Molecular Basis for the Association of Microcephalin (MCPH1) Protein with the Cell Division Cycle Protein 27 (Cdc27) Subunit of the Anaphase-promoting Complex*

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Namit Singh1, Timothy D. Wiltshire1, James R. Thompson*, Georges Mer1,2, and Fergus J. Couch3

From the Departments of 1Biochemistry and Molecular Biology, 2Laboratory Medicine and Pathology, and 3Physiology and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota 55905

Background: Recognition of phosphorylated proteins by BRCT domains is key to cell signaling events.

Results: Microcephalin (MCPH1) can recognize phosphorylated Cdc27 via its tandem BRCT domains.

Conclusion: Single amino acid changes are detrimental to the MCPH1-pCdc27 interaction in vitro and in vivo.

Significance: Our findings reveal the structural and biochemical basis for MCPH1-phosphoprotein interaction.

Microcephalin (MCPH1), the first gene identified as causative for primary recessive autosomal microcephaly, is aberrantly expressed in autism-like disorders and human malignancy of breast and ovarian origin. MCPH1, the encoded protein product, has been implicated in various cellular processes including the DNA damage checkpoint, DNA repair, and transcription. Although our understanding of the cellular context in which MCPH1 operates continues to develop, a structural understanding of the C-terminal tandem BRCT domains of MCPH1 remains unexplored. Here, we identify cell division cycle protein 27 (Cdc27), a component of the anaphase-promoting complex (APC/C), as a novel interacting partner of MCPH1. We provide in vitro and in vivo evidence that the C-terminal tandem BRCT domains of MCPH1 (C-BRCTs) bind Cdc27 in a phosphorylation-dependent manner. To characterize this interaction further, we determined the structure of MCPH1 C-BRCTs in complex with a phosphorylated Cdc27 peptide (pCdc27) using X-ray crystallography. Based on this structure, we identified single amino acid mutations targeted at the binding interface that disrupted the MCPH1-pCdc27 interaction. Collectively, our data define the biochemical, structural, and cellular determinants of the novel interaction between MCPH1 and Cdc27 and suggest that this interaction may occur within the larger context of MCPH1-APC/C.

Although first uncovered in an in vitro screen as a transcriptional repressor of telomerase reverse transcriptase (hTERT) (1), microcephalin (MCPH13/BRIT1) has emerged as a “guardian of the genome” due to its ability to preserve genomic integrity. MCPH1 achieves this in part by facilitating the localization of DNA damage and repair proteins to sites of DNA damage, by mediating both homologous recombination and non-homologous recombination repair processes, and by activation of the DNA damage checkpoint. Loss of MCPH1 leads to an absence of DNA damage response proteins, including the BRCA2/Rad51 complex, from irradiation-induced foci and failure to activate Chk1/Chk2 cell cycle checkpoints (2–9).

Human MCPH1 is an 835-amino acid protein that is predicted to be largely disordered except at the very N and C termini. Whereas a single BRCT domain is present at the N terminus, the C terminus consists of tandem BRCT domains (C-BRCTs). The BRCT domains are evolutionarily conserved protein-protein interaction modules. A subset of BRCT domains, the tandem BRCT repeats, has been shown to directly recognize phosphopeptide targets (10, 11). Although several direct and indirect interacting partners of MCPH1 have been identified, phosphorylation-dependent direct binding proteins that map to the C-BRCTs remain largely undiscovered. Indeed, the histone variant γH2A.X is the only protein that has emerged as a direct binding target of MCPH1 C-BRCTs (12). However, because this interaction originates under DNA damage conditions as MCPH1 has no detectable affinity for unmodified H2A.X, the cognate binding partner(s) of MCPH1 C-BRCTs in the absence of damage remain unknown.

Tandem BRCT domains, including those derived from human BRCA1, MDC1, and TopBP1, and Schizosaccharomyces pombe Brc1 and Crb2, have served as models for studying how BRCT domains interact with phosphopeptides. In vitro phosphopeptide interaction screens along with the crystal structures of BRCA1- and MDC1-phosphopeptide complexes have helped define a consensus BRCT binding phosphopeptide interaction site (13–23). This consensus binding site conforms to the pS/XXX rule where pS is a phosphoserine and X is any residue, with position pS+3 usually being a phenylalanine (BRCA1) or tyrosine (MDC1) or an aliphatic residue (TopBP1, Brc1, and Crb2), and possibly an aspartate in the sequence recognized by NBS1 tandem BRCT domains (24). In the case of TopBP1 the C-terminal tandem BRCT domains can bind either

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1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. Tel.: 507-266-0451; Fax: 507-284-3383; Email: mer.georges@mayo.edu.

3 The abbreviations used are: MCPH1, microcephalin; APC/C, anaphase-promoting complex; BisTris, bis(2-hydroxyethyl)methanol; Cdc27, cell division cycle protein 27; ITC, isothermal titration calorimetry; PDB, Protein Data Bank.
phosphoserine- or phosphothreonine-containing peptides (23). Both BRCA1 and MDC1 have been suggested to bind internal and terminal sequences, although the terminal motif containing the free carboxylate binds with higher affinity (19, 25). The interaction between MCPH1 and γH2AX appears to follow this trend as γH2AX also presents a C-terminal carboxylate motif (Tyr-142). Therefore, to identify new potential binding targets for the MCPH1 C-BRCTs we narrowed our search to proteins that present the pS/T\(\text{R}\)ylate motif (Tyr-142).

In this study, we have identified Cdc27, a component of the anaphase-promoting complex (APC/C) (26), as a novel binding partner of MCPH1. APC/C is an E3 ubiquitin ligase that controls the cell cycle. A combination of in vitro and in vivo experiments revealed that the interaction between MCPH1 and Cdc27 is dependent on the phosphorylation of Cdc27 and is mediated by the C-terminal BRCT domains of MCPH1. Following the determination that the interaction between MCPH1 and pCdc27 is direct in nature, we determined the crystal structure of the MCPH1-pCdc27 peptide complex. The co-crystal structure highlights the key elements of how MCPH1 is bound to Cdc27. Finally, structure guided predictions allowed us to make precise single point mutations that disrupt the interaction between MCPH1 and Cdc27 both in vitro and in vivo.

**EXPERIMENTAL PROCEDURES**

**Preparation of Proteins and Peptides**—The C-terminal BRCT domains of human MCPH1 (residues 635–835) were cloned into the pTEV *Escherichia coli* expression vector to generate a tobacco etch virus protease-cleaveable His\(_6\) fusion protein. The construct was initially transfected in BL21 Rosetta (DE3) cells that were grown at 37 °C until the optical density reached 0.7 at which point cells were transferred to 18 °C, induced with isopropyl β-d-1-thiogalactopyranoside, and cultured overnight at 18 °C. Recombinant MCPH1 was enriched following standard nickel-nitrilotriacetic acid purification protocols for Histagged proteins (Qiagen). The protein was further purified by size exclusion chromatography using a Superdex 75 column (GE Healthcare). Finally, the integrity and purity of the protein preparation were assessed by SDS-PAGE. All peptides were synthesized and HPLC-purified at our institutional facilities. Peptide masses were verified by mass spectrometry.

MCPH1 wild type and deletion mutants were cloned in pCS3–6×Myc vector or pIRE2-FLAG vector. The ΔN mutant lacks the N-terminal BRCT domain, BRCTΔ2 is missing the second BRCT domain, and the BRCTΔ3 mutant is missing the third BRCT domain.

**Crystallization and Structure Determination of the MCPH1-pCdc27 Complex**—MCPH1 was prepared at a concentration of 15 mg/ml and incubated with a pCdc27 tetrapeptide on ice at a 1:3 molar ratio. Several crystallization conditions were screened using the above mixture at 22 °C. The optimized crystallization condition consisted of 0.3 M NaCl, 0.1 M BisTris, pH 7.1, and 30% PEG3350. The crystals were cryoprotected in their mother liquor supplemented with 25% glycerol (v/v) following which they were flash frozen in liquid nitrogen. Data were collected using a Rigaku Cu (Ka) rotating anode and R axis detector. Data were indexed, integrated, and scaled using d*TREK (27) and Crystal Clear (Rigaku). A molecular replacement solution containing two molecules of MCPH1 was obtained using a poly-Ala model derived from the structure of BRCA1 tandem domains (Protein Data Bank (PDB) accession code 1T29) using Phaser 2.1.1 (28). The programs COOT (29) and PHENIX (30) were used during repeated cycles of manual model building and refinement. Noncrystallographic restraints were included until the R-factor dropped below 0.30 and then removed for further refinement using PHENIX.

**Isothermal Titration Calorimetry (ITC)**—ITC measurements were carried out at 10 °C using a VP-ITC titration calorimeter (MicroCal). All protein and peptide samples were prepared in 50 mM Tris-HCl, pH 7.5, 40 mM NaCl. In a typical experiment, the peptide solution (1.0–2.0 mM) was placed in the calorimeter injection syringe and delivered as a series of 3–6-μl injections every 5 min to the reaction cell containing 1.42 ml of MCPH1 C-BRCTs at concentrations of 20–35 μM. Measurements were paired with control experiments for heat of mixing and dilution values (31). Data were analyzed with Levenberg-Marquardt nonlinear regression using a single binding site model (MicroCal).

**Co-immunoprecipitation Assays**—293T cells were purchased from American Tissue Type Culture and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO\(_2\). For co-immunoprecipitation assays, nuclear extracts were prepared from 293T cells. Briefly, freshly harvested cells were allowed to swell on ice for 10 min in hypotonic buffer (20 mM HEPES, pH 7.8, 1.5 mM MgCl\(_2\), 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT), homogenized, and extracted in high salt buffer (20 mM HEPES, pH 7.8, 25% glycerol, 1.5 mM MgCl\(_2\), 350 mM NaCl, 0.5 mM DTT, Complete protease (Roche Applied Science), and phosphatase inhibitor mixture (Pierce)) for 30 min at 4 °C. Following centrifugation, no salt buffer (20 mM HEPES, pH 7.8, 20% glycerol, 0.1% Tween 20, and 0.2% PMSF) was added to the nuclear lysates, which were incubated overnight at 4 °C with 2 mg of antibody. The samples were incubated for 1 h with protein G-agarose beads (Invitrogen), washed three times, boiled in Laemmli buffer, and resolved by SDS-PAGE. Western blotting was carried out using anti-FLAG (Sigma), anti-GFP (Abcam), or anti-Myc (Santa Cruz Biotechnology) antibody. Endogenous proteins were detected by anti-MCPH1 (Cell Signaling) or anti-Cdc27 (Santa Cruz Biotechnology) antibody. For phosphatase treatment experiments, nuclear extracts were incubated with 400 units of A-phosphatase (NEB) with 2 mM Mn\(^{2+}\) and with or without 50 mM EDTA for 30 min at 30 °C before immunoprecipitation was carried out. Irradiated cells were harvested 3 h after exposure to 10 Grays of radiation using a cesium 137 source at a rate of 8.03 Grays/min. MG132 treatment was done by addition of 10 μM MG132 to cultured cells for 5 h before harvesting.

**In Vitro Pulldown Assays**—Wild-type and mutant His-MCPH1-C-BRCTs were bound to 50 ml of 50% nickel-nitrilotriacetic acid resin (Qiagen) slurry in PBS, washed three times, and incubated with lysate of cells expressing GFP-Cdc27 overnight at 4 °C in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0, and 0.5% (v/v) Nonidet-P40) supplemented with protease (Roche Applied Science) and phosphatase (Pierce) inhibitors. Beads were spun down at 5 °C,
washed three times with PBS, and boiled in Laemmli buffer. Samples were resolved on a 4–15% gradient gel (Bio-Rad) that was then transferred onto a PVDF membrane and probed overnight with anti-GFP antibody (Abcam).

**RESULTS**

**MCPH1-Cdc27 Interaction Is Phosphorylation-dependent and Mediated by the C-terminal Tandem BRCT Domains**—To discover a novel binding partner for MCPH1 C-BRCTs, we adhered to three established principles of BRCT-phosphopeptide interactions. First, the absolute requirement is that the serine/threonine residue of the candidate target protein has been shown to be phosphorylated. Second, there is the need for a free terminal carboxylate group. And third, the search was limited to proteins that present the pSXXY/F-COO⁻/H motif because BRCA1 and MDC1 prefer a phenylalanine or a tyrosine at the +3 position and because this position is a tyrosine in H2A.X, a target of MCPH1. In light of the recent discovery that phosphorylation of Cdc27 at Ser-821 leads to the creation of a tandem BRCT binding proficient pSDEF-COO⁻/H site (32), we hypothesized that suitably modified Cdc27 could be a potential MCPH1-binding partner. Using reciprocal immunoprecipitation experiments, we found that MCPH1 and Cdc27 interact in vivo and that the interaction occurs in the absence of DNA damage (Fig. 1A). Furthermore, this interaction between MCPH1 and Cdc27 was confirmed under endogenous conditions (Fig. 1B). Because BRCT domains have been shown to interact with proteins in a phosphorylation-dependent as well as -independent manner (33), we evaluated whether phosphorylation of Cdc27 was required for this association by pretreating cell lysates with λ-phosphatase. Phosphatase treatment substantially altered the interaction between MCPH1 and Cdc27, suggesting that this interaction is phosphorylation-dependent (Fig. 1C). Co-incubation of the lysate with EDTA, which can inactivate metal-dependent phosphatases, restored the interaction between MCPH1 and Cdc27 to original levels, lending further credence to a phosphorylation-dependent interaction (Fig. 1C).

Next, we asked whether the MCPH1-Cdc27 interaction was influenced by DNA damage. This question arose from concerns that exposure to DNA damage might result in additional post-translational modification of MCPH1 or Cdc27 that might directly influence binding and might create a competing binding target for MCPH1 C-BRCTs, thereby indirectly affecting the MCPH1-pCdc27 interaction. To find out whether the MCPH1-Cdc27 interaction was modulated by irradiation, we carried out immunoprecipitation experiments in the presence and absence of radiation. No significant change was observed in the amount of Cdc27 associated with MCPH1 (Fig. 1, D and E), suggesting that irradiation did not influence the MCPH1-pCdc27 interaction. In contrast, as stated above, an appreciable decrease in binding was noted on treatment with phosphatase (Fig. 1C). In combination, these results suggested that whereas phosphorylation affected this interaction, irradiation-induced phosphorylation did not.

Because MCPH1 contains three BRCT domains that may recognize phosphorylated targets, we evaluated the role of each domain in the interaction with Cdc27 (6, 11). Immunoprecipitation experiments using in-frame deletion constructs devoid of either the N-terminal or the C-terminal BRCTs confirmed that the loss of the C-terminal tandem BRCT domains, but not...
the N-terminal BRCT domain, influenced the interaction with Cdc27 (Fig. 2A). Because Cdc27 presents a suspected MCPH1 interaction motif at its C terminus, we asked whether MCPH1 might bind Cdc27 phosphorylated at Ser-821. Pulldown assays showed that a single amino acid mutation (S821A) within full-length Cdc27 disrupted the interaction with MCPH1 (Fig. 2B). Therefore, we conclude that phosphorylation of Cdc27 at Ser-821 plays a key role in mediating an interaction with the C-terminal BRCT domains of MCPH1. Because Cdc27 is a component of APC/C that is essential for the metaphase-to-anaphase transition, we asked whether the MCPH1-Cdc27 interaction takes place in the context of the MCPH1-APC/C interaction. Immunoprecipitation experiments showed that MCPH1 also interacts with Cdc16, another component of APC/C (Fig. 2C). This points to a potential association between MCPH1 and APC/C that derives in part from the MCPH1-Cdc27 interaction.

Biochemical Basis for MCPH1-Cdc27 Interaction—Although the above findings are consistent with a direct interaction between MCPH1 and pCdc27, an indirect association could not be ruled out. Therefore, we asked whether the interaction between MCPH1 and pCdc27 was direct in nature. ITC experiments revealed a direct interaction between recombinant MCPH1 C-BRCTs (residues 646–835) and a phosphopeptide derived from the C-terminal tail of Cdc27 (residues 814–824; TQLHAAEpSDEF-COO\(^{-}\)) with an apparent \(K_d\) measured at 0.71 ± 0.1 \(\mu\)M, within the reported range of other BRCT-phosphopeptide interactions (Fig. 3A). Furthermore, we found that a minimally phosphorylated Cdc27 tetrapeptide (residues 821–824; pSDEF-COO\(^{-}\)) could also bind MCPH1 C-BRCTs with an apparent \(K_d\) of 0.81 ± 0.1 \(\mu\)M, suggesting a limited role for residues N-terminal to the phosphoryserine (Fig. 3A). MCPH1 did not show any discernable binding to the unphosphorylated SDEF-COO\(^{-}\) peptide.

Whereas the phosphate was absolutely essential for the MCPH1-Cdc27 peptide interaction, the contribution of the C-terminal carboxyate group to this binding was less certain. Indeed, previous studies have revealed that the free carboxyate is not an absolute requirement for BRCT-phosphopeptide interactions (33). To shed light on the role of the free C-terminal carboxyate in the MCPH1-Cdc27 interaction, we synthesized a peptide with the free carboxyate blocked by amidation (pSDEF-CONH\(_2\)) and measured the ability of the peptide to bind MCPH1. ITC measurements showed extremely weak binding of MCPH1 to the blocked peptide (\(K_d\), not determined) (Fig. 3A). Therefore, these findings revealed that both the Cdc27 phosphate and the terminal carboxylate are indispensable for Cdc27-MCPH1 complex formation and that the minimal tetrapeptide (pSDEF-COO\(^{-}\)) containing both of these elements is sufficient to bind MCPH1.

In the context of full-length proteins, phosphorylation of Cdc27 is important for its tight interaction with MCPH1. However, from the immunoprecipitation assays presented in Figs. 1 and 2, there is residual binding after phosphatase treatment as well as with Cdc27 constructs that lack the C-terminal BRCT domains. This indicates that other regions of MCPH1 interact with Cdc27 either directly or via other subunits of APC/C.

Crystal Structure of MCPH1-pCdc27 Complex—To explore the MCPH1-pCdc27 interaction in depth, we crystallized the tandem BRCT domains of MCPH1 with the pCdc27 tetrapeptide (pSDEF-COO\(^{-}\)). The needle-shaped crystals diffracted to a resolution of 2.6 Å in a \(P1\) space group. Although MCPH1 C-terminal BRCT domains share limited sequence homology with other BRCT domains, the structure was solved by molecular replacement using an initial search model derived from a structure of BRCA1 tandem BRCT domains with no phosphopeptide (13). The molecular replacement solution consisted of two MCPH1 C-BRCT molecules in the asymmetric unit. In the two molecules, we observed unaccounted electron density corresponding to pCdc27 in the \(F_o - F_c\) omit electron density map contoured at 3 \(\sigma\) level (Fig. 3B). The final structure was refined to R-factors \(R_{work}/R_{free}\) of 21.8%/26.2%. The crystallographic statistics are provided in Table 1.

MCPH1 C-BRCTs consist of two compact lobes that are partitioned by a 33-amino acid long linker (Fig. 3, C and D). Each lobe shows the characteristic mixed \(\alpha/\beta\) BRCT topology with two \(\alpha\)-helices stacked across one face of the four parallel \(\beta\)-strands and a third \(\alpha\)-helix stacked across the opposite face. MCPH1 C-BRCTs are comparable in length to the 195-amino acid long MDC1 tandem BRCT domains (19). Although their overall folds are similar, sequence alignment reveals only 22% identity. The root mean square difference between these structures of MCPH1 and MDC1 (PDB accession code 2ETX) for 171 C\(^\text{C}\) atoms is 3.0 Å. In MCPH1, the two BRCT domains are...
connected by a single α-helix (αL) followed by a 17-amino acid long linker, whereas in MDC1 the connector region consists of 2 α-helices, αL1 and αL2, followed by a much shorter loop.

Phosphate Recognition and Phosphopeptide Binding by MCPH1—The key determinant of the enhanced recognition of phosphorylated over the corresponding nonphosphorylated peptide is the phosphate itself. Comparison of the MCPH1 C-BRCT-pCdc27 and MDC1 C-BRCT-pH2A.X structures highlighted the conserved and nonconserved elements in phosphopeptide recognition. MCPH1 detects the presence of the phosphate via direct hydrogen bonding to Thr-653, Ser-654, and Asn-696 (Fig. 4A). To our knowledge, asparagine is rarely relied upon to discriminate between a phosphorylated and a nonphosphorylated state (34). In other tandem BRCT domains, such as those of BRCA1 and MDC1, there is a lysine in place of MCPH1 Asn-696 (Fig. 4B). The absence of an electropositive residue to tether the phosphate is a unique feature of the MCPH1-pCdc27 complex. We note that the pCdc27 peptide conformations and MCPH1 C-BRCT-pCdc27 interaction interfaces are virtually identical in the two complexes in the asymmetric unit.

To gain further insight into the role played by Asn-696, we mutated it into an aspartate. ITC titrations revealed that the binding affinity of this MCPH1 mutant for pCdc27 was substantially diminished (34). Another key element in the recognition of the pCdc27 peptide by MCPH1, as suggested by the binding experiments, is the C-terminal aromatic residue (+3) of pCdc27. The co-crystal structure of MCPH1 C-BRCT-pCdc27
suggests that this carboxylate is anchored via conserved Arg-693 of MCPH1 through direct hydrogen bonds (Fig. 4, A and C). Whereas the guanidinium group of Arg-693 forms bipartite hydrogen bonds to the C-terminal carboxylate of Phe-824 (3.0 Å and 2.9 Å), its main chain carbonyl supplements this interaction by hydrogen bonding to the main chain amide proton of the peptide (2.7 Å). Mutation of Arg-693 to methionine profoundly affected binding (KD undetermined) between the peptide and MCPH1 (Fig. 4C). Analysis of the MCPH1 C-BRCT-pCdc27 complex further reveals that MCPH1 Lys-814 may have a role to play in terminal carboxylate recognition by providing electrostatic stabilization (Fig. 4A). This is in contrast to MDC1 where no such interaction is present and, surprisingly, a negatively charged Glu-2063 resides at the analogous position.

Single Point Mutations in MCPH1 Can Disrupt the MCPH1-pCdc27 Interaction—Next, we explored the influence of single amino acid substitutions that disrupted in vitro binding to the pCdc27 peptide, on binding to the full-length protein. As shown in Fig. 4D, when introduced into the recombinant MCPH1 BRCT domains, the R693M and N696D mutations displayed diminished binding to full-length wild-type Cdc27 pro-
MCPH1 and Anaphase-promoting Complex Cdc27 Subunit

FIGURE 5. Model for MCPH1-Cdc27 interaction in vivo. A, comparison of MCPH1, Cdc27, ANAPC11, and Cdc23 mitotic phenotypes obtained from MitoCheck (41, 42). B, model schematic hinting at the potential biological outcome of the MCPH1-Cdc27 (APC/C) interaction. In the figure, the C-BRCTs of MCPH1 are shown to interact with Cdc27 (and APC/C) (26) via pS821, by virtue of which MCPH1 either regulates APC/C activity/stability or acts as an APC/C substrate.

The ability of MCPH1 to differentiate between the phosphorylated and the corresponding nonphosphorylated state resides in the tandem BRCT domains. First identified as a repeating unit in BRCA1 (35–38), the tandem BRCT domains are ubiquitous and conserved motifs across phyla that recognize the phosphoserine/phosphothreonine mark on proteins with high selectivity (10, 11, 39). However, the phosphoserine/phosphothreonine mark alone is not sufficient to impart such specificity and residues, particularly those C-terminal to the phosphoserine, have been found to contribute. Based on the overall binding preferences, a consensus binding site for tandem BRCT has emerged, with position pS+3 being key for the specificity of interaction.

Having previously determined that the C-BRCTs of MCPH1 can directly bind γH2A.X via the pSQEY-COO− sequence (12), we explored the presence of the pSXXY/F motif in other proteins to identify potential MCPH1 binding partners. In light of the recent discovery that Cdc27 is phosphorylated at Ser-821, a potential MCPH1 binding target emerged. Due to the obvious similarities in the pCdc27 and γH2A.X terminal sequences (pSDEF-COO− versus pSQEY-COO−), we hypothesized that MCPH1 may also bind Cdc27. In this study, we confirm that MCPH1 can interact with Cdc27 both in vitro and in vivo. Detailed analysis of the MCPH1-pCdc27 interaction using x-ray crystallography and ITC revealed that MCPH1 relies on a three-pronged strategy to engage pCdc27. This is achieved by the direct recognition of the phosphate, the terminal carboxylate, and van der Waal interactions originating from the aromatic residue present at the +3 position. MCPH1 contacts the serine-phosphate using a network of hydrogen bonds that emanate from Thr-653, Ser-654 and Asn-696. A striking feature of this interaction with the phosphate group is the absence of an electropositive residue in its immediate vicinity. Indeed, a survey of the different phosphopeptide-protein structures reveals that arginine or lysines are ubiquitous at the binding interface. Therefore, one would expect strong evolutionary pressure to conserve such a site and, consistent with such a hypothesis, all known phosphopeptide binding structures present a conserved lysine/arginine at that position. However, neither human MCPH1 nor its orthologs are known to present a lysine at the aforementioned position (Fig. 4B). Interestingly, Thr-653 and Ser-654 are conserved across all species whereas Asn-696 is not strictly conserved. We found that Bos taurus relies on a serine residue instead of the asparagine to interact with the phosphate. This suggests that the presence of an electropositive residue is not essential and that more than one neutral amino acid could substitute for the lysine while preserving its interaction with the phosphate.

Although this study is focused on the MCPH1-Cdc27 interaction, our data also suggest that MCPH1 interacts with APC/C. Specifically, we show that MCPH1 forms a complex with Cdc16 from APC/C as well as Cdc27. In addition, among the components of APC/C, Cdc26 is an alternate candidate protein that presents the canonical BRCT binding sequence. The key difference between Cdc27 and Cdc26 derives from the residue present at the +1 position relative to the phosphoserine. Whereas in the case of Cdc27 it is an aspartate, it is a leucine in Cdc26. Because the MCPH1 C-BRCT-pCdc27 structure suggests that this amino acid is solvent-exposed with no obvious contacts with MCPH1, we hypothesize that it provides only a small contribution to the overall binding. Therefore, based on the concepts derived from the crystal structure of MCPH1 C-BRCT-pCdc27, we suggest that Cdc26 may represent an alternate MCPH1 binding target. Our data therefore allude to the possibility that MCPH1 could interact directly with more than one component of APC/C, namely Cdc27 and Cdc26, via its C-terminal end. This may also explain why the S821A mutation in Cdc27, although detrimental, is not sufficient to completely disrupt the interaction with
MCPH1, as MCPH1 may interact with APC/C (and therefore its component Cdc27) via Cdc26. Apart from its interaction with Cdc27, MCPH1 tandem BRCT domains were also found to interact with γH2A.X under conditions of genotoxic stress. On DNA damage, MCPH1 relocates to γH2A.X-containing nucleosomes in what is thought to be a direct association (12, 40). Because irradiation results in the creation of a competing partner for MCPH1 C-BRCTs in the histone H2A.X, we asked whether DNA damage could influence the MCPH1-pCdc27 interaction. We hypothesized that if there were a relocalization of MCPH1 from the Cdc27-bound pool to γH2A.X, one would expect a concomitant decrease in the levels of Cdc27 being pulled down by MCPH1 following irradiation. However, if one does not observe such a change, then it could be ascribed to the presence of different pools of MCPH1, one associated with γH2A.X and another with Cdc27. Because we did not note any change in the Cdc27-bound fraction of MCPH1 after subjecting the cells to 10-Gy radiation, our finding lends credence to the latter hypothesis. Our finding also rules out a direct influence of DNA damage on the MCPH1-pCdc27 interaction by additional post-translational modifications. Biochemical and structural evidence suggests that only the last four terminal Cdc27 amino acids are critically required for the interaction with MCPH1 and the absence of an SQ/TQ phosphorylation motif within this site may explain the limited influence of DNA damage. Therefore, we believe that although Cdc27 may be additionally phosphorylated upon irradiation, the location of these sites outside of the "central" BRCT binding region may explain why these modifications Cdc27 continues to engage MCPH1.

Finally, what are the implications of the MCPH1-Cdc27 interaction? Because this interaction probably occurs within the ambit of the MCPH1-APC/C association, we envisage one of two outcomes. One possibility is that due to the similar nature of the loss-of-gene phenotype, MCPH1 may modulate the activity of APC/C. MitoCheck is a publicly accessible database that acts as a repository of the various mitotic defects associated with cells when genes are depleted by siRNA (41, 42). This resource therefore allows for an unbiased assessment of which genes, when depleted, result in a similar phenotype. Using MitoCheck we identified a phenotypic correlation between MCPH1 and Cdc27, as the loss of MCPH1 or Cdc27 results in similar mitotic phenotypes (Fig. 5A). Interestingly, knockdown of two other APC/C components known to interact with Cdc27, ANAPC11 and Cdc23, showed parallel phenotypes (Fig. 5A). The correlation between MCPH1 and APC/C could imply that MCPH1 activates or stabilizes APC/C (Fig. 5B). This would explain why a reduction in MCPH1 levels mirrors the phenotype of the APC/C hypomorphs. An alternate possibility is that MCPH1 is degraded by APC/C. Under this model Cdc27 may function as one of the subunits that mediate the interaction between MCPH1 and APC/C, leading to proteosome-dependent degradation of MCPH1 (Fig. 5B). Although one cannot rule out the interaction of MCPH1 with other APC/C subunits, immunoprecipitation experiments using single point mutants hint at the importance of Cdc27 in mediating the MCPH1-APC/C interaction. Although further studies are required to delineate the cellular consequence of this interaction, our work provides the first glimpse into how MCPH1 interacts with Cdc27 and sheds light on the structural basis for its interaction with Cdc27.

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REFERENCES

1. Lin, S. Y., and Elledge, S. J. (2003) Multiple tumor suppressor pathways negatively regulate telomerase. Cell 113, 881–889

2. Xu, X., Lee, J., and Stern, D. F. (2004) Microcephalin is a DNA damage response protein involved in regulation of CHK1 and BRCA1. J. Biol. Chem. 279, 34091–34094

3. Lin, S. Y., Rai, R., Li, K., Xu, Z. X., and Elledge, S. J. (2005) BRIT1/MCPH1 is a DNA damage-responsive protein that regulates the Brca1-Chk1 pathway, implicating checkpoint dysfunction in microcephaly. Proc. Natl. Acad. Sci. U.S.A. 102, 15105–15109

4. Alderton, G. K., Galbiati, L., Griffith, E., Surinya, K. H., Neitzel, H., Jack- son, A. P., Jeggo, P. A., and O’Driscoll, M. (2006) Regulation of mitotic entry by microcephalin and its overlap with ATR signaling. Nat. Cell Biol. 8, 725–733

5. Rai, R., Dai, H., Multani, A. S., Li, K., Chin, K., Gray, I., Lahad, J., P., Liang, J., Mills, G. B., Meric-Bernstam, F., and Lin, S. Y. (2006) BRIT1 regulates early DNA damage response, chromosomal integrity, and cancer. Cancer Cell 10, 145–157

6. Peng, G., Yim, E. K., Dai, H., Jackson, A. P., Burgt, I., Pan, M. R., Hu, R., Li, K., and Lin, S. Y. (2009) BRIT1/MCPH1 links chromatin remodeling to DNA damage response. Nat. Cell Biol. 11, 865–872

7. Wu, X., Mondal, G., Wang, X., Wu, J., Yang, L., Pankratz, V. S., Rowley, M., and Couch, F. J. (2009) Microcephalin regulates BRCA2 and Rad51-associated DNA double-strand break repair. Cancer Res. 69, 5531–5536

8. Peng, G., and Lin, S. Y. (2009) BRIT1/MCPH1 is a multifunctional DNA damage-responsive protein mediating DNA repair-associated chromatin remodeling. Cell Cycle 8, 3071–3072

9. Tubelis, A., Marhold, J., Zentgraf, H., Heilig, C. E., Niitzel, H., Dum- mun, B., Rauch, A., Ho, A. D., Bartek, J., and Krämer, A. (2009) Micro- cephalin and pericentrin regulate mitotic entry via centrosome-associated checkpoint. J. Cell Biol. 185, 1149–1157

10. Manke, I. A., Lowery, D. M., Nguyen, A., and Yaffe, M. B. (2003) BRCT repeats as phosphopeptide-binding modules involved in protein targeting. Science 302, 636–639

11. Yu, X., Chini, C. C., He, M., Mer, G., and Chen, J. (2003) The BRCT domain is a phospho-protein binding domain. Science 302, 639–642

12. Wood, J. L., Singh, N., Mer, G., and Chen, J. (2007) MCPH1 functions in an H2AX-dependent but MDC1-independent pathway in response to DNA damage. J. Biol. Chem. 282, 35416–35423

13. Shiozaki, E. N., Gu, L., Yan, N., and Shi, Y. (2004) Structure of the BRCT repeats of BRCA1 bound to a BACH1 phosphopeptide: implications for signaling. Mol. Cell 14, 405–412

14. Clapperton, J. A., Manke, I. A., Lowery, D. M., Ho, T., Haire, L. F., Yaffe, M. B., and Smardon, S. J. (2004) Structure and mechanism of BRCA1 BRCT domain recognition of phosphorylated BACH1 with implications for cancer. Nat. Struct. Mol. Biol. 11, 512–518

15. Williams, R. S., Lee, M. S., Hau, D. D., and Glover, J. N. (2004) Structural basis of phosphopeptide recognition by the BRCT domain of BRCA1. Nat. Struct. Mol. Biol. 11, 519–525

16. Botuyan, M. V., Nominé, Y., Yu, X., Juranic, N., Macura, S., Chen, J., and Mer, G. (2004) Structural basis of BACH1 phosphopeptide recognition by BRCA1 tandem BRCT domains. Structure 12, 1137–1146

17. Varma, A. K., Brown, G. R., Birrane, G., and Ladias, J. A. (2005) Structural basis for cell cycle checkpoint control by the BRCA1-CIP complex. Biochemistry 44, 10941–10946

18. Lee, M. S., Edwards, R. A., Thede, G. L., and Glover, J. N. (2005) Structure of the BRCT repeat domain of MDC1 and its specificity for the free COOH terminus of the γH2AX histone tail. J. Biol. Chem. 280, 32053–32056
MCPH1 and Anaphase-promoting Complex Cdc27 Subunit

19. Stucki, M., Clapertonn, J. A., Mohammad, D., Yaffe, M. B., Smerdon, S. J., and Jackson, S. P. (2005) MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell 123, 1213–1226

20. Nominé, Y., Botuyan, M. V., Bajzer, Z., Owen, W. G., Caride, A. I., Wisalewski, E., and Mer, G. (2008) Kinetic analysis of interaction of BRCA1 tandem breast cancer C-terminal domains with phosphorylated peptides reveals two binding conformations. Biochemistry 47, 9866–9879

21. Kilkenny, M. L., Doré, A. S., Roe, S. M., Nestoras, K., Ho, J. C., Watts, F. Z., and Pearl, L. H. (2008) Structural and functional analysis of the C2b2-BRCT domain reveals distinct roles in checkpoint signaling and DNA damage repair. Genes Dev. 22, 2034–2047

22. Williams, J. S., Williams, R. S., Dovey, C. L., Guenther, G., Tainer, J. A., and Russell, P. (2010) pH2A binds BrC1 to maintain genome integrity during S-phase. EMBO J. 29, 1136–1148

23. Leung, C. C., Gong, Z., Chen, J., and Glover, J. N. (2011) Molecular basis of BACH1/FANCJ recognition by TopBP1 in DNA replication checkpoint control. J. Biol. Chem. 286, 4292–4301

24. Xu, C., Wu, L., Cai, G., Botuyan, M. V., Chen, J., and Mer, G. (2008) Structure of a second BRCT domain identified in the nijmegen breakage syndrome protein Nbs1 and its function as an MDC1-dependent localization of Nbs1 to DNA damage sites. J. Mol. Biol. 381, 361–372

25. Campbell, S. J., Edwards, R. A., and Glover, J. N. (2010) Comparison of the structures and peptide binding specificities of the BRCT domains of MDC1 and BRCA1. Structure 18, 167–176

26. Schreiber, A., Stengel, F., Zhang, Z., Enchev, R. I., Kong, E. H., Morris, E. P., Robinson, C. V., da Fonseca, P. C., and Barford, D. (2011) Structural basis for the subunit assembly of the anaphase-promoting complex. Nature 470, 227–232

27. Pfugrath, J. W. (1999) The finer things in x-ray diffraction data collection. Acta Crystallogr. D Biol. Crystallogr. 55, 1718–1725

28. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674

29. Emsley, P., and Cowtan, K. (2004) COOT: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132

30. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, I. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221

31. Turnbull, W. B., and Daranas, A. H. (2003) On the value of c: can low affinity systems be studied by isothermal titration calorimetry? J. Am. Chem. Soc. 125, 14859–14866

32. Coster, G., Hayouka, Z., Argaman, L., Strauss, C., Friedler, A., Brandeis, M., and Goldberg, M. (2007) The DNA damage response mediator MDC1 directly interacts with the anaphase-promoting complex/cyclosome. J. Biol. Chem. 282, 32053–32064

33. Leung, C. C., and Glover, J. N. (2011) BRCT domains: easy as one, two, three. Cell Cycle 10, 2461–2470

34. Joughin, B. A., Tidor, B., and Yaffe, M. B. (2005) A computational method for the analysis and prediction of protein:phosphopeptide-binding sites. Protein Sci. 14, 131–139

35. Bork, P., Hofmann, K., Bucher, P., Neuwald, A. F., Altschul, S. F., and Koonin, E. V. (1997) A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. FASEB J. 11, 68–76

36. Callebaut, I., and Monro, J. P. (1997) From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. FEBS Lett. 400, 25–30

37. Williams, R. S., Green, R., and Glover, J. N. (2001) Crystal structure of the BRCT repeat region from the breast cancer-associated protein BRCA1. Nat. Struct. Biol. 8, 838–842

38. Joo, W. S., Jeffrey, P. D., Cantor, S. B., Finnin, M. S., Livingston, D. M., and Pavletich, N. P. (2002) Structure of the 53BP1 BRCT region bound to p53 and its comparison to the BRCA1 BRCT structure. Genes Dev. 16, 583–593

39. Rodriguez, M., Yu, X., Chen, I., and Songyang, Z. (2003) Phosphopeptide binding specificities of BRCA1 COOH-terminal (BRCT) domains. J. Biol. Chem. 278, 52914–52918

40. Jeffers, L. J., Coull, B. J., Stack, S. J., and Morrison, C. G. (2008) Distinct BRCT domains in Mcp1/Brf1 mediate ionizing radiation-induced focus formation and centrosomal localization. Oncogene 27, 139–144

41. Neumann, B., Walter, T., Hériché, J. K., Bulkescher, J., Erfle, H., Conrad, C., Rogers, P., Poser, I., Held, M., Liebel, U., Cetin, C., Sieckmann, F., Pau, G., Kabbe, R., Wünsche, A., Satagopam, V., Schmitz, M. H., Chapuis, C., Gerlich, D. W., Schneider, R., Els, R., Huber, W., Peters, J. M., Hyman, A. A., Durbin, R., Pepperkok, R., and Ellenberg, J. (2010) Phenotypic profiling of the human genome by time-lapse microscopy reveals cell division genes. Nature 464, 721–727

42. Hutchins, J. R., Toyoda, Y., Hegemann, B., Poser, I., Hériché, J. K., Sykora, M. M., Augsburg, M., Hudecz, O., Buschhorn, B. A., Bulkescher, J., Conrad, C., Comartin, D., Schleifer, A., Sarov, M., Pozniakovsky, A., Slabicki, M. M., Schloissnig, S., Steinmacher, I., Leuehncher, M., Seykor, A., Lawo, S., Pelletier, L., Stark, H., Nasmyth, K., Ellenberg, J., Durbin, R., Buchholz, F., Mechtler, K., Hyman, A. A., and Peters, J. M. (2010) Systematic analysis of human protein complexes identifies chromosome segregation proteins. Science 328, 593–599

43. Delano, W. F. (2010) The PyMOL Molecular Graphics System, version 1.3r1, Schrödinger, LLC, New York