Supplemental Information

Supplemental Methods

Cloning, Expression, and Purification of UbcH5c

Human UbcH5c was cloned using BamHI and EcoRI restriction sites into the N-terminal GST fusion vector pGEX6P-1 (GE Healthcare). GST-UbcH5c was expressed in *E. coli* Rosetta2 cells. Cells were grown to an OD_{600nm} of 0.6 in LB media containing 50 μg/mL carbenicillin and 12.5 μg/mL chloramphenicol, induced by addition of 0.5 mM IPTG, grown overnight at 16° C, and harvested by centrifugation. Cell pellets were resuspended in buffer A (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT), homogenized using a Microfluidics M-110Y Microfluidizer (MFIC Corp.), and then the suspension was centrifuged at 27,000 *g* for 1 h. Supernatants were passed through a 0.2 μm filter and applied to a column of Glutathione Sepharose 4B (GE Healthcare). The column was washed with buffer B (50 mM HEPES pH 7.2, 150 mM NaCl, 1 mM DTT), followed by buffer C (50 mM HEPES pH 7.2, 150 mM NaCl, 1 mM DTT, 0.05% Tween-20), and buffer D (50 mM Tris pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). UbcH5c was released by overnight on-column cleavage with 200 units of GST-PreScission protease (GE Healthcare) in buffer D at 4°C. Following cleavage, UbcH5c was applied to a SP HP column (GE Healthcare) in buffer E (20 mM sodium citrate pH 5.8, 1 mM DTT), and eluted using a gradient from 0 – 1 M NaCl in buffer E. UbcH5c then further purified using a Superdex S75 16/60 gel-filtration column (GE Healthcare) that had been previously equilibrated in buffer B. Fractions containing UbcH5c were pooled, concentrated using 3,000-MWCO centrifugal concentrators (Millipore) to 5 mg/mL, and stored at 4° C.

Cloning, Expression, and Purification of Bmi1/Ring1b Complexes

Human Bmi1 (residues 1-109, hereafter "Bmi1") was cloned using NdeI and XhoI restriction sites into the pET24-b vector (EMD Biosciences), producing Bmi1 with a C-terminal His_6 fusion. Human Ring1b (residues 1-116, hereafter "Ring1b") was cloned using BamHI and EcoRI restriction sites into pGEX6P-1, as described above for UbcH5c. Ring1b was also cloned into a modified form of the pET52-b vector (EMD Biosciences), which contained an N-terminal His_6 tag, a TEV protease cleavage site, and an Avi tag (GeneCopoeia, Inc.). Complexes of GST-tagged Ring1b/Bmi1 were made by coexpression in *E. coli* Rosetta2 cells, which were grown to an OD_{600nm} of 0.6 in LB media containing 50 μg/mL carbenicillin, 50 μg/mL kanamycin, and 12.5 μg/mL chloramphenicol, and 10 μM $ZnCl_2$. Expression of protein was induced by addition

of 0.5 mM IPTG, and cells were grown overnight at 16° C. Cell pellets were resuspended in buffer A, homogenized, centrifuged and filtered as described above. Filtered supernatants were applied to a column of Ni-NTA Superflow (Qiagen, Inc.), washed extensively with buffer A containing 30 mM imidazole, and eluted with buffer A containing 250 mM imidazole. Eluted fractions were concentrated, the buffer was exchanged into buffer B, and the material was applied to a column of Glutathione Sepharose 4B. The column was washed and proteins were eluted by overnight cleavage with PreScission protease as described above. The Ring1b/Bmi1 complex was further purified by chromatography using a Superdex 75 16/60 gel-filtration column that was equilibrated in buffer B plus 10% glycerol. Fractions containing the complex were pooled, concentrated and stored as described above.

For production of biotinylated protein, complexes of Avi-tagged Ring1b/Bmi1 were coexpressed with the *E. coli* biotin ligase gene BirA (pET52-b vector) in *E. coli* BL21(DE3) cells. Cells were grown and induced as described for the GST-Ring1b/Bmi1 complexes above, except that 50 μM D-Biotin was added at the time of induction. Biotin-Ring1b/Bmi1 was resuspended, homogenized, centrifuged, filtered, and purified by Ni-NTA chromatography as described above. Following elution from Ni-NTA columns, the material was dialyzed overnight into buffer F (50 mM HEPES pH 7.2, 25 mM NaCl, 1 mM DTT) along with 500 units of AcTEV protease (Invitrogen, Inc.). Following dialysis, samples were applied to a SP HP column in buffer F, and then eluted using a gradient from 0–1 M NaCl in buffer F. The biotin-Ring1b/Bmi1 was further purified by gel-filtration chromatography, pooled, concentrated and stored as described for the Ring1b/Bmi1 complexes above.

Supplemental Figures

Supplemental Figure 1

Overlay of the Bmi1/Ring1b RING-RING heterodimer from the Bmi1/Ring1b-UbcH5c crystal structure (this study) with previous crystal structures of Bmi1/Ring1b (PDBs: 2H0D, and 2CKL). Bmi1/Ring1b overlay closely, with an average backbone r.m.s.d. of 0.44 Å over 673 atoms (PDB: 2H0D) and 0.40 Å over 685 atoms (PDB: 2CKL), respectively.

Supplemental Figure 2

Characterization of purified human nucleosomes. (**A**) Protein content of nucleosomes. Samples of purified recombinant proteins (≈ 1 ug individiual histones, 2 ug H2A/H2B dimer and $(H3.1/H4)_2$ tetramer) and 4 ug nucleosomes, as indicated, were run on a 4-12% NuPAGE® Bis-Tris precast gel, and stained with SafeStain (Invitrogen, Inc.). (**B**) Nucleic acid content of nucleosomes. 4 ug nucleosomes was digested with Proteinase K and run on a 1.2% E-gel (Invitrogen, Inc.). Lane markers: 1 = 1-kb ladder (New England Biolabs, Inc.), 2 = 100-bp TrackIt ladder (Invitrogen, Inc.), 3 = Nucleosomes. Complete digestion releases a DNA fragment of ≈ 400 bp, corresponding approximately to tri-nucleosomes.

Supplemental Figure 3

Tandem mass spectrum of a ubiquitin modified histone H2A peptide. Bmi1/Ring1b was incubated with ATP, E1, UbcH5c, and nucleosomes as described earlier, and products were separated by SDS-PAGE. A 22-kDa band (corresponding to the molecular weight of H2A-ubiquitin) was excised from the gel, subjected to tryptic digestion, and analyzed on a hybrid LTQ-Orbitrap (ThermoFisher) mass spectrometer. An ion at 725.119 m/z was detected, corresponding to the triply charged state of the H2A peptide ^{100}VTIAQGGVLPNIQAVLLPKK^{119} containing two additional glycine residues from the C-terminus of ubiquitin. This ion was subjected to collision-induced dissociation (CID), and b- and y-ions were detected corresponding to H2AK118ub. Although K119 is the site of *in vivo* modification described previously, the presence of two glycines on the sidechain of K119 would prevent trypsin cleavage after K119; thus, only peptides with K118-modification were detected from this parent ion. It is likely that either site can be modified by Bmi1/Ring1b activity *in vitro*. Our mass spectral data do not indicate with high confidence either that K119 is modified or that it is not.

Supplemental Figure 4

F-EMSA titration. A fixed concentration (70 nM) of 5'-FAM DNA probe was incubated with increasing concentrations of Bmi1/Ring1b (39.1 nM to 20 μM) at room temperature for 10 min. Samples were mixed 1:1 with 50% glycerol, loaded onto 6% TBE gels, and separated by electrophoresis as described in the Materials and Methods. Gels were imaged using a Typhoon image scanner with excitation and emission at 488 and 526 nm, respectively. Symbols: * = Bmi1/Ring1b-probe complex, ° = free probe.

Supplemental Figure 5

Bmi1 and Ring1b contain basic residues not found in other RING-domain E3 ligases. (**A**) Electrostatic potential molecular surfaces of the RING-RING dimers of Bmi1/Ring1b, BRCA1/BARD (PDB: 1JM7), and cIAP2 (homodimer, PDB: 3EB5). Surfaces were generated using the Adaptive Poisson-Boltzmann Solver (APBS) plugin to PyMol (DeLano Scientific, LLC), were colored according to solvent-accessible surface area, and were contoured to ± 4.0 kT/e. A large basic saddle region is formed by residues at the base of Ring1b (left) and Bmi1 (right). BRCA1/BARD contains a basic region on BRCA1 only, while BARD is neutral. cIAP2 is less basic overall. (**B**) Sequence alignment of RING-domains from several E3 ligases. Conserved Zn^{2+}-binding cysteine and histidine residues are highlighted in beige. A conserved acidic residue required for E2-binding is highlighted in pink. Non-conserved basic residues on the Ring1b and Bmi1 are highlighted in blue. (**C**) Location of mutations made in Bmi1 and Ring1b. Residues that make up the "basic saddle" on Bmi1 and Ring1b are colored in blue, while basic residues that localize to a region on the opposite face of the heterodimer are colored in orange.

Supplementary Figure 6

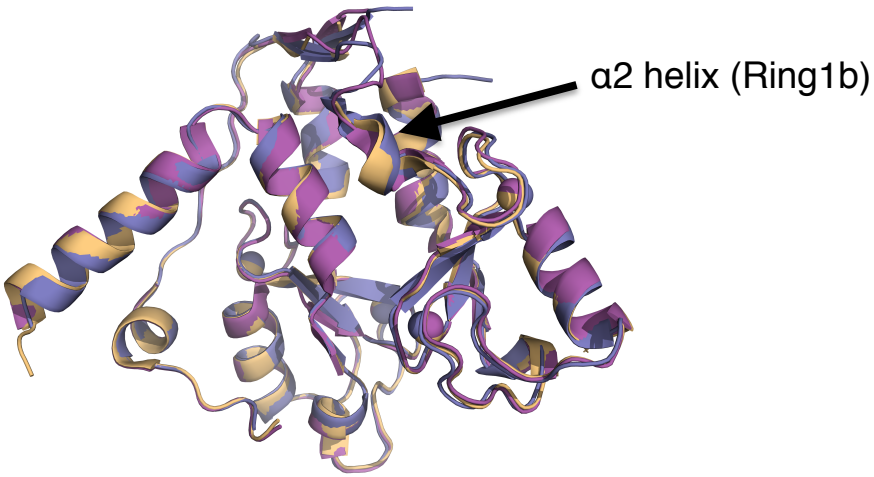
Gel-filtration traces of Bmi1/Ring1b mutant complexes. Samples of purified protein were injected onto a Superdex 75 16/60 gel-filtration column that had been equilibrated with buffer B as described in the Materials and Methods section. Bio-Rad gel filtration standards were run over the same column in identical fashion, to calibrate the column. All Bmi1/Ring1b mutant complexes eluted at a volume corresponding to a molecular weight of ≈ 27 kDa, indicating that each complex is a 1:1 heterodimer between a Bmi1 subunit and a Ring1b subunit. *Top*: Traces for Ring1b mutants. *Bottom*: Traces for Bmi1 mutants.

Supplementary Figure 7

Representative members of the two clusters arising from biochemically-driven docking of Bmi1/Ring1b-UbcH5c against the nucleosome. Models are shown as Cα/phosphate traces, with Bmi1 colored orange, Ring1b colored light blue, UbcH5c colored gray, and the nucleosome colored pink/red. (**A**) The top ten models by intermolecular energy belonging to the largest cluster (197 out of 200 trajectories), with the UbcH5c catalytic cysteine and histone H2A acceptor lysine rendered as spheres. (**B**) The only two models belonging to the second-most populated cluster, which are rotated 180° about the UbcH5c catalytic cysteine relative to the cluster shown in **A**.

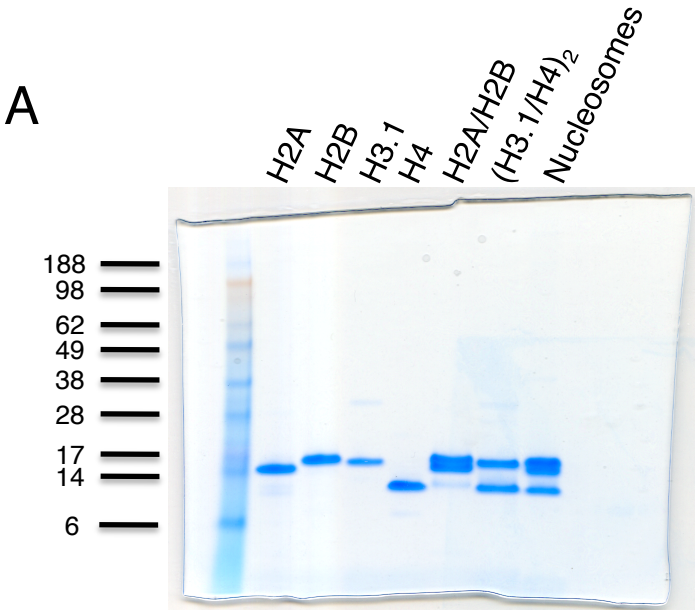
Supplemental Figure 1

UbcH5c complex
PDB: 2H0D
PDB: 2CKL



Supplemental Figure 2

A



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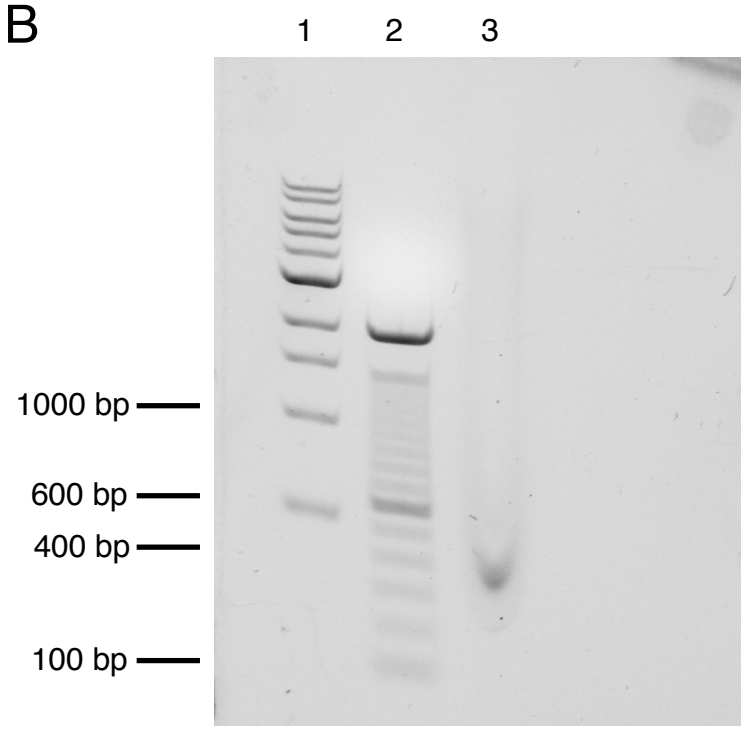
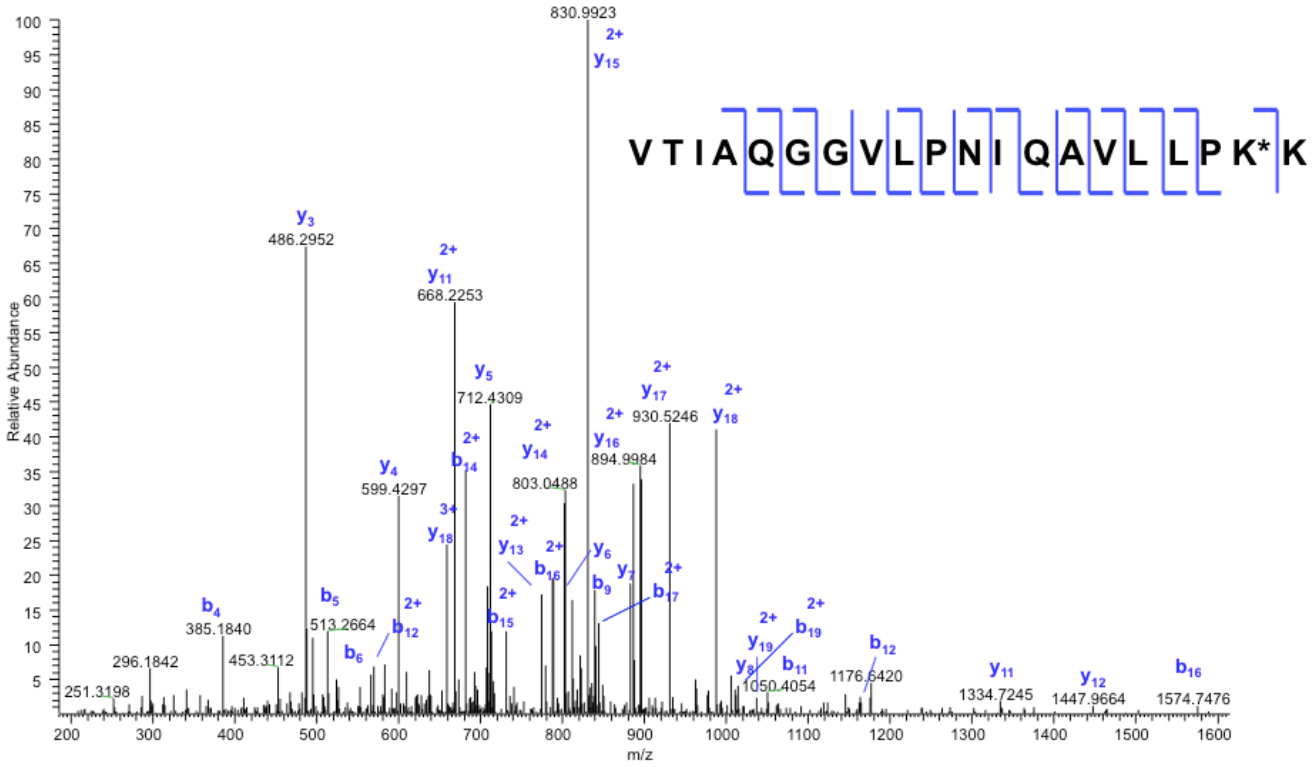
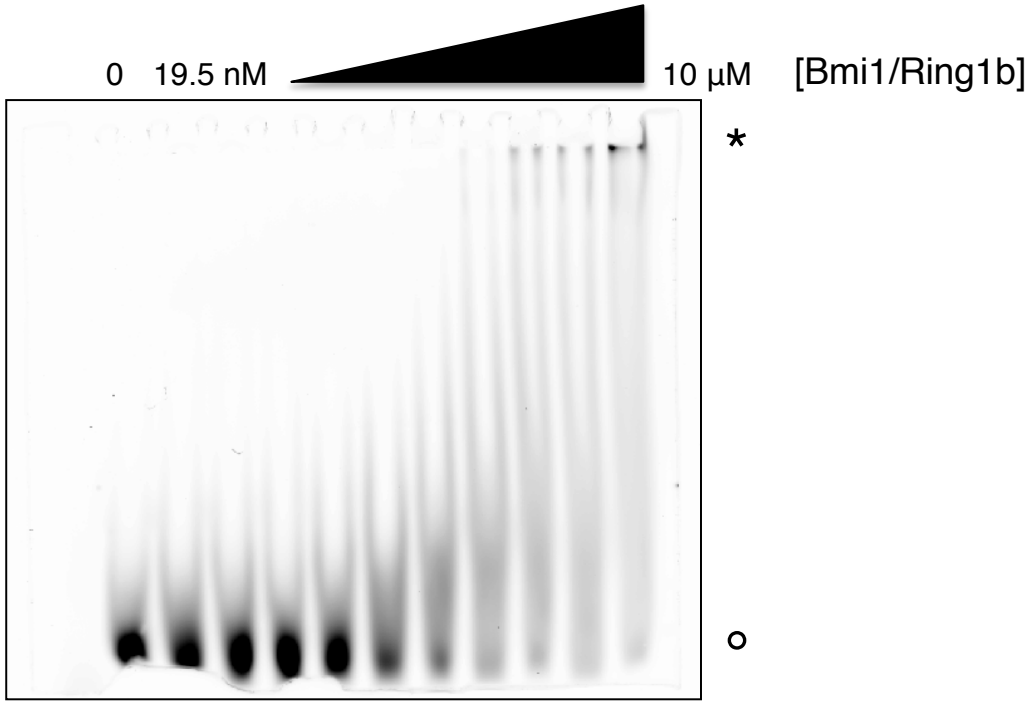


Supplemental Figure 3



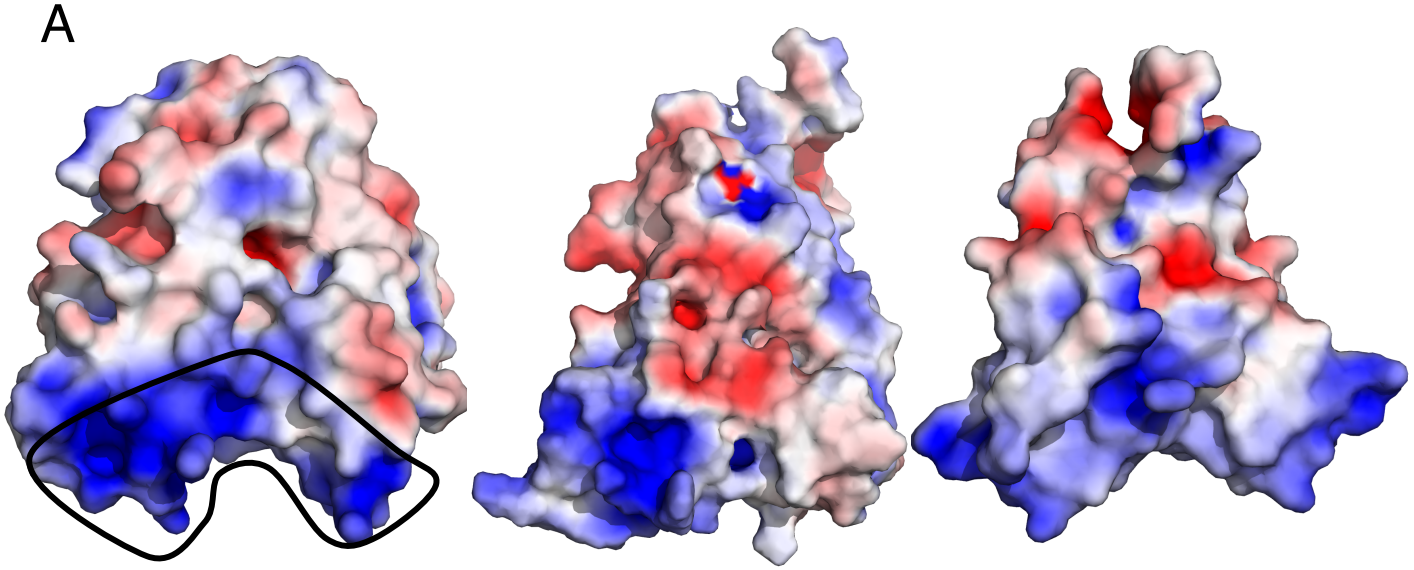
* *Site of ubiquitin modification*

Supplemental Figure 4



Supplemental Figure 5

A



Bmi1/Ring1b BRCA1/BARD cIAP2 (homodimer)

B

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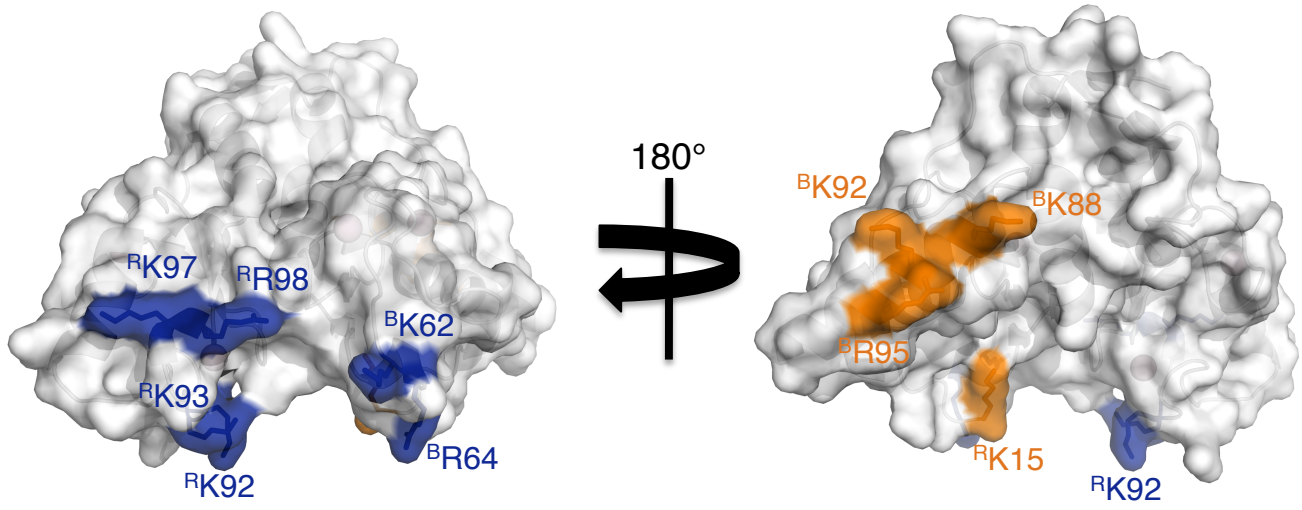
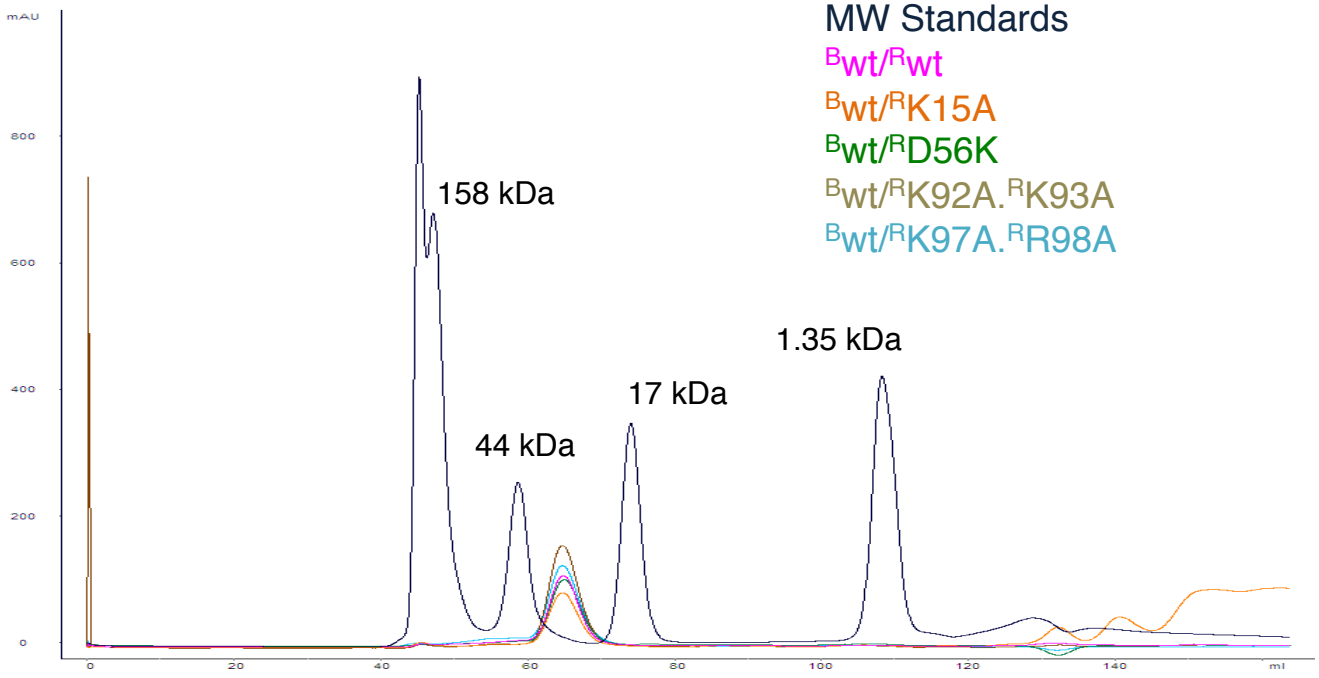
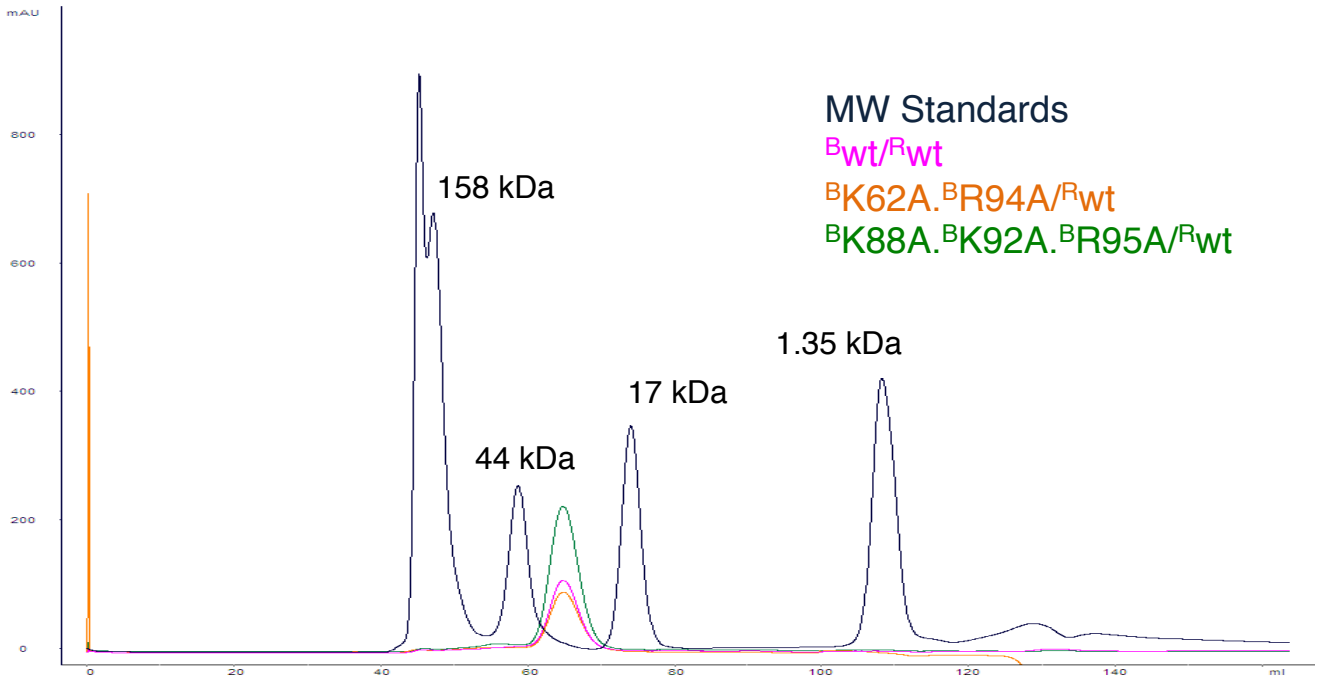
BARD1_human    41 LDRL-------E-KLLRCSRC---TNILRE---P-VCLGGCEHIF-CSNCVSDCI-----GTGCPVCYTPA---WI-QDL--K---INR--  99
Bmi1_human      9 ITE---L----N-PHLMCVLC---GGYFID---A-TTIIECLHSF-CKTCIVRYLE--T-SKYCPICDVQV---HKTRPL--LNIRSDK--  73
BRCA1_human    15 INA---M----Q-KILECPIC---LELIKE---P-VS-TKCDHIF-CKFCMLKLLNQKKGPSQCPLCKNDI---TK-RSL--Q---EST--  77
Ring1a_human   39 PRS---L----H-SELMCPIC---LDMLKN---T-MTTKECLHRF-CSDCIVTALR--SGNKECPTCRKKLV--SK-RSL--R---PDP--  101
Ring1b_human   42 PRS---L----H-SELMCPIC---LDMLKN---T-MTTKECLHRF-CADCIITALR--SGNKECPTCRKKLV--SK-RSL--R---PDP--  104
cIAP1_human   562 LRRL-------QE-ERTCKVC---MDKEVS---V-VF-IPCGHLVVCQECAPSL-------RKCPICRGII---KG-T---------VR--  614
cIAP2_human   548 LRRL-------QE-ERTCKVC---MDKEVS---I-VF-IPCGHLVVCKDCAPSL-------RKCPICRSTI---KG-T---------VR--  600
xIAP_human    441 LRR---L----Q-EEKLCKIC---MDRNIA---I-VF-VPCGHLVTCKQCAEAV-------DKCPMCYTVI---TF-K---------QK--  493
c-Cbl_human   364 TQEQYELYCEMGSTFQLCKIC---AENDKD---V--KIEPCGHLM-CTSCLTSWQE--SEGQGCPFCRCEI---KG-TEP-IV---VDP--  433
CNOT4_human     6 -DA---K----E-DPVECPLC---MEPLEIDDIN-FFPCTCGYQI-CRFCWHRIRT--DENGLCPACRKPY---PE-DPA-VYKPLSQE--  73
RAG1_human    283 FPEH--F----V-KSISCQIC----EHILAD--P-VE-TNCKHVF-CRVCILRCLK--VMGSYCPSCRYPC---FP-TDL--E--SPV---  344
MAT1_human      1 ----------M-DDQGCPRCKTTKYRNPS---LKLMVNVCGHTL-CESCVDLLFV--RGAGNCPECGTPL---RK-SNF-RVQLFEDP--  66
PML_human      48 SEE---E----F-QFLRCQQC---QAEAKC---P-KL-LPCLHTL-CSGCL----E--ASGMQCPICQAPWP LGADTPALDNV---FFESL  112
```

C



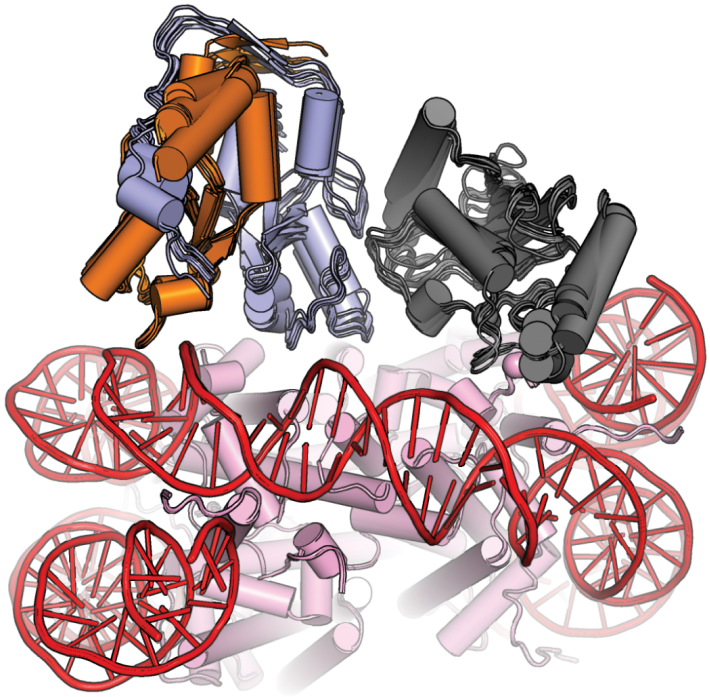
Supplemental Figure 6





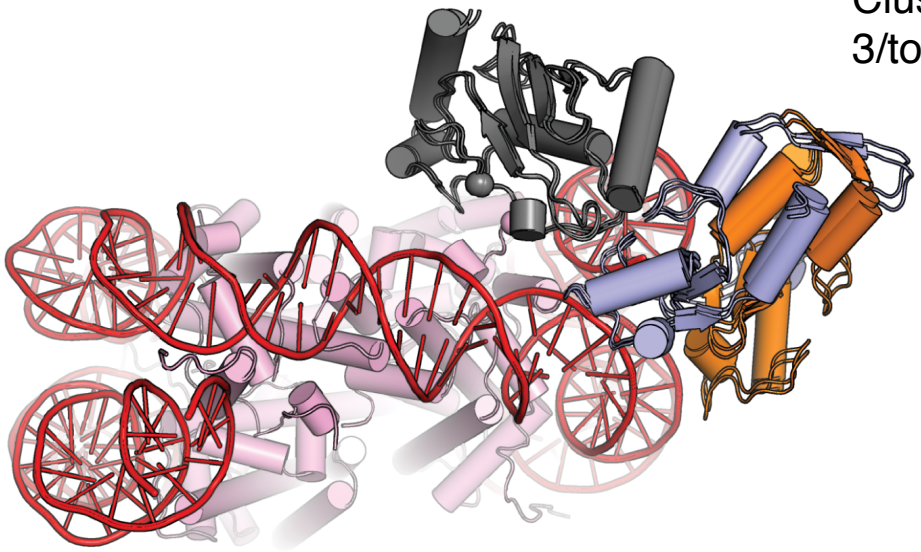
Supplemental Figure 7

A



Cluster #1
197/top 200 models

B



Cluster #2
3/top 200 models