Microcephalin Regulates BRCA2 and Rad51-Associated DNA Double-Strand Break Repair

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Abstract

Microcephalin (MCPH1) is a BRCA1 COOH terminal (BRCT) domain containing protein involved in the cellular response to DNA damage that has been implicated in autosomal recessive primary microcephaly. MCPH1 is recruited to sites of DNA double-strand breaks by phosphorylated histone H2AX (γH2AX), but the mechanism by which MCPH1 contributes to the repair process remains to be determined. Here, we show that MCPH1 binds to BRCA2 and regulates the localization of BRCA2 and Rad51 at sites of DNA damage. The interaction occurs through the NH2 terminus of BRCA2 and the COOH terminal BRCT domains of MCPH1. Disruption of the interaction between MCPH1 and BRCA2 has no effect on the ability of BRCA2 to form a complex with Rad51 but is associated with substantially reduced levels of both BRCA2 and Rad51 at sites of DNA double-strand breaks. Uncoupling of MCPH1 from BRCA2 also interferes with Rad51-dependent and BRCA2-dependent homologous recombination repair activity. These results suggest that the role of MCPH1 in the DNA damage response is in part associated with the ability to localize BRCA2 to sites of DNA double-strand breaks.

Introduction

Microcephalin (MCPH1) is a member of the BRCA1 COOH terminal (BRCT) domain family of proteins that are involved in the cellular response to DNA damage (1, 2). MCPH1 contains a single NH2 terminal and two COOH terminal BRCT domains. Biallelic mutations in the MCPH1 gene are associated with primary microcephaly (ref. 3; OMIM 251200) and premature chromosome condensation (PCC) syndrome (refs. 4, 5; OMIM 606858). Mutations in other DNA damage response genes, including Nijmegen condensation syndrome (ref. 4), Nijmegen breakage syndrome (NBS1; ref. 6), ataxia-telangiectasia (7), and ataxia-telangiectasia and Rad3-related protein (ATR; ref. 8) are also associated with substantially reduced levels of both BRCA2 and Rad51 at sites of DNA double-strand breaks. The recent observation that MCPH1 that interacts with Condensin II through the CAPG2 subunit is required for homologous recombination activity in response to DNA double-strand breaks in mouse embryonic fibroblasts (MEF) reconstituted with MCPH1 (15).

Materials and Methods

Plasmids and antibodies. FLAG-tagged BRCA2 fragments were cloned in pEV3S-FLAG3 vector. Glutathione S-transferase (GST)-tagged BRCA2 fragments (B2F1–B2F7) in pEGB and FLAG-tagged fragments (B2F1–B2F7) in pcDNA3.1 were provided by Dr. J. Chen (Yale University). The anti-BRCA2 rabbit polyclonal antibody was raised against residues 92 to 214 of MCPH1. The anti-BRCA2 rabbit polyclonal antibody was raised against the COOH terminal of BRCA2 (residues 2418–2501). The antiserum was affinity purified with AminoLink Plus immobilization and purification kit (Pierce). Anti-γH2AX, 53BP1, MDC1, and Rad51 polyclonal antibodies were gifts from Dr. J. Chen (Yale University). Mouse anti-Flag (M2) antibody was purchased from Sigma. Mouse anti-HA antibody was purchased from Roche. Mouse anti-Myc antibody was purchased from Sigma.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). X. Wu and G. Mondal are joint primary authors.

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doi:10.1158/0008-5472.CAN-08-4834
Short interfering RNA. Short interfering RNAs (siRNA) against MCPH1 were synthesized by Dharmacon, Inc. The siRNA duplexes were as follows: MCPH1 siRNA1 sense strand, 5’ C TCT CTG TGT GAA GCA CCA; MCPH1 siRNA2 sense strand, 5’AA CTT CAG AAG AGA GGC GT. BRCA2 siRNAs (SMARTpool) were purchased from Dharmacon. Transfections were performed with 200 nmol/L siRNA using FuGene-6 (Roche) according to the manufacturer's instructions.

Commmunoprecipitation. 293T cells were purchased from American Type Culture Collection and maintained in DMEM supplemented with 10% bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO2 (v/v). VU423 FANCDD1 (FA-D1) and A9113 423-2-33 BRCA2-reconstituted FANCDD1 cells were grown under similar conditions (16). Cells were lysed with NETN buffer [20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Nonidet P-40] containing protease inhibitors (Complete, Boehringer Mannheim Biologicals, Inc.) 2 h after exposure to 10 Gy of irradiation or in the absence of irradiation (data not shown). Whole-cell lysates obtained after centrifugation were incubated with 2 μg antibody and Protein A or Protein G sepharose beads (Amersham Biosciences) for 2 h at 4°C. For GST pull downs of GST-tagged proteins, cleared cell lysates were incubated with glutathione agarose beads (Sigma).

Immunofluorescence. 293T cells cultured on glass coverslips were irradiated (10 Gy), incubated for 3 h, and analyzed for formation of DNA damage repair foci. Cells were fixed with cold methanol for 20 min, permeabilized in 0.05% Triton X-100 for 15 min, incubated in blocking buffer (16) and incubated with primary antibodies diluted in blocking buffer for 1 h at room temperature. After washing in PBS, cells were incubated with Alexa Fluor 488 and 568–conjugated goat anti-mouse and goat anti-rabbit IgG secondary antibodies (Molecular Probes). Cells were mounted with 4,6-diamidino-2-phenylindole (Vector). After a further wash in PBS, coverslips were mounted in Prolong (Molecular Probes). Cells were visualized and imaged using a Zeiss LSM510 confocal microscope. For each experiment, 100 nuclei transfected with expression constructs or siRNAs were evaluated. Effects on foci were consistently observed in >80% of transfected cells in each experiment.

Homologous recombination repair assay. The homologous recombination repair assay was carried out as described previously (17). Briefly, the efficiency of homology-directed repair was assessed using an 1-Scel expression plasmid (pHBASce) and an 1-Scel repair reporter plasmid (DR-GFP) composed of two differentially mutated green fluorescent protein (GFP) genes, one of which contains a unique I-SceI restriction site. Here, HeLa cells or V-C8 BRCA2 null hamster lung fibroblast cells that were stably transfected with a single copy of DR-GFP (16) were cotransfected with 1-Scel and BRCA2 or MCPH1 siRNAs, and the number of GFP-expressing cells was assessed by flow cytometry after 48 h. Transfection efficiency was defined by immunofluorescence analysis of FLAG-tagged BRCA2 constructs and was used to normalize homologous recombination repair levels. Statistical significance relative to wild-type BRCA2 or vector control was determined using paired t tests and Fisher's protected least significant difference pair-wise comparison procedure.

Results

MCPH1 interacts with BRCA2. Whereas it has been noted that MCPH1 is one of the first signaling proteins recruited to nuclear foci after DNA damage, there is also evidence that MCPH1 functions downstream of Chk1 and BRCA1 (13) and that MCPH1 is retained at sites of damage beyond the initial recognition stage (12). This suggests that MCPH1 contributes to DNA repair at multiple levels. To elucidate the involvement of MCPH1 in the response to DNA double-strand breaks, we explored potential interactions between MCPH1 and components of DNA damage signaling and repair pathways. Extracts from 293T cells ectopically expressing Myc-tagged MCPH1 were exposed to 10 Gy of irradiation and screened for interactions between MCPH1 and a series of proteins involved in the DNA repair process. We reproducibly found that myc-tagged MCPH1 coimmunoprecipitated with BRCA2 and Rad51 (Fig. 1A and B). In separate experiments, a significant amount of endogenous MCPH1 associated with endogenous BRCA2 and Rad51 (Fig. 1C and D). These interactions were also evident in the absence of irradiation. Together, these results suggest that MCPH1 interacts with BRCA2-Rad51 complexes in the presence and absence of DNA damage.

The MCPH1 BRCT domains are required for interaction with the NH2 terminus of BRCA2. To identify regions of BRCA2 that are responsible for the MCPH1 interaction, we generated a series of constructs encoding fragments of BRCA2 (Fig. 2A; Supplementary Table S1). The fragment B2F1, encompassing BRCA2 residues 1 to 472, accounted for the interaction between BRCA2 and MCPH1 (Fig. 2A). Deletion mutants of B2F1 mapped the MCPH1 interaction region to residues 234 to 335 (B2F2; Fig. 2B). Further deletion analysis within B2F2 showed that residues 294 to 335 in B2F2ΔΔ were sufficient for the interaction (Fig. 2C). However, deletion of residues 293 to 323 in B2F2Δ3 had no effect on the interaction (Fig. 2D). These findings indicate that a previously undefined BRCA2 NH2 terminal domain and the MCPH1 BRCT2 domain are necessary for the interaction of BRCA2 with MCPH1.

Localization of BRCA2 at sites of DNA damage is dependent on MCPH1. Whereas MCPH1 is one of the first proteins recruited to sites of radiation-induced DNA damage, the interaction of MCPH1 with BRCA2 and Rad51 suggests that MCPH1 may function at multiple levels within the DNA damage signaling pathway. Here, we focused on determining the influence of MCPH1 on distal DNA damage signaling pathways. Specifically, we evaluated whether MCPH1 influenced the presence of BRCA2 and Rad51 at sites of DNA damage. As expected, MCPH1 was rapidly recruited to DNA damage foci after irradiation, where it colocalized with 53BP1, BRCA2, and Rad51, and MCPH1 in 293T cells were assessed by coimmunoprecipitation and immunoblotting.

Figure 1. MCPH1 interacts with BRCA2 and Rad51. A, whole-cell lysates (WCL) from 293T cells transfected with Myc-MCPH1 and treated with 10 Gy of irradiation were immunoprecipitated (IP) with anti-BRCA2 and anti-Rad51 antibodies and immunoblotted with anti-Myc (MCPH1) antibody. B, lysates immunoprecipitated with Myc were immunoblotted for BRCA2, Rad51, and Myc-tagged MCPH1. C and D, interactions between endogenous BRCA2, Rad51, and MCPH1 in 293T cells were assessed by coimmunoprecipitation and immunoblotting.

Cancer Res 2009; 69: (13). July 1, 2009 5532 www.aacrjournals.org
γH2AX (Supplementary Fig. S2a and c), and Rad51 (Fig. 3A) in all cells examined. However, when MCPH1 was depleted by siRNAs (Supplementary Fig. S2b and c), a substantial reduction in the number and intensity of Rad51 foci (Fig. 3A) and BRCA2 foci (Supplementary Fig. S2d) was consistently observed. In contrast, the MCPH1 siRNAs had little influence on recruitment of 53BP1 (Fig. 3A; Supplementary Fig. S2c) and MDC1 (Supplementary Fig. S2d) to foci, suggesting that the proximal DNA damage response pathway remained intact. This is consistent with recent findings that MCPH1 functions in an γH2AX-dependent but MDC1-independent DNA damage response pathway (11). Together, these results indicate that MCPH1 is an important mediator of the BRCA2-associated and Rad51-associated double-strand DNA break repair process.

To determine whether the influence of MCPH1 on DNA damage repair foci is dependent on the interaction of MCPH1 with BRCA2 and Rad51, we assessed the effect of MCPH1 mutants with deletions of the NH2 terminal BRCT1 and COOH terminal BRCT2 and BRCT3 domains on radiation foci. Absence of the NH2 terminal BRCT domain had no effect on localization of 53BP1 (Fig. 3A; Supplementary Fig. S2c) and MDC1 (Supplementary Fig. S2d) to foci, suggesting that the proximal DNA damage response pathway remained intact. This is consistent with recent findings that MCPH1 functions in an γH2AX-dependent but MDC1-independent DNA damage response pathway (11). Together, these results indicate that MCPH1 is an important mediator of the BRCA2-associated and Rad51-associated double-strand DNA break repair process.

MCPH1 does not influence formation of BRCA2-Rad51 complexes. To determine the influence of MCPH1 on the established interaction between BRCA2 and Rad51, we generated a deletion mutant (BRCA2-D328-351) of full-length BRCA2 (Supplementary Table S1) that disrupts part of the MCPH1 interaction site defined by the deletion mapping studies (Fig. 2C and D). Similarly to BRCA2 siRNA, this mutant had no influence on the localization of MCPH1, MDC1, and 53BP1 to sites of DNA damage (Fig. 3C; Supplementary Fig. S4a and b) but substantially reduced the number and intensity of BRCA2 and Rad51 foci (Fig. 3C; Supplementary Fig. S4b). Likewise, the B2F12 fragment that contains the MCPH1 interaction site and binds to MCPH1 inhibited the localization of MCPH1, BRCA2, and Rad51 to DNA damage foci (Supplementary Fig. S5a) in a dominant negative manner. In contrast, the B2F12ΔR6 construct, in which the MCPH1 interaction domain is disrupted, had no influence on MCPH1, BRCA2, or Rad51 focus formation (Supplementary Fig. S5b). These results suggest that the presence of Rad51 and BRCA2 at DNA damage foci is dependent on the interaction between BRCA2 and MCPH1.

Importantly, we also found that the BRCA2-Δ328-351 deletion mutant retained the ability to coimmunoprecipitate with Rad51 after DNA damage (Fig. 3D). Similarly, deletion of the MCPH1, BRCT2, and BRCT3 domains that mediate the interaction with BRCA2 and the presence of BRCA2 and Rad51 at foci had no effect.
Figure 3. The MCPH1-BRCA2 interaction is required for localization of Rad51 at sites of DNA damage. 

A, MCPH1 colocalizes with Rad51 after DNA damage. 293T cells either not transfected (Control) or transfected with a Myc-tagged MCPH1 expression construct (WT-MCPH1) were stained with anti-Rad51 (red) and anti-Myc (green) antibodies 3 h after exposure to 10 Gy of radiation to visualize ionizing radiation–induced nuclear foci. Likewise, 293T cells transfected with MCPH1 siRNA for 48 h were exposed to 10 Gy of radiation and stained with anti-Rad51 (red) and anti-53BP1 (green) antibodies after 3 h to visualize depletion of Rad51 foci.

B, cells transfected with plasmids encoding Myc-tagged MCPH1 deletion constructs were treated and evaluated for MCPH1 and BRCA2 foci by staining for Myc (red) and BRCA2 (green). Separately, the same cells were evaluated for Rad51 and Myc-tagged MCPH1 foci by staining for Myc (red) and BRCA2 (green). 

C, 293T cells transfected with FLAG-tagged wild-type BRCA2 (FLAG-WT-BRCA2) or FLAG-tagged BRCA2Δ328-351 deletion mutant were incubated for 48 h, exposed to 10 Gy of radiation, and stained after 3 h for FLAG (red) and BRCA2, MCPH1, Rad51, or MDC1 (green).

D, lysates (WCL) from 293T cells transfected with Flag-tagged wild-type BRCA2 (WT-BRCA2) and the Flag-tagged BRCA2Δ328-351 deletion construct were coimmunoprecipitated with anti-Rad51 antibody and immunoblotted with anti-FLAG antibody. Lysates from cells expressing wild-type (WT-MCPH1) and deletion mutants of MCPH1 were coimmunoprecipitated with anti-BRCA2 antibody and immunoblotted with Rad51 antibody and anti-Myc antibody to verify MCPH1 expression. VU423 FANCD1 cells (FA-D1) and A913 423/2-33 FANCD1 cells reconstituted with BRCA2 (FA-D1 + BRCA2) were transfected with Myc-MCPH1, immunoprecipitated with anti-Rad51 antibody, and immunoblotted with anti-Myc and anti-BRCA2 antibodies. Lysates were immunoblotted with anti-Rad51 antibody to verify Rad51 expression.
on the BRCA2-Rad51 complex (Fig. 3D). We further explored this
effect using BRCA2-deficient FANCD1 cells and FANCD1 cells
reconstituted with wild-type BRCA2. After DNA damage, MCPH1
coimmunoprecipitated with Rad51 from the BRCA2 reconstituted
cells, but not from the BRCA2-deficient cells (Fig. 3D), suggesting
that the Rad51 interaction with MCPH is dependent on BRCA2.
Together, these results indicate that MCPH1 interacts with and
controls the presence of the BRCA2-Rad51 complex at sites of DNA
damage.

**MCPH1 influences BRCA2-dependent homologous recombi-
nation repair.** Given the direct involvement of the BRCA2-Rad51
complex in homologous recombination repair of DNA double-
strand breaks, these results suggest that MCPH1 may influence
homologous recombination repair. We evaluated the influence of
MCPH1 on homologous recombination using an *in vivo* assay
(18–22) that depends on repair of a double-strand break at a
unique I-Sce1 restriction endonuclease site (Fig. 4A). Depletion of
MCPH1 from HeLa cells by siRNA significantly reduced homolo-
gous recombination repair activity (Fig. 4B). To determine whether the interaction of MCPH1
with BRCA2 accounts for the influence of MCPH1 on homologous
recombination, we evaluated a series of BRCA2 deletion constructs
(Fig. 2; Supplementary and Table S1) using the *in vivo* assay.

Expression of B2F12 and B2F12Δ5, which contain the MCPH1
interaction domain, dominantly negatively inhibited homologous
recombination activity (*P* = 0.002; Fig. 4C). In contrast, three
BRCA2 fragments (B2F12ΔR6, B2F12ΔR7, and B2F12ΔR8) that do
not interact with MCPH1 did not significantly disrupt homologous
recombination activity (Fig. 4C). To confirm these effects in the
context of full-length BRCA2, the influence of the BRCA2-Δ328-351
deletion mutant on homologous recombination activity relative to
wild-type BRCA2 and a known deleterious missense mutant
(D2723H) was assessed in BRCA2-null V-C8 cells containing the
DR-GFP construct. Consistent with the results described above, the
BRCA2-Δ328-351 deletion mutant caused a partial but statistically
significant reduction in activity relative to wild-type BRCA2
(*P* = 0.009) similarly to D2723H (*P* = 0.001; Fig. 4D). These data indicate
that the interaction between MCPH1 and BRCA2 mediates the
homologous recombination repair activity of the BRCA2-Rad51
complex.

**Discussion**

Although several studies suggest that MCPH1 is one of the first
proteins recruited to sites of irradiation-induced foci where it is
thought to mediate the response to DNA damage (10–14), little is
known about how MCPH1 influences DNA repair. Recent studies

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**Figure 4.** The MCPH1-BRCA2 interaction influences homologous recombination repair. A, diagram of the homologous recombination repair reporter assay. B, homologous recombination repair activity in a HeLa DR-GFP cell line is reduced relative to vector controls when MCPH1 is depleted by MCPH1 siRNA. C, homologous recombination repair activity in a HeLa DR-GFP cell line cotransfected with I-Sce1 plasmid and BRCA2 deletion constructs with the MCPH1 interaction domain (B2F12, B2F12Δ5) and without the interaction domain (B2F12ΔR6-8). D, homologous recombination repair activity in a V-C8 DR-GFP cell line, using BRCA2-Δ328-351 and BRCA2-D2723H full-length mutant constructs.
have suggested that MCPH1 controls the cell cycle response to DNA damage by binding and mediating Chk1 and Cdc25a activity (13) and by regulating both the S and G2–M checkpoints. In addition, the interaction between MCPH1 and Condensin II is apparently not associated with PCC but is required for homologous recombination repair of DNA double-strand breaks (15), suggesting that MCPH1 may have a role in the process of chromosome condensation and perhaps chromatin remodeling in response to DNA damage.

Here, we report that the presence of BRCA2-Rad51 complexes at sites of DNA double-strand breaks is dependent on an interaction between the NH2 terminus of BRCA2 and the COOH terminal tandem-BRCT domains of MCPH1. Importantly, BRCA2 and Rad51 form complexes in the absence of MCPH1, albeit not at sites of DNA damage, suggesting that MCPH1 is required for the recruitment and/or retention of BRCA2-Rad51 complexes to repair foci. We also show that MCPH1 can regulate homologous recombination repair of DNA double-strand breaks in a BRCA2-dependent manner. These findings are consistent with the observation that MCPH1−/− MEFs exhibit defects in homologous recombination repair of DNA damage (15). Thus, MCPH1 seems to contribute to homologous recombination repair through interactions with BRCA2 and the Condensin II complex (15).

Our findings extend our knowledge of MCPH1 and suggest that it forms an important link between the initial sensors and effectors of the DNA damage response, as defined by its rapid recruitment to DNA repair foci through an interaction with γH2AX, and the process of DNA repair, as established by its ability to regulate the localization of BRCA2-Rad51 complexes to sites of damage. Further efforts are needed to better understand how MCPH1 integrates these components of the DNA damage response signaling pathway.

Interestingly, it has recently been shown that Akt1 can repress homologous recombination through cytoplasmic retention of BRCA1 and Rad51 (23). It remains to be seen whether Akt1 signaling influences MCPH1-dependent recruitment and/or retention of BRCA2-Rad51 complexes at sites of damage. Furthermore, MEFs deficient in the SIRT1 histone deacetylate have been shown to exhibit significantly reduced recruitment of BRCA1, Rad51, and 53BP1 to DNA double-strand breaks and reduced homologous recombination activity (24). Given the role of SIRT1 in chromatin remodeling and the recent finding that SIRT1 localizes to DNA breaks to promote repair (25), further studies aimed at understanding the relationship between SIRT1 and components of the DNA signaling pathway, such as MCPH1 and BRCA2 are needed.

The ability of MCPH1 to regulate BRCA2 activity suggests that down-regulation or mutation of MCPH1 could lead to defects in DNA repair similar to those associated with BRCA2 inactivation. Importantly, down-regulation of MCPH1 has been observed in a number of tumors. Thus, MCPH1 may function as a tumor suppressor. Whereas mutation of both MCPH1 alleles is associated with PCC and primary microcephaly (5), mutation of a single allele may predispose or contribute to cancer development. Future studies aimed at detecting mutations in this gene and studies using knockout mice will test whether MCPH1 functions as a tumor suppressor in vivo. In addition, because MCPH1 seems to mediate BRCA2 function, it may be possible to use poly(ADP-ribose) polymerase inhibitors or DNA cross-linking agents that seem to be particularly effective against BRCA2-deficient cells to treat tumors with reduced levels of MCPH1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 12/18/08; revised 3/30/09; accepted 4/23/09; published OnlineFirst 6/23/09.

Grant support: NIH grants CA102701 and CA116167 (F.J. Couch).

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Cancer Res 2009; 69: (13). July 1, 2009 5536 www.aacrjournals.org

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Cancer Res  Published OnlineFirst June 23, 2009.

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doi:10.1158/0008-5472.CAN-08-4834

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