Supporting Information

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SI Text

SI Materials and Methods. For bacterial expression, gene fragments corresponding to NTD + OB1 (2479–2658), OB1 (2669–2801), OB2 + OB3 (2802–3192), OB2 (2802–3051), OB3 (3052–3192), and $BRCA2_{[BRC1\text{-}8]}$ (987–2113) were amplified from full-length BRCA2 and subcloned into pET24a vector containing a His-lipoyl domain and tobacco etch virus (TEV) protease site. For transient transfection experiments, BRCA2 and $BRCA2_{CTD}$ (2281–3418) were cloned into pCDNA3.1 (+) (Clontech). BRCA2 ΔCTD was created by replacing codon 2151 with a stop codon. All the plasmids constructed were verified by sequencing. Different domains constructs of BRCA2 and p53 were expressed in BL21 cells and purified to high homogeneity by using several procedural steps. Pull-down experiments, ITC, NMR, and fluorescence titrations were carried out as described (1–3).

Protein expression and purification. For large-scale protein expression, BL21 cells were transformed with OB2, OB3, or OB2 + OB3 expression plasmids. A single colony was used to inoculate the overnight culture. Then 4 L of expression cultures at 37 °C and 250 rpm were induced with 1 mM IPTG at an $A_{600} \sim 0.6$ and allowed to express for 16 h at 22 °C. The cells were harvested and sonicated in cell-cracking buffer of 50 mM sodium phosphate, pH 7.5, 300 mM NaCl, 14 mM β-mercaptoethanol. After centrifugation, the soluble fraction was loaded onto a Hi-trap Ni column (GE Healthcare) and eluted with a 10–250 mM imidazole gradient over 10-column volumes. The pooled fractions were digested with TEV protease overnight at 4 °C and then diluted with equilibration buffer followed by loading onto the Ni column. The flow-through was concentrated and purified further on a Superdex 75 26/60 preparative gel filtration (Amersham Biosciences). The proteins were >95% pure as judged by SDS-PAGE analysis. All the purification steps were carried out at 4 °C. Coexpression with chaperones (Takara) was employed for better solubility and expression levels. The expression yield of OB2 was roughly half the amount obtained from the purification of OB3 and that of OB2 + OB3 was significantly lower compared to OB2 and OB3. $BRC_{1\text{-}8}$ was purified as described (4), with the additional step of Ni purification and TEV protease digestion being included in the purification procedure. p53 and different domain constructs were expressed and purified as described (5).

The genes corresponding to OB2 mut and OB3 mut were purchased from GenScript and subcloned into pET24a vector containing a His-lipoyl domain and TEV protease cleavage site. Protein expression and purification were carried out as described above for wild-type domains.

Binding experiments. We incubated 20 μL of Ni-nitrilotriacetic acid Sepharose beads (Qiagen) with 5 μg His_6, 5 μg His_6-NTD + OB1, or His_6-OB3 + OB2 in Ni-binding buffer (25 mM imidazole, 25 mM Tris-HCl, 150 mM NaCl, 3 mM DTT, pH 7.4, 0.1% Nonidet P-40) for 1 h followed by rocking with different truncated p53 proteins at 4 °C for 4 h. After gentle centrifugation, the beads were washed five times with Ni-binding buffer. Bound proteins were eluted in 60 μL of SDS-PAGE sample buffer containing 250 mM imidazole and 5 mM DTT and were separated in SDS-PAGE gels, and p53 was detected by immunoblotting.

Fluorescence titrations were carried out as described earlier (1). 5′ flourescein-labeled ssDNA $(dT)_{15}$ was used. The experiments were carried out in a buffer containing 20 mM Hepes, pH 7.4, 150 mM NaCl, and 2 mM DTT at 20 °C. For the p53 TAD binding, alexa fluor 546-labeled p53 (1–93) was used. Isothermal titration calorimetry experiments were performed as described (2).

NMR spectra were acquired with a Bruker DRX spectrometer operating at 500 MHz ^1H frequency equipped with a cryogenic inverse probe head and Z-axis gradients. Heteronuclear single quantum coherence (HSQC) spectra were recorded with ^{15}N-labeled p53 TAD (1–93) in a buffer of 50 mM MES pH 6.8, 100 mM NaCl, 5 mM DTT, and 5% (vol/vol) D_2O at 25 °C. In both the control (^{15}N-labeled p53 TAD) and sample (^{15}N-labeled p53 TAD + OB3) runs, concentration of p53N and OB3 were ~100 μM. Spectra were internally referenced on the basis of the position of the water peak. Similarly, $^1H^{15}N$ HSQC spectra of p53 DNA binding domain (92–312) were recorded in the presence and absence of $BRCA2_{[BRC1\text{-}8]}$ (75 μM ^{15}N-labeled p53 DBD and BRC repeats were used in experiments).

Bioinformatics. The human $BRCA2_{CTD}$ model was built with Modeller by using the crystal structure of mouse $BRCA2_{CTD}$ as a template [Protein Data Bank (PDB) ID code 1MJE] (6, 7). The crystal structure of replication protein A in complex with p53 TAD2 (PDB ID code 2B3G) was used for superimposition with the human $BRCA2_{CTD}$ model (8). Pairwise structure superimpositions were performed by using Topmatch (9). All structural figures were created with Pymol (10).

Cell lines, transfections, and apoptosis assay. H1299 cells expressing wild-type p53 upon induction under tetracycline-regulated promoter were maintained in RPMI 1640 (Invitrogen) containing 10% FCS, 2 μg/mL G418, 2.5 μg/mL puromycin, and 4.5 μg/mL tetracycline (tet) as described (3). Twenty-four hours following transfection of BRCA2, $BRCA2_{CTD}$, and p21-luciferase reporter vector, cells were harvested and assayed for luciferase activity by using the Dual-Luciferase Reporter Assay system (Promega) in accordance with the manufacturer's instructions. Wells were prepared in triplicate, and error bars represent 1 standard deviation. The DNA amounts were normalized by using empty pcDNA3.1 vector. Cells were also transfected with 0.1 μg of pCMV-Renilla vector in order to normalize the transfection efficiency.

The apoptosis assay was carried out as described (11) . H1299 cells were seeded at 1.0×10^5 cells per well in a 6-well plate. After 24 h, p53 was induced by placing the cells in tetracycline-free medium, or, in the case of control experiments, cells were grown in tetracycline-containing medium. Cells were transfected with 1 μg of BRCA2 or pcDNA (+) vector. After 24 h, cells were assayed for caspase-3 activity by using a caspase-3 activity kit (Promega) according to manufacturer's instructions. Cells were also transfected with 0.1 μg of pcDNA-EGFP vector in order to normalize the transfection efficiency.

Cell-cycle analysis was carried out as previously described (12). Briefly, cells were harvested 24 h posttransfection and fixed in 80% ethanol, stained with propidium iodide (50 μg/mL) (Invitrogen), and the DNA content was measured by FACS by using FACSCalibur (BD Biosciences) and analyzed by using FLOWJO software.

1. Rajagopalan S, Jaulent AM, Wells M, Veprintsev DB, Fersht AR (2008) 14-3-3 activation of DNA binding of p53 by enhancing its association into tetramers. *Nucleic Acids Res* 36:5983–5991.
2. Rajagopalan S, Andreeva A, Teufel DP, Freund SM, Fersht AR (2009) Interaction between the transactivation domain of p53 and PC4 exemplifies acidic activation domains as single-stranded DNA mimics. *J Biol Chem* 284:21728–21737.
3. Rajagopalan S, Sade RS, Townsley FM, Fersht AR (2010) Mechanistic differences in the transcriptional activation of p53 by 14-3-3 isoforms. *Nucleic Acids Res* 38:893–906.
4. Shivji MK, et al. (2006) A region of human BRCA2 containing multiple BRC repeats promotes RAD51-mediated strand exchange. *Nucleic Acids Res* 34:4000–4011.
5. Ang HC, Joerger AC, Mayer S, Fersht AR (2006) Effects of common cancer mutations on stability and DNA binding of full-length p53 compared with isolated core domains. *J Biol Chem* 281:21934–21941.
6. Sali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* 234:779–815.
7. Yang H, et al. (2002) BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure. *Science* 297:1837–1848.
8. Bochkareva E, et al. (2005) Single-stranded DNA mimicry in the p53 transactivation domain interaction with replication protein A. *Proc Natl Acad Sci USA* 102:15412–15417.
9. Sippl MJ, Wiederstein M (2008) A note on difficult structure alignment problems. *Bioinformatics* 24:426–427.
10. DeLano WL (2002) *The PyMOL Molecular Graphics System* (DeLano Scientific, San Carlos, CA).
11. Khoo KH, Mayer S, Fersht AR (2009) Effects of stability on the biological function of p53. *J Biol Chem* 284:30974–30980.
12. Dubrez L, et al. (2001) Cell cycle arrest in sufficient for p53-mediated tumor regression. *Gene Ther* 8:1705–1712.

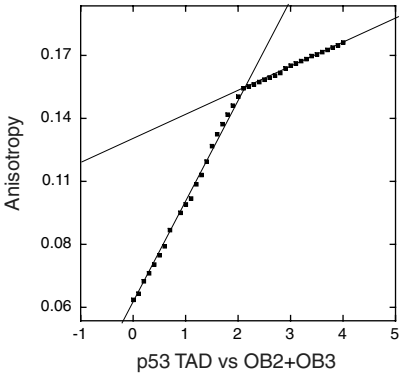

Fig. S1. Stoichiometric titration of p53 TAD with OB2 + OB3.

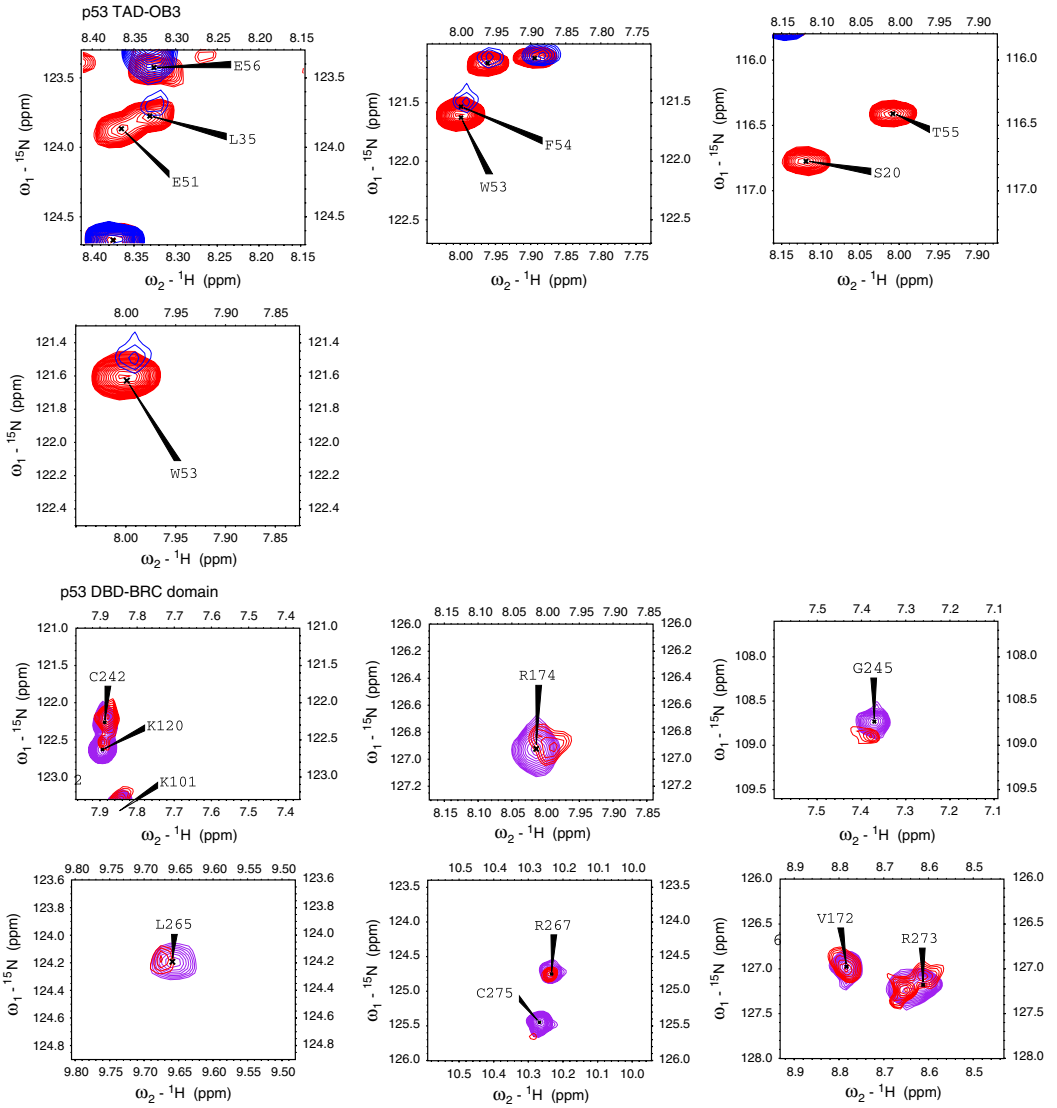

Fig. S2. Selected residues in p53 TAD and DBD that either disappeared or had significant chemical shift perturbations are shown. Color coding is the same as in Figs. 2 and 4 of the main text.

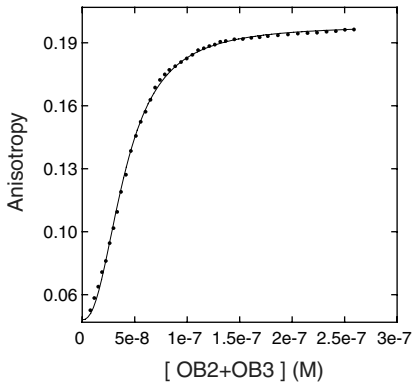

Fig. S3. ssDNA binding titration profile of OB2 + OB3.

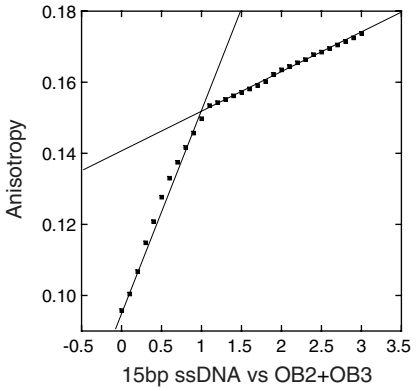

Fig. S4. Stoichiometric titration of ssDNA with OB2 + OB3.

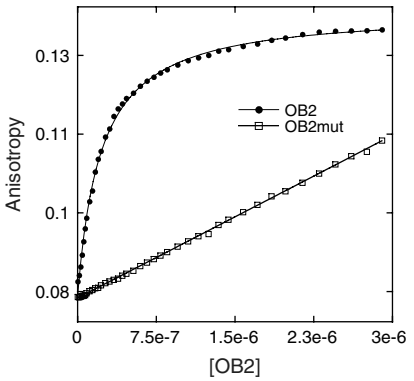

Fig. S5. ssDNA binding titration profile of OB2 and OB2 mut.

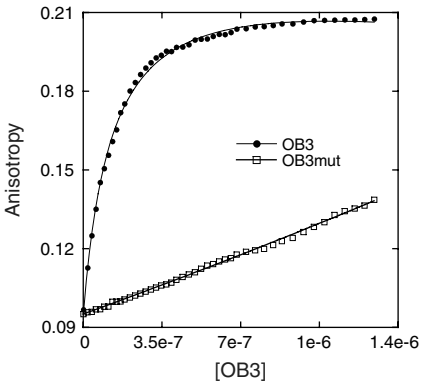

Fig. S6. ssDNA binding titration profile of OB3 and OB3 mut.

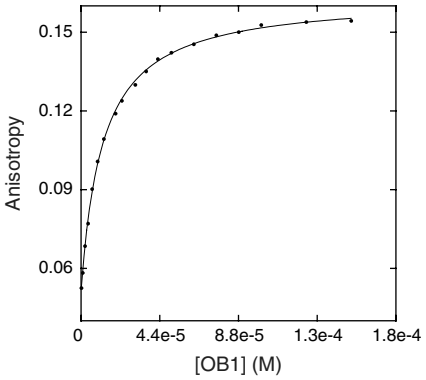

Fig. S7. ssDNA binding titration profile of OB1.

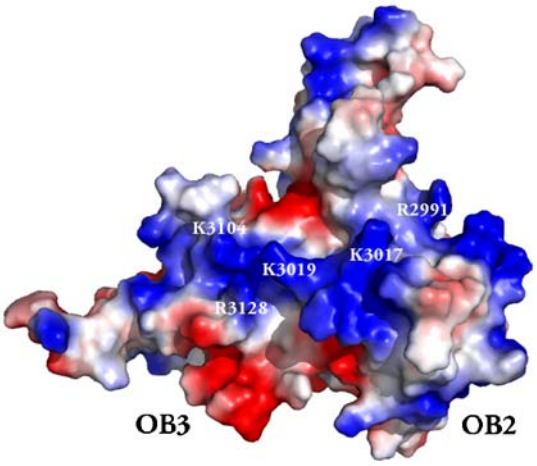

Fig. S8. Electrostatic surface representation of the homology model of human BRCA2 OB2 + OB3 domains. Red surface indicates negative potential and blue positive. Labels indicate the approximate position of the positively charged residues mutated in this study.

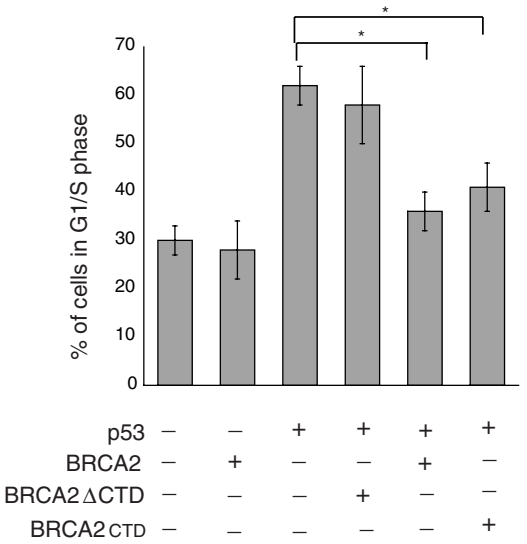

Fig. S9. Cell-cycle distribution was measured 24 h posttransfection by using flow cytometry. Percentage of cells in G0/G1 phase of cell cycle is shown. Cells transfected with BRCA2 or $BRCA2_{CTD}$ but not BRCA2 ΔCTD exhibited G0/G1 growth arrest rescue. A significant percentage of cells (~30%) had undergone apoptosis by 24 h in nontransfected cells.