

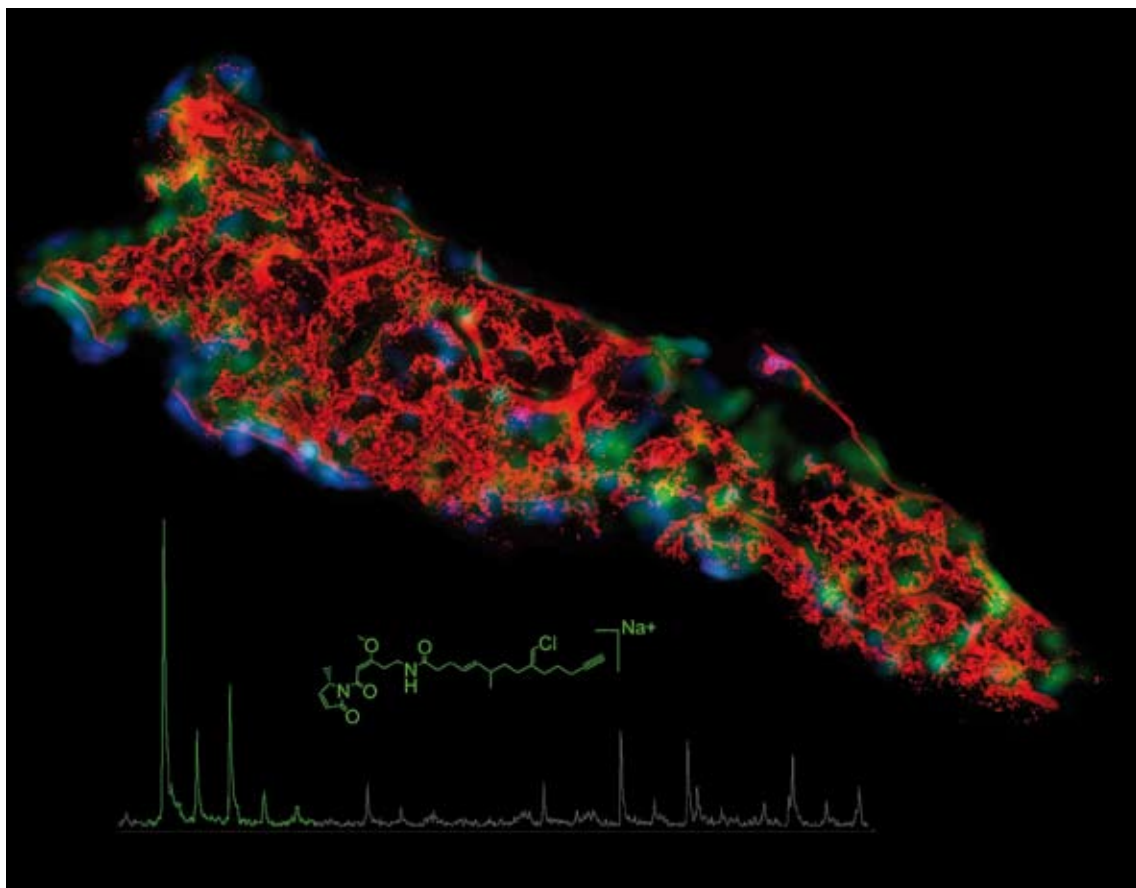
# Molecular BioSystems

This article was published as part of the

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# Re-engineering a split-GFP reassembly screen to examine RING-domain interactions between BARD1 and BRCA1 mutants observed in cancer patients†

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Received 14th February 2008, Accepted 26th March 2008

First published as an Advance Article on the web 15th April 2008

DOI: 10.1039/b802481b

Identification of protein–protein interactions is critical for understanding protein function and regulation. Split protein reassembly is an *in vivo* probe of protein interactions that circumvents some of the problems with yeast 2-hybrid (indirect interactions, false positives) and co-immunoprecipitation (loss of weak and transient interactions, decompartmentalization). Split GFP reassembly, also called Bimolecular Fluorescence Complementation (BiFC), is especially attractive because the GFP chromophore forms spontaneously on protein folding in virtually every cell type tested. However, cellular fluorescence evolves slowly in bacteria and fails to evolve at all for some interactions. We aimed to use split-GFP reassembly to examine the determinants of association for a heterodimeric four-helix bundle, and we chose the N-terminal RING domains of BARD1 and the tumor suppressor BRCA1 as our test system. The wild-type interaction failed to give fluorescence with the split sg100 GFP variant. We found that split folding-reporter GFP (a hybrid of EGFP and GFPuv) evolves fluorescence much faster (overnight) with associating peptides and also evolves fluorescence for the BRCA1/BARD1 wild-type pair. Six cancer-associated BRCA1 interface mutants were examined with the system, and only two resulted in a significant reduction in complex reassembly. These results are generally in accord with Y2H studies, but the differences highlight the utility of complementary approaches. The split frGFP system may also be generally useful for other proteins and cell types, as the split-Venus system has proven to be in mammalian cells.

## Introduction

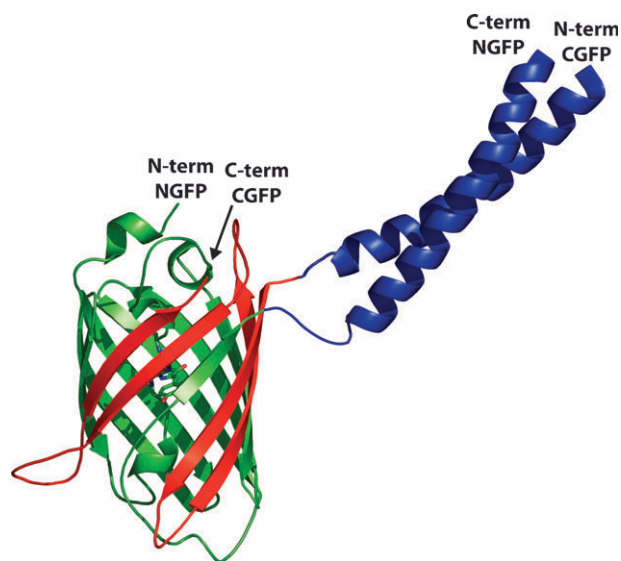
Protein–protein interactions are involved in every level of cellular function, from signal transduction to gene expression and regulation. To understand protein function, identifying interacting partners is of prime importance. A number of methods are available to study protein–protein interactions *in vitro* and *in vivo*. Co-immunoprecipitation and TAP-tag, protein arrays, mass spectrometry, yeast two-hybrid analysis, and split protein complementation assays are among the most prevalent today.<sup>1,2</sup> The split protein complementation approach has become a valuable tool to study protein–protein interactions in a range of cells. In this approach, a protein is split into two fragments which are then fused to potential interacting partners. If the fused proteins interact, the split fragments associate and/or fold, and the function of the split protein is restored. Dihydrofolate reductase (DHFR),<sup>3</sup>  $\beta$ -lactamase,<sup>4</sup>  $\beta$ -galactosidase,<sup>5</sup> and green fluorescent protein (GFP)<sup>6</sup> have been used successfully for this purpose.

Ghosh *et al.* first reported the split GFP fluorescence complementation method in 2000.<sup>6</sup> In their system, GFP variant sg100 was dissected in a loop between residues 157 and 158. When expressed *in trans* in bacteria, the fragments do not associate to give reassembled GFP. When the fragments were fused with strongly-interacting leucine zipper peptides and coexpressed, the GFP reassembled and green fluorescence was observed (Fig. 1). In 2005, Magliery *et al.* engineered two compatible plasmid vectors for expression of the fragment fusions that can be co-maintained in *E. coli*.<sup>7–9</sup> The two plasmids (pET11a-Z-NGFP and pMRBAD-Z-CGFP) have different origins of replication (ColE1 and p15A, respectively), antibiotic resistances (Amp<sup>R</sup> and Kan<sup>R</sup>) and inducible promoters (T7 and *araBAD*). Upon co-transformation and induction with IPTG and arabinose for 16 h at 37 °C and 24–48 h at room temperature, or overnight at 30 °C and three days at room temperature, cellular fluorescence was observed. Using a library of antiparallel leucine zippers, the method was shown to be very sensitive, detecting interactions as weak as 1 mM in  $K_D$  due to the irreversibility of the GFP reassembly. However, some known protein interactions cannot be trapped using this system (such as barnase and barstar; C. G. Wilson, T. J. Magliery and L. Regan, unpublished work), and the slow evolution of cellular fluorescence is problematic.

Kerppola and colleagues have also applied this method to a variety of proteins in mammalian cells, using EGFP and several spectral variants.<sup>10,11</sup> (They call the method

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† This article is part of a *Molecular BioSystems* 'Emerging Investigators' issue highlighting the work of outstanding young scientists at the chemical- and systems-biology interfaces.

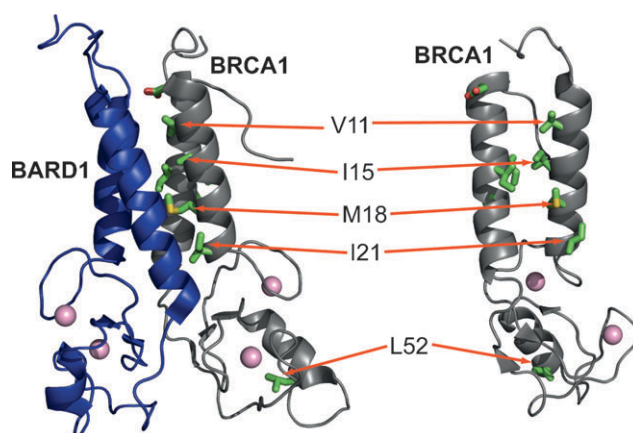


**Fig. 1** Schematic of split-GFP fragment reassembly by leucine zippers.<sup>8</sup> NGFP (residues 1–157) is green, CGFP (158–238) is green, and the zipper peptides are blue.

Bimolecular Fluorescence Complementation, or BiFC). It has also been applied in plants and *C. elegans*.<sup>12–15</sup>

We wished to use this system to examine the association of heterodimeric four-helix bundles. As a test case, we chose the RING domains of the tumor suppressor BRCA1 and its binding partner BARD1, since they are known to associate in bacteria and their binding has been explored by yeast 2-hybrid (Y2H) analysis. Mutations of BRCA1 are associated with familial breast and ovarian cancer, and the protein is involved in various cellular functions such as double-stranded DNA break repair,<sup>16</sup> cell cycle regulation, chromatin remodeling, transcriptional regulation<sup>17–19</sup> and protein ubiquitylation.<sup>20,21</sup> BRCA1 (1863 aa) principally interacts with BARD1 (777 aa) through N-terminal RING domains that together constitute an E3 ubiquitin ligase, the targets of which are largely unknown (although the estrogen receptor appears to be one<sup>22</sup>). Klevit and co-workers reported the solution NMR structure of the heterodimer complex, which is essentially composed of four Zn<sup>2+</sup> binding sites associated through a four-helix bundle interface (Fig. 2).<sup>23</sup> The truncated BRCA1/BARD1 RING/RING complex has E3 ubiquitin ligase activity *in vitro*.<sup>21,24,25</sup>

About 20% of clinically-relevant mutations of BRCA1 occur within the N-terminal 100 residues of BRCA1.<sup>18</sup> Two classes of known N-terminal cancer-associated mutations have been reported: RING motif mutations in residues involved in Zn<sup>2+</sup> binding sites, and helical packing residues involved in the RING domain binding interface.<sup>26–28</sup> Altering Zn<sup>2+</sup> ligating residues prevents BRCA1 from folding properly and impairs its function. How the interface mutations cause loss of function of BRCA1, what effects they have on interaction of BRCA1 with BARD1, and how they contribute to tumorigenesis is not fully clear yet. One curious observation is that virtually no cancer-associated BARD1 mutants are known, although some truncation isoforms have been observed.<sup>29–31</sup> However, a Y2H library approach detected BARD1 interface



**Fig. 2** Solution structure of BRCA1 and BARD1 RING domain heterodimer complex (1JM7).<sup>23</sup> (Left) BARD1 is at left in blue and BRCA1 at right in grey. The bound Zn<sup>2+</sup> ions are rendered as pink spheres. The positions of cancer-associated interface mutations, as well as the cancer-associated L52F mutation, are rendered in sticks. (Right) The BRCA1 RING domain is rendered alone for clarity. Indicated mutations are examined in this study. Rendered with PyMOL (Delano Scientific).

mutants that reduce association.<sup>32</sup> On the other hand, none of the cancer-associated interface mutations of BRCA1 detectably reduce association by the same Y2H screen, although some of them reduce the association with an E2 ubiquitin ligase, and some Zn<sup>2+</sup> binding site mutations do appear to abrogate complex formation.<sup>33</sup> It is not entirely clear how distal mutations at the interface ablate E2 binding but do not perturb complex formation, although NMR shifts are seen into the helical subdomain upon E2 binding (*e.g.*, K20).<sup>26</sup> Mutations at Zn<sup>2+</sup>-ligating residues (C39A, H41A, C61G, C61A and C64A) result in a stable complex *in vitro*,<sup>28</sup> although there is little detectable complex *in vivo* by immunoprecipitation for C61G or C64G.<sup>26</sup> Using yeast 2-hybrid, C39R, H41R and C64G appear to bind, but C61G does not.<sup>33</sup>

We attempted to observe split-GFP reassembly driven by the BRCA1 and BARD1 RING domains in *E. coli* using the pET11a/pMRBAD plasmid system with the original sg100 GFP variant. Regardless of which protein was fused to which GFP fragment, no fluorescence was observed on plates even after growing cells overnight at 30 °C and four days at room temperature. We therefore set out to optimize the split GFP system for more reliable, robust, faster fluorescence complementation, at least with BRCA1 and BARD1. In 1999, Waldo *et al.* reported an engineered GFP variant known as folding reporter GFP (frGFP) for use in a screen for soluble proteins.<sup>34</sup> The frGFP is a hybrid of EGFP<sup>35</sup> (F64L S65T) and the “Cycle 3” mutations (F99S M153T V163A) of GFPuv reported by Stemmer and co-workers.<sup>36</sup> We engineered the same variant from EGFP and GFPuv and used it to make a split frGFP system using the pET11a/pMRBAD plasmids. In this study, we report that the split frGFP system results in a very robust and bright fluorescence complementation within 24 h with the leucine zipper peptides. Moreover, we observed fluorescence for the pET11a-BARD1-NfrGFP/pMRBAD-BRCA1-CfrGFP combination. Several BRCA1 RING

domain mutants were assayed with the screen, and some but not all appear to abrogate binding.

## Results

### Split sg100 system

Magliery *et al.* developed<sup>8,9</sup> an improved plasmid system for GFP reassembly in *E. coli*, dissecting the sg100 GFP between residues 157–158, and resulting in C-terminal fusion to the NGFP fragment and N-terminal fusion to the CGFP fragment. We replaced the leucine zipper peptides in the original constructs with N-terminal RING domains of BRCA1 (1–109) and BARD1 (26–140) in both possible orientations (*i.e.*, pET11a-BRCA1-NGFP/pMRBAD-BARD1-CGFP, pET11a-BARD1-NGFP/pMRBAD-BRCA1-CGFP). Upon co-transformation, plates were incubated at 30 °C for one day and at room temperature for three days. Green fluorescence was visible for the leucine zipper positive control after three days, but no cellular fluorescence was observed for BRCA1/BARD1 in either orientation, or for a non-cognate negative control (Fig. 3).

### Split folding-reporter GFP

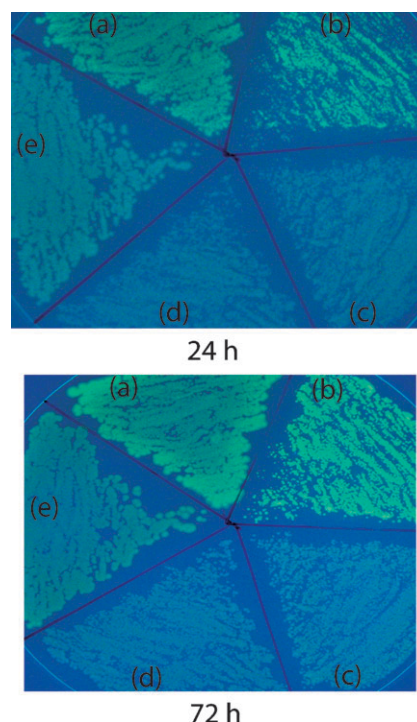
We generated the frGFP from two other variants of GFP available in our lab. The 5' fragment (for residues 1–84) from EGFP and the 3' fragment (for residues 85–238) from GFPuv were PCR amplified, and the full-length folding reporter GFP (frGFP) gene with all five mutations was assembled using overlap PCR. Cells expressing frGFP were visibly brighter than those with sg100 itself and were about as bright as with EGFP or GFPuv under a hand-held UV lamp (not shown).

Using the pET11a/pMRBAD plasmid system, the sg100 fragments were replaced with the corresponding fragments of EGFP and, separately, of frGFP, using the positive-control leucine zipper peptides as fusions. The length of time to cellular fluorescence was not improved by EGFP over sg100. However, overnight incubation (~18 h) at 30 °C was enough to observe bright fluorescence with frGFP (Fig. 4). Incubation for longer times from 24–72 h showed little or no change in the intensity of fluorescence for split frGFP with zipper peptides or with BARD1/BRCA1 (see below).

Leucine zipper peptides of pET11a-Z-NfrGFP and pMRBAD-Z-CfrGFP vectors were replaced with the N-term-



**Fig. 3** Fluorescence complementation with the split sg100 system. Cells were grown for 24 h at 30 °C followed by two days at room temperature. (a) pET11-link-NGFP/pMRBAD-link-CGFP, negative control; (b) pET11-BARD1-NGFP/pMRBAD-BRCA1-CGFP; (c) pET11-BRCA1-NGFP/pMRBAD-BARD1-CGFP; (d) pET11a-Z-NGFP/pMRBAD-Z-CGFP, positive control.

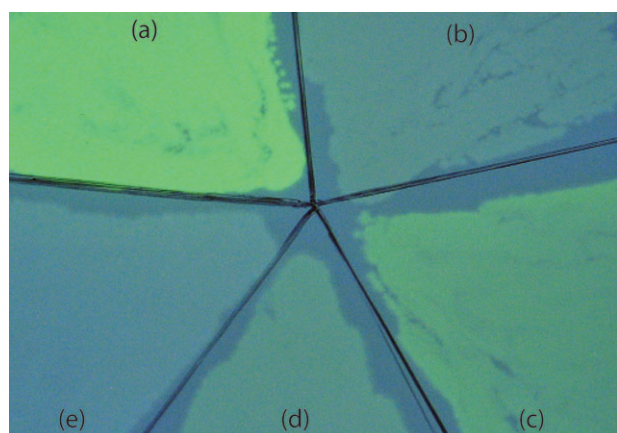


**Fig. 4** Comparative fluorescence complementation for split sg100 and split frGFP systems. Cells were incubated overnight at 30 °C (left) followed by additional 48 h at room temperature (right). (a) pET11a-BARD1-NfrGFP/pMRBAD-BRCA1-CfrGFP, new system; (b) pET11a-Z-NfrGFP/pMRBAD-Z-CfrGFP, positive control, new system; (c) pET11a-link-NGFP/pMRBAD-link-CGFP, negative control; (d) pET11a-BARD1-NGFP/pMRBAD-BRCA1-CGFP, old system; (e) pET11a-Z-NGFP/pMRBAD-Z-CGFP, positive control, old system.

inal RING domains of BRCA1 (1–109) and BARD1 (26–140) to obtain four different constructs (pET11-BRCA1-NfrGFP/pMRBAD-BARD1-CfrGFP, pET11-BARD1-NfrGFP/pMRBAD-BRCA1-CfrGFP). In contrast to the experiment with sg100, 24 h incubation at 30 °C resulted in cellular fluorescence for pET11-BARD1-NfrGFP/pMRBAD-BRCA1-CfrGFP and the zipper positive control (Fig. 4 and 5). No fluorescence was observed for negative controls (non-cognate pET11-BARD1-NfrGFP/pMRBAD-Z-CfrGFP and pET11-Z-NfrGFP/pMRBAD-BRCA1-CfrGFP) or for the other cognate fusion orientation (pET11-BRCA1-NfrGFP/pMRBAD-BARD1-CfrGFP). Fluorescence was observed only when BARD1 is fused to the C-terminus of NfrGFP and BRCA1 is fused to the N-terminus of CfrGFP, possibly due to differences in expression or to the conformational impossibility of binding or GFP reassembly in the other orientation.

### BRCA1 cancer-associated mutations

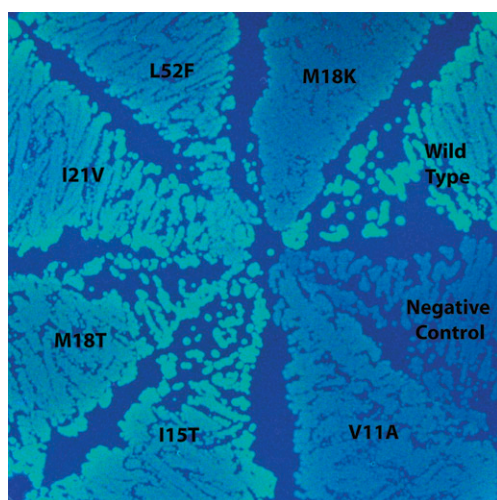
Our goal is to use the split-GFP system to study the determinants of heterodimeric four-helix bundle association. We aim to completely map the determinants of BRCA1/BARD1 interaction using a library approach. As a test, we constructed a small number of cancer-associated mutants of BRCA1, which have been examined by Y2H methods, *in vitro* methods,



**Fig. 5** Fluorescence complementation with split frGFP system depends on fusion orientation for BRCA1/BARD1. Cells were incubated overnight 18 h at 30 °C. (a) pET11a-Z-NfrGFP/pMRBAD-Z-CfrGFP, positive control; (b) pET11a-BARD1-NfrGFP/pMRBAD-Z-CfrGFP, negative control; (c) pET11a-BARD1-NfrGFP/pMRBAD-BRCA1-CfrGFP; (d) pET11a-BRCA1-NfrGFP/pMRBAD-BARD1-CfrGFP; (e) pET11-Z-NfrGFP/pMRBAD-BRCA1-CfrGFP, negative control.

or both. We selected five mutations (see Fig. 1) which are mostly buried in the helical interface (V11A, I15T, M18T, M18K and I21V) and one mutation in the RING motif away from the interface (L52F). These mutations were introduced into the BRCA1 N-terminal RING domain gene sequence of pMRBAD-BRCA1-CfrGFP vector by site directed mutagenesis using overlap PCR.

All six mutants were co-transformed with the pET11a-BARD1-NfrGFP plasmid expressing wild-type BARD1 (Fig. 6). Bright fluorescence was observed for both positive controls (pET11-Z-NfrGFP/pMRBAD-Z-CfrGFP and wild-type pET11a-BARD1-NfrGFP/pMRBAD-BRCA1-CfrGFP), and no fluorescence was observed for a non-cognate negative



**Fig. 6** Interaction of BARD1 with cancer-associated mutants of BRCA1 observed by split frGFP reassembly. Fluorescence was observed after 24 h of incubation at 30 °C. Mutants are labeled as mutations in N-terminal RING domain of BRCA1 which were fused with CfrGFP in the pMRBAD vector. NfrGFP was fused with the N-terminal RING domain of wild-type BARD1 in the pET11a vector.

control. The V11A and M18K mutations showed little or no fluorescence relative to the negative control. The I15T, I21V and M18T mutations did not markedly reduce the fluorescence, although the L52F mutation away from the interface did reduce fluorescence.

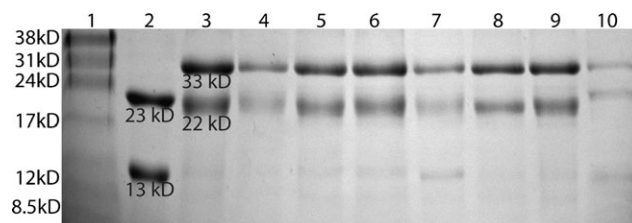
### Pull-down assay

The N-terminus of the NGFP fragments in our systems is fused to a hexahistidine tag. The re-assembled complex can be purified using IMAC methods from the soluble fraction of the cellular lysate. If the fragments do not assemble, a small amount of the His<sub>6</sub>-NGFP-analyte is found in the soluble fraction but most is found in the insoluble fraction, because the individual GFP fragments are unfolded and poorly soluble. IMAC purification of reassembled complex after several days of growth is a very sensitive test of interaction, because the reassembly of GFP is irreversible, and the soluble, refolded complex builds up over time even for weak or transient interactions.

After NiNTA purification of the cleared lysate, strong bands corresponding to the complex components are observed from the positive zipper control by SDS-PAGE (with Coomassie staining, Fig. 7). The complex dissociates on the SDS gel due to denaturation of the proteins. Virtually nothing is observed for the non-cognate negative control, although weak Ni-NTA binders from the *E. coli* proteome purify more strongly in the absence of a His<sub>6</sub>-tagged protein. For wild-type BRCA1/BARD1 and all of the mutants except V11A and M18K, strong bands corresponding to the complex components are observed. A much smaller amount of complex is purified for V11A and M18K. In whole-cell lysate, there is no obvious difference in the band patterns for any of the BRCA1 variants, suggesting that there is not a marked difference in the expression of the unassembled fragments (not shown).

### Discussion

Our lab takes combinatorial approaches to understand the folding and association of proteins. For example, we use a cell-based screen developed by Magliery and Regan to study the association of the homodimeric four-helix bundle, Rop.<sup>37</sup> We wished to use the split-GFP approach introduced by Ghosh and Regan and improved by Magliery *et al.* to examine the determinants of heterodimeric four-helix bundle association,



**Fig. 7** SDS-PAGE of purified, reassembled complexes by IMAC. Lane 1, MW markers; 2, positive control (pET11a-Z-NfrGFP/pMRBAD-Z-CfrGFP); 3, wild-type BRCA1; 4, V11A; 5, I15T; 6, M18T; 7, M18K; 8, I21V; 9, L52F; 10, negative control (pET11a-BARD1-NfrGFP/pMRBAD-Z-CfrGFP). The calculated molecular weights of the constructs are H<sub>6</sub>-NfrGFP-Z, 23 kDa; Z-CfrGFP, 13 kDa; H<sub>6</sub>-NfrGFP-BARD1, 33 kDa; BRCA1-CfrGFP, 22 kDa.

and we selected the BRCA1/BARD1 RING-domain complex as a test case. BRCA1/BARD1 has the same helical topology as the well-studied Rop protein, it has been expressed in *E. coli* as a complex and characterized structurally, and the association of the RING domains and mutants thereof have been examined by Y2H and *in vitro* methods. Moreover, mutations in the RING domains and at the helical interface between them are associated with familial breast and ovarian cancer, although the effects of those mutations are not entirely clear. Most notably, none of the interface mutants appear to abrogate complex formation by Y2H, although some do seem to perturb binding of the complex to an E2 ubiquitin ligase away from the helical interface.

### Improving the split-GFP system for BRCA1/BARD1

Split-GFP reassembly is a complementary approach to Y2H and *in vitro* methods. By its mechanism, it demands a direct interaction and is not prone to false positives caused by autoactivation of gene expression by the “bait” protein sometimes observed in Y2H. It can detect weak and transient interactions that might be lost by *in vitro* methods. It is amenable to use in virtually any type of cell, making it especially useful for library approaches in *E. coli*, where transformation efficiency is excellent. However, the time to reassembly and chromophore formation is long in the original implementation of the screen, and some known interactions have not successfully led to reassembly. In particular, we found that the BRCA1/BARD1 interaction could not be detected in bacteria using the original fragments of the sg100 variant of GFP.

We set out to improve the screen in bacteria specifically for the BRCA1/BARD1 complex, starting with the EGFP variant and improving it with mutation. We found that EGFP did not perform better than sg100, but that the three additional mutations in GFPuv dramatically improve its performance in reassembly. Reassembly can be observed for the BRCA1/BARD1 complex; for that complex and for strongly-associating leucine zippers, fluorescence is observed in a much shorter time. The improved reassembly of frGFP is likely a result of improved protein folding. Rational mutation to other known fast-folding GFP variants may yield further improvements. For example, a “superfolder” variant of frGFP was engineered by Pedelacq *et al.*,<sup>38</sup> and it shows excellent reassembly at a different dissection point even in the absence of fused, interacting proteins.<sup>39</sup> (It is unlikely that it would spontaneously associate if dissected at 157–158, since no other GFP variants self-assemble when dissected at this position.) Shyu and colleagues have recently demonstrated that split Venus, a fast-folding variant of YFP, shows improved reassembly in mammalian cells with certain proteins.<sup>40,41</sup> However, none of these proteins were specifically engineered for fast *bimolecular* folding, which we are now selecting for using directed evolution of the frGFP fragments.<sup>42</sup>

### BRCA1/BARD1 interaction

Our results indicate that three cancer-associated interface mutants of BRCA1 bind to wild-type BARD1 about as well as wild-type BRCA1. However, two interface mutants, V11A and M18K, do not promote reassembly, and one mutation in

the RING domain away from the helical interface reduces reassembly somewhat. These data together show that the interface is fairly insensitive to mutation but that underpacking and charge burial can be sufficiently deleterious to prevent binding. Our results are generally consistent with those of Solomon and co-workers,<sup>33</sup> who examined cancer-associated mutations of BRCA1 RING domain using Y2H. They found that none of the interface mutants has reduced binding, although some Zn<sup>2+</sup>-ligating positions resulted in loss of binding. They also found (by Y2H) that some of the interface mutations abrogated binding of the complex to and E2 ligase, even though that interface is far from the helical interface. Of course, it is not known if all of the cancer-associated mutations are causative, and it is conceivable that the interface mutations result in a non-productive association that perturbs the E2 binding site at a distance. It is curious that several similar interface mutations of BARD1 were engineered that abrogate binding by Y2H, but that no cancer-associated BARD1 interface mutations are known.

Our results suggest that more investigation is warranted, including split-GFP assay of other cancer-associated mutations and *in vitro* biophysical characterization (for example, by gel filtration chromatography). The apparent inconsistency between Y2H, co-IP and gel filtration chromatography for the Zn<sup>2+</sup>-ligating positions also highlights the necessity of using complementary techniques for detecting protein interactions to fully understand a given system. Y2H, split-protein reassembly and *in vitro* methods have different read-outs, operate in different concentration regimes and are not equally useful at all interaction strengths. For example, Y2H has an indirect read-out (gene expression) and the bait and prey fusions are typically overexpressed. In co-IP, proteins are typically at physiological concentrations, but weak interactions can be lost. Split protein reassembly has a very direct read-out of association and is applicable to very weak interactions. One potential limitation of *in vivo* methods (Y2H and split-GFP) is that weak expression of the fusion can be mistaken for lack of interaction, and we cannot rule that out for the V11A or M18K cases without *in vitro* characterization of binding of the purified, unfused complex. However, our lysate and IMAC purification data is consistent with the fluorescence results. The confluence of data from *in vivo* and *in vitro* methods gives the most comprehensive picture of the actual association event.

Due to the amenability of the split-GFP screen to bacteria, our improved system is especially robust for the examination of libraries of BRCA1 and BARD1 variants to comprehensively probe the determinants of interaction. It is also easily compatible with high-throughput screening for small molecules that restore complex formation in disabled mutants, such as V11A, M18K or possibly at the Zn<sup>2+</sup>-ligating sites, potentially as leads to novel therapeutics.

## Experimental

### Plasmid construction

The plasmids pET11a-Z-NGFP, pMRBAD-Z-CGFP, pET11a-link-NGFP and pMRBAD-link-CGFP have unique restriction sites for convenient subcloning of bait and prey

proteins. They are useful in any *E. coli* strain that expresses T7 polymerase.<sup>8</sup> The N-terminus of the NGFP fusion contains a hexahistidine tag for direct purification of reassembled complex. Cloning into these plasmids was carried out as described.<sup>9</sup> Plasmids encoding the N-terminal RING domains of BARD1 (amino acids 26–140) and BRCA1 (1–109) were kindly provided by Rachel Klevit (University of Washington). BRCA1 and BARD1 were PCR amplified with primers containing *Xho*I and *Bam*HI sites to subclone into pET11-Z-NGFP and *Aat*II and *Bsr*GI sites to subclone in to pMRBAD-Z-CGFP. Oligonucleotide primers were obtained from IDT (Coralville, IA).

### Construction and cloning of GFP variants

The frGFP gene was constructed from EGFP and GFPuv. N-terminal EGFP (1–84) was PCR amplified with primers AATAATAAT CATATG ATGGTGAGCA AGGGCGAGGA G and GTA CATAACCTTC GGGCATGGC G GACTTGAAGA AGTCGTGCTG C. C-terminal GFPuv (85–238 aa) was PCR amplified with primers GCCATGCC CGAAGGTATGTGA C and AATATA GGATCCTTATTTGTAGAGCTCATCCATG CC. Full length frGFP is constructed from these fragments by overlap PCR and amplified with two terminal primers with *Nde*I and *Bam*HI cloning sites to subclone into pET11a.

The frGFP was dissected as residues 1–157 and 158–238. The N-terminal fragment was PCR amplified with primers, AAT AAT AAT CATATG GCTAGT CATCAC CACCAT CAC CAC GGC GTGAGC AAGGGC GAGGAG CTG, AATAAT CTCGAG CCAGAGCCAGAGCCACC TTGTTTGTCTGCC GTGATG, and cloned into pET11a-Z-NGFP, replacing NGFP (sg100) with NfrGFP. The C-terminal fragment was PCR amplified with AATAAT GACGTC GGGTGGAAG CCGT A AGAATGGAAT CAAAGCTAAC TTC and AATATA GCGGCCGC TTA TTTGTA GAGCTCATCCATGC and cloned into pMRBAD-Z-CGFP, replacing CGFP with CfrGFP. These new plasmid vectors were used to subclone the N-terminal RING domains of BRCA1 and BARD1 in place of leucine zipper peptides. Similar protocols, as described for sg100 system, were used to construct pET11a-BRCA1-NfrGFP, pMRBAD-BARD1-CfrGFP, pET11a-BARD1-NfrGFP and pMRBAD-BRCA1-CfrGFP.

### Screening

All the screening experiments were carried out following the protocols described by Regan and co-workers.<sup>8,9</sup> Compatible pairs of plasmids (e.g., pET11a-BARD1-NfrGFP and pMRBAD-BRCA1-CfrGFP) were cotransformed into BL21(DE3) *E. coli* electrocompetent cells by electroporation. Cells were grown overnight to a saturation at 37 °C in LB supplemented with 100 µg mL<sup>-1</sup> ampicillin and 35 µg mL<sup>-1</sup> kanamycin. Five to 10 µL of 1 : 1000 dilutions of saturated culture were plated on LB agar media supplemented with 20 µM IPTG, 0.2% arabinose and antibiotics. Plates were incubated at 30 °C for 18 to 24 h. For the sg100 system, cells were grown at 30 °C for 24 h and 48–72 h at room temperature. In each case green fluorescence was observed on a

transilluminator (UVP Inc.) using long wavelength (365 nm) UV irradiation.

### BRCA1 mutants

All BRCA1 mutants were made by the overlap site directed mutagenesis method. V11A, I15T, M18T, M18K and I21V all five of these are within *Nco*I and *Bcl*II restriction sites which are 117 bases apart from each other. Two synthetic oligonucleotides with the required point mutations and with 18 bases overlap were used to generate a gene sequence between *Nco*I and *Bcl*II sites. Five sets of oligonucleotides were used for this purpose: Vafw: GCTGATT TATCTGCTCT ACGCGTTGAA GAA-GCCCAA ATGTCATTAA TGCTATGCAG, VAre: CCTTGATCAA CTCTAGACAG ATGGGACACT CTAAGATTTT CTGCATAGCA TTAATGACAT; Itfw: GCTGATT TATCTGCTCT ACGCGTTGAA GAAGTACAAA ATGTCACTAA TGCTATGCAG, IAre: CCTTGATCAA CTCTAGACAG ATGGGACACT CTAAGATTTT CTGCATAGCA TTAATGACAT; Mfww: GCTGATT TATCTGCTCT ACGCGTTGAA GAAGTACAAA ATGTCATTAA TGCTACGCAG, MAre: CCTTGATCAA CTCTAGACAG ATGGGACACT CTAAGATTTT CTGCATAGCA TTAATGACAT; Mkfw: GCTGATT TATCTGCTCT ACGCGTTGAA GAAGTACAAA ATGTCATTAA TGCTAAACAG, MAre: CCTTGATCAA CTCTAGACAG ATGGGACACT CTAAGATTTT CTGTTAGCA TTAATGACAT; Ivfw: GCTGATT TATCTGCTCT ACGCGTTGAA GAAGTACAAA ATGTCATTAA TGCTATGCAG, IvAre: CCTTGATCAA CTCTAGACAG ATGGGACACT CTAAGACTTT CTGCATAGCA TTAATGACAT (bold letter shows the mutation). Two terminal primers (AATAATTAA CCATG GCTGATT TATCTGCTCTACGC and AATAATAATCCTTGATCAACTCTAGACAG ATG) were used to amplify the extended sequence with *Nco*I and *Bcl*II sites. The plasmid pMRBAD-BRCA1-CfrGFP is used as the vector to clone in these DNA fragments.

For L52F mutation, a modified overlap method is used to insert the point mutation. Primers used for this purpose are: (5'fw) CTAACC GGTTCCCTT AGCTcG ACTCGGC ACGGTAACAAAAGTGTCTA T; (5're) CTTTCTT CTGGTTGAAT AGTTTCAGCA TG; (3'fw) CATGCTG AAACATCA ACCAGAAGAA AG; (3're) CATAGT CACACG TACGAC GCGAGA GC AGAATTCTTATGTACA TTATTGTAGAGCTC. Primers GATTGGCCAAGGAATC-GAGCTGAGCCG and GTATCAGTGTGCATGCTGCGCTCTCG were used for final amplification of the whole sequence between *Mlu*I and *Bsr*GI restriction enzyme sites. All inserted mutations were confirmed by DNA sequencing from Genewiz, Inc (South Plainfield, NJ).

### Affinity purification of fusion proteins and interacting partners

BL21(DE3) cells containing compatible plasmids were grown overnight to saturated culture. Two mL of LB both containing kanamycin and ampicillin was inoculated with 40 µL of saturated culture for each sample and grown at 37 °C to OD<sub>600</sub> ~0.60. Cells were diluted 1 : 1000 and 100 µL was plated on screening plates supplemented with 10 µM IPTG, 0.2% arabinose, 100 µg mL<sup>-1</sup> ampicillin and 35 µg mL<sup>-1</sup>

kanamycin. After growing for 24 h at 30 °C and 48 h at room temperature, cells were resuspended in 1X phosphate buffered saline (PBS). The OD<sub>600</sub> of 100-fold diluted cells were measured to normalize the cell densities. Cells were then harvested by centrifugation and each pellet was resuspended in 2.5 mL of lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 100 μM ZnCl<sub>2</sub>, 0.1% Tween 20, 5 mM β-mercaptoethanol, 10 mM imidazole, pH 8.0) containing 0.5 mg mL<sup>-1</sup> HEW lysozyme, DNase, RNase, PMSF, and 0.5 mM MgCl<sub>2</sub>. After sonication and centrifugation, cleared lysate was collected, mixed with 100 μL of Ni-NTA agarose (Qiagen) equilibrated with lysis buffer and left at 4 °C for 2 h with gentle shaking. The resin was washed twice with 5 volumes of wash buffer (lysis buffer containing 20 mM imidazole) and purified proteins were eluted with 300 μL of elution buffer (lysis buffer containing 250 mM imidazole).

## Acknowledgements

We are grateful to Rachel Klevit for BRCA1 and BARD1 RING domain gene-containing plasmids. This work was supported by The Ohio State University.

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