrafish	0.6423	0.0009	

The numbers of meiotic spermatocysts were quantitated as the percentage of the total number of spermatocysts in 50 tubules from 10 zebrafish of each *brca2* genotype. The numbers of spermatocysts per 50 tubules were compared between *brca2* genotypes by the unpaired *t* test (GraphPad Software, www.graphpad.com/quickcalcs/ttest1.cfm). The numbers of meiotic spermatocysts were compared between *brca2* genotypes using the unpaired *t* test with Welch's correction (GraphPad Prism version 5.0).

Table S3. Analysis of age at tumor onset in *brca2*^{Q658X} homozygous;*tp53*^{M214K} homozygous zebrafish, *brca2*^{Q658X} heterozygous;*tp53*^{M214K} homozygous zebrafish, and the parent line of *tp53*^{M214K} homozygous zebrafish

	brca2$^{Q658X-/-}$;*tp53*$^{M214K-/-}$	*brca2*$^{Q658X+/-}$;*tp53*$^{M214K-/-}$	*tp53*$^{M214K-/-}$
Age at tumor onset, mo	7.0	6.5	7.0
	7.0	8.0	10.0
	8.0	8.0	11.0
	7.0	8.5	11.5
	9.0	8.5	11.5
	9.5	10.0	12.0
	11.0	11.0	12.0
	11.0	11.0	12.0
		11.0	12.5
			12.5
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			15.5
			16.0
			16.0
			16.5
			17.0
			17.0
			17.0
			17.0
			17.0
			18.5
			18.5
Total	8	9	32
Mean age, mo	8.7	9.2	14.3

	brca2$^{Q658X-/-}$;*tp53*$^{M214K-/-}$	*brca2*$^{Q658X+/-}$;*tp53*$^{M214K-/-}$
tp53 $^{M214K-/-}$	$P < 0.0001$	$P < 0.0001$
brca2$^{Q658X+/-}$;*tp53*$^{M214K-/-}$	$P = 0.5643$	

Data sets were compared using the unpaired *t* test (GraphPad Prism version 5.0).

Table S4. Analysis of tumors in zebrafish from an incross of *brca2*^{Q658X} heterozygous;*tp53*^{M214K} heterozygous zebrafish and in a subset of zebrafish from the parent line of *tp53*^{M214K} homozygous zebrafish

Specimen	Age, mo	Sex	Diagnosis	Primary site(s)
brca2$^{Q658X-/-}$;*tp53*$^{M214K-/-}$				
1	7.0	M	MPNST	Coelom
			MPNST	Eye
2	7.0	F	MPNST	Ovary
3	7.0	M	MPNST	Coelom
			MPNST	Eye
4*	8.0	ND	Eye mass with proptosis	Eye
5	9.0	M	MPNST	Coelom
6	9.5	ND	MPNST	Coelom
			MPNST	Subpharyngeal
			Cutaneous hemangiosarcoma	Tail
7	11.0	M	Seminoma	Testis
8	11.0	F	Rhabdomyosarcoma	Head
brca2$^{Q658X+/-}$;*tp53*$^{M214K-/-}$				
9	6.5	F	MPNST	Coelom
10	8.0	F	MPNST	Ovary
11	8.0	F	MPNST	Ovary
12	8.5	F	MPNST	Coelom
			MPNST	Eye
13	8.5	M	Sarcoma	Coelom
14	10.0	F	Sarcoma	Ovary
15	11.0	M	MPNST	Coelom
			MPNST	Eye
16	11.0	F	MPNST	Coelom
17	11.0	F	Intestinal adenocarcinoma	Intestine
			Cutaneous hemangioma	Body wall
brca2$^{Q658X+/+}$;*tp53*$^{M214K-/-}$				
18	10.0	F	Primitive neuroectodermal tumor	Coelom
19	11.0	F	MPNST	Body wall
tp53$^{M214K-/-}$ parent line				
20	7.0	F	MPNST	Liver
21	10.0	F	MPNST	Caudal coelom/ovary
22	11.0	F	MPNST	Caudal coelom/ovary
23	11.5	M	Rhabdomyosarcoma	Tail
24	11.5	F	MPNST	Coelom
25	12.0	M	MPNST	Eye
			Rhabdomyosarcoma	Tail

MPNST, malignant peripheral nerve sheath tumor; ND, not determined.
*Specimen 4 was not available for histological analysis.