A Novel Non-canonical Forkhead-associated (FHA) Domain-binding Interface Mediates the Interaction between Rad53 and Dbf4 Proteins

Background: The interaction between the checkpoint kinase Rad53 and Dbf4 is critical to suppress late origin firing and to stabilize stalled forks during replication stress.

Results: The FHA1 domain of Rad53 interacts with the BRCT domain of Dbf4 through a novel non-canonical interface.

Significance: Understanding how FHA domains interact with their binding partners is key to elucidate how they relay information along signaling pathways.

Forkhead-associated (FHA) and BRCA1 C-terminal (BRCT) domains are ubiquitous phosphoepitope recognition modules but can also mediate non-canonical interactions. The latter are rare, and only a few have been studied at a molecular level. We have identified a crucial non-canonical interaction between the N-terminal FHA1 domain of the checkpoint effector kinase Rad53 and the BRCT domain of the regulatory subunit of the Dbf4-dependent kinase that is critical to suppress late origin firing and to stabilize stalled forks during replication stress. The Rad53-Dbf4 interaction is phosphorylation-independent and involves a novel non-canonical interface on the FHA1 domain. Mutations within this surface result in hypersensitivity to genotoxic stress. Importantly, this surface is not conserved in the FHA2 domain of Rad53, suggesting that the FHA domains of Rad53 gain specificity by engaging additional interaction interfaces beyond their phosphoepitope-binding site. In general, our results point to FHA domains functioning as complex logic gates rather than mere phosphoepitope-targeting modules.

Forkhead-associated (FHA) domains are ubiquitous phosphoepitope-binding modules present in a number of proteins that play critical roles in the DNA damage and replication stress response (1). The replication checkpoint preserves the genomic integrity of the cell by stabilizing stalled forks, boosting DNA repair enzyme levels, and pausing the cell cycle (2). Rad53 (the budding yeast homolog of the tumor suppressor Chk2) is an effector kinase with integral roles in the replication checkpoint (3). In contrast to other FHA domain-containing proteins, Rad53 has two FHA domains (FHA1 and FHA2) that mediate independent interactions of Rad53 with upstream and downstream branches of the checkpoint. One of the binding partners of FHA1 is Dbf4 (4), the regulatory subunit of the initiator kinase Cdc7 (5, 6). The association of Rad53 and Dbf4 mediates the Rad53-dependent phosphorylation of Dbf4 and the consequent inhibition of Cdc7, thus preventing late origin firing (7–10). This interaction involves the N-terminal region of Dbf4 that folds as a modified BRCA1 C-terminal (BRCT) domain (4, 11–13).

BRCT domains are also commonly found in DNA damage and replication stress response proteins, where they occur as single or multiple repeats (14). Tandem BRCT repeats are phosphoepitope-binding modules with the phosphoepitope-binding pocket residing at the interface between the two domains (15). Conversely, single BRCT domains have diverse functions, and their mechanisms cannot be extrapolated from one protein to another due to low sequence identity. Furthermore, their specific functions remain elusive because many BRCT domains, like the one found in Dbf4, require additional structural elements to become functional (12, 16, 17). The BRCT domain of Dbf4 is immediately preceded by an α-helix that stabilizes the domain, and thus, it has been previously referred to as HBRCT (12, 18). The HBRCT domain of Dbf4 is necessary and sufficient for the interaction with Rad53, yet it does not contain any threonine residue that could serve as the phosphorylation site recognized by Rad53 (7, 9, 12). A phospho- phosphorylated peptide derived from the N-terminal sequence of the HBRCT domain of Dbf4 including the canonical pTXXE FHA-binding motif can interact weakly with the FHA1 domain of Rad53 (19); however, mutation of this threonine in full-
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length Dbf4 does not disrupt the interaction with the FHA1 domain of Rad53 (12, 19).

Phosphothreonine (pThr) binding to FHA domains has been extensively studied using short phosphopeptides (20). However, there is some evidence indicating that interactions with full-length partners may involve additional interfaces. The FHA domain of *Saccharomyces cerevisiae* Dun1 requires the presence of a second pThr for specific binding (21), prompting the comparison of FHA domains to logic gates (22), whereas that of *Mycobacterium tuberculosis* Rv1827 uses its pThr-binding pocket to mediate both phosphorylation-dependent and phosphorylation-independent interactions (23). Dbf4 interacts preferentially with the FHA1 domain of Rad53 (4), whereas Rad9 interacts with its FHA2 domain (24), indicating that defined features in each FHA domain determine their binding specificities. In the present study, we exploited this characteristic of Rad53 to analyze how FHA domains increase binding specificity for their targets. We have uncovered a novel interface defined by one of the lateral surfaces of the FHA1 domain and characterized its interaction with the HBRCT domain of Dbf4. We found that the FHA1 domain of Rad53 can bind simultaneously to the HBRCT domain of Dbf4 and a pThr-containing phosphopeptide, suggesting that a bipartite interaction may modulate the interaction between Rad53 and Dbf4 in vivo. This dual binding mechanism and its functional implications for the replication checkpoint are discussed.

EXPERIMENTAL PROCEDURES

**Cloning, Expression, and Purification**—A codon-optimized version of the HBRCT domain of Dbf4 (amino acids 105–220) was synthesized by GeneArt® (Invitrogen) and subcloned into an expression vector including a His<sub>6</sub>-SUMO tag. HBRCT-W116D/M120A was also subcloned in the pSUMO vector. Plasmids encoding wild-type and His<sub>6</sub>-SUMO-tagged HBRCT variants were transformed in Rosetta<sup>TM</sup> cells (Novagen). Cultures were grown to *A<sub>600</sub>* = 0.7, induced by addition of 1 mM isopropyl β-d-thiogalactopyranoside, and incubated overnight at 16 °C with orbital agitation.

Two variants of the FHA1 domain of Rad53, including amino acids 14–164 and 4–165, were subcloned in pPROEX-HTa vectors (Invitrogen). The two variants were produced in Rosetta<sup>TM</sup> cells (Novagen). Protein expression was induced with 1 mM isopropyl β-d-thiogalactopyranoside, and the cultures were subsequently incubated for 4 h at 30 °C with orbital agitation. Cell pellets were resuspended in 20 ml of buffer containing 20 mM Tris (pH 8.0), 500 mM NaCl, 1.4 mM β-mercaptoethanol, and 5% glycerol supplemented with 15 mM imidazole and lysed by sonication. Lysates were cleared by centrifugation at 39,000 × g, and the supernatants were loaded onto HiTrap nickel-chelating HP columns (GE Healthcare). His<sub>6</sub>-SUMO-HBRCT and His<sub>6</sub>-FHA1 variants were eluted with linear gradients to 300 mM imidazole and titrated overnight at 30 °C with orbital agitation. Cell pellets were resuspended in 20 ml of buffer containing 20 mM Tris (pH 8.0), 500 mM NaCl, 1.4 mM β-mercaptoethanol, and 5% glycerol supplemented with 15 mM imidazole and lysed by sonication. Lysates were cleared by centrifugation at 39,000 × g, and the supernatants were loaded onto HiTrap nickel-chelating HP columns (GE Healthcare). His<sub>6</sub>-SUMO-HBRCT and His<sub>6</sub>-FHA1 variants were eluted with linear gradients to 300 mM imidazole. Tags were removed with tobacco etch virus protease, and aliquots of purified HBRCT and FHA1 were stored at −80 °C in 20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 5 mM DTT, and 5% glycerol.

**NMR Spectroscopy**—All experiments were conducted using a Bruker 700-MHz AV NMR spectrometer with a TCI CryoProbe. HBRCT samples were uniformly labeled by expressing the domain (residues 105–220) in M9 medium containing 15NH<sub>4</sub>Cl and D-[<sup>13</sup>C]-glucose (HBRCT or FHA1) or 15NH<sub>4</sub>Cl alone (HBRCT). Labeled HBRCT was purified and stored as described (12). All experiments with labeled HBRCT were conducted at 306 K in 20 mM Tris (pH 8.0), 100 mM NaCl, 5 mM DTT, 1 mM EDTA, and 5% glycerol. The FHA1 domain of Rad53 (residues 14–164) was similarly labeled to produce 15N,13C-FHA1 or 15N-FHA1. The protein was purified as described above and stored in 10 mM sodium phosphate buffer (pH 6.5), 1 mM EDTA, and 5 mM DTT.

Assignment of resonances for 15N,13C-HBRCT (0.5 mM) was done using standard triple resonance experiments as described (26). The 15N,13C-FHA1 sample (0.3 mM) was first mixed with an equimolar amount of unlabeled HBRCT prior to collecting triple resonance experiments. All data were processed with NMRPipe (27), and resonance assignment was carried out using SPARKY (42).

**Peptide Titration Experiments**—Lyophilized phosphorylated peptide derived from Cdc7 (pPep, 480DGESPDTDDEVS); and a non-phosphorylated control (Pep, 480DGESTDDDEVS) were synthesized by Biomatik. The peptides were resuspended in 10 mM sodium phosphate buffer (pH 6.5) and titrated into a sample containing 0.12 mM 15N-FHA1 with an equimolar amount of HBRCT. Heteronuclear single quantum correlation (HSQC) spectra were collected after each addition, and the titrant volume of peptide added was <20% of the sample volume.

**Trypsin Proteolysis**—9 µl of HBRCT (20 µM in storage buffer) was mixed with 1 µl of 50 mM MgCl<sub>2</sub> and 1 µl of trypsin (0.195–12.5 µg/ml) and incubated for 1 h at room temperature. The reaction products were subsequently resolved on 15% SDS-polyacrylamide gels.

**Statistical Analysis**—Peak intensity losses for 15N-FHA1 in the HSQC spectrum after the addition of HBRCT were analyzed based on a method published previously (29). The peak fit height was measured with SPARKY using a Lorentzian fit to integrate the peaks. Overlapping peaks were rejected (Glu–26, Ile–32, Thr–39, Lys–57, Lys–64, Cys–74, Leu–99, Gln–114, Ser–120, and Asn–158). The remaining values were normalized using the most intense peak (Arg–164) prior to being compared with a similarly analyzed data set from a sample lacking
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Bioinformatics Analysis—To identify patterns of amino acid conservation in the FHA1 and FHA2 domains of Rad53, representative protein sequences were retrieved using PSI-BLAST database using PSI-BLAST, and sequences containing both FHA1 and FHA2 domains. The ConSurf server was used to visualize conserved surface residues (31). A single Protein Data Bank (PDB) file containing both FHA1 and FHA2 domains was retained. A total of 28 sequences were identified (UniProt IDs P2216, G3AR58, G3BDW6, H8X5Q1, B9WDY3, C5M9H1, B5RTA3, G8J57, C4Y319, A5D0Y0, F2QUG7, C4R6G9, K0K5L2, 7SAKQ, Q6CKF2, G8R9N2, Q75CE9, 1GY5M, C5DF05, Q6FKO1, H2AZ96, A7TKN4, G8BV8, G8ZMW0, C5DX25, G0WBA8, G0V58, and E7NNR4), with no further edited to contain only regions corresponding to the FHA1 and FHA2 domains.

Yeast Two-hybrid Analysis—Two-hybrid analysis was carried out as described previously (12) using pEG-Dbf4-FL as the bait and the indicated pJG-FHA1 variants as the prey. Point mutations within FHA1 were generated by QuikChange (Invitrogen). Yeast strains DY-27 (MATa, ade1-1, can1-100, his3-11,15, trp1-1, ura3-1, sml1::HIS3, rad53::URA3), whereas yeast strain DY-145 (MATa, ade1-1, can1-100, his3-11,15, trp1-1, ura3-1, sml1::HIS3) was transformed with empty vector pRS315. Growth spotting assays were performed as described previously (32). Protein expression levels in whole-cell extracts were analyzed by Western blotting using goat anti-Rad53 antibody (Santa Cruz Biotechnology sc6749) and Alexa Fluor 488-conjugated donkey anti-goat secondary antibody (Invitrogen A11055).

RESULTS

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The RAD53 open reading frame, along with 500 bp each of flanking upstream and downstream sequences, was PCR-amplified using genomic DNA from yeast strain DY-27 (MATa/α, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/lys2Δ0, met15Δ0/met15Δ0, ura3Δ0/ura3Δ0) as the template and cloned into the XbaI and PstI sites of the CEN vector pRS315 to create pRS315-Rad53. The SexAI-BglII fragments of the pJG-FHA1 variants (pJG-FHA1NE, pJG-FHA1NEVF, and pJG-FHA1VF) were used to replace the equivalent fragments in pRS315-Rad53 to generate pRS315-Rad53NE, pRS315-Rad53NEVF, and pRS315-Rad53VF, respectively. pRS315-Rad53 variants along with empty pRS315 were transformed into yeast strain DY-147 (MATa, ade1-1, can1-100, his3-11,15, trp1-1, ura3-1). Whereas the HBRCT-W116D/M120A and HBRCT-L109A/W112S variants with increasing amounts of trypsin. We chose these variants because point mutations at these positions disrupt the interaction with Rad53, presumably by destabilizing the interaction between helix α0 and the BRCT core (12). We found that the HBRCT variant was degraded homogeneously, whereas the HBRCT-W116D/M120A and HBRCT-L109A/W1125 variants with increasing amounts of trypsin. This region of the domain indeed forms a helix in solution.

We then checked whether the N-terminal helix is an integral part of the domain. Because the loop connecting this helix to the BRCT core includes several positively charged residues, its susceptibility to trypsin proteolysis should vary depending on whether helix α0 is anchored to the domain or free to move. We treated the HBRCT, HBRCT-W116D/M120A, and HBRCT-L109A/W1125 variants with increasing amounts of trypsin. We chose these variants because point mutations at these positions disrupt the interaction with Rad53, presumably by destabilizing the interaction between helix α0 and the BRCT core (12). The first step resulted in the formation of a fragment of similar size to a BRCT domain. The second step degraded the domain without the accumulation of any intermediate fragments, thereby confirming that the additional N-terminal helix is anchored to the BRCT core of the domain.

The HBRCT Domain of Dbf4 and the FHA1 Domain of Rad53 Interact in Vitro—The interaction between Rad53 and Dbf4 is weak and transient, and it has been studied only by yeast-two hybrid analysis (4, 12, 19). However, yeast-two hybrid analysis does not provide any information as to whether an interaction is direct or mediated by a cellular adaptor. To test whether the interaction between Rad53 and Dbf4 is direct, we sought to characterize the interaction using purified proteins.
Once we determined that HBRCT is a stable domain in solution, we overexpressed and purified the HBRCT (residues 105–220) and FHA1 (residues 14–164) domains. We collected HSQC spectra of uniformly labeled^{15}NFHA1 on its own or in the presence of equimolar amounts of unlabeled HBRCT domain. The main effect caused by the addition of the HBRCT domain was peak broadening for several^{15}NFHA1 residues, which is often a reliable indicator of binding, as previously shown for other weakly interacting protein-protein complexes (29, 33, 34).

Additionally, 10 new peaks appeared in the HSQC spectrum of^{15}NFHA1-HBRCT. We assigned these peaks and found that they corresponded to Gln-16, Leu-19, Ile-20, Glu-21, Lys-22, Phe-23, Ser-24, Gln-25, Glu-26, and Gln-27, which are all located at the N terminus of the domain (Fig. 2A and supplemental Fig. S1). Importantly, these peaks were absent in the HSQC spectrum of^{15}NFHA1-HBRCT, whereas it maintained 95% of the original height upon addition of the non-interacting BRCT domain, thereby confirming that peak broadening was caused by the interaction of the two domains.

We integrated known peaks in the^{15}NFHA1 and^{15}NFHA1-HBRCT spectra and calculated their intensity loss (Fig. 2C). We defined perturbed residues as those whose peak height was reduced by at least 1 S.D. from the mean. Nine residues in the
core of the FHA1 domain met this criterion, and they were located on strands 1 (Ile-37), 7 (Asn-112), 9 (Asn-121 and Leu-123), 10 (Gly-127), and 11 (Phe-146, Ile-147, and Asn-148). With the exception of Asn-121 and Leu-123, all of these residues reside on the same lateral surface of the FHA1 domain (Fig. 2D). Thr-15, Phe-18, and Gln-27, located at the N terminus of the FHA1 domain, also met this criterion, but they are not present in the FHA1 fragment used for the crystallization studies (20); thus, they are not shown in Fig. 2D. Therefore, the comparative HSQC analyses suggested that the FHA1-HBRCT interaction affected two main areas of the FHA1 domain, namely its N terminus and one of the lateral surfaces.

The HBRCT Domain of Dbf4 Interacts with the Lateral Surface of the FHA1 Domain—We decided to explore whether either of these two surfaces defined the interface of the FHA1-HBRCT complex. If Rad53 interacts with Dbf4 through one of these surfaces, we would expect this area to be conserved among Rad53 homologs. Because Dbf4 binds preferentially to the FHA1 domain of Rad53 (4), we postulated that any conserved features of this interface should be present in FHA1 (but not FHA2) domains.

We retrieved representative protein sequences homologous to yeast Rad53 from the non-redundant UniRef90 database using PSI-BLAST (30). 28 full-length Rad53 homologs containing both FHA1 and FHA2 domains were retained and used to compare the degree of conservation of residues in FHA1 and FHA2. In addition to residues associated with the phosphoepitope-binding sites on FHA1 and FHA2, we found a second conserved surface patch on FHA1 (Fig. 3). This conserved surface spans strands 1 (Arg-35, Thr-39, and Gly-41), 7 (Asn-112), 10 (Gln-126, Gly-127, Asp-128, and Glu-129), and 11 (Val-144 and Phe-146), consistent with residues identified by NMR on the lateral surface of the domain. Val-144 and Phe-146 in strand 11 are not strictly conserved; however, these two positions are always occupied by bulky hydrophobic amino acids. Therefore, we included them in the conserved surface. A corresponding conserved region was not found in FHA2, suggesting that this conserved surface may mediate FHA1-specific interactions.

Given the agreement between the NMR and bioinformatics analyses, we probed the in vivo biological relevance of this surface using yeast two-hybrid experiments. For these analyses, we used a construct of Rad53 including residues 1–165...
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FIGURE 3. The FHA1 domain of Rad53 has a conserved lateral surface. A and B, surface representation of the FHA1 domain of Rad53 (PDB ID 1G6G) with residue conservation shown as S.D. values from the mean, where blue indicates conserved, and yellow indicates variable. The FHA1 domain has two highly conserved areas on opposite faces of the molecule: the phosphopeptide-binding pocket (A) and the lateral β-sheet composed of strands β2-β1-β11-β10-β7-β8 (B). The view in B is a 180° rotation from A. C, ribbon diagram of the FHA1 domain of Rad53 (PDB ID 1G6G) shown in the same orientation as in B and colored as in Fig. 2D. Residues defining the conserved lateral region of FHA1 domain are shown as purple sticks and are labeled.

Instead of the shorter fragment (residues 14–164) used for the NMR studies. Given the extent of the conserved lateral surface, we were concerned that the effect of single point mutations might result in changes too subtle to produce noticeable effects in the yeast two-hybrid assay, and thus, we generated variants of the FHA1 domain including both single and multiple mutations. Indeed, single point mutations on this surface had minimal effect (supplemental Table S1). Of the three mutation pairs (I37A/T39A, V144N/F146A, and N112A/E129A) generated, only the N112A/E129A pair weakened the interaction with Dbf4 significantly (Fig. 4A and supplemental Table S1). However, combination of the N112A/E129A pair with either of the other two pairs completely abrogated the interaction (Fig. 4B and supplemental Table S1). Because all FHA1 variants had comparable expression levels (Fig. 4A, A, and B), we attributed the reduced β-galactosidase activity to a defect in the Rad53-Dbf4 interaction.

We then examined the ability of these Rad53 variants to rescue the growth defects of a rad53Δ strain when exposed to agents that cause replicative stress. In the presence of hydroxyurea (HU), a ribonucleotide reductase inhibitor that impairs DNA replication fork progression and induces the replication checkpoint, the rad53Δ strain had no detectable growth at either HU concentration. However, growth could be completely rescued by a plasmid encoding wild-type Rad53 or variants of Rad53, including the paired mutations V144N/F146A (Rad53-NE) and N112A/E129A (Rad53-NEVF), at 50 mM HU and partially rescued at 150 mM HU (Fig. 4D). Conversely, Rad53-N112A/E129A/V144N/F146A (Rad53-NEVF) only complemented the growth defect at 50 mM HU but failed to rescue at higher HU concentrations. We obtained similar results when we examined growth in the presence of methyl methanesulfonate, a DNA-alkylating agent that causes fork stalling. The growth defects observed in the presence of HU or methyl methanesulfonate were not related to lower protein expression levels of these variants (Fig. 4D), supporting the idea that this surface of the FHA1 domain is important for checkpoint function.

Additionally, we tested the effect of the loop connecting strands β10 and β11 and found that the V134D/I140A mutation weakened the interaction with Dbf4 significantly (Fig. 4C and supplemental Table S1). Because these two residues reside on a loop rather than one of the strands, it is possible that their conformation is more sensitive to point mutations and hence the increased effect. However, alternative explanations cannot be ruled out at this point.

Although mutations on the lateral surface of the domain abrogated the interaction with Dbf4 (Fig. 4), we wanted to confirm that the N-terminal region of the FHA1 domain (residues 15–29) was not necessary for the interaction with Dbf4. Therefore, we generated a variant of Rad53 corresponding to the minimal FHA1 fold (residues 22–165). This FHA1 variant interacted with the HBCT domain of Dbf4 similarly to longer fragments of Rad53, confirming that the region immediately preceding the FHA1 domain is not necessary for the FHA1-HBCT interaction (supplemental Table S1). Collectively, these results indicate that Rad53 likely interacts with Dbf4 through the lateral surface of the FHA1 domain defined by the β-sheet composed of strands β2-β1-β11-β10-β7-β8 (Fig. 2D).

The pThr-binding Pocket of FHA1 Is Not Required for the Interaction with Dbf4—It has been shown that a point mutation (R70A) in the phosphopeptide-binding pocket of the FHA1 domain abrogates the interaction with Dbf4 through yeast two-hybrid analysis (4). The phosphorylated peptide consisting of residues 98–113 from Dbf4 (including pThr-105) interacts with the FHA1 domain weakly (19). However, mutation of Thr-105 (or any other threonine within the HBCT domain) does not disrupt the interaction with Rad53 (12), suggesting that the HBCT domain does not contain a phosphopeptide targeted by Rad53. To probe the effect of the R70A mutation on the FHA1-HBCT interaction, we compared the HSQC spectra of 15N-FHA1 and 15N-FHA1-R70A. Although a number of peaks exhibited chemical shift changes, the addition of the HBCT domain to the 15N-FHA1-R70A sample still caused an overall decrease in intensity and the appearance of the 10 peaks indicative of complex formation (Fig. 5), thereby confirming that FHA1-R70A interacts with the HBCT domain of Dbf4 in vitro. It is possible, however, that Rad53 interacts with Dbf4 through a
bipartite interface involving both the recognition of a phosphopeptidome and the interaction of the HBRCT domain with the lateral surface of the FHA1 domain. Among many other phosphorylated substrates, the FHA1 domain of Rad53 recognizes a phosphoepitope within Cdc7 (37). We reasoned that Cdc7 might modulate the interaction between Rad53 and Dbf4, and thus, we tested whether a Cdc7-derived peptide (480DGESpTDEDDVVS491, herein referred to as pPEP) could outcompete the interaction of Rad53 with Dbf4.

To this end, we monitored complex formation using chemical cross-linking with the amine-reactive cross-linker BS3. Upon treatment of the individual proteins with BS3, we could detect HBRCT dimers and, to a much lesser extent, FHA1 dimers (Fig. 6, control lanes). Treatment of FHA1-HBRCT complex mixtures with BS3 resulted in three cross-linked products. Two of them corresponded to the HBRCT and FHA1 dimers identified when each protein was treated with BS3 individually, whereas the third was found only when both proteins were present and had a molecular weight consistent with complex formation. The presence of both proteins in this cross-linked species was confirmed by LC-MS/MS (data not shown). The phosphorylated Cdc7 peptide (pPEP), but not its non-phosphorylated form (480DGESpTDEDDVVS491, PEP), could be cross-linked to the FHA1 domain of Rad53 (Fig. 6A). Conversely, neither pPEP nor PEP could be cross-linked to the HBRCT domain of Dbf4 (Fig. 6B), indicating that BS3 cross-linking captured a specific and phosphorylation-dependent interaction between the FHA1 domain and the peptide. Titration of pPEP (or PEP) into equimolar mixtures of FHA1-HBRCT did not prevent the appearance of the cross-linked product corresponding to the FHA1-HBRCT complex (Fig. 6C). This result indicates that pPEP and HBRCT can bind the FHA1 domain simultaneously, thereby suggesting that they bind different regions of the FHA1 domain.

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FIGURE 4. The lateral surface of the FHA1 domain of Rad53 is required for interaction with the HBRCT domain of Dbf4. A and B, yeast two-hybrid analysis using Dbf4 as the bait and the FHA1 domain of Rad53 (WT), empty vector (Empty), or variants of FHA1 containing double (A) or multiple (B) mutations within the interaction surface predicted from NMR and bioinformatics analysis as the prey. The double and multiple mutants are labeled as IT (I37A/T39A), VF (V144N/F146A), NE (N112A/E129A), ITVF (I37A/T39A/V144N/F146A), and IT (I37A/T39A/V144N/F146A). The interaction is shown as β-galactosidase activity units and, in each case, represents the mean of three independent measurements. Error bars represent S.D. C, yeast two-hybrid analysis using Dbf4 as the bait and the FHA1 domain of Rad53, empty vector, or the FHA1-V134D/I140A variant containing a double mutant in the β10-β11 loop (VI). To control for the two-hybrid bait and prey expression levels in A–C, whole cell extracts were prepared from transformants following prey induction and analyzed by Western blotting using rabbit anti-LexA antibody (bait) and mouse anti-HA monoclonal antibody (prey), along with Alexa Fluor 647-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies, respectively. Prior to detection, the membrane was stained with Ponceau S to assess relative protein loading. D, sensitivity of Rad53 variants to genotoxic stress was tested in the presence of HU or methyl methanesulfonate (MMS). The growth of a rad53Δ, sml1Δ strain in the presence of plasmids encoding Rad53, Rad53-NE, Rad53-NEVF, and Rad53-VF was compared on synthetic complete without leucine (SC − Leu) plates containing increasing concentrations of HU or methyl methanesulfonate by spotting 10-fold serial dilutions. Rad53, sml1Δ, and rad53Δ, sml1Δ strains transformed with an empty plasmid were used as controls for normal sensitivity or hypersensitivity to genotoxic agents, respectively. Rad53 expression was assessed using goat anti-Rad53 antibody and Alexa Fluor 488-conjugated donkey anti-goat secondary antibody.
We confirmed these findings using NMR. To this end, we incubated a mixture of $^{15}$NFHA1 and HBRCT with increasing amounts of pPEP. We collected HSQC spectra at increasing concentrations of peptide corresponding to increasing ratios of pPEP to FHA1. We found that the presence of pPEP caused chemical shift perturbations consistent with phosphopeptide binding, whereas the non-phosphorylated version of the peptide (PEP) did not induce any changes in the $^{15}$NFHA1 HSQC spectrum (Fig. 7). Importantly, the peaks indicative of the interaction between FHA1 and HBRCT were present at all pPEP concentrations, although the peak corresponding to Gln-16 could not be monitored in this experiment because it overlapped with the peak corresponding to Glu-117 during the titration. Collectively, these experiments demonstrate that Rad53 interacts with a phosphopeptide and the HBRCT domain of Dbf4 through different surfaces of the FHA1 domain and that both interactions can occur simultaneously.

**DISCUSSION**

We have shown that the HBRCT domain of Dbf4 interacts with the lateral surface of the Rad53 FHA1 domain, rather than its phosphopeptide-binding surface. This lateral surface is not conserved in the FHA2 domain of Rad53, indicating that the FHA1 domain of Rad53 gains specificity by engaging additional interaction surfaces. Additionally, FHA1 can simultaneously bind a phosphopeptide and the HBRCT domain. Therefore, the Rad53-Dbf4 interaction *in vivo* may occur through a bipartite interaction. The phosphorylated peptide consisting of residues 98–113 of Dbf4 includes a canonical $^{105}$pTXXE$^{108}$ FHA-binding motif and interacts with the FHA1 domain weakly (19). Mutation of Thr-105 in the context of full-length Dbf4 does not, however, disrupt the interaction between Dbf4 and Rad53 (12). Chen *et al.* (19) also showed that point mutations on the surface of the HBRCT domain defined by helices α0 and α3 abrogate the interaction with Rad53, suggesting that Thr-105 may contribute to defining this interface rather than providing a pThr target site. Indeed, this surface of the HBRCT domain has good shape and charge complementarity with the lateral surface of the FHA1 domain.

Several FHA domains have been shown to mediate bipartite interactions. For instance, the FHA domain of Dun1 requires the simultaneous interaction with two pThr residues to recognize its target (21), and KIF13B interacts simultaneously with the ArfGAP and PH1 domains of CENTA1 (38). Therefore, FHA domains have been compared with logic gates able to integrate multiple inputs and generate a single output (22). When phosphopeptide binding is considered as the output, the FHA1 domain from Rad53 acts as an “OR” logic gate because it can only bind to one pThr residue within a multiply phosphorylated peptide. In contrast, the FHA domain from Dun1 is considered an “AND” logic gate because it must bind to two pThr residues simultaneously (21). If we extend the logic gate model to Dbf4-dependent kinase (DDK) targeting, the FHA1 domain of Rad53 also behaves as an AND logic gate, requiring simultaneous binding of a phosphopeptide and engagement of the HBRCT domain of Dbf4 through its lateral interface for their normal targeting.

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4 A. Almawi and A. Guarné, unpublished data.
physiological interaction (Fig. 8). An AND logic gate would also explain why disabling either contact point abrogates the Rad53-Dbf4 interaction in a yeast two-hybrid assay and would have important ramifications during the checkpoint response. DDK-mediated phosphorylation of Mcm2 has a role in the response to replicative stress, likely as a result of replication fork stabilization (39). Therefore, one of the logic gate inputs may guarantee the specificity of Rad53 targeting the HBRCT domain of Dbf4, whereas the second may ensure the stress dependence of the interaction by recognizing a phosphoepitope generated by a stress-activated kinase.

Logic gates have emerged as critical components of biological signaling networks, thereby implying that FHA domains function as highly specialized modules that integrate complex input signals (22, 40). The Rad53-Dbf4 interface unveiled in this work is reminiscent of the BRCA1-Chk2 interaction, where the tandem BRCT repeat of BRCA1 must recognize two distal surfaces in the FHA domain of Chk2 simultaneously (41). In the BRCA1-Chk2 complex, the interaction involves the pThr-binding site and a conserved hydrophobic patch on one of the lateral surfaces of the FHA domain. Disruption of either contact point prevents the interaction, and mutation of the hydrophobic patch has been linked to Li-Fraumeni syndrome, which predisposes patients to multi-organ cancers (41). However, Dbf4 and BRCA1 do not interact with the same lateral surface of the FHA domains of Rad53 and Chk2. Furthermore, the hydrophobic patch found in the FHA domain of Chk2 is not conserved in FHA1 from Rad53, revealing the extreme plasticity of FHA domains to enhance binding specificity. The interaction of Rad53 with Dbf4 or Chk2 with BRCA1 leads to inhibition of late origin firing and double-strand break repair, respectively. Therefore, it is not surprising that cells have developed strict selectivity mechanisms to modulate these two interactions. However, although the need for bipartite interactions increases Rad53 and Chk2 binding specificities, it also underlines the experimental difficulty in detecting dual input FHA domains, as eliminating one binding site abrogates the interaction, effectively masking the presence of a second binding site. Our work suggests that FHA domains may have a broader spectrum of interactions that has remained elusive due to the intrinsic limitations of studying AND logic gates, and it provides a novel approach to study functional interactions mediated by this highly prevalent signaling domain.
Characterization of the Rad53-Dbf4 Interface

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