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E2–BRCA1 RING interactions dictate synthesis of mono- or specific polyubiquitin chain linkages

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An E3 ubiquitin ligase mediates the transfer of activated ubiquitin from an E2 ubiquitin-conjugating enzyme to its substrate lysine residues. Using a structure-based, yeast two-hybrid strategy, we discovered six previously unidentified interactions between the human heterodimeric RING E3 BRCA1-BARD1 and the human E2s UbcH6, Ube2e2, UbcM2, Ubc13, Ube2k and Ube2w. All six E2s bind directly to the BRCA1 RING motif and are active with BRCA1-BARD1 for autoubiquitination *in vitro*. Four of the E2s direct monoubiquitination of BRCA1. Ubc13-Mms2 and Ube2k direct the synthesis of Lys63- or Lys48-linked ubiquitin chains on BRCA1 and require an acceptor ubiquitin attached to BRCA1. Differences between the mono- and polyubiquitination activities of the BRCA1-interacting E2s correlate with their ability to bind ubiquitin noncovalently at a site distal to the active site. Thus, BRCA1 has the ability to direct the synthesis of specific polyubiquitin chain linkages, depending on the E2 bound to its RING.

Inherited germline mutations of the human breast and ovarian cancer susceptibility protein BRCA1 predispose an individual to early onset of breast and ovarian tumors. The only biochemical activity associated with BRCA1 is found at its RING domain, which functions as a ubiquitin ligase. Numerous cancer-associated BRCA1 missense mutations occur within the RING domain[1], and these eliminate ubiquitin ligase activity. Hence, loss of ubiquitin ligase activity in the RING domain of BRCA1 is closely associated with breast-cancer susceptibility. We therefore set out to further define the function of this BRCA1 domain during the process of ubiquitination.

An E3 ubiquitin ligase functions at the crossroad between activation of ubiquitin and its covalent attachment to substrate proteins. The process is initiated by an E1 ubiquitin-activating enzyme, which activates the C-terminal Gly76 of ubiquitin and transfers it to the active site cysteine of E2 ubiquitin-conjugating enzyme. Specificity for ubiquitination is believed to be dictated primarily by an E3, which binds both an E2~ubiquitin complex and a substrate to mediate the transfer of ubiquitin from the E2 to a lysine residue on the E3-bound substrate.

Identification of the substrates of an E3 and the type of ubiquitin modification that it synthesizes on those substrates is important in understanding the function of the E3. However, identifying substrates of an E3 is not straightforward, as the mechanisms underlying substrate specificity, E2-E3 interactions, and selection of polyubiquitin linkage types are not well understood. Some of these processes might be determined by the E2. For example, certain E2s function in the synthesis of specific ubiquitin chain linkages; the Ubc13-Mms2 heterodimer[2,3] and Ube2k[4] direct the synthesis of Lys63- or Lys48-linked polyubiquitin chains, respectively. There are over 30 E2s encoded in the human genome, and, similar to Ube2k and Ubc13-Mms2, each has the potential to play a unique role in the process of ubiquitination. The type of ubiquitin modification that can occur on a substrate may depend on the E2 with which the E3 associates. Understanding the function of an E3 therefore depends in part on knowledge of the E2s with which it interacts.

An absolute requirement for a functional E2-E3 pair in any context is a direct physical interaction between them. E2-E3 interactions are believed to be highly selective, with only a single E2 expected to function with a given E3, despite a growing number of reports of an E3 interacting with more than one E2 (refs. 5–7). Defining the complement of E2s that can interact with a given E3 may provide a practical advantage for identifying putative substrates of that E3 and the type of ubiquitin modification that can be attached to the substrate. It is likely that many E3s have more than one substrate, and there is no guarantee that each E2 capable of interaction with the E3 will transfer ubiquitin to every substrate. Thus, a strategy capable of identifying all interacting E2-E3 pairs based on binding would provide the crucial subset of all possible E2s that should be considered in future studies of that E3. Common approaches for identifying protein-protein interactions, such as co-immunoprecipitation or pull-down assays, may not be effective for evaluating all E2-E3 pairings, as E2-E3 interactions are transient and of modest affinity.

To overcome the difficulties in identifying weakly associating E2-E3 pairs, we designed a yeast two-hybrid strategy based on the three-dimensional structure of the heterodimeric RING E3 BRCA1-BARD1 and used it to identify E2-BRCA1 interactions. Specifically, a fused human BRCA1-BARD1 bait construct was used to screen a library of human E2 prey constructs. The screen identified not only the known interacting E2 partners for BRCA1-BARD1, UbcH5 and UbcH7 (ref. 1), but also six new E2 partners for BRCA1. When paired with different E2s, BRCA1-mediated ubiquitination yielded different

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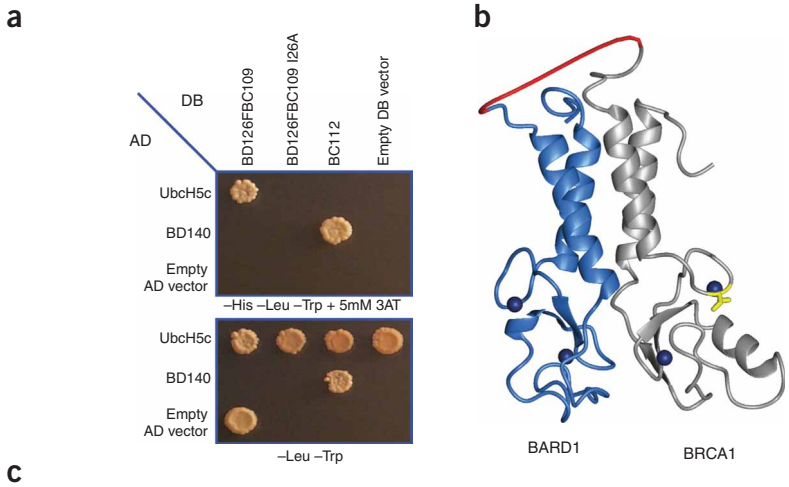

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Bait \ Prey	Rad6b	UbcH10	UbcH5a	UbcH5b	UbcH5c	UbcH6	Ube2e2	UbcM2	Ube2g1	Ube2h	Ubc9	Ube2k	UbcH7	UbcH8	UbcH12	Ube2f	Ubc13	Cdc34	Ube2s	Ube2t	Ube2u	Ube2w	Ube2z	Ube2v1	Uev1b	Mms2	Ube2v3	Ube2q2	Ft1	TSG101	Empty AD vector
BD126FBC109	–	–	+	+	+	+	+	+	–	–	+	+	+	–	–	–	+	–	–	–	+	+	–	–	–	–	–	+	–	–	–
BD126FBC109 I26A	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Empty DB vector	ND	ND	–	–	–	–	–	–	ND	ND	–	–	–	ND	ND	ND	–	ND	ND	ND	+	–	ND	ND	ND	ND	ND	+	ND	–	–

Figure 1 A fused BRCA1-BARD1 construct interacts with multiple E2s in a yeast two-hybrid analysis. (**a**) Proof-of-principle screen showing that UbcH5c interacts with the RING-RING fusion BD126FBC109 but not with the Ile26Ala (I26A) mutant of BRCA1 or the RING of BRCA1 alone (BC112). Top, yeast that can grow under selective conditions indicate an interaction between the respective bait (Gal4 DNA-binding domain (DB)) and prey (Gal4 activation domain (AD)) fusions. Bottom, the yeast are viable under control conditions. (**b**) Model of BD126FBC109 based on the structure of the RING-RING heterodimer of BRCA1-BARD1 (PDB 1JM7). A 5-residue linker is shown schematically in red connecting the C-terminal residue of the BARD1 RING to the N-terminal residue of BRCA1. Ile26 of the BRCA1 RING is highlighted in yellow. (**c**) Summary of directed yeast two-hybrid screens with the respective bait DNA-binding fusions and prey activation domain fusions. +, growth present on selective medium; –, growth absent; ND, not determined. Positive interactions specific for the bait BD126FBC109, and not empty vector controls, are highlighted in red.

products, including monoubiquitination and specific polyubiquitin chains. Comparison of the functional E2s defined residues important for recognition by BRCA1, which provide a mechanistic rationale that distinguishes BRCA1-interacting E2s that build polyubiquitin chains from those that transfer a single ubiquitin. We also show that E2 proteins can function synergistically to build specific chain linkages, as E2s such as the Ubc13-Mms2 heterodimer selectively build Lys63-linked chains on a monoubiquitinated substrate in a BRCA1-dependent fashion. These results suggest a potential division of labor among the various E2 proteins and provide a direct functional link for BRCA1 in the process of DNA repair.

RESULTS

Design of a structure-based BRCA1-BARD1 bait

Structural studies of E2-E3 complexes have revealed the E2-interacting regions for E3s with RING, U-box and HECT domains[7–9]. In the case of the heterodimeric BRCA1-BARD1, the RING motif of BRCA1 interacts directly with the E2s UbcH5c and UbcH7, although only UbcH5c shows activity in either substrate-independent or auto-ubiquitination assays[1]. Although no physical interactions between UbcH5c or UbcH7 and the BARD1 RING were detected, the N-terminal portion of BARD1 is required for the E3 ligase activity of BRCA1 (ref. 10), indicating that it is the heterodimeric structure that imparts functional activity. A directed yeast two-hybrid experiment in which only the BRCA1 subunit was used as bait did not result in a positive interaction with a UbcH5c prey construct (**Fig. 1a**), consistent with the idea that structural integrity of the heterodimeric RING is crucial. We therefore designed a fused version of the BRCA1-BARD1 RING heterodimer that would present a structurally relevant bait molecule in the context of a yeast two-hybrid experiment.

Design of the fusion protein was based on the BRCA1-BARD1 RING-RING heterodimer structure, comprising the first ~110 residues of each subunit[11]. In the solution structure, the C-terminal end of the BARD1 RING domain is within 10 Å of the N-terminal end of the BRCA1 subunit. A Gly_2-Ser-Gly_2 linker was used to link the RING domain of BARD1 (residues 26–126) to the RING domain of BRCA1 (residues 2–109) to create the fusion construct BD126FBC109 (**Fig. 1b**). To confirm that the fusion protein retained both structure and function, we expressed it in bacteria and purified and tested it in a substrate-independent assay with UbcH5c. The ubiquitin ligase activity of BD126FBC109 was identical to that of its nonfused counterpart, BC112-BD140 (BRCA1 residues 1–112 and BARD1 residues 26–140; **Supplementary Fig. 1** online). The $^{1}H,^{15}N$-HSQC NMR spectrum of the fusion protein is highly similar to that of the non-fused heterodimer, confirming the structural integrity of the 'heterodimer' fusion construct (**Supplementary Fig. 1**). BD126FBC109 was therefore fused to the DNA-binding domain of Gal4 to generate our yeast two-hybrid bait.

As proof of principle, BD126FBC109 was tested for interaction with UbcH5c in a directed yeast two-hybrid screen. The BD126FBC109 bait showed an interaction with the UbcH5c prey, whereas a bait comprising the identical BRCA1 residues alone was unable to support growth (**Fig. 1a**). The BRCA1-alone bait supported growth in the presence of a BARD1 prey, confirming that this bait molecule is capable of protein-protein interactions. Inclusion of the ligase-inactive BRCA1 RING mutation I26A (which disrupts the interaction with UbcH5c and UbcH7)[1], in the context of the fused bait construct BD126FBC109-I26A, abrogated the two-hybrid interaction with UbcH5c (**Fig. 1a**). Together, these results confirm that the bait design recapitulates the salient features of the BRCA1-BARD1 E3 interaction with the E2 UbcH5c, namely that the interaction requires the intact heterodimeric structure and can be disrupted by the I26A mutation.

Identification of BRCA1-interacting E2s

To identify additional human E2s that can interact with BRCA1-BARD1, we used BD126FBC109 as bait in a directed yeast two-hybrid screen with 24 human E2s and six human ubiquitin-conjugating E2 variants (UEVs) as prey. Growth of yeast cells on selective medium in the presence of BD126FBC109 above the background level of the empty-vector controls was supported by 11 E2s: UbcH5a (also called Ube2d1), UbcH5b (Ube2d2), UbcH5c (Ube2d3), UbcH6 (Ube2e1), Ube2e2, UbcM2 (Ube2e3), Ube2k (E2-25K), UbcH7 (Ube2l3), Ubc13 (Ube2n1), Ubc9 (Ube2i), and Ube2w (**Fig. 1c** and **Supplementary Fig. 2** online). When these E2s were retested against the

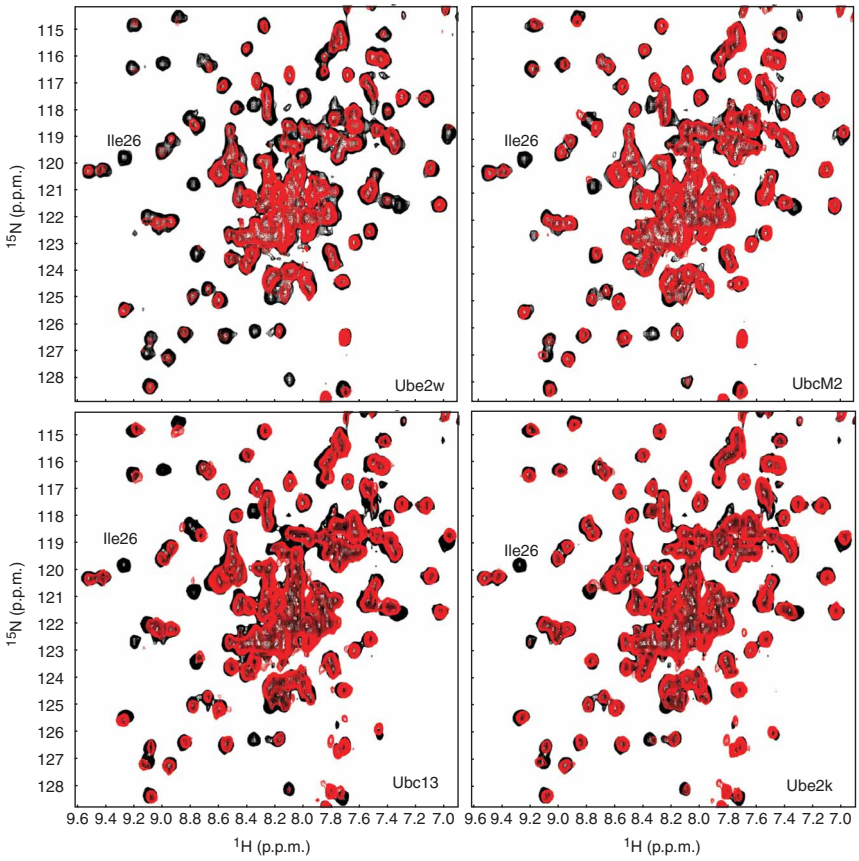

Figure 2 Confirmation of direct E2–BRCA1-BARD1 interactions by NMR. Overlay of an expanded region of the ^{1}H,^{15}N-HSQC-TROSY spectrum of [^{15}N]BC112-BD140 in the absence (black) or presence (red) of 0.5-mol equivalents of an unlabeled E2. The resonance of Ile26 of BRCA1 is labeled. Spectra collected after addition of UbcH6 and Ube2e2 are not shown, as their effects on [^{15}N]BC112-BD140 are indistinguishable from those of UbcM2. Spectra collected after addition of Ubc9 are not shown, as no effect was observed.

BRCA1 RING mutant BD126FBC109-I26A, all but Ubc9 lost the ability to interact with BD126FBC109-I26A. These results suggest that UbcH6, Ube2e2, UbcM2, Ube2k, Ubc13, and Ube2w bind directly to the RING motif of BRCA1, similar to UbcH5c.

To confirm the observed yeast-two hybrid interactions, we used two-dimensional ^{1}H,^{15}N-HSQC-TROSY NMR to detect direct protein-protein interactions by collecting spectra of ^{15}N-labeled BC112-BD140 (non-fused) in the presence or absence of each purified E2. We previously used this approach, which can detect even modest-affinity protein-protein interactions, to map the interactions between BRCA1-BARD1 and the E2s UbcH5c and UbcH7 (ref. 1). NMR spectra were acquired in the presence of UbcH6, Ube2e2, UbcM2, Ube2k, Ubc13, Ubc9, and Ube2w (representative spectra are shown in **Fig. 2**). Addition of any of these purified E2s except Ubc9 resulted in detectable perturbations to the BRCA1-BARD1 NMR spectrum, confirming a direct interaction with the BRCA1-BARD1 heterodimer. Ubc9 was the only E2 that showed a positive yeast two-hybrid interaction but did not interact with BRCA1-BARD1, as judged by NMR. We therefore conclude that Ubc9 does not directly interact with BC112-BD140. As further corroboration, HSQC spectra were collected in the presence of an E2 or UEV that did not support growth in the two-hybrid context. Addition of Rad6, Ube2v1 or Mms2 caused no detectable perturbations in the NMR spectrum of BRCA1-BARD1 (data not shown), confirming that the observed spectral effects are specific.

The HSQC NMR experiments also revealed information on the nature of the E2-E3 interaction. Perturbations elicited by small (substoichiometric) amounts of each E2 were remarkably similar to each other (**Fig. 2**) and to the perturbations previously reported for UbcH5c[1]. In particular, the first peak affected upon addition of each E2 was Ile26 of BRCA1, confirming its central role in E2 binding, as suggested by the directed yeast two-hybrid screens with the I26A mutant bait. Quantitatively similar broadening in the BRCA1-RING peaks was observed for each E2 at early titration points (**Supplementary Fig. 3** online). Thus, although there are small differences in the details of each E2 interaction, the data indicate that UbcH6, Ube2e2, UbcM2, Ube2w, Ube2k, and Ubc13 all bind with similar affinity to the same surface of the BRCA1 RING as does UbcH5c.

E2 selection by BRCA1

The eight E2s found to interact with the RING of BRCA1 are a diverse group. UbcH5, UbcH7, Ubc13 and Ube2w are class I E2s, as they possess only the Ubc core domain. Ube2k is a class II E2, possessing a C-terminal extension from the Ubc core domain. UbcH6, Ube2e2 and UbcM2 are class III E2s, possessing unique N-terminal extensions from their nearly identical core Ubc domains. A sequence alignment of the BRCA1-interacting E2s was compared with a noninteracting set (**Fig. 3**). There are many conserved features among all E2s, as they share the same core domain and the ability to interact with E1s. To understand how numerous E2s interact with the same surface on the RING of BRCA1, we looked for unifying features among the set of BRCA1-interacting E2s that are not shared with other (noninteracting) E2s.

To further define the E2 surface that interacts with BRCA1, we used NMR chemical shift mapping. Two-dimensional HSQC-TROSY spectra of ^{15}N-labeled UbcH5c with increasing amounts of unlabeled BC112-BD140 were collected and compared, and the UbcH5c resonances most perturbed by BRCA1 were identified. The two structural elements of UbcH5c affected most were helix 1 (residues Arg5, Ile6, Lys8, Asp12 and Leu13) and loop L2 (residues Ala96, Thr98, Ile99, Ser100 and Val102; **Supplementary Fig. 4** online), consistent with the idea that these elements comprise the major interaction site for the BRCA1 RING. The surface-exposed residues of the BRCA1 interaction site are highlighted in the sequence alignment in **Figure 3**. Three of these positions—UbcH5c residues Arg5 and Lys8 (in helix 1) and Ala96 (in loop L2)—are strongly conserved within the BRCA1-interacting group of E2s. Alignment of noninteracting human E2 sequences revealed none that contain all three conserved residues.

The ability of the BRCA1-BARD1 heterodimer to ubiquitinate itself[10,12] was used to test the functional importance of the putative E2 binding determinants. For the autoubiquitination assays, a heterodimer comprising the first 304 amino acid residues of BRCA1 (BC304)

		Helix 1		Loop L1		Loop L2
		* * *				* * *
UbcH5a	1	MALK**R**IQ**K**ELS**D**LQRD	:57	PTDYPFKPP	:92	QWSP**A**L**T**v**S**KVLL
UbcH5b	1	MALK**R**IH**K**ELN**D**LARD	:57	PTDYPFKPP	:92	QWSP**A**L**T**I**S**KVLL
UbcH5c	1	MALK**R**IN**K**ELS**D**LARD	:57	PTDYPFKPP	:92	QWSP**A**L**T**I**S**KVLL
UbcH6	47	TSAK**R**IQ**K**ELA**D**ITLD	:103	TPEYPFKPP	:138	NWSP**A**L**T**I**S**KVLL
Ube2e2	55	TSAK**R**IQ**K**ELA**E**ITLD	:111	SPDYPFKPP	:146	NWSP**A**L**T**I**S**KVLL
UbcM2	61	TSAK**R**IQ**K**ELA**E**ITLD	:117	SSDYPFKPP	:152	NWSP**A**L**T**I**S**KVLL
Ube2k	7	QRIK**R**EF**K**EVL**K**SEET	:63	PETYPFNPP	:99	QWAA**A**M**T**L**R**TVLL
UbcH7	2	AASR**R**LM**K**ELE**E**IRKC	:58	PAEYPFKPP	:94	NWKP**A**T**K**T**D**QVIQ
Ubc13	3	GLPR**R**II**K**ETQ**R**LLAE	:59	PEEYPMAAP	:94	KWSP**A**L**Q**I**R**TVLL
Ube2w	14	WFPK**R**LQ**K**ELL**A**LQND	:72	SSRYPFDSP	:109	DWSP**A**L**S**v**Q**SVCL
Rad6b	4	PARR**R**LM**R**DFK**R**LQED	:60	SEEYPNKPP	:95	RWSP**T**Y**D**v**S**SILT
UbcH10	30	PVGK**R**LQ**Q**ELM**T**LMMS	:86	PSGYPYNAP	:121	KWSA**L**Y**D**v**R**TILL
Ube2g1	8	LLLR**R**QL**A**ELN**K**NPVE	:62	PKDYPLRPP	:110	RWLP**I**H**T**v**E**TIMI
Ube2h	6	PGKR**R**MD**T**DVV**K**LIES	:58	PDKYPFKSP	:94	TWTA**L**Y**D**I**T**NIFE
Ubc9	10	AQER**K**AW**R**KDH**P**FGFV	:65	KDDYPSSPP	:102	DWRP**A**I**T**I**K**QILL
UbcH8	2	MASM**R**VV**K**ELE**D**LQKK	:58	PPEYPFKPP	:94	NWKP**C**T**K**T**C**QVLE
UbcH12	29	AAQL**R**IQ**K**DIN**E**LNLP	:83	GQGYPHDPP	:118	DWKP**V**L**T**I**N**SIIY
Ube2f	32	VRDK**L**LV**K**EVA**E**LEAN	:88	PDAYNMVPP	:129	GWAP**T**R**T**L**K**DVVW
cdc34	8	SSQK**A**LL**L**ELK**G**LQEE	:65	PIDYPYSPP	:113	RWNP**T**Q**N**v**R**TILL
Ube2s	11	HIIR**L**VY**K**EVT**T**LTAD	:67	GKDFPASPP	:102	DWTA**E**L**G**I**R**HVLL
Ube2t	2	QRAS**R**LK**R**ELH**M**LATE	:58	PERYPFEPP	:97	AWRP**S**L**N**I**A**TVLT
Ube2u	4	RAYL**L**LH**R**DFC**D**LKEN	:60	TSEYNYAPP	:98	KWNT**N**Y**T**I**S**SILL
Ube2z	1	M**S**IY**K**EPP**P**GMFV	:47	PPDYPPIHP	:91	AWSP**A**Q**S**I**S**SVLI

Figure 3 Sequence alignment of helix 1, loop L1 and loop L2 of BRCA1-interacting and noninteracting E2s. Most of the BRCA1-interacting residues reside in helix 1 and loop L2 of UbcH5c (**Supplementary Fig. 4**). Each E2 was aligned relative to the sequence of UbcH5c. The top group of E2s includes those that interact directly with the RING of BRCA1. The bottom group includes those that were tested in the yeast two-hybrid screen but did not interact with BRCA1. Surface-exposed residues of UbcH5c involved in direct interaction with BRCA1 are marked with an asterisk and in bold. Shaded residues are aligned with Arg5, Lys8 and Ala96 of UbcH5c, which are strictly conserved among the BRCA1-interacting set and poorly conserved among the noninteracting E2s.

and amino acids 26–327 of BARD1 (BD327) was used. UbcH5 residues Arg5 and Lys8 are not good candidates for mutational analysis of functional E2-E3 interactions, as they are on helix 1, which is also recognized by the E1 (ref. 13) and is therefore necessary for ubiquitin-E2 thiolester formation. To assess the role of Ala96 in BRCA1-mediated E2 recognition, we mutated Ala96 of UbcH5c to serine or aspartate and tested the abilities of the mutant E2s to facilitate autoubiquitination of BRCA1. UbcH5c-A96S showed only weak activity with BRCA1, and UbcH5c-A96D showed no detectable activity (**Fig. 4**). Both UbcH5c mutants retained the ability to form a thiolester with ubiquitin comparable to that of wild-type UbcH5c (**Supplementary Fig. 5** online), consistent with their proper folding. Furthermore, a comparison of the HSQC spectra of wild-type UbcH5c and UbcH5c-A96D revealed no major structural changes in UbcH5c in the presence of the A96D mutation (**Supplementary Fig. 6** online). The HSQC spectrum of ^{15}N-labeled UbcH5c-A96D showed no detectable shifts after addition of unlabeled BC112-BD140 (**Supplementary Fig. 6**). Hence, the observed loss of BRCA1 autoubiquitination activity by UbcH5c-A96D can be attributed to its inability to interact with the RING of BRCA1. Together, these results demonstrate that Ala96 of UbcH5c is a crucial determinant for binding to BRCA1, and we propose that the analogous alanine residues in the other BRCA1-interacting E2s have a similar role. On the basis of this analysis, we conclude that the remaining human E2s tested in our directed yeast two-hybrid screen are unlikely to bind directly to the RING domain of BRCA1.

Characterization of BRCA1 autoubiquitination

The BRCA1-interacting E2s were tested for activity with the BRCA1-BARD1 E3. The BRCA1 subunit is efficiently polyubiquitinated when UbcH5c is used as the E2. UbcH6, Ube2e2, UbcM2, and Ube2w also transfer ubiquitin to BRCA1, but the predominant product is monoubiquitinated BRCA1 (**Fig. 5a**). The core Ubc domains of the class III E2s UbcH6, Ube2e2 and UbcM2 are over 95% identical to each other, which may explain why each of these E2s has similar properties with BRCA1. The Ubc domains of these three E2s also have the highest level of identity with UbcH5c, at ~64%.

Given their high degree of similarity with UbcH5c, it is notable that the class III E2s do not polyubiquitinate BRCA1. The ability of UbcH5c to polyubiquitinate BRCA1 depends on a noncovalent interaction between ubiquitin and the β-sheet surface of UbcH5c[12]. We previously showed that mutation of UbcH5c (S22R) on the β-sheet surface disrupts the noncovalent interaction and results in an E2 that transfers only a single ubiquitin to BRCA1, similar to what was observed for UbcH6, Ube2e2, UbcM2, and Ube2w. We therefore tested UbcM2 and Ube2w, E2s that monoubiquitinate BRCA1, for their ability to bind to ubiquitin noncovalently. No evidence was found for such interactions in HSQC NMR binding experiments, even with saturating amounts of added E2 (D.M. Wenzel, D.E.C. and R.E.K., unpublished data). Sequence comparison with the Ubc domain of UbcM2 indicates that UbcH6 and Ube2e2 are also unlikely to bind free ubiquitin. Similar to the results with UbcH5c and UbcH5c-S22R, the inability of UbcH6, Ube2e2, UbcM2 and Ube2w to build polyubiquitin chains with BRCA1-BARD1 is associated with the inability to interact with ubiquitin in a noncovalent manner.

Three E2s—UbcH7, Ube2k, and Ubc13—were unable to transfer ubiquitin to BRCA1 (**Fig. 5a**) despite their ability to bind the same surface on BRCA1 as the active E2s. *In vitro*, the Ubc13-Mms2 heterodimer can synthesize unanchored, Lys63-linked polyubiquitin chains. The chain-building activity of Ubc13-Mms2 depends on the ability of Mms2, a ubiquitin E2 variant, to bind an acceptor ubiquitin noncovalently[14]. Because a subset of BRCA1-interacting E2s monoubiquitinate BRCA1, we tested whether a single ubiquitin attached to BRCA1 can serve as an acceptor ubiquitin for Ubc13-Mms2–mediated synthesis of polyubiquitin chains. We conducted autoubiquitination assays with BRCA1-BARD1 in the combined presence of equimolar amounts of Ube2w, Ubc13 and Mms2. The combination of Ube2w with Ubc13-Mms2 produced a ladder of ubiquitinated-BRCA1 bands (**Fig. 5b**). The addition of multiple ubiquitins to BRCA1 requires the presence of all three E2s, as any combination of

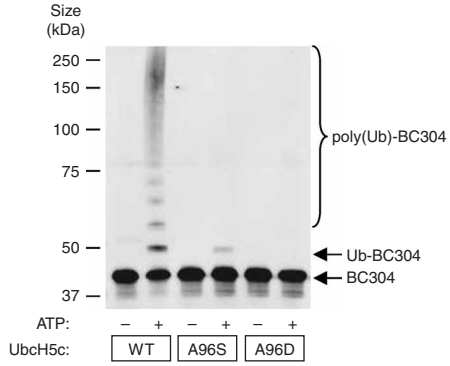

Figure 4 Mutation of conserved alanine in loop L2 of UbcH5c eliminates autoubiquitination of BRCA1. Purified wheat E1, ubiquitin (Ub) and Flag–BC304-BD327 were mixed with the indicated mutant or wild-type UbcH5c. Ubiquitination was measured 0 (–) and 60 min (+) after addition of ATP. Reaction products were visualized by western blotting for Flag-BC304. Slower-migrating bands above the main Flag-BC304 band are ubiquitinated forms of BC304.

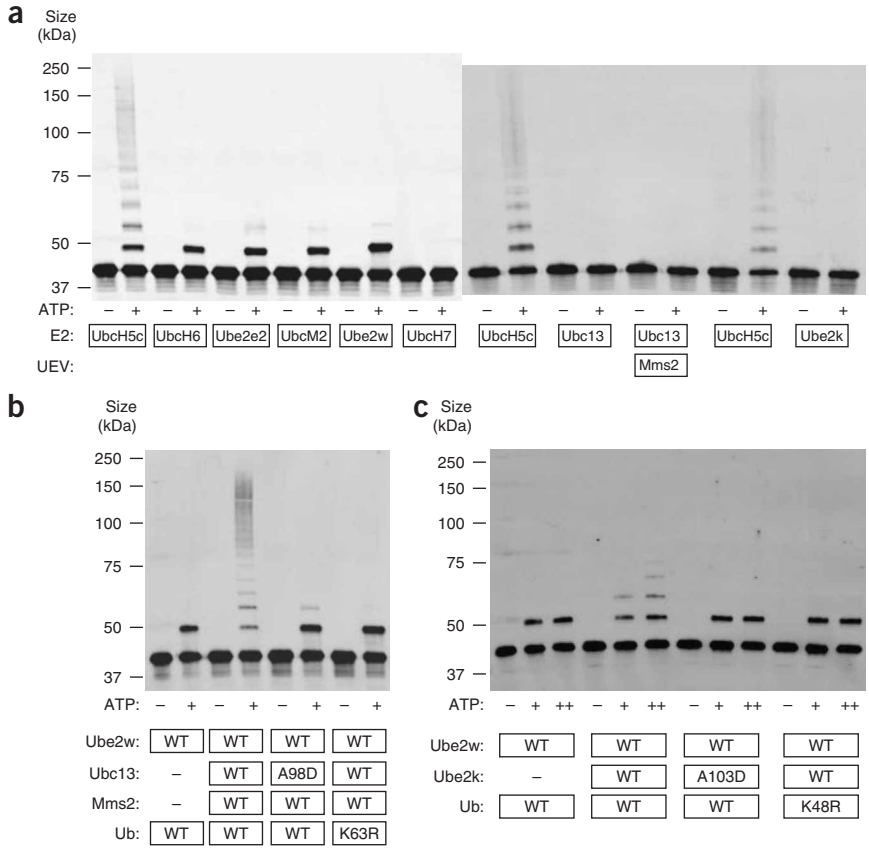

Figure 5 Autoubiquitination activity assays of BRCA1 with its interacting E2s. Flag–BC304-BD327 was assayed for autoubiquitination activity with each of the E2s found to interact with the RING of BRCA1. Each panel shows a western blot for Flag with unmodified and ubiquitinated BC304 0 (–), 1 h (+), and 2 h (++) after addition of ATP, for each indicated E2. Similar ubiquitination products to those found on BRCA1 were also detected for BARD1 in all assays (data not shown). (**a**) Autoubiquitination of BRCA1 with each E2. Ubc13 was tested with and without its heterodimeric partner, Mms2. Assays with Ube2k were performed with human E1; for comparison, UbcH5c was also assayed with human E1 (shown in the lanes to the immediate left of Ube2k). (**b**,**c**) Autoubiquitination assays in the presence of two E2s. Ube2w with Ubc13-Mms2 (**b**) or Ube2w with Ube2k (**c**) were assayed in combination for auto-polyubiquitination activity with BRCA1, with equal concentrations of each E2. Ubc13-Mms2 and Ube2k are active for polyubiquitination of BRCA1-ubiquitin. The polyubiquitination activity is dependent on interaction of the E2s with the RING of BRCA1 (Ubc13-A98D (**b**) and Ube2k-A103D (**c**)). Polyubiquitin chains formed by Ubc13-Mms2 are linked through Lys63 (**b**; ubiquitin-K63R) and chains formed by Ube2k are linked through Lys48 (**c**; ubiquitin-K48R). All E2s were efficiently activated with ubiquitin at their active site (data not shown) despite differences in BRCA1 autoubiquitination.

two of the three E2s resulted in only monoubiquitination when Ube2w was present, or no ubiquitination with only Ubc13-Mms2 (**Supplementary Fig. 7** online). These results indicate that BRCA1 is first monoubiquitinated by Ube2w, and the BRCA1-ubiquitin then becomes a substrate for Ubc13-Mms2. Use of ubiquitin-K63R resulted in only monoubiquitination of BRCA1 with the mixture of Ube2w, Ubc13 and Mms2 (**Fig. 5b**), indicating that this combination of E2s synthesizes a Lys63-linked polyubiquitin chain attached to BRCA1.

Because Ubc13-Mms2 can build unanchored polyubiquitin chains in the absence of an E3, we tested whether the chains attached to BRCA1 require a direct E2-E3 interaction between BRCA1 and Ubc13. To disrupt binding to BRCA1-BARD1, we mutated the conserved loop L2 Ala98 in Ubc13 to aspartate, a mutation similar to UbcH5c-A96D. The mutant Ubc13-A98D–Mms2 complex was unaffected in its ability to create E3-independent, unanchored polyubiquitin chains (**Supplementary Fig. 8** online). However, Ubc13-A98D–Mms2 was unable to synthesize a polyubiquitin chain attached to monoubiquitinated BRCA1. These results indicate that polyubiquitination of BRCA1 by Ubc13-Mms2 requires a functional RING-E2 interaction between BRCA1 and Ubc13, and not solely the attachment of a ubiquitin to BRCA1.

The C-terminal tail of Ube2k has been defined as a ubiquitin-associated domain on the basis of sequence homology. The tail is required for the activity of Ube2k and for the synthesis of Lys48-linked polyubiquitin chains *in vitro*[4]. Though it has been inferred that Ube2k can bind free ubiquitin[15], we confirmed this experimentally by HSQC-NMR (D.M. Wenzel, D.E.C. and R.E.K., unpublished data). We tested whether Ube2k, like Ubc13-Mms2, can also recognize monoubiquitinated BRCA1 as an acceptor ubiquitin for polyubiquitin chain elongation. When mixed with Ube2w in a BRCA1 autoubiquitination assay, Ube2k extended a polyubiquitin chain from a monoubiquitinated BRCA1 (**Fig. 5c**). The polyubiquitin chain synthesized on BRCA1 was linked through Lys48, as ubiquitin-K48R abrogated product formation. Chain-building was dependent on the RING-E2 interaction between Ube2k and BRCA1, as the Ube2k loop L2 mutant A103D also abolished product formation (**Fig. 5c**).

DISCUSSION

We have identified six new E2 interactions for the BRCA1-BARD1 ubiquitin ligase. Crucial determinants of E2-BRCA1 interactions are Ile26 of BRCA1 and a conserved alanine in loop L2 of the E2, as mutation at either site abrogates both binding and activity. BRCA1 can facilitate either monoubiquitination or polyubiquitination though Lys6 (refs. 16,17), Lys48 or Lys63 of ubiquitin, depending on the E2 present. Our studies provide some mechanistic conclusions about how different E2s function with the E3 BRCA1-BARD1. Ubc13-Mms2, Ube2k, and UbcH5c each build polyubiquitin chains on BRCA1 and share a common ability to bind ubiquitin not only at their active site cysteine but also at a distal site. The nature and location of the noncovalent ubiquitin-binding sites are different for UbcH5c, Ubc13-Mms2 and Ube2k, and presumably differences in the ubiquitin binding geometry determine the specific chain linkages each E2 produces with, and on, BRCA1. UbcH6, Ube2e2, UbcM2, and Ube2w transfer only a single ubiquitin to BRCA1. UbcM2 and Ube2w do not bind ubiquitin noncovalently, and it is unlikely that UbcH6 and Ube2e2 bind ubiquitin, because of their homology to the Ubc domain of UbcM2. These results are consistent with our previous report that the S22R UbcH5c mutation, which abrogates noncovalent ubiquitin-UbcH5 binding, yields only monoubiquitinated BRCA1 as

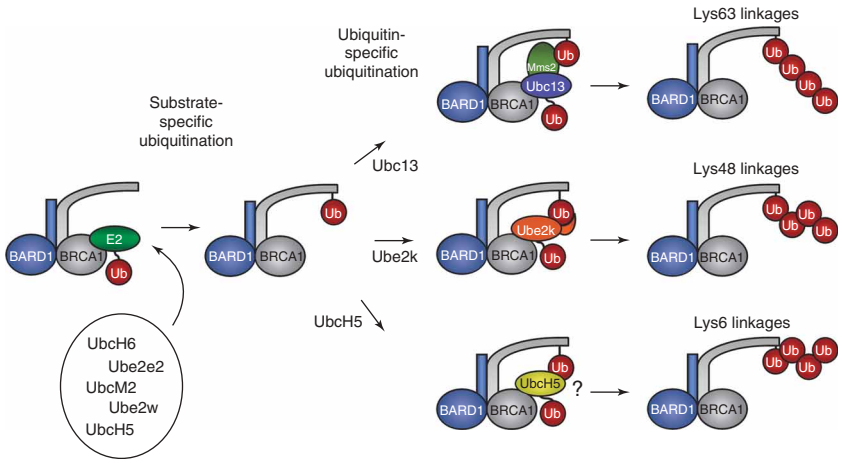

Figure 6 Model of BRCA1-BARD1 autoubiquitination mechanisms. BRCA1-interacting E2s are classified according to their mode of ubiquitin transfer. Substrate-specific E2s UbcH6, Ube2e2, UbcM2 and Ube2w transfer ubiquitin directly to BRCA1. Ubiquitin-specific E2s Ubc13-Mms2 and Ube2k bind ubiquitin noncovalently and require BRCA1-ubiquitin for synthesis of Lys63- or Lys48-linked polyubiquitin chains. UbcH5 can both recognize BRCA1 for ubiquitin transfer and extend a polyubiquitin chain. UbcH7 is not included, as it does not autoubiquitinate BRCA1. Noncovalent interaction with ubiquitin is a common property among the ubiquitin-specific E2s and is likely to be necessary for polyubiquitin chain elongation.

its product. Thus, there is a clear correlation between the ability of an E2 to bind ubiquitin at a site other than its active site and its ability to build polyubiquitin chains with BRCA1-BARD1.

Neither Ubc13-Mms2 nor Ube2k transfers ubiquitin directly to BRCA1; rather, they require BRCA1-ubiquitin as a substrate. We classify Ubc13-Mms2 and Ube2k as ubiquitin-specific E2s with BRCA1-BARD1, as they conjugate ubiquitin to another ubiquitin in our system. We classify UbcH6, Ube2e2, UbcM2 and Ube2w as substrate-specific E2s with BRCA1, as they transfer ubiquitin directly to BRCA1 but not to ubiquitin itself (no chains are produced). Among the BRCA1-interacting E2s, UbcH5c is unique in its ability to both transfer the first ubiquitin to BRCA1 and extend the chain. The salient features of the three E2 classes are summarized in **Figure 6**. UbcH7 did not transfer ubiquitin to BRCA1 under any assay conditions tested, suggesting that it selects for a substrate that was not present in our autoubiquitination assay or may function by a different mechanism. Notably, Ubc13 shows the same properties as UbcH7 in the absence of Mms2, consistent with the notion that UbcH7 may require additional unidentified factors for activity with BRCA1-BARD1.

A question that arises from this work is how an E3 selects the E2s with which it will interact and function. Common E2 features recognized by E3s are helix 1, loop L1 and loop L2. Studies of E2-E3 interactions of c-Cbl with UbcH7 (ref. 8), CHIP with Ubc13-Uev1 (ref. 7), and CNOT4 with UbcH5b[18] indicate that loop L1 has a major role, with loop L1 residues Phe63 of UbcH7, Met64 of Ubc13, and Lys63 of UbcH5b implicated in interactions with c-Cbl, CHIP, and CNOT4, respectively. The E2 features recognized by BRCA1 seem to differ somewhat. For example, mutation of Lys63 in UbcH5b to aspartate abrogates the interaction with CNOT4 (ref. 19). The analogous position is not conserved among the BRCA1-interacting set of E2s, as Ube2w has aspartate at this position, suggesting it is not a determinant of E2 binding to BRCA1. Consistent with this, loop L1 residues are not highly perturbed upon binding to BRCA1-BARD1 in NMR mapping studies, whereas loop L2 shows large spectral perturbations (**Supplementary Fig. 4**). Among the loop L2 residues that show large chemical shifts, only Ala96 is strictly conserved among interacting E2s and poorly conserved among the E2s that do not interact with BRCA1. The other structurally characterized E3s also use the residue corresponding to UbcH5c Ala96 when interacting with an E2. The ability of c-Cbl, CHIP and CNOT4 to discriminate elements of loop L1 may allow them to select a subset of the BRCA1-interacing E2s. For example, the preference of CNOT4 for a lysine at the UbcH5 Lys63 position suggests that other potential CNOT4-interacting E2s include UbcH6, Ube2e2, UbcM2, and UbcH7, but not Ube2w.

How BRCA1 chooses among its interacting E2s for ubiquitination of specific substrates is not yet known. Among the set of human E2s that interact with BRCA1-BARD1, five[6,20–22] are known to be nuclear and therefore potentially available to act with nuclear BRCA1-BARD1. Our results indicate that their similar intrinsic affinities allow BRCA1-interacting E2s to generate products in the presence of each other: the simultaneous presence of Ubc13-Mms2 does not adversely affect the ability of Ube2w to monoubiquitinate BRCA1, and vice versa. Similarly, Ubc13-Mms2 is able to recognize monoubiquitinated BRCA1 generated by UbcM2 or UbcH5c-S22R when these E2s are present at equimolar concentrations (data not shown). Thus, although each E2 binds the same surface of BRCA1, the transient nature of the interaction allows both E2s to access the BRCA1-RING to carry out their respective functions. Together, these observations suggest that regulation of a specific E2–BRCA1-BARD1 activity must be accomplished by regulation of the abundance of an E2 protein or by other yet-to-be-determined molecular components.

The ability of BRCA1 to synthesize different types of ubiquitination products depending on the E2 present implies that it can target different substrates for different fates. For example, BRCA1 can synthesize Lys6-linked polyubiquitin with UbcH5. Although the functional consequence of Lys6-linked polyubiquitin chains is unknown, UbcH5 has been found associated with BRCA1-BARD1 complexes in response to DNA damage[23], suggesting that this E2-E3 pairing is physiologically important. Several investigations into the function of BRCA1-BARD1 strongly implicate the complex in multiple DNA damage repair pathways. Notably, BRCA1 seems to have an important yet undefined role in the process of DNA double-strand break repair by homologous recombination[24]. In this context, our discovery that BRCA1 both binds to and is active with the E2 Ubc13-Mms2 is particularly noteworthy. A recent investigation of Ubc13 function in higher eukaryotes has suggested a role for Ubc13 in double-strand break repair by homologous recombination[25]. HeLa cells depleted of Ubc13 by short interfering RNA are deficient in their ability to promote homologous recombination–mediated double-strand break repair, a defect similar to that seen in cells lacking BRCA1. Notably, the ionizing radiation–induced ubiquitination activity in BRCA1 immunoprecipitates is severely reduced in Ubc13-depleted cells. The authors of that study noted that the simplest interpretation of their observations is that BRCA1 cooperates with Ubc13 to promote ubiquitination at sites of DNA damage. Their attempts to detect a Ubc13-BRCA1 interaction were unsuccessful, but our studies now provide direct evidence that BRCA1 and

Ubc13-Mms2 can both physically and functionally interact to promote Lys63-linked polyubiquitin chains on a monoubiquitinated substrate. In the context of our discovery and previous results, we propose that Ubc13 functionally interacts with BRCA1 in response to DNA double-strand breaks, leading to ubiquitination events necessary for efficient repair of DNA double-strand breaks by homologous recombination.

BRCA1 is also implicated in transcription-coupled repair of DNA damage, in which a fraction of the RNA polymerase II large subunit is ubiquitinated and degraded by the proteasome. BRCA1 depletion prevents proteasome-mediated degradation of RNA polymerase II[26], and BRCA1 can ubiquitinate the large subunit of RNA polymerase II *in vitro*[26,27]. Our finding that BRCA1 can promote Lys48-linked polyubiquitin chains with Ube2k is consistent with its ability to ubiquitinate RNA polymerase II and promote its degradation.

Binding to both a substrate and an E2 is a necessary function for all E3 ligase complexes. Standard models for the ubiquitin transfer cascade suggest that all E2s perform the same general function by simply mediating transfer of activated ubiquitin from the E1 and concluding with E3-directed ubiquitin attachment to putative substrates. With over 30 E2s in the human genome, we felt that E2s may serve specialized and distinct functions and set out to identify all the E2s that can interact with BRCA1-BARD1. We discovered ten such E2s, all of which can interact with the BRCA1 RING and six of which were previously unknown interaction partners. We did not expect such a large number of E2s to function with a single E3, as prevailing models assume a high degree of specificity in the E2-E3 interaction. The existence of multiple E2s that interact with an E3 may allow specificity in the ubiquitination of various substrates, the topology of the ubiquitin chains and therefore the fates of those substrates. It will be important to determine which E2s have the ability to transfer ubiquitin to a given substrate, making it prudent to include all BRCA1-interacting E2s in assays of potential substrates of BRCA1-BARD1. Ultimately, the identification of all possible E2s that can interact with a given E3 will lead to a more complete understanding of substrate ubiquitination and its consequences.

METHODS

Bacterial protein expression and purification. To generate N-terminal His_6-tagged proteins, the primers 5′-TAATGCACCATCATCATCATCA-3′ and 5′-ATACTACTACTACTACCACGTA-3′ were annealed and ligated into pET24a+ (Novagen) using the NdeI restriction site, generating the plasmid pET24H. To create a DNA construct encoding BD126FBC109, the following primers were used to amplify the respective human BARD1 and BRCA1 RING domains: 5′-GGCTGGCCATATGGAACCGGATGGTCGC-3′, 5′-CGCGGATCC GCCATCTTCTTTCAAATCTGACAGCT-3′, 5′-CGCGGATCCGGCGGCGATT TATCTGCTCTTCGCGTTG-3′ and 5′-CGACGCGTCGACTTATTTTGCAAAA TTATAGCTGTTTGC-3′. The resulting PCR products were digested with NdeI, BamHI and SalI (New England Biolabs) and sequentially ligated into pET24H. Genes encoding Ube2k, Ubc13 and Rad6b were cloned into pET24H. Genes encoding UbcH6, Ube2e2, Ube2w, Mms2 and Uev1a were cloned into pET24a+. The gene encoding Ubc9 was cloned into pET28a (Novagen).

All proteins used for NMR or ubiquitination assays were expressed in BL21 Star (DE3) (Invitrogen), except UbcH7, Mms2 and Uev1a, which were expressed in Rosetta (DE3) cells (Novagen). Bacteria were grown in either rich LB or minimal MOPS medium supplemented with [^{15}N]ammonium chloride (Cambridge Isotope Labs). Plasmid constructs, expression and purification of BC112-BD140 and Flag–BC304-BD302 were described previously[12]. His_6-Ube2k, His_6-Ubc13 and His_6-Rad6b were purified by Ni^{2+}-affinity chromatography, according to the manufacturer's instructions (Sigma), followed by size-exclusion chromatography. UbcH5c, UbcH6, Ube2e2, UbcM2, Ube2w, UbcH7, Ubc9, Mms2 and Uev1a were purified by cation exchange (SP Sepharose) and eluted with a 0–0.5 M NaCl gradient in 30 mM 2-(*N*-morpholino)ethanesulfonic acid and 1 mM EDTA (pH 6.0). E2-rich fractions were pooled and purified further by size-exclusion chromatography in 25 mM sodium phosphate buffer (pH 7.0) and 0.15 M NaCl. pET28N-His_6-Uba1 (E1) was purified by Ni^{2+} affinity and anion exchange (Poros HQ) and eluted with a 0–3 M NaCl gradient. Ubiquitin was purified as described[28].

Directed yeast two-hybrid screens. DNA encoding BD126FBC109 was subcloned into pGBKT7 (Clontech) using NdeI and SalI restriction sites. The I26A mutation was introduced using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing (Biochemistry Sequencing Facility, University of Washington). Genes encoding each of the E2s in **Figure 1c** were inserted into pENTR-D-Topo (Invitrogen) according to the manufacturer's instructions.

UbcH6, Ube2e2, Ubc9, Ube2m3, Ube2s, Ube2t and Ube2w were PCR-amplified from a Matchmaker human ovary complementary DNA library (Clontech). Plasmids encoding Ube2g1, Ube2u, Ube2z, Ube2v2, Ube2v3, Ube2q2, Ft1 and TSG101 were purchased from Open Biosystems. Plasmids encoding Rad6b, UbcH10, Ube2k, UbcH5b, UbcH12, Ube2h, UbcH8, Ubc13, Cdc34, UbcM2, Uev1a and Mms2 were gifts (see Acknowledgments). Using the Gateway vector conversion system (Invitrogen), the gateway cassette was inserted into pACT2 (Clontech). Recombinational cloning was performed with the LR Clonase kit (Invitrogen), resulting in a pACT2 vector encoding each E2.

Respective bait (pGBKT7) and prey (pACT2) plasmids were cotransformed into the yeast strain AH109 (Clontech). Positive transformants were selected on minimal SD –Leu –Trp medium (Clontech). A single colony for each bait-and-prey combination was suspended in 100 µl of sterile water in a 96-well plate. Using a replica plater (Sigma), we spotted yeast cells from the single colony onto selective medium (SD –His –Leu –Trp with 0, 1, 2.5, 5 or 10 mM 3-amino-1,2,4-triazole (3AT; Sigma)) or nonselective control medium (SD –Leu –Trp). Yeast were incubated at 30 °C for 7 d and then photographed.

NMR spectroscopy. Titrations of uniformly ^{15}N-labeled BRCA1-BARD1 (BC112-BD140) heterodimer (initial concentration, 0.3 mM) with each unlabeled E2 were performed at 35 °C in 25 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl and 10% D_2O. Sequential additions of 0, 0.125, 0.25, 0.5 or 1 equivalent of E2 to BRCA1-BARD1 were made. ^{1}H,^{15}N-TROSY spectra were recorded on a Bruker DMX 500 MHz spectrometer and processed and analyzed using NMRPipe[29] and NMRView[30].

Autoubiquitination activity assays. Reaction mixtures (150 µl) for BRCA1-directed ubiquitination assays contained 1.6 µM Flag–BC304-BD302 heterodimer, 1.2 µM specified E2, 20 µM ubiquitin, and 0.5 µM wheat Uba1. All reactions with Ube2k were assayed with 0.2 µM human E1 (BioMol). Reactions were initiated by adding 5 mM ATP and 10 mM $MgCl_2$. Samples were collected at 0, 30, 60 and 120 min after addition of ATP. Reaction products were resolved on a NuPAGE 4%–12% Bis-Tris gradient gel (Invitrogen) and transferred onto polyvinylidene fluoride membranes (Bio-Rad). The membranes were probed with mouse antibody to Flag (Sigma) followed by goat anti-mouse secondary antibody conjugated to Alexa Fluor 680 (Molecular Probes). Blotted proteins were detected using an Odyssey infrared imaging system (Licor).

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

D.E.C., P.S.B. and R.E.K. designed and analyzed the experiments. D.E.C. conducted all experiments except those in **Supplementary Figures 4** and **6**, which were conducted by P.S.B. The paper was written by D.E.C. and R.E.K. with editorial assistance from P.S.B.

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