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Extending the Interaction Repertoire of FHA and BRCT Domains

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<http://dx.doi.org/10.5772/51670>

1. Introduction

All living organisms are connected by the necessity to replicate their DNA. However, the process of unwinding the parental DNA to serve as a template at replication forks is dangerous. The ssDNA generated by helicases is inherently cytotoxic; not only because it is more prone to damage, but it can also be an inappropriate target for nucleases and recombination proteins leading to loss of genetic material or gross chromosomal rearrangements [1]. Normally, the replication machinery rapidly restores this single-stranded template DNA to its more stable double-stranded form. However, replication forks are prone to stalling if they encounter obstacles that the DNA polymerase is unable to bypass, such as sites of damage or DNA sequences with complex secondary structures [2], resulting in long stretches of ssDNA remaining exposed [3]. Replication stress, therefore, represents an important mechanism that erodes the genetic integrity of organisms. Not surprisingly, replication stress has been linked to aging in budding yeast, which can likely be extrapolated to higher eukaryotes as well [4]. Furthermore, inducing replication stress in normal human fibroblasts results in pathogenic changes in copy number due to duplication or deletion events [5]. Therefore, the ability of eukaryotes to detect, stabilize and resolve stalled replication forks using the replication checkpoint represents an important safeguard for genomic stability.

The replication checkpoint response relies on a cascade of kinases that either remain localized to the stalled fork or disseminate the stress signal to distal sites resulting in the suppression of late origin firing, pausing of the cell cycle, and increasing the expression of DNA repair enzymes [6]. Overall, this checkpoint involves individual proteins and protein complexes coming together to assemble intricate supramolecular complexes triggered by stalled replication forks. For simplicity's sake, this chapter will focus on the *Saccharomyces cerevisiae* system and nomenclature. However, regardless of the organism being con-

sidered, the general recurring theme is that most stress dependent protein interactions involve either BRCT or FHA domains. In fact, BRCT and FHA domains are rarely found in cytosolic proteins, but they are overrepresented in nuclear proteins involved in DNA replication, as well as the detection and response to DNA damage [7, 8]. Both domains share an ability to specifically recognize phosphorylated epitopes, although with different specificities. BRCT domains primarily recognize phospho-serine (pSer) containing epitopes, while FHA domains exclusively recognize phospho-threonine (pThr). However, phosphorylation-independent interactions have recently been described for both domains.

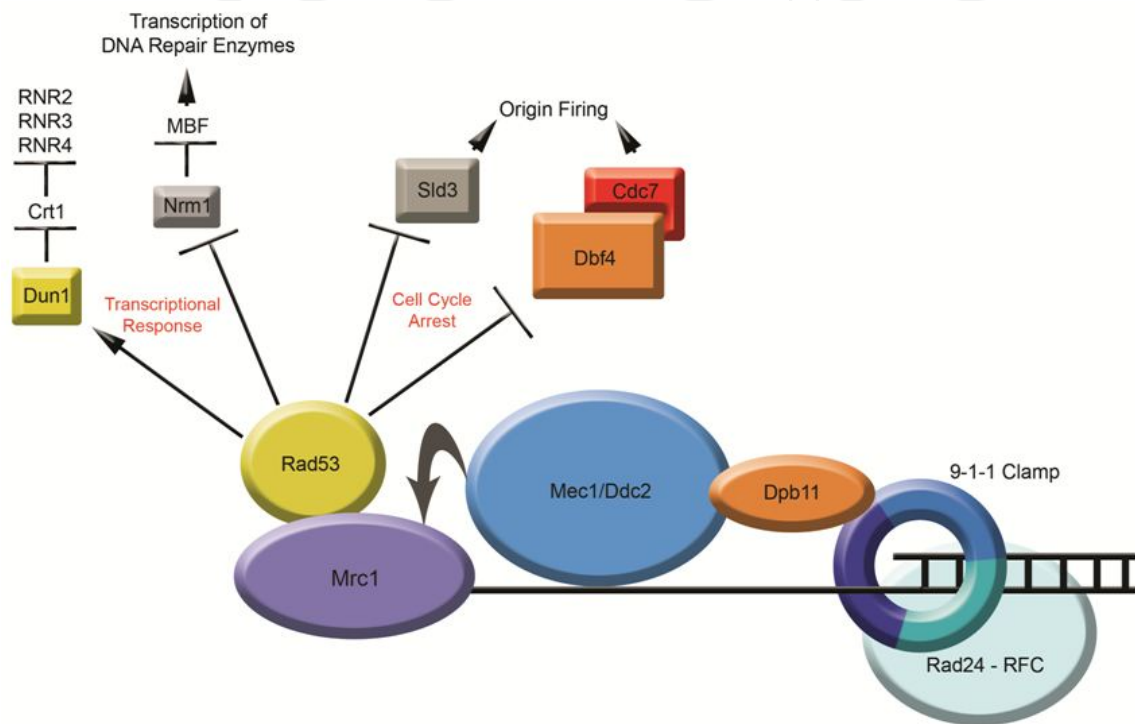


Figure 1. BRCT and FHA domains mediate protein interactions that relay the replication stress signal. A stalled replication fork is represented as a region of ssDNA recognized by a variety of checkpoint proteins colored in shades of blue. Proteins that mediate interactions involved in relaying the stress signal are colored orange if they contain BRCT domains or yellow if they contain FHA domains. The curved arrow indicates a phosphorylation event that takes place on Mrc1 to allow for Rad53 recruitment, while the straight arrows indicate interactions that take place at distal sites to the stalled fork.

Several BRCT-containing proteins are essential for the assembly of the pre-replication complex. For instance, the regulatory subunit of the Cdc7 kinase, Dbf4, contains a single BRCT domain [9, 10] and Dpb11, whose function is essential to activate pre-replication complexes, contains two BRCT pairs [8]. The Rfc1 subunit of the RFC complex, which functions in loading the sliding clamp onto ssDNA/dsDNA junctions during replication [11], also contains a BRCT domain required for DNA binding [12]. Most replication proteins containing BRCT domains are also involved in the replication checkpoint response, suggesting that BRCT domains may have fundamental roles in preserving DNA integrity. During the DNA damage response, BRCT domains are often used to recognize the site of damage, as they can bind to

DNA breaks directly [13, 14] or indirectly by recognizing phosphorylated histone H2A that marks areas of damage [15-20]. In the case of replication stress, at least two first responders are recruited to the ssDNA gap at stalled forks (Figure 1). The first is the protein kinase Mec1 and its targeting subunit Ddc2 that mediates the interaction with RPA-coated ssDNA [21]. The second is the 9-1-1 clamp that encircles the DNA at the ssDNA/dsDNA junction [22]. This requires the action of a clamp loader, which is composed of Rad24 and Rfc2-5. Interestingly, this complex is the alter ego of the clamp loader RFC, which differs only in having Rfc1 instead of Rad24 [23]. In contrast to the sliding clamp, the 9-1-1 complex is held statically by protein-protein interactions at the stalled fork [24].

After the recognition of DNA damage or stalled forks, both FHA- and BRCT-containing proteins feature prominently in bridging protein-protein interactions that disseminate the stress signal (Figure 1). For instance, Dpb11 bridges the interaction between the 9-1-1 clamp and Mec1, leading to the full activation of the Mec1 kinase [24, 25]. Mec1 has many roles at the stalled fork including facilitating the activation of the next downstream kinase in the pathway, Rad53 [26]. This is accomplished after Mec1 phosphorylates Mrc1—a protein naturally associated with the stalled replisome [27]. This creates phospho-epitopes that act as beacons for the FHA domain of Rad53. Multiple copies of Rad53 are thus recruited, increasing the local concentration of this kinase, which can then be autophosphorylated *in trans* or phosphorylated by additional kinases present at the stalled fork [28]. Hyperphosphorylation of Rad53 presents phospho-epitopes to other FHA-containing proteins such as Dun1, which leads to increased synthesis of nucleotides [28-30]. Additionally, Rad53 uses its own FHA domains to bind a variety of substrates, including the regulatory subunit of the Dbf4-dependent kinase (DDK) complex [31], consequently suppressing the firing of late origins [32-34]. Rad53 also modulates the activity of Nrm1 leading to a burst of expression of DNA repair enzymes contributing to recovery of the stalled fork [35]. BRCT domains also feature in this recovery process. For example, an important scaffolding protein involved in coordinating the recruitment of repair enzymes to the stalled fork, Rtt107, has six BRCT domains [20].

These themes are echoed in other DNA damage response pathways, where BRCT and FHA domains are known to mediate important interactions. Not surprisingly, mutations in the BRCT and FHA domains of critical damage repair proteins such as Chk2 [36], Nbs1 [37] and BRCA-1 [38, 39], lead to cancer predisposing syndromes. Why does nature rely so heavily on BRCT and FHA domains to respond to stress? The answer may seem to lie in the ability of BRCT and FHA domains to recognize phospho-epitopes, since they are an important cue during replication stress and the DNA damage response, when a number of kinases are awakened. However, their power could also lie in the plasticity of these two domains that can use multiple interaction surfaces to mediate additional interactions beyond phospho-epitope recognition. Such plasticity could, in turn, mediate the interaction network sustaining the formation of the large protein complexes required to promote genome stability in eukaryotes. Along with the well-characterized phosphate recognition ability of FHA and BRCT domains, these varied and unique alternative interaction surfaces will be considered in this chapter. Interactions occurring during the replication checkpoint will be discussed, but examples from other cellular pathways will also be included.

2. Interaction Modes of FHA Domains

An FHA domain consists of an 11-stranded β sandwich connected by loops that often contain short helical regions. The phospho-epitope binding groove is located at the apical surface of the β -sandwich, with the N- and C-termini at the opposite end of the domain. Unlike BRCT domains, that are often present in multiple copies in a single protein, FHA domains are almost always singular. Only two proteins are known to possess two FHA domains in the same polypeptide: Rad53 from *S. cerevisiae* and Rv1747 from *Mycobacterium tuberculosis* (*M. tuberculosis*). In the case of Rad53, these domains (FHA1 and FHA2) are found at opposite ends of the protein and have independent functions [40], reinforcing the idea that FHA domains function as single units. This, however, does not diminish the power of the FHA domain as a scaffold to build large protein complexes in response to stress. FHA domains can bind partners in a phosphorylation dependent or independent manner, the latter of which can utilize either the phospho-epitope binding pocket or alternative surfaces.

2.1. Phospho-epitope dependent interactions

FHA domains recognize phosphorylated proteins with a strict specificity for pThr-containing epitopes. The majority of interactions between FHA domains and their phosphorylated partners have been studied using short peptides including a central phosphorylated threonine [41-46]. These phospho-peptides bind to the apical surface of the FHA domain in an extended conformation using two pockets that determine their binding specificity. The β 3- β 4 and β 4- β 5 loops from the FHA domain primarily define the pThr-binding site, where a conserved arginine and serine (Arg70 and Ser85 in Rad53) provide critical contacts with the phosphate group. An extensive hydrogen-bond network mediated by non-conserved residues further stabilizes the interaction with the phosphorylated threonine. The second pocket recognizes the third residue C-terminal to the phospho-threonine (pThr+3), and is usually defined by the β 6- β 7 and β 10- β 11 loops of the FHA domain (Figure 2A and B).

A unique aspect of the pThr-binding pocket in FHA domains is its ability to distinguish between phospho-threonine and phospho-serine residues, a talent not shared by other phospho-epitope recognition modules. For instance, MH2 domains share a common ancestor with FHA domains and, yet, MH2 domains can also bind phospho-serine with high affinity [42, 47]. The difference is that the pThr-binding pocket in the FHA domain includes a well-defined hydrophobic nook that provides a docking site for the methyl moiety of the phosphorylated threonine (Figure 2C). While most residues in this nook are not conserved, the hydrophobic nature of this pocket is strictly maintained, thus providing a number of Van der Waals interactions that orient the phospho-threonine such that its phosphate moiety is locked in the most favorable geometry for the interaction with the domain [48]. Thus, the pThr pocket of an FHA domain can be thought of as a glove where only a phospho-threonine can fit (Figure 2C). Despite this rigid mode of binding, certain FHA domains can accommodate deviations. For instance, the FHA2 domain from Rad53 can bind pTyr-containing peptides with low affinity [47] and the FHA domain found in Dun1 has a unique preference for phosphorylated substrates including two pThr residues [49].

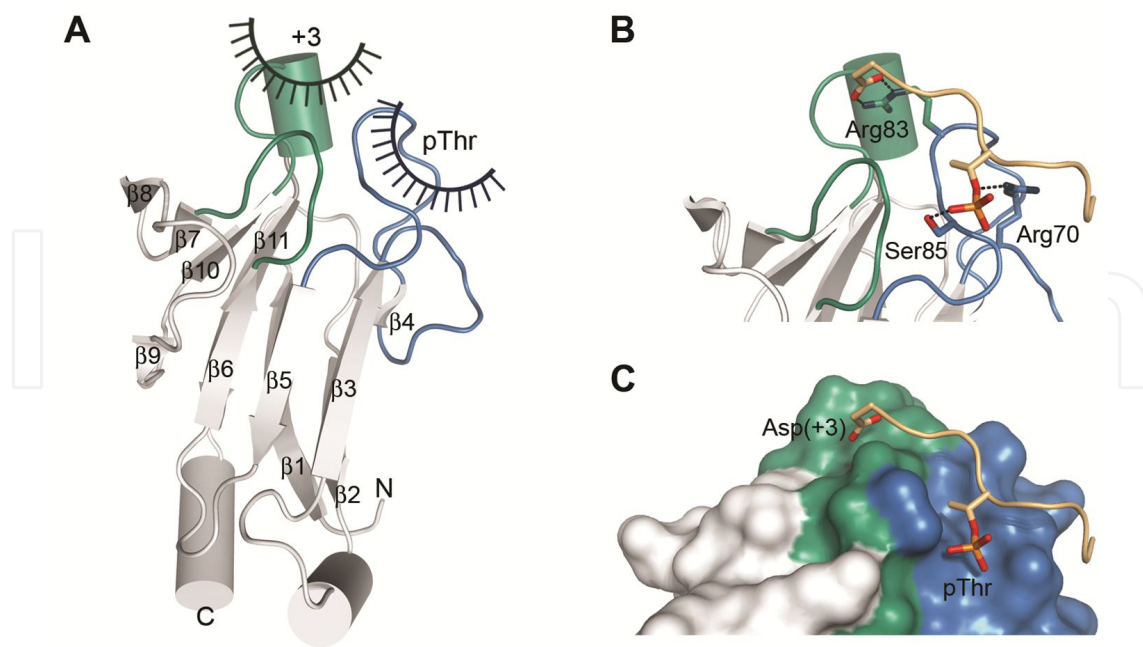


Figure 2. Phospho-peptides interact with FHA domains using at least two pockets. (A) Ribbon diagram of the FHA1 domain from Rad53 (PDB ID: 1G6G) with the loops defining the pThr (β 3- β 4 and β 4- β 5) and pThr+3 (β 6- β 7 and β 10- β 11) binding pockets highlighted in blue and green, respectively. The N- and C-terminal residues of the domain, which lie on the opposite side of the β -sandwich, are labeled for clarity. (B) Detailed view of a phospho-peptide bound to the pThr and pThr+3 pockets. The side chains of two conserved residues important for the recognition of the phosphate group (Ser85 and Arg70), as well as that of a non-conserved residue important for the specificity of the pThr+3 pocket are shown as sticks. Hydrogen bonds are indicated with dashed lines. (C) Surface representation, shown in the same orientation and color-coding as panel B, indicating the presence of defined pockets for both the phosphate and methyl moieties of the pThr.

In contrast to the pThr pocket, the residues defining the pThr+3 pocket are not conserved allowing for different domains to have different target-sequence specificities. This is called the “pThr+3 rule” wherein different FHA domains have different specificities for the pThr+3 residue. While this provides a convenient way to classify FHA domains, it should be noted that the specificity of the pThr+3 pocket is not fixed. For example, the FHA1 domain from Rad53 prefers aspartic acid as the pThr+3 residue using short peptides *in vitro*, but binds to a bulky, hydrophobic isoleucine in its partner Mdt1 *in vivo*, with the pThr+3 residue (Asp or Ile) occupying physically different pockets in each case [45]. Although FHA domain interactions have been disrupted *in vivo* by mutating the pThr residue, similar experiments with the pThr+3 residue are not available and, hence, the importance of the pThr+3 pocket is unclear. It has been proposed that this ancillary pocket may only have relevance for determining the specificity of the phospho-epitope in small peptides, whereas full-length partners may use different binding mechanisms [50]. Consequently, a detailed understanding of how FHA domains recognize phosphorylated binding partners will necessitate the structural analysis of FHA domains bound to full-length proteins rather than short phosphorylated peptides.

FHA domains often bind only weakly to pThr-containing peptides [43, 51], supporting the idea that additional contact points beyond the phospho-epitope binding site are necessary to form high avidity complexes with their partners. For example, the interaction between the FHA domain from Chk2 and the tandem BRCT repeat from BRCA-1 requires an addi-

tional hydrophobic patch on the surface of one of the FHA β -sheets. Mutating either the phosphate binding pocket or this hydrophobic patch destroys the interaction with BRCA-1 even though structurally these two sites are more than 20 Å apart [52]. Reinforcing the importance of this additional interaction surface *in vivo*, mutation of an isoleucine (Ile157Thr) within this hydrophobic patch results in the cancer predisposing Li-Fraumeni syndrome [52]. While this hydrophobic surface is not a common feature of all FHA domains—not even amongst Chk2 homologues [52], it is possible that unique patches exist within the surfaces of the β -sandwich of other FHA domains that provide auxiliary contacts to enhance binding to phosphorylated target proteins. Due to the inherent difficulty in obtaining uniformly phosphorylated proteins, solving the structures of FHA domains interacting with their full-length phosphorylated partners is a lofty goal. This is further compounded by the fact that FHA domains cannot be fooled by phospho-mimetic mutations—a trick commonly used to study phosphorylation-dependent interactions—at least when using small peptides [43]. The structure of the Ki67 FHA domain interacting with NIFK1 has recently shed light onto this problem. This interaction was recapitulated with a very long phospho-peptide (consisting of 44 amino acids), which, in addition to occupying the phospho-epitope binding site, also wraps around and extends one of the β -sheets in the Ki67 FHA domain by providing an additional β -strand [50] (Figure 3).

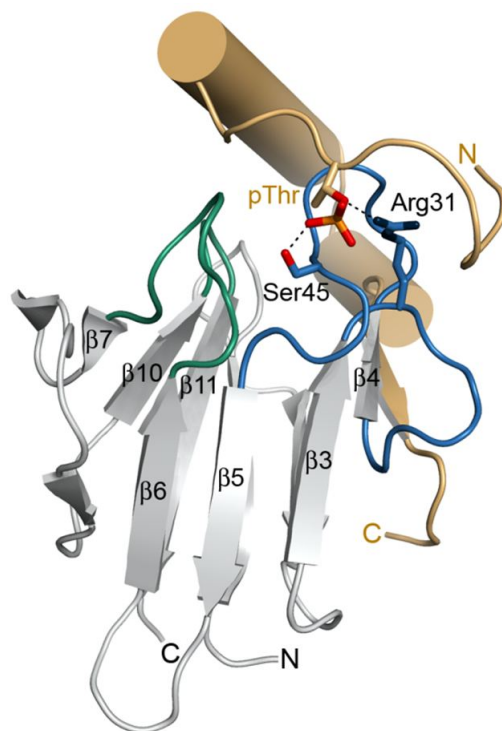


Figure 3. Phosphorylated binding partners of FHA domains can occupy extensive interaction surfaces. Ribbon diagram of the FHA domain of Ki67 bound to a phospho-peptide encompassing residues 226-269 of human NIFK (PDB ID: 2AFF). This long phospho-peptide interacts with three distinct surfaces on the FHA domain, but does not occupy the pThr+3 pocket identified in the structures of FHA domains bound to short phospho-peptides. The phospho-threonine occupies the canonical pThr-binding pocket defined by the β 3– β 4 and β 4– β 5 loops (blue), the α helix following the pThr covers a hydrophobic surface partially defined by the β 4– β 5 and β 10– β 11 loops (green), and the β -strand at the C-terminus of the peptide extends the β -sheet defined by β 7– β 10– β 11– β 1– β 2– β 4.

This long phospho-peptide does not conform to the “pThr+3” rule. Instead, binding to the FHA domain induces the formation of an α helix that nestles in a hydrophobic pocket formed by the $\beta 4$ – $\beta 5$ and $\beta 10$ – $\beta 11$ loops [50], underscoring the need for additional structural information using full-length phosphorylated binding partners.

2.2. Phospho-epitope independent interactions

Although FHA domains were initially identified as pThr binding domains, it has been predicted that they can also mediate phosphorylation-independent interactions. Members of the kinesin-3 family, a class of motor proteins that transport vesicles to the tips of axons in neural cells [53], contain an N-terminal FHA domain in addition to their motor domain and coiled-coil regions [53]. One member of this family, KIF13B, uses its FHA domain to transport PIP3-rich vesicles in order to facilitate axon development. This involves the formation of a tetrameric complex with CENPA1, which has been studied through X-ray crystallography [54]. This complex has two CENPA1 molecules and two kinesin molecules, with the FHA domain of each kinesin involved in two simultaneous interactions (Figure 4). The first is with the ArfGAP domain of one of the CENPA1 molecules, which contacts the FHA loops that normally recognize a pThr. However, this interaction is phosphorylation-independent because the FHA domain of KIF13B lacks the conserved residues for phospho-threonine recognition [54]. The second CENPA1 molecule in the tetramer uses its Pleckstrin Homology 1 (PH1) domain to contact a surface on the β -sandwich of the same KIF13B FHA domain. This situation is reminiscent of the interaction between Chk2 and BRCA-1, and suggests that auxiliary contacts mediated by the β -sandwich may enhance both phosphorylation dependent and independent interactions. Phosphorylation independent interactions are not exclusive to FHA domains that lack the pThr-recognition residues. Another member of the kinesin-3 family, KIF1A, has a canonical phospho-epitope binding site and, yet, is also suspected of using this pocket for a phosphorylation-independent interaction [55]. Similarly, the FHA domain of *S. cerevisiae* Rad53 has a canonical phosphoepitope binding site, but is presumed to interact with the BRCT domain of Dbf4 in a phosphorylation-independent manner, though the molecular determinants of this interaction are unclear [10].

Some bacterial proteins can also interact with the phospho-epitope binding site of an FHA domain in a phosphorylation-independent manner. Although bacteria primarily rely on histidine kinases and their associated regulatory responders for phosphorylation-dependent signaling, some also utilize eukaryotic-like Ser/Thr Protein Kinases (STPKs) [56]. Like eukaryotes, bacteria can use STPKs to respond to stress, but they also participate in other processes such as pathogenicity, thereby providing important drug targets [56]. The best characterized bacterium in this regard is *M. tuberculosis*, the causative agent of tuberculosis, which tops the charts in the prokaryotic kingdom with eleven STPKs [57]. Proteins that work downstream of bacterial STPKs often contain FHA domains, with *M. tuberculosis* having five such proteins [58]. One of them, Rv1827, is of special interest because it engages a phospho-epitope present in its N-terminal tail intramolecularly [59]. This effectively occludes the phospho-epitope binding site preventing the interaction of other binding partners [59]. Intriguingly, at least three different binding partners can compete with this intramolec-

ular phospho-epitope even though none of them includes a phosphorylation site [59]. Therefore, the interactions mediated by the FHA domain of *M. tuberculosis* Rv1827 reveal two recurrent features: the ability of an intramolecular interaction to negatively regulate the interactions of an FHA domain, and the use of an FHA phospho-epitope binding site to engage in phosphorylation-independent interactions. These features are also reminiscent of the interactions mediated by the FHA domains found in KIF1A [55], reinforcing the idea that FHA domains may use competing interactions to fine-tune cellular processes.

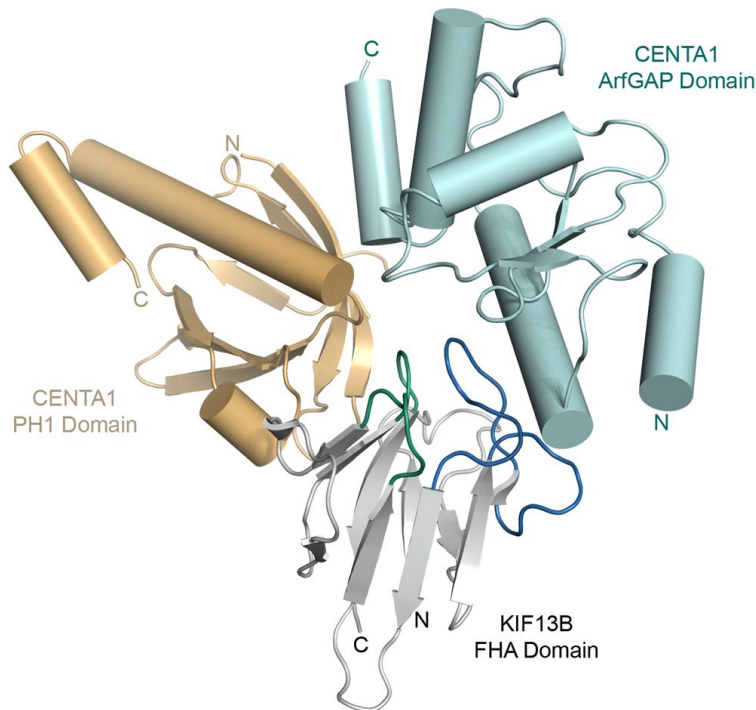


Figure 4. The FHA domain of kinesin KIF13B mediates two phosphorylation independent interactions simultaneously. Ribbon diagram of the FHA domain of KIF13B bound to CENTA1 (PDB ID: 3MDB). In the crystal structure, the FHA domain of KIF13B (white) contacts the PH1 (tan) and the ArfGAP (cyan) domains of two adjacent CENTA1 molecules simultaneously. The interaction between the FHA and PH1 domains is mediated by one of the faces of the β -sandwich, while the loops that normally define the pThr-binding pocket are involved on the recognition of the ArfGAP domain.

3. Interaction Modes of BRCT Domains

BRCT domains are named after the breast cancer associated protein 1 (BRCA-1) C-terminus because they were originally identified at this end of BRCA-1. However, the BRCT is an ancient domain that originates in prokaryotic NAD⁺ ligases where it is used to bind to DNA. Eukaryotes obtained the BRCT domain through horizontal gene transfer, and while some eukaryotic BRCT domains still retain DNA binding function, the vast majority have evolved to recognize protein partners instead [60]. BRCT domains are defined by a central four-stranded parallel β -sheet surrounded by three helices: α 1 and α 3 on one side, and α 2 on the other of the β -sheet [61]. However, additional secondary structure elements have been de-

scribed in the loop regions. There are also BRCT domains that lack elements, notably helix $\alpha 2$ [61]. Rap1 is the most extreme example of this, having only three strands in its central β -sheet and all helices packed against the same side of the sheet, leaving the other side exposed [62]. This unique structure is highly flexible and relatively unstable [62].

BRCT domains can occur both as single or multiple units, which usually consist of two BRCT domains and are referred to as tandem BRCT repeats. The structural diversity of BRCT domains is perhaps best illustrated by the human homologue of Dpb11 (human TopBP1), which contains eight BRCT domains that function as single, double or triple BRCT units [63-65]. Similarly to FHA domains, BRCT domains are overrepresented in DNA damage response proteins where they recognize phosphorylated targets generated during damage recognition and repair [7]. While the molecular intricacies of phospho-epitope recognition by tandem BRCT repeats have been extensively studied, phosphorylation-independent interactions mediated by BRCT tandems or the functions of single BRCT domains remain poorly characterized. Elegant proteolysis studies have shown that mutations in the BRCT tandem repeat of BRCA-1, with a causal link to early onset breast and ovarian cancer, destabilize the BRCT fold [39], suggesting that BRCT domains may work as protein scaffolds. These studies also revealed the hypersensitivity of BRCT domains to mutations, an effect that was attributed to its minimal size (95-100 amino acids) [66] and, consequently, the fact that every residue contributes to either maintaining the domain fold or mediating interactions with BRCT-binding partners [67].

3.1. Tandem BRCT Domains: The Two-Knob Hypothesis

The tandem BRCT repeat was formed through a gene duplication event, in which the binding pocket originally used to bind to the phosphate backbone of DNA evolved to recognize a phospho-epitope in a target protein [60]. Being an α/β fold, the BRCT domain has a topological switch point; a region along the C-terminal edge of the β -sheet whereupon a groove is formed in the connecting loops. Tandem BRCT repeats use the topological switch point—termed the P1 pocket—of their first BRCT domain to bind phospho-serine (pSer) residues in their interaction partners (Figure 5). Similar to FHA domains, this interaction involves the side chain of a conserved serine residue, but in BRCT domains the phosphate moiety of the pSer residue is further stabilized by the interaction with the side chain of a conserved lysine, as well as the backbone atoms from the glycine immediately following the conserved serine [68].

Similar to FHA domains, tandem BRCT repeats also subscribe to a “pSer+3 rule” to enhance phospho-epitope binding specificity [68]. However, the pSer+3-binding pocket—termed the P2 pocket—only forms in tandem BRCT repeats as it is defined by residues at the interface between the first and second BRCT domains. When two BRCT domains coalesce to define a tandem repeat, the central β -sheet of both domains adopt a parallel arrangement that defines an intervening hydrophobic three-helix bundle form by $\alpha 2$ from the N-terminal and $\alpha 1$ and $\alpha 3$ from the C-terminal BRCT domains (Figure 5) [69]. Helical bundles are known to facilitate molecular interactions [70] and, in the case of tandem BRCT domains, it allows for the recognition of a bulky hydrophobic residue at the pSer+3 position [68]. Indeed, the high specificity of tandem BRCT repeats for their phospho-epitope

pes is primarily due to the presence of the P2 pocket that imposes the need for a second knob in the phospho-epitope, thus precluding non-specific binding.

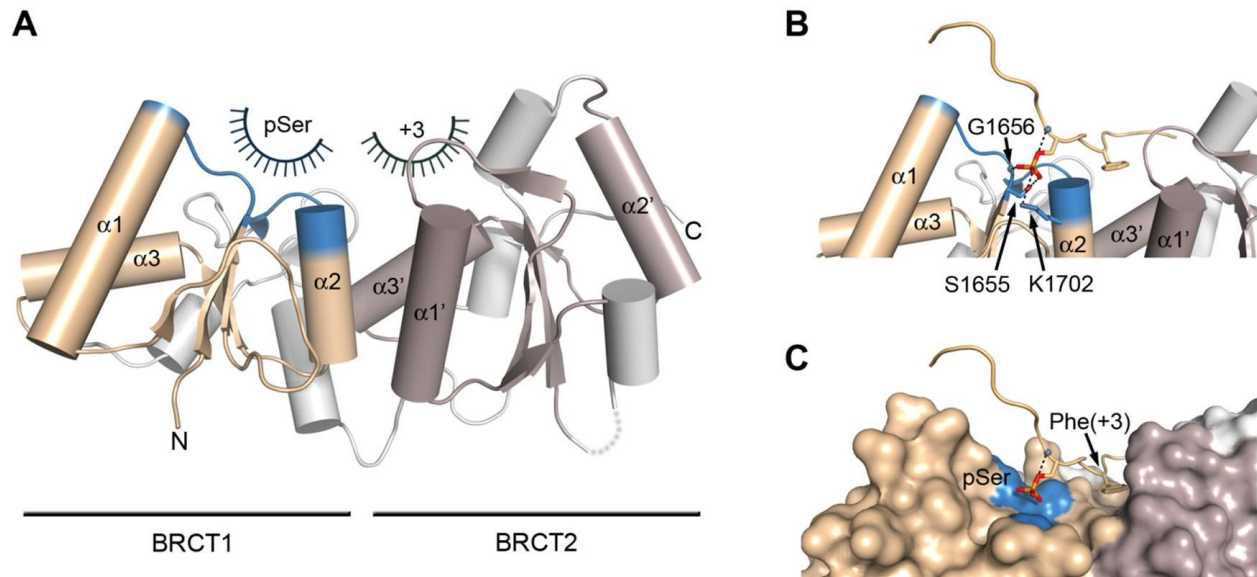


Figure 5. Phospho-epitope recognition by tandem BRCT repeats. (A) Ribbon diagram of the tandem BRCT domain from BRCA-1 (PDB ID: 1T2V) with secondary structure elements defining the BRCT fold shown in tan (BRCT1) and brown (BRCT2), and those not common to the BRCT fold shown in white. The pSer-binding site is located entirely within BRCT1, whereas the pSer+3 pocket is located in a three-helical bundle created at the interface between the two domains. (B) The side chains of Ser1655, Lys1702, as well as the main chain nitrogen of Gly1656 stabilize the phosphate moiety of the pSer. Additionally, the phosphate group engages in hydrogen bonds with the backbone amino groups represented as blue spheres. (C) The surface representation, shown in the same orientation as in panel B, reveals that the pSer binds to a shallow pocket that could not accommodate any other phosphorylated residue, while the conserved aromatic residue at the pSer+3 position is nestled into a well-defined hydrophobic pocket.

In certain tandem BRCT repeats, the P1 pocket is found in the C-terminal rather than the N-terminal BRCT, however due to the absence of the P2 pocket it is presumed that this mode of interaction is weaker than the canonical binding mode [71]. In fact, BRCT repeats containing a P1 pocket on the C-terminal BRCT are known to mediate phosphorylation-independent protein–protein interactions [72], suggesting that the binding specificity for a phospho-epitope may not be as critical. In the structure of the tandem BRCT repeats found in 53BP1 bound to the DNA-binding domain of p53 [73, 74], the inter-domain linker is critical to mediate the interaction between 53BP1 and p53. Similarly, in the structure of the *Schizosaccharomyces pombe* Crb2 homodimer [75], the linker connecting the two BRCT domains mediates protein dimerization. Collectively, these structures underscore the fact that tandem BRCT repeats define single functional units with multiple interaction surfaces.

3.2. BRCT “Super-domains”: Expanding the two-knob model

The individual units in a tandem BRCT repeat are dependent on each other for structural stability due to the hydrophobicity of the α helices that define the intervening helix bundle [69]. Thus, a tandem BRCT repeat can actually be considered one single domain module, distinct from single BRCT domains. Beyond single and tandem BRCT arrangements,

a number of BRCT structures over the past decade have revealed many unexpected tertiary structures formed by the combination with other functional domains. This is not only in the number of BRCT domains involved, such as the structure of the triple BRCT repeat from the human TopBP1 [76], but also in the diversity of domains that can be ensnared by a BRCT neighbor. This includes the Fibronectin Type III domain (FN3) found at the N-terminus of a tandem BRCT domain in the *S. cerevisiae* protein Chs5 [77], or the FHA domain that does likewise in *S. pombe* Nbs1 [78, 79].

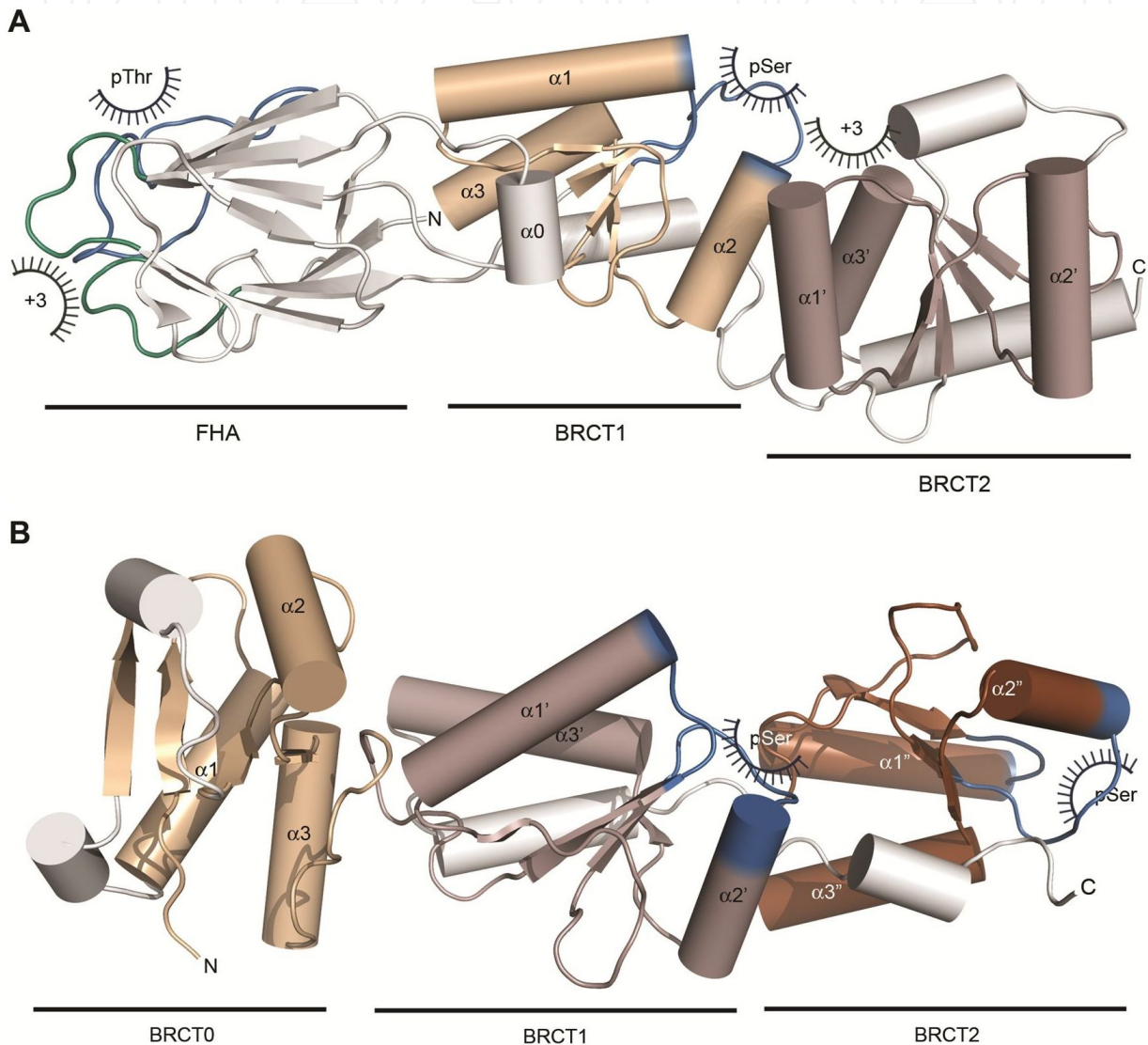


Figure 6. BRCT super-domains. (A) Ribbon diagram of the FHA–BRCT–BRCT super-domain found in Nbs1 (PDB ID: 3ION). Four knobs are present: pThr and pThr+3 pockets in the phospho-epitope binding site in the FHA domain and pSer and pSer+3 pockets in the phospho-epitope binding site defined by the tandem BRCT repeat. Helix α_0 in the FHA domain interacts with helices α_1 and α_3 in the BRCT1 domain, however the relative orientation of these helices does not resemble that of the characteristic three-helix bundle found at the interface of tandem BRCT repeats. (B) Ribbon diagram of the triple BRCT repeat found in human TopBP1 (PDB ID: 2XNH) with the structural elements defining each BRCT domain shown in different shades of brown. The pSer binding sites present in the second and third BRCT domains are highlighted in blue, while additional structural elements not common to the BRCT fold are shown in white.

The crystal structure of the FHA–BRCT–BRCT super-domain of Nbs1 emphasizes the ability of BRCT domains to build scaffolds capable of multiple interaction modes. While the two BRCT domains in Nbs1 associate to form a canonical tandem BRCT repeat with a phospho-epitope binding site, the FHA domain interacts with the hydrophobic core of the first BRCT domain leaving helices $\alpha 1$ and $\alpha 3$ exposed to the solvent (Figure 6) [78, 79]. In contrast to other BRCT domains, these two helices are amphipathic and, hence, break the theme of BRCT domains using hydrophobic three-helix bundles to build super-domains. Surprisingly, the FHA and the first BRCT domain (FHA–BRCT1) form the most stable unit of Nbs1, whereas the second BRCT (BRCT2) is quite flexible despite forming a canonical tandem repeat with the first BRCT domain [78, 79]. Given its tertiary structure, the FHA–BRCT–BRCT super-domain could bind two phospho-epitopes simultaneously, suggesting that the interactions of Nbs1 with its binding partners may be highly regulated.

The recent structure of the triple BRCT repeat in TopBP1 (BRCT0/1/2) provides another example of a BRCT super-domain deviating from the canonical three-helix bundle interface (Figure 6). In this case, neither BRCT0/1 nor BRCT1/2 associate to form canonical tandem BRCT repeats, primarily due to the unusually short inter-domain linkers that connect adjacent BRCTs [76]. Beyond connecting adjacent BRCT domains, the inter-domain linkers in some tandem BRCT repeats actively mediate protein-protein interactions [73, 74] and, in extreme cases, it is the linker rather than the BRCT domains that mediates the interaction. For example, the damage response protein XRCC4 interacts exclusively with the inter-domain linker connecting the two BRCT domains of ligase IV [80]. Collectively, these structures demonstrate that not only the BRCT repeat, but also the length and composition of the inter-domain linker joining the two domains affect the binding plasticity of BRCT repeats and their ability to form higher order structures with diverse binding specificities.

3.3. Single BRCT Domains: Is One the Loneliest Number?

The association of multiple BRCT domains with other functional domains within a single polypeptide chain is becoming a common theme found in many DNA damage response proteins. This poses the question as to whether the increased binding specificity, and hence the underlying ability to fine tune interactions during the checkpoint response, is the driving force for BRCT domains to build such complex structures. Surprisingly, the majority of eukaryotic proteins that possess BRCT domains have at least one that functions solo. The exposed α helices ($\alpha 1$, $\alpha 2$ and $\alpha 3$) in single BRCT domains are amphipathic, with a hydrophobic face interacting with the central β -sheet and a polar face exposed to the solvent, unlike their tandem counterparts where both faces are chiefly hydrophobic. This enhances the stability of single BRCT domains but does not shed light on their functions.

There is mounting evidence indicating that both single and tandem BRCT domains may require additional secondary structural elements to form functional units [10, 12, 81, 82]. Some of these structural elements are required for structural stability, such as the additional C-terminal α -helix in the PARP-1 BRCT [81], while others enhance function (Figure 7). An example of the latter comes from the largest subunit of the budding yeast clamp loader, Rfc1. Rfc1 has a single BRCT domain that is required for DNA-binding [12]. However, as an isolated unit, this do-

main is unable to recognize DNA despite having a positively charged patch positioned at the conserved P1 pocket [12]. The inclusion of an N-terminal extension recapitulates DNA binding with a K_D in the nM range. The extension encompasses an additional α helix that directly contacts DNA [12]. Rather than being a structural element integrated into the BRCT fold, however, this helix is predicted to act as an auxiliary element to enhance function [12]. Based on this model, both the BRCT domain and its auxiliary helix likely bind synergistically to the DNA leading to a robust interaction (Figure 7). Similarly, the presence of an additional N-terminal helix necessary to bind DNA is also predicted in the single BRCT domain from the translesion polymerase Rev1 [12, 82]. It is conceivable that these additional structural elements play the dual role of stabilizing the BRCT fold and enhancing its function. In fact, this is the case of *S. cerevisiae* Dbf4, the regulatory subunit of the Cdc7 kinase, where an α -helix immediately precedes the β 1 strand and its presence is important for the stability of the domain, as well as the interaction with Rad53 during the checkpoint response [10, 83].

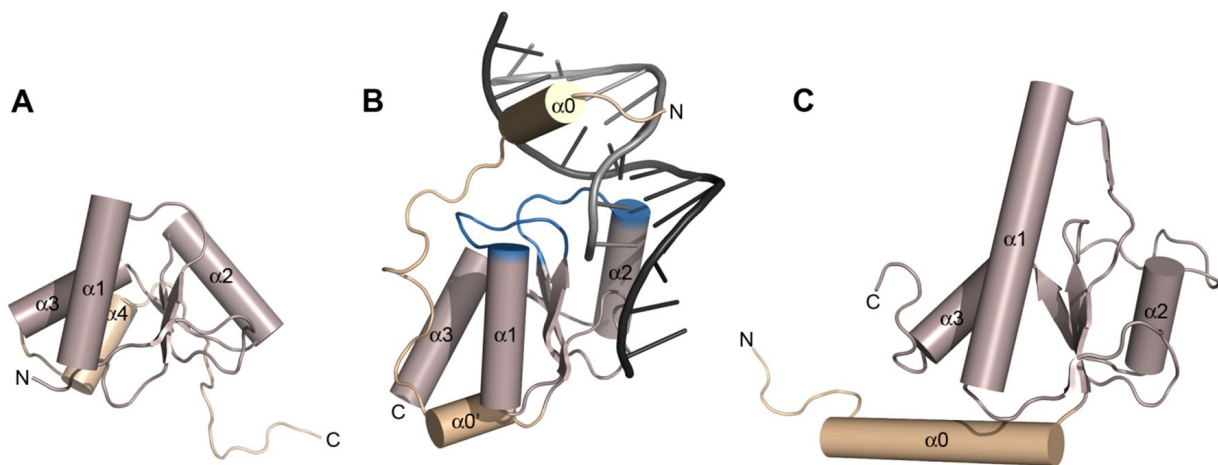


Figure 7. Single BRCT domains including additional structural elements. (A) Ribbon diagram of the BRCT domain of PARP-1 (PDB ID: 2LE0), highlighting an additional C-terminal α helix (α 4) necessary for the structural integrity of the domain. (B) Ribbon diagram of the molecular model of Rfc1 bound to DNA (PDB ID: 2K7F). Similar to Dbf4, this model predicts two additional helices at the N-terminus of the BRCT domain of Rfc1. Helix α 0 is not part of the BRCT fold, but it is essential for DNA binding by Rfc1. Conversely, the predicted helix α 0' does not appear to be involved in DNA binding but its relative orientation and interaction with the BRCT core resembles that of helix α 0 in Dbf4. (C) Ribbon diagram of the HBRCT domain of Dbf4 (PDB ID: 3QBZ), highlighting an additional N-terminal α helix (α 0) necessary to mediate the interaction between this domain of Dbf4 and the FHA1 domain of Rad53. Helix α 0 is an integral part of the fold as it anchors itself to the BRCT core through hydrophobic interactions, thereby introducing the idea of BRCT domains being building blocks that can be decorated to form super-structures with broader binding specificities.

4. Dbf4/Rad53: A Case Study for phosphorylation-independent BRCT and FHA Interactions

The DDK complex, formed by the association of the Cdc7 kinase and its regulatory subunit Dbf4, is required for initiating DNA replication and, hence, it is essential for the life of all eukaryotes [84]. Like many other replication proteins, DDK is also involved in the replica-

tion stress checkpoint [31-34, 85, 86]. The ability of Dbf4 to crossover into the stress response pathway is partly due to a conserved motif at the N-terminus of the protein (motif N). Based on sequence alignments, it was a matter of debate whether motif N was a bona fide BRCT domain [9, 87], however concerns were laid to rest when the crystal structure of the N-terminal region of *S. cerevisiae* Dbf4 was determined [10]. The structure revealed that this region of Dbf4 folds as a modified BRCT domain that requires an additional N-terminal α -helix to form a stable unit (Figure 7). A fragment of Dbf4 consisting of the canonical BRCT domain but missing the additional helix did not support binding to the FHA1 domain of the checkpoint effector kinase, Rad53 [9, 10, 31, 88]. It was proposed that this additional helix (α_0) defines, at least in part, the interaction interface [10]. Due to the functional and structural relevance of the α_0 helix, this domain of Dbf4 is referred to as H-BRCT to signify the location of the additional helix. In contrast to the additional N-terminal helix identified in the BRCT domain of Rfc1 that is completely independent from the BRCT domain, helix α_0 in Dbf4 is physically latched onto the BRCT domain in the crystal structure of Dbf4 [10] (Figure 7). This is through hydrophobic residues from α_0 interacting with a hydrophobic pocket between the central β -sheet and α_1 of the BRCT domain [10]. Therefore, helix α_0 is in a sense decorating the surface of the BRCT and maintained as an integral part of the domain [10, 83]. This serves as a note of caution when studying BRCT domains in general, as the functional and the structurally stable forms of the domain may not necessarily coincide. Thus, functional BRCT units can only be reliably ascertained through empirical assays with different sized protein fragments and not through structure-guided sequence alignments.

The interaction between the H-BRCT domain of Dbf4 and the first FHA domain of Rad53 also poses an interesting paradigm during the checkpoint response because Dbf4 and Rad53 interact in a phosphorylation independent manner using domains notable for recognizing phosphorylated epitopes. This interaction was initially proposed to depend on the recognition of a phospho-epitope in Dbf4 because a point mutation in the conserved arginine (R70A) involved in phospho-threonine recognition by FHA1 effectively abolished the interaction between the two proteins in a yeast two-hybrid experiment [31]. Yeast two-hybrid experiments conducted in the past have demonstrated that phosphorylation-dependent interactions with Rad53 can indeed be detected, likely due to the activity of endogenous kinases [89]. However, all the threonine residues located within or surrounding the H-BRCT domain of Dbf4 can be mutated without abrogating the interaction with Rad53 [10]. Several possible scenarios can explain the critical role of Arg70 in mediating the interaction with Dbf4. First, Arg70 may be part of the interaction interface despite the lack of a phospho-epitope in Dbf4. This scenario would be reminiscent of *M. tuberculosis* Rv1827 that can bind several binding partners using the apical surface of the FHA domain containing the conserved arginine in a phosphorylation-independent manner [59]. Alternatively, the R70A mutation could destabilize the FHA1 fold, in which case the Dbf4-binding defect associated with this mutation would be indirect. This scenario seems unlikely, given that FHA are stable domains and that Arg70 is solvent exposed (Figure 2). Lastly, Dbf4 could have a dual interaction with Rad53, where two independent interactions would need to occur simultaneously to form a high-avidity complex. In this case, the H-BRCT domain of Dbf4 could interact with the FHA1 domain of Rad53 in a phos-

phorylation-independent manner, while FHA1 recognizes a phospho-epitope located in another region of the DDK complex. While this idea awaits validation, other dual interactions have been previously observed in the structures of other FHA domains, including that of KIF13B and Chk2 [52, 54]. This seems to suggest that simultaneous phosphorylation dependent and independent interactions may be a broader mechanism to regulate interactions mediated by FHA domains than previously anticipated.

5. Conclusion

Through the use of short phospho-peptides, the basic mechanism underlying pThr recognition by FHA domains has been elucidated. However, it is clear that full-length binding partners harboring pThr-epitopes will likely contact the FHA domain at multiple sites in addition to the pThr pocket, many of which will be unique to that particular interaction. Additionally, proteins are capable of interacting with FHA domains in a phosphorylation independent manner, using the phospho-epitope binding site as well as alternative interfaces, most often the surfaces of the β -sandwich. While phosphorylation-dependent interactions have a clear mode for turning the interaction on and off, regulation of phosphorylation-independent interactions remains unexplored. If these interactions are mediated by the phospho-epitope binding site it may simply be a matter of availability of phosphorylated binding partners that can compete with the unmodified protein. However, if the canonical phosphate binding residues are absent or the interaction takes place on an alternative surface, the mechanism for control is less clear.

BRCT domains have previously been divided in tandem repeats, which can interact with phosphorylated partners in a well-defined manner, and the enigmatic single domains. The plethora of interaction mechanisms used by single BRCT domains can seem overwhelming, but like FHA domains a common theme is now emerging. The BRCT fold may serve as a structural core upon which more complex and unique assemblies can be built. In this way, specific interaction surfaces can be created allowing for the BRCT domain to gain function. Therefore, BRCT domains can use extra secondary structural elements, either integrated into the fold as in Dbf4 or as an auxiliary element as in Rfc1, or entire domains such as in the case of Chs5 and Nbs1—or even in tandem BRCT repeats—to modulate their functions.

Functional and structural analyses of FHA and BRCT domains during the last decade have unveiled a complex repertoire of interactions mediated by these two domains. Once regarded as mere phospho-epitope binding units, we now know that they can mediate very sophisticated interactions regulated by multiple binding knobs. Further structural and functional analysis of protein complexes mediated by these two domains will delineate the common mechanisms that regulate the DNA damage response, and will extend the lessons learned from studying the replication stress pathway in yeast to a variety of stress response networks that rely on BRCT and FHA domains across all kingdoms of life.

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