Supplementary Information.

Secondary mutations as a mechanism of cisplatin resistance in *BRCA2*-mutated cancers

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Table of Contents

1. Supplemental Figures.

Sakai et al., Supplemental Figure1

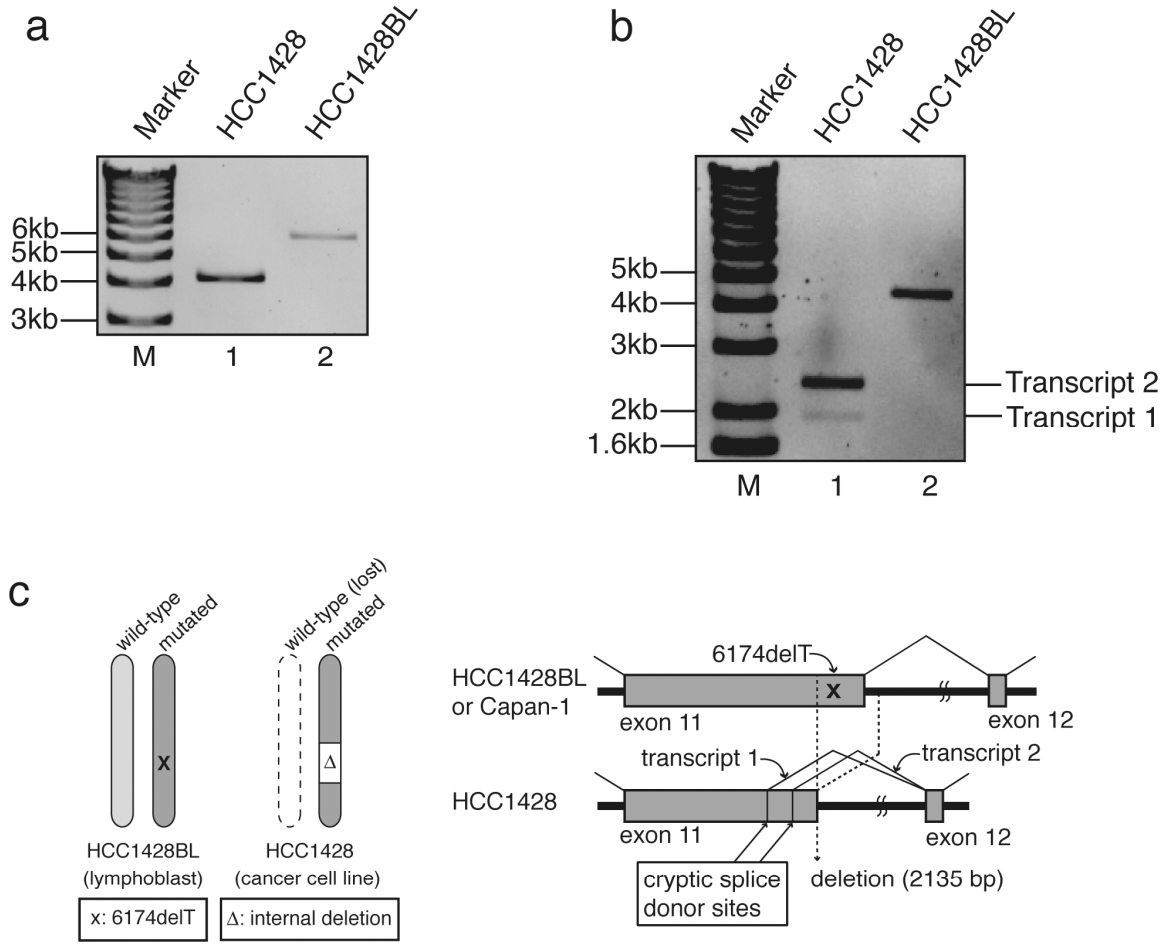

Figure S1. *BRCA2* of HCC1428BL lymphoblast line and HCC1428 breast cancer cell line. **a**, Ethidium bromide-stained agarose gel electrophoresis of the genomic PCR products from HCC1428 and HCC1428BL. The primers, B2F3785 and B2R6932, were used for the amplification. The molecular weight size standard is a 1000-bp ladder. A smaller PCR product was detected in HCC1428. **b**, Ethidium bromide-stained agarose gel electrophoresis of the RT-PCR products from HCC1428 and HCC1428BL. The primers, B2F3785 and B2R8452, were used for the amplification. The molecular weight size standard is a 1000-bp ladder. Two smaller RT-PCR products (transcripts 1 and 2) were detected in HCC1428. **c**, Schematic presentation of *BRCA2* alleles. HCC1428BL had wild-type and mutant (6174delT) alleles. HCC1428 lost the wild-type allele and retained the mutant allele with an additional internal deletion. The HCC1428's mutant allele had a 2135-bp deletion, which activates two cryptic splice donor sites in exon 11, resulting in the expression of transcripts 1 and 2.

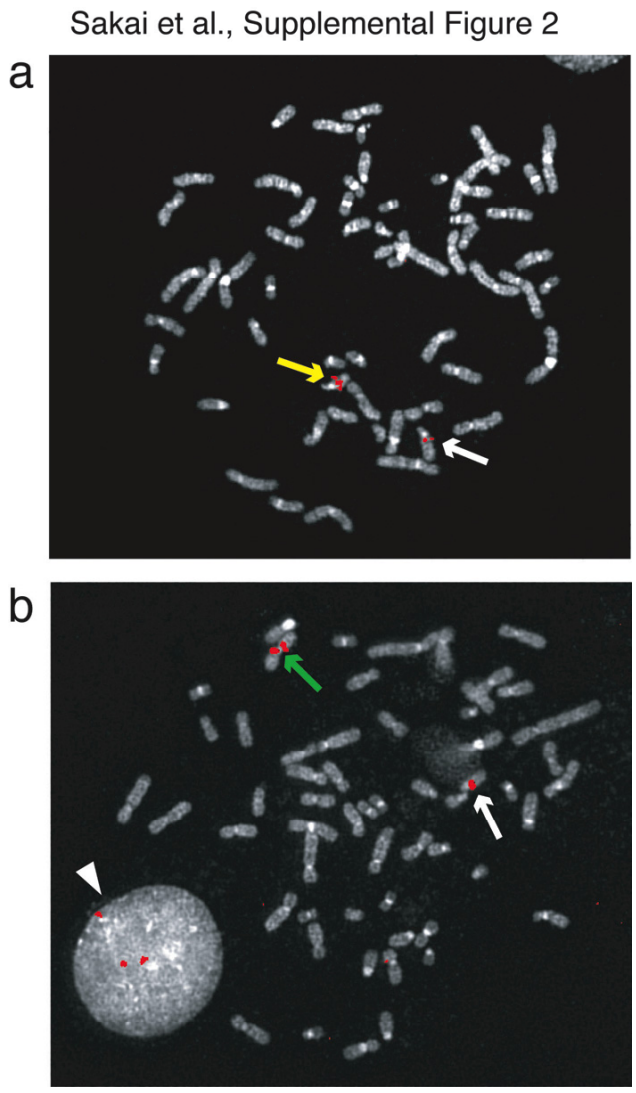

Figure S2. Multiple copies of *BRCA2* gene in Capan-1. Representative pictures of BRCA2 FISH on metaphase chromosomes of Capan-1 cells. **a**, This cell has a short chromosome 13 (yellow arrow) and a normal-looking chromosome 13 (white arrow), both of which have one BRCA2 signal (red). Therefore, the cell has at least 2 copies of *BRCA2* gene. **b**, This cell has a normal-looking chromosome 13 (white arrow) with one BRCA2 signal (red) and an isochromosome 13 (green arrow) with two *BRCA2* signals (red). Therefore, the cell has at least 3 copies of *BRCA2* gene. An interphase nucleus (white arrow head) also shows three BRCA2 signals (red).

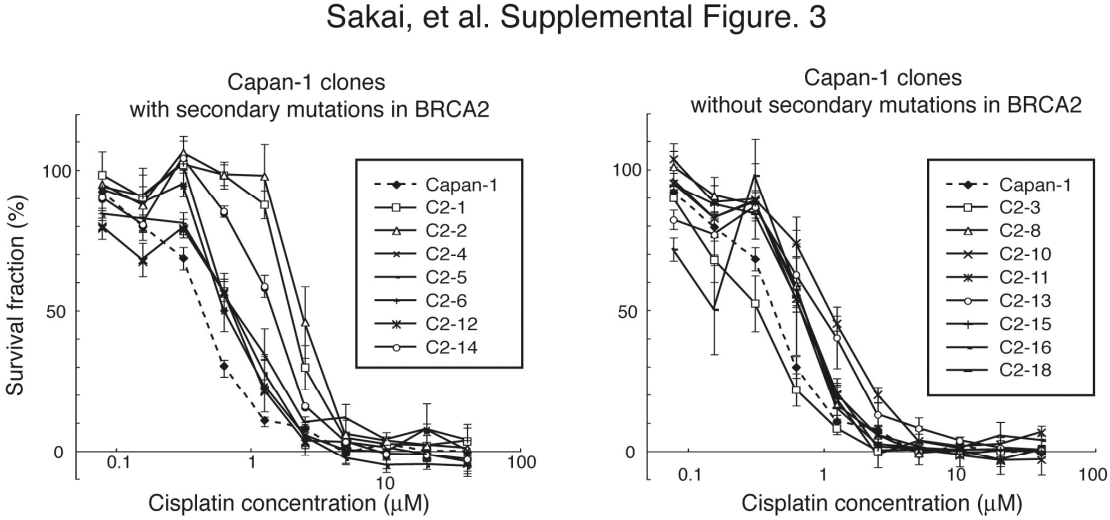

Figure S3. Cisplatin-selected clones of a pancreatic cancer cell line, Capan-1. Fifteen subclones of Capan-1 were generated by selecting the cells in the presence of cisplatin. Fourteen clones (except C2-3) were resistant to cisplatin compared to the parental Capan-1 cells. The cells were treated with cisplatin at the indicated concentrations for 10 days, and survival fraction was measured by crystal violet assay. Mean values of at least three independent experiments ± SEM are shown.

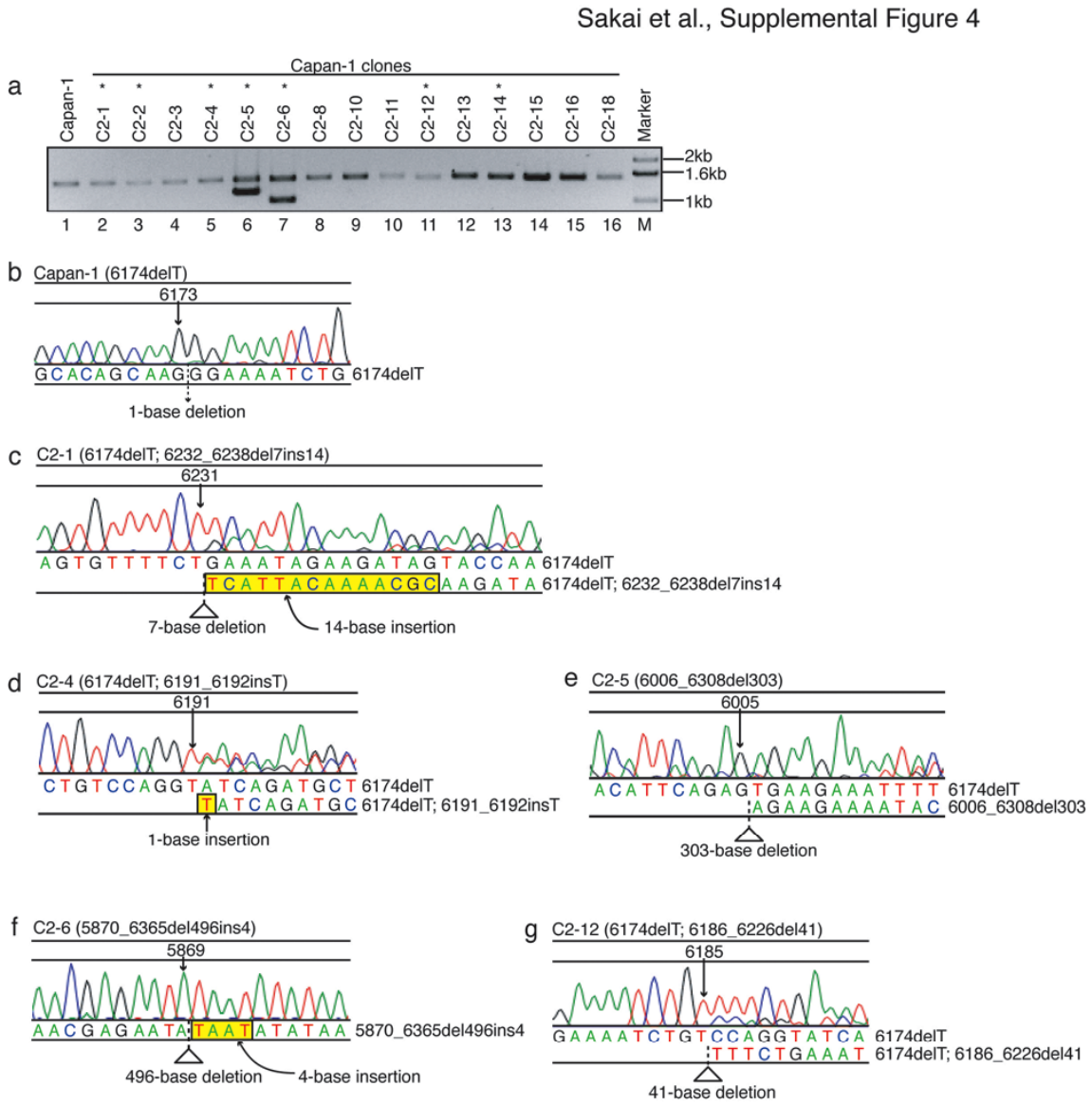

Figure S4. *BRCA2* sequences of transcripts in Capan-1 clones. **a**, Ethidium bromide-stained agarose gel electrophoresis of the RT-PCR products from Capan-1 and its clones. The primers, B2F5391 and B2R6932, were used for the amplification. The molecular weight size standard is a 1000-bp ladder. Asterisk (*) indicates clones with restored BRCA2 protein expression. In C2-5 and C2-6, smaller RT-PCR products, in addition to normal size products, were detected. **b**, Sequence of *BRCA2* transcript in parental Capan-1 cells. A *BRCA2* mutation (6174delT) was detected. **c**, *BRCA2* sequence in clone C2-1. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6232_6238del7ins14) were detected. C2-2 showed the same pattern (data not shown). **d**, C2-4. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6191_6192insT) were detected. C2-14 showed the same pattern (data not shown). **e**, C2-5. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6006_6308del303) were detected. The signals derived from the original sequence were much weaker than those from 6006_6308del303 sequence,

consistent with the ratio of intensities of 1.6kb (the original sequence, weak) and 1.3kb (6006_6308del303, strong) bands in lane 6 of Fig S4A. **f**, C2-6. The two RT-PCR products shown in Fig. S4A lane 7 were purified from the gel, and sequenced separately. In the smaller RT-PCR product (1.1kb), the sequence with an additional mutation (5870_6365del496ins4)) was detected. In the normal size product (1.6kb), the original sequence (6174delT) was detected (data not shown). **g**, C2-12. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6186_6226del41) were detected. All of these mutations were confirmed in genomic DNA as shown in Fig S5.

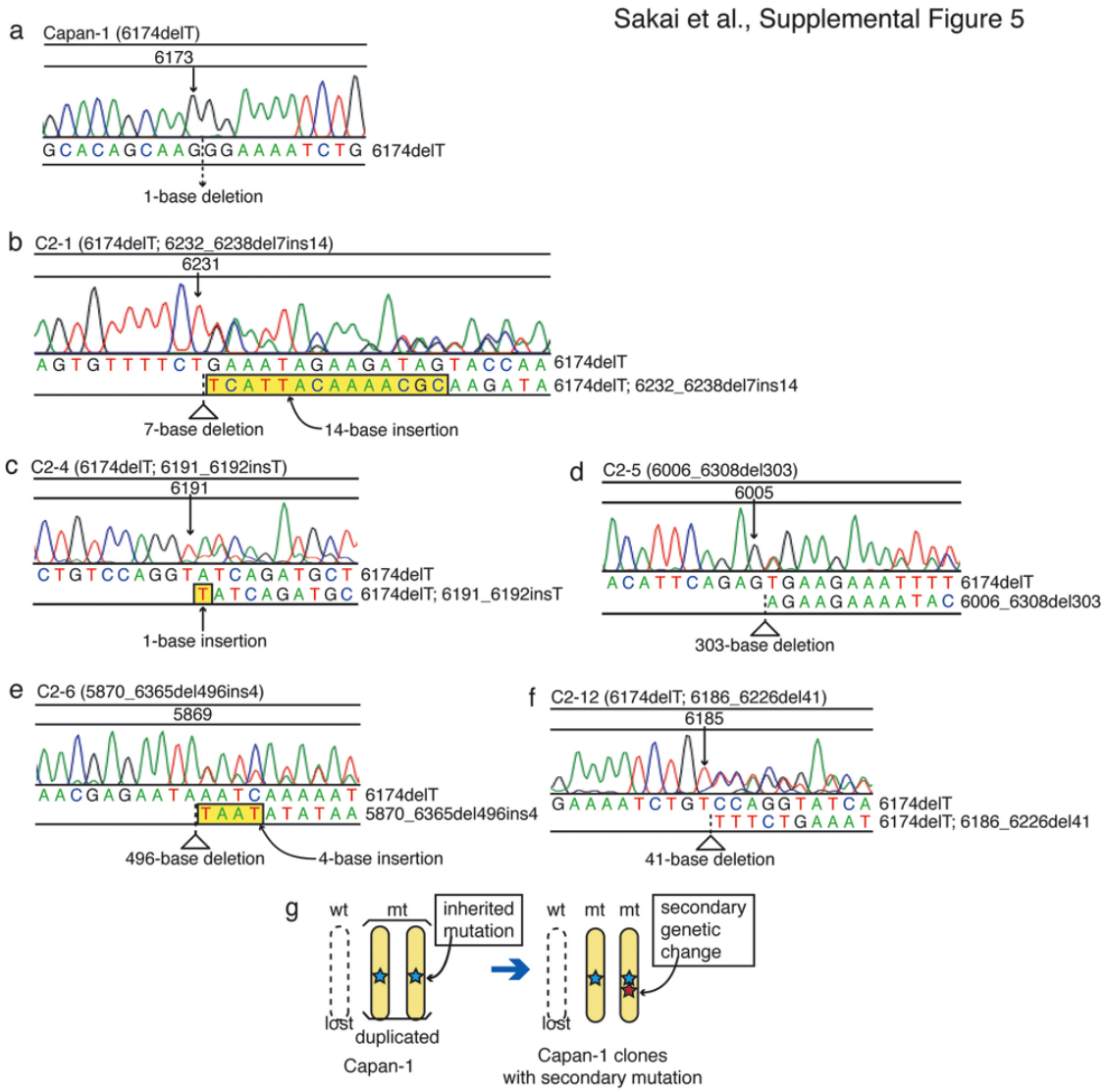

Figure S5. ***BRCA2*** **sequences of genomic DNAs from Capan-1 clones.**
a, Parental Capan-1 cells. A *BRCA2* mutation (6174delT) was detected. **b**, C2-1. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6232_6238del7ins14) were detected. C2-2 showed the same pattern (data not shown). **c**, C2-4. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6191_6192insT) were detected.

C2-14 showed the same pattern (data not shown). **d**, C2-5. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6006_6308del303) were detected. **e**, C2-6. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (5870_6365del496ins4) were detected. **f**, C2-12. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6186_6226del41) were detected. **g**, Schematic model of the secondary mutations in Capan-1 clones. Capan-1 has lost a wild-type *BRCA2* allele, but has retained an allele with the inherited mutation (6174delT). This mutant allele has been duplicated (or triplicated), according to the FISH data shown in Fig. S2. In Capan-1 clones with secondary mutations, the secondary genetic change occurs only on one of the mutant *BRCA2* copies. This model explains why we see mixed sequences of *BRCA2* in all of the Capan-1 clones with secondary *BRCA2* mutations.

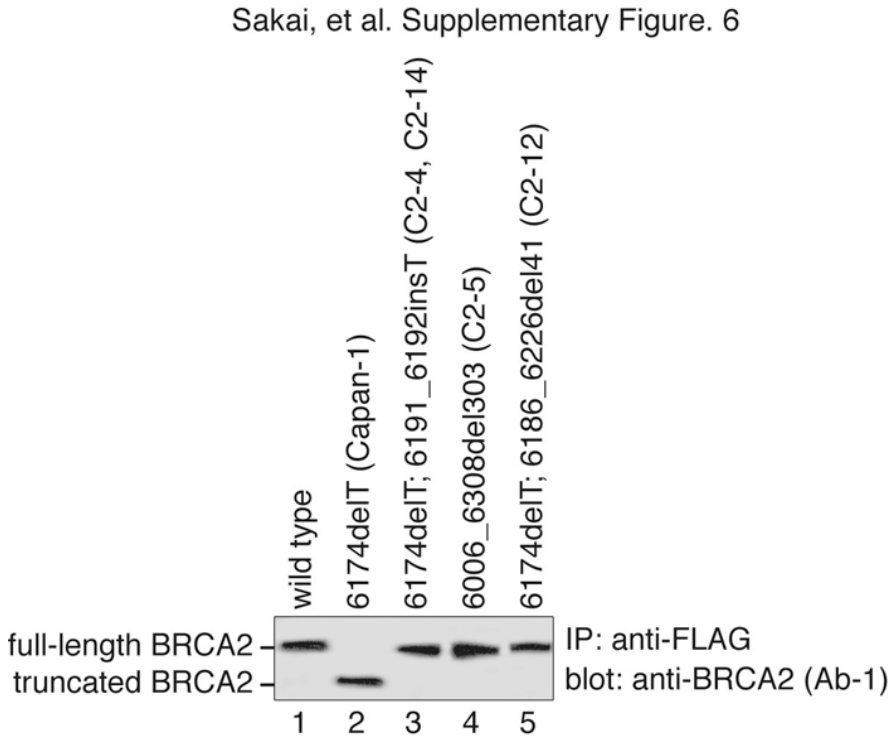

Figure S6. Expression of FLAG-BRCA2 mutant proteins used for the DR-GFP homologous recombination assay. FLAG-tagged BRCA2 variants used for the DR-GFP homologous recombination assay shown in Fig 3b were transiently expressed in 293T cells, immunoprecipitated with anti-FLAG (M2) (Sigma), and analyzed by Western blotting using a BRCA2 antibody (Ab-1, EMD Biosciences).

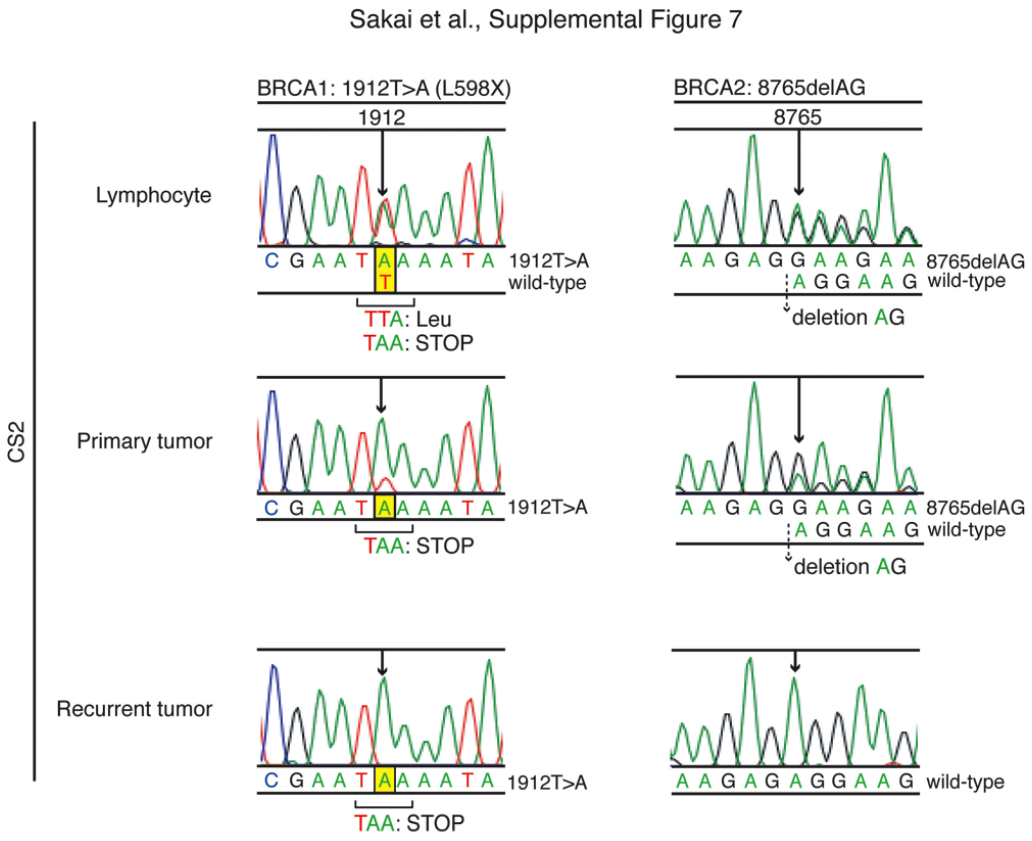

Figure S7. DNA sequences of *BRCA1* and *BRCA2* in peripheral blood lymphocytes, and pre-treatment and recurrent tumors from a patient (CS2) with *BRCA1-BRCA2*-doubly mutated ovarian cancer. The lymphocytes showed heterozygous mutations of both *BRCA1* (1912T>A) and *BRCA2* (8765_8766delAG). Before treatment, the primary tumor showed LOH of *BRCA1* (loss of wild-type *BRCA1*), and hetrozygosity of *BRCA2*. The post-treatment recurrent tumor showed LOH of *BRCA1* (loss of wild-type *BRCA1*) and LOH of *BRCA2* (loss of mutant *BRCA2*).

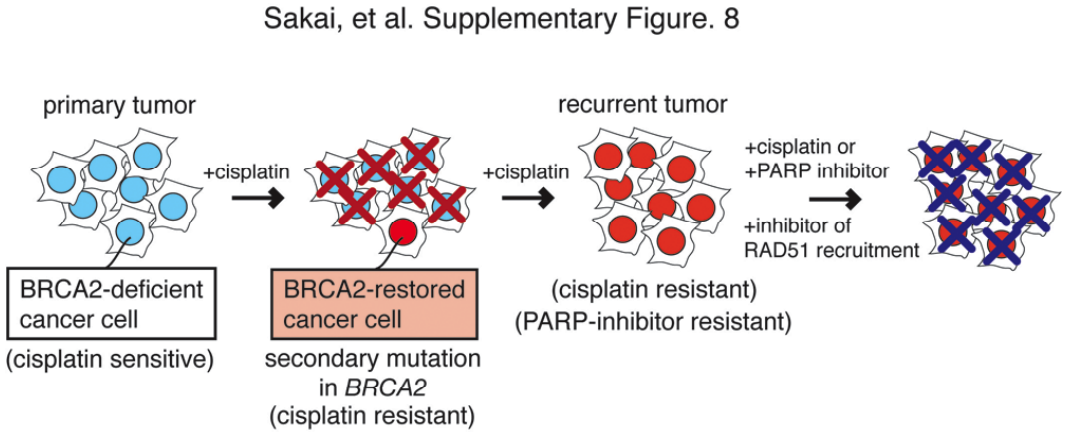

Figure S8. Schematic model for involvement of a secondary *BRCA2* mutation in development of resistance to cisplatin in a *BRCA2*-mutated tumor. See text for detail.

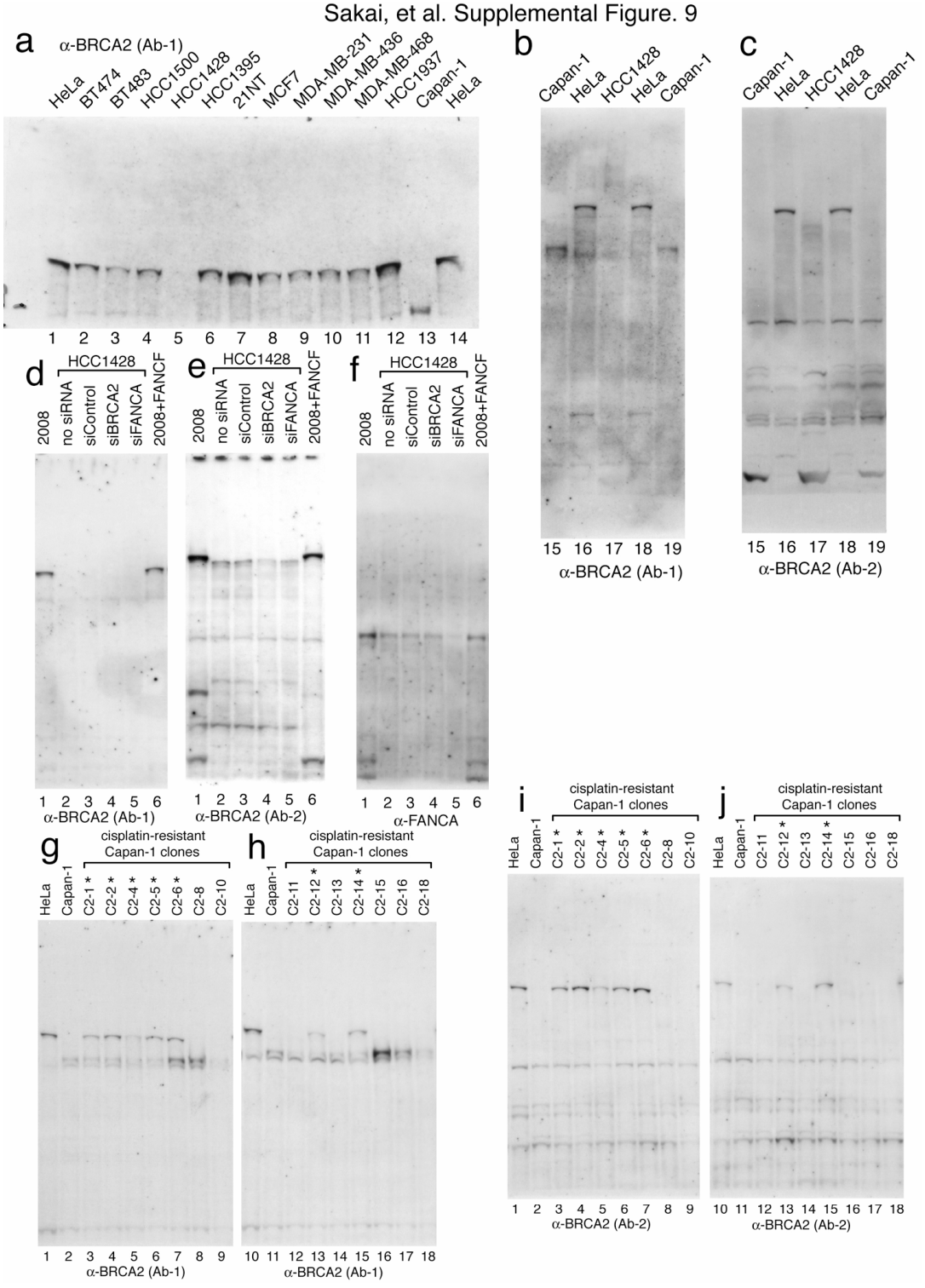

Figure S9. Non-cropped pictures of blots.
(a, b, c) Non-cropped pictures of blots presented in Figure 1a.
(d, e, f) Non-cropped pictures of blots presented in Figure 1f.
(g, h, i, j) Non-cropped pictures of blots presented in Figure 2a.

2. Supplemental Tables

Table S1. Summary of characterization of cisplatin-selected Capan-1 clones. *BRCA2* sequence, BRCA2 protein expression, and cisplatin sensitivity of Capan-1 and its clones are summarized.

Table S1. Characterization of cisplatin-selected Capan-1 clones.

Name of clone	originated from	BRCA2 mutation inherited	BRCA2 mutation additional	Type of secondary mutation	BRCA2 protein expression	Cisplatin sensitivity	IC50 (mean±SEM) (uM)
C2-1	plate #1	6174delT	6232_6238del7ins14	second site deletion and insertion	+	R	2.09±0.15
C2-2	plate #1	6174delT	6232_6238del7ins14	second site deletion and insertion	+	R	2.49±0.39
C2-3	plate #2	6174delT	not found	N/A	-	S	0.34±0.08
C2-4	plate #3	6174delT	6191_6192insT	second site insertion	+	R	0.73±0.16
C2-5	plate #3	6174delT	6006_6308del303	in-frame deletion surrounding nt6174	+	R	0.72±0.08
C2-6	plate #3	6174delT	5870_6365del496ins4	in-frame deletion surrounding nt6174	+	R	0.86±0.15
C2-8	plate #4	6174delT	not found	N/A	-	R	0.75±0.12
C2-10	plate #5	6174delT	not found	N/A	-	R	1.16±0.06
C2-11	plate #6	6174delT	not found	N/A	-	R	0.69±0.05
C2-12	plate #7	6174delT	6186_6226del41	second site deletion	+	R	0.71±0.08
C2-13	plate #8	6174delT	not found	N/A	-	R	1.07±0.28
C2-14	plate #9	6174delT	6191_6192insT	second site insertion	+	R	1.48±0.10
C2-15	plate #10	6174delT	not found	N/A	-	R	0.74±0.07
C2-16	plate #11	6174delT	not found	N/A	-	R	0.73±0.18
C2-18	plate #12	6174delT	not found	N/A	-	R	0.71±0.07
Capan-1	N/A	6174delT	N/A	N/A	-	S	0.46±0.03

abbreviations: N/A; not applicable,

ND; not done, R; resistant, S;

sensitive

Table S2. Evaluation of homology-directed repair of the FLAG-BRCA2 mutants.

	Vector	Wild-type	6174delT	C2-4, C2-14	C2-5	C2-12
Mean GFP positive cells ± SEM	214 ± 8	1073 ± 69	316 ± 9	1114 ± 39	1771 ± 25	1540 ± 27
Relative transfection efficiency (compared to wild-type BRCA2 construct) determined by immunofluorescence with anti-FLAG antibody	~	1.0	1.1	0.7	0.9	1.3
Normalized GFP positive cells	~	1073	297	1620	2024	1173
Fold induction relative to vector ± SEM	1.0 ± 0.1	5.0 ± 0.3	1.4 ± 0.1	7.6 ± 0.3	9.5 ± 0.1	5.5 ± 0.1

Raw data of Figure 3b.

Table S3. Summary of genetic changes in clinical specimens of recurrent *BRCA2*-mutated ovarian cancer treated with platinum.

Table S3. Summary of genetic changes in clinical specimens of recurrent *BRCA2*-mutated ovarian cancer treated with platinum

Patient	specimen	Clinical platium response at the time of sampling	Mutated gene	Inherited mutation	LOH	Secondary genetic change
	Paired specimens					
CS9	pre-platinum	sensitive	*BRCA2*	6174delT	Yes (loss of WT)	No
	post-platinum	sensitive	*BRCA2*	6174delT	Yes (loss of WT)	No
CS15	pre-platinum	sensitive	*BRCA2*	2699delT	Yes (loss of WT)	No
	post-platinum	sensitive	*BRCA2*	2699delT	Yes (loss of WT)	No
CS2	pre-platinum	sensitive	*BRCA1*	1912T>A (L598X)	Yes(loss of WT)	No
			BRCA2	8765_8766delAG	No	No
	post-platinum	refractory	*BRCA1*	1912T>A (L598X)	Yes (loss of WT)	No
			BRCA2	8765_8766delAG	Yes (loss of mt)	Yes (loss of mt)
	Non-paired post-treatment specimens					
UW174	post-platinum	refractory	*BRCA2*	5578_5579delAA	Yes (loss of WT)	No
UW3548	post-platinum	refractory	*BRCA2*	6174delT	Yes (LOH of SNPs) gain of wild-type sequence	Yes (back mutation)

abbreviations: LOH; loss of heterozygosity, mt: mutant allele, SNP; single nucleotide polymorphism, WT; wild-type allele

Table S4. Primers used for *BRCA2* sequencing and PCR.

#	Primer	Seq 5' to 3'
1	B2Pro1F	TCCGAATCCTAAGAATGCAAA
2	B2Pro1R	GCGAGGGCTTGGTAGTATCT
3	B2Pro2F	CACCCAGGCCTGACTTCC
4	B2Pro2R	CACGCTGGACTGGGACTG
5	B2Pro3F	GGTAGTGGGTTGGGACGAG
6	B2Pro3R	ATGGCGTCATCTGGGACA
7	B2Pro4F	CTAGCCACGCGTCACTGGT
8	B2Pro4R	TCTGCAAAACAGAAGACCAAAA
9	B2Pro5F	ACTGGCCCCTTGACTAGCAG
10	B2Pro5R	GCCAGGCTGGTCTCAAACT
11	B2F-125	TCTGAAACTAGGCGGCAGAG
12	B2R32	AATGTTGGCCTCTCTTTGGA
13	B2F420	TGAAAGTCCTGTTGTTCTAC
14	B2R586	TTGACCAAGACATATCAGGA
15	B2F806	CATCAGGGAATTCATTTAAAGT
16	B2R848	ATGTGGTCTTTGCAGCTATTTA
17	B2F1178	GAATGGTCTCAACTAACCCT
18	B2F1561	TCAGGTCATATGACTGATCC
19	B2F1934	GAAGCTGTTCACAGAATGAT
20	B2R2208	TGCAGCCAAGACCTCTTCTT
21	B2F2314	ACTCCTACTTCCAAGGATGT
22	B2R2365	GCAGGCATGACAGAGAATCA
23	B2F2689	GAAAGGAATAATCTTGCTTTAG
24	B2F3016	GGAGGTAGCTTCAGAACAGC
25	B2F3173	AACTGAGCAAGCCTCAGTCAA
26	B2F3398	CAAGCTACATATTGCAGAAG
27	B2R3548	TCAAATTGCTTGCTGCTGTC
28	B2F3785	CAAGTAAATGTCATGATTCTGTTG
29	B2F4190	AAGCATGTCATGGTAATACTTC
30	B2R4382	GGAAAAGTTATGCAATTCTTCTGG
31	B2R4528	GTTGTCCCTGGAAGGTCACT
32	B2F4600	ATTGCAAAGGAATCTTTGGA
33	B2R4840	TAGGTGGCACCACAGTCTCA
34	B2F4985	CAGTCATTGAAAATTCAGCCTTAG
35	B2R5202	TTCGGAGAGATGATTTTTGTCAT
36	B2F5391	AAATGCATACCCACAAACTG
37	B2R5534	CTAAATGCAGGTGGCCCTAC

38	B2F5741	GCACGCATTCACATAAGGTTT
39	B2F5796	TAACCAAAATATGTCTGGATTGGAG
40	B2R5859	TTCCAAACTAACATCACAAGGTG
41	B2F5836	TCACCTTGTGATGTTAGTTTGGA
42	B2F5925	TGGGATTTTTAGCACAGCAA
43	B2R5928	CCCACAAGTATTTGCAGATGAG
44	B2R5990	CTTGCGTTTTGTAATGAAGCA
45	B2R6076	TGAGCTGGTCTGAATGTTCG
46	B2F6193	CAAGTTTCCATTTTAGAAAGTTCCT
47	B2R6202	TGGAAACTTGCTTTCCACTTG
48	B2F6592	GAAACTTTTTCTGATGTTCCTGTGA
49	B2R6496	CCAACTGTTGTTTGTCTTGTTGA
50	B2R6692	GCAATTTCTACTGCTTCTGTTTCA
51	B2R6932	GGAGTGCTTTTTGAAGCCTTT
52	B2F7000	CCCTTTCGCACAACTAAGGA
53	B2F7402	GTAACTTTCACAAAGTGTGAAGA
54	B2F7810	CTGTGTGACACTCCAGGTGT
55	B2R8452	CACAACCAACATTTCCTCCA
56	B2F8565	GGAGGCCCAACAAAAGAGAC
57	B2R8923	CAATACGCAACTTCCACACG
58	B2F8960	TGAGTATTTGGCGTCCATCA
59	B2F9362	CTGCAAGCAACCTCCAGTG
60	B2F9905	GGAGTTGTGGCACCAAATACGAAAC
61	B2F10259	CATTTGCAAAGGCGACAATA
62	B2R10303	CTGGAAAGGTTAAGCGTCAA
63	B2F10396	GTGTATCGGGCAAAAATCGT
64	B2F10663	TCTTTGGATTTGATCACTACAAGT
65	B2R10797	GCTAAACTAAAAGGAATTATCTGCATC
66	B2F10933	CAATTCTTCATCCTTAAGTCAGCA
67	B2F11129	TTGCTTTCAAATTGGCACTG
68	B2R11158	CAGTGCCAATTTGAAAGCAA
69	B2F.IVS10-237	TACACCACCACACCCAGCTA
70	B2F.IVS12-221	TGCTGATTTCTGTTGTATGCTTG
71	B2R.IVS13+278	TGATTCGGAGCAATTTCCTT
72	B2F.IVS16-59	TTGAATTCAGTATCATCCTATGTGG
73	B2R.IVS17+135	GAGAACAGCAGTGTGGGATG
74	B2F.IVS18+1419	CTCAGAGGCTTGTTGGGAAA
75	B2R.IVS18+1769	AGGCCAGGTACAGTGGTTCA
76	B2F.IVS18+2497	TGCCATGTTTTATAGATTTGTCTTT

77	B2R.IVS18+2914	TCACATCTGCACCTTTCTCTG
78	B2F.IVS19-174	GGGGTTTCATCATGTTGGTC
79	B2R.IVS20+179	TGTCCCTTGTTGCTATTCTTTG
80	B2F.IVS20-1695	GGGAGGCCTCAGGAAACTTA
81	B2R.IVS20-1271	TGATTCCAACCCTCATGGAT
82	B2R.IVS21-206	GGAGGATCATTTTTGCCGTA
83	B2F.IVS24+127	GCCTCTTTGAACCTCTGATTTT
84	B2R.IVS24+551	CGGTGGCGGGTATATGTAGT
85	B2F.IVS25-492	CCAGTTGTGATTTCTCAAGCA
86	B2R.IVS25-76	AAAGCAGAAATGAAAAGTTTGGA

Table S5. Primers used for *BRCA1* sequencing and PCR.

#	Primer	Seq 5' to 3'
1	B1F3	CTCGCTGAGACTTCCTGGAC
2	B1R418	TCCAAACCTGTGTCAAGCTG
3	B1F878	AGCTGAGAGGCATCCAGAAA
4	B1R972	GCTGTAATGAGCTGGCATGA
5	B1F1311	TCACATGATGGGGAGTCTGA
6	B1F1641	CCTACATCAGGCCTTCATCC
7	B1R2114	GTTTCTGCTGTGCCTGACTG
8	B1F2095	CAGTCAGGCACAGCAGAAAC
9	B1F2280	TTTGTCAATCCTAGCCTTCCA
10	B1F2494	GGAAGGCAAAAACAGAACCA
11	B1R2504	TTTTGCCTTCCCTAGAGTGC
12	B1F2717	CCAGTCATTTGCTCCGTTTT
13	B1R2743	GGATTTGAAAACGGAGCAAA
14	B1R2921	CTGACCAACCACAGGAAAGC
15	B1F2997	GGCAACGAAACTGGACTCAT
16	B1R3247	TTGCTTGAGCTGGCTTCTTT
17	B1R3361	TTCAATTTTGGCCCTCTGTT
18	B1F3524	GCCTATGGGAAGTAGTCATGC
19	B1F3653	CAAAAGCGTCCAGAAAGGAG
20	B1R3876	ACAGACACTCGGTAGCAACG
21	B1F4100	GTCTGAAAGCCAGGGAGTTG
22	B1F4305	CAGAGGGATACCATGCAACA
23	B1F4530	GGCCTTTCTGCTGACAAGTT
24	B1F5120	GTTTGCCAGAAAACACCACA
25	B1R5668	AGCTCCTGGCACTGGTAGAG

26	B1F101-378	ATTGGTGTTGGTTTGGTCGT
27	B1F101-159	TCTTTAAAAATAAAGGACGTTGTCA
28	B1R199+143	TTCAGTTAAGAAAATCAGCAATTACA
29	B1R199+451	TCTCGATCTCCTGACCTCGT
30	B1F200-352	GATGAGATGTGCACCCACAG
31	B1F200-206	GCTCACTGAAGGTAAGGATCG
32	B1R253+190	TTACCAGGAACTATGATTACAACCAA
33	B1R253+451	AGGTGGATCACAAGGTCAGG
34	B1F.IVS10-294	CTGATGGCCAATCTGCTTTT
35	B1R.IVS11+33	ACTGGGGCAAACACAAAAAC
36	B1F.IVS15-47	TTCAACATTCATCGTTGTGTAAA
37	B1R.IVS16+1	CAAATTCTTCTGGGGTCAGG