

SHORT COMMUNICATION

Distinct BRCT domains in Mcph1/Brit1 mediate ionizing radiation-induced focus formation and centrosomal localization

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Microcephalin (*MCPH1*/*BRIT1*) forms ionizing radiation-induced nuclear foci (IRIF) and is required for DNA damage-responsive S and G₂-M-phase checkpoints. *MCPH1* contains three BRCT domains. Here we report the cloning of chicken *Mcph1* (*cMcph1*) and functional analysis of its individual BRCT domains. Full-length *cMcph1* localized to centrosomes throughout the cell cycle and formed IRIF that colocalized with γ -H2AX. The tandem C-terminal BRCT2 and BRCT3 domains of *cMcph1* were necessary for IRIF formation, while the N-terminal BRCT1 was required for centrosomal localization in irradiated cells. Centrosomal targeting of *cMcph1* was independent of ATM, *Bracl* or Chk1. *cMcph1* formed IRIF in ATM- and *Bracl*-deficient cells, but not in H2AX-deficient cells. Inability to form *cMcph1* IRIF impaired the cellular response to DNA damage. These results suggest that the role of microcephalin in the vertebrate DNA damage response is controlled by interaction of the C-terminal BRCT domains with γ -H2AX.

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The tumour suppressor BRCA1 is involved in numerous activities that maintain genome stability (Starita and Parvin, 2003). Crucial to the functions of BRCA1 are a pair of C-terminal domains, which can recognize phosphopeptides and mediate protein–protein interactions in the response to DNA damage (Manke *et al.*, 2003; Yu *et al.*, 2003). These BRCA1 C-terminal (BRCT) repeats are found in a large superfamily of proteins involved in cellular responses to genotoxic stress (Glover *et al.*, 2004).

One member of this BRCT domain-containing superfamily is microcephalin (*MCPH1*). *MCPH1* is encoded by *MCPH1*/*BRIT1* (hereafter *MCPH1*), a gene mutated in primary microcephaly (OMIM 251200) (Jack-

son *et al.*, 2002) and in premature chromosome condensation syndrome (OMIM 606858) (Neitzel *et al.*, 2002; Trimborn *et al.*, 2004). *MCPH1* has been described as a transcriptional repressor of human telomerase reverse transcriptase, from which the alternative gene name of *BRIT1*, for BRCT-repeat inhibitor of hTERT expression, was derived (Lin and Elledge, 2003). *MCPH1* is involved in the DNA damage-responsive S and G₂-M-phase checkpoints, although the precise mechanism is not yet clear (Xu *et al.*, 2004; Lin *et al.*, 2005; Alderton *et al.*, 2006). *MCPH1* colocalizes in ionizing radiation-induced nuclear foci (IRIF) with MDC1/NFBD1 (Xu *et al.*, 2004) and phosphorylated H2AX (γ -H2AX) (Lin *et al.*, 2005), a chromatin modification that occurs in a large region around a DNA double-strand break (Rogakou *et al.*, 1999). A recent study (Rai *et al.*, 2006) found that depletion of *MCPH1* in human cells blocked formation of IRIF by NBS1, 53BP1, phosphorylated ATM and MDC1, but not γ -H2AX. DNA damage-induced chromatin association of several DNA damage response proteins was also blocked by *MCPH1* RNAi and a high level of chromosome aberrations was observed. *MCPH1* aberrations were frequently detected in breast, ovarian and prostate cancer (Rai *et al.*, 2006). Together, these data suggest an important role for *MCPH1* in DNA damage responses and in preventing cellular transformation.

We identified the chicken *MCPH1* orthologue by database analysis, cloned it by RT-PCR and confirmed the sequence (DDBJ/EMBL/GenBank accession no. DQ788861). Zebrafish *Mcph1* sequence was derived by database analysis and these sequences were aligned with known mammalian *Mcph1* sequences using ClustalW. As shown in Supplementary Figure S1, all vertebrate sequences analysed showed similar organization, with a highly conserved single N-terminal BRCT domain and a pair of C-terminal BRCT domains, which will be referred to here as BRCT1, BRCT2 and BRCT3, respectively. The conserved BRCT1 and BRCT2–BRCT3 regions are separated by a poorly conserved region that does not contain any distinct structural motifs, as determined by SMART domain search at <http://smart.embl-heidelberg.de/>. These findings suggest that key regions for microcephalin function lie within the BRCT domains.

We expressed recombinant, tagged *cMcph1* in chicken DT40 cells and examined its subcellular localization. As

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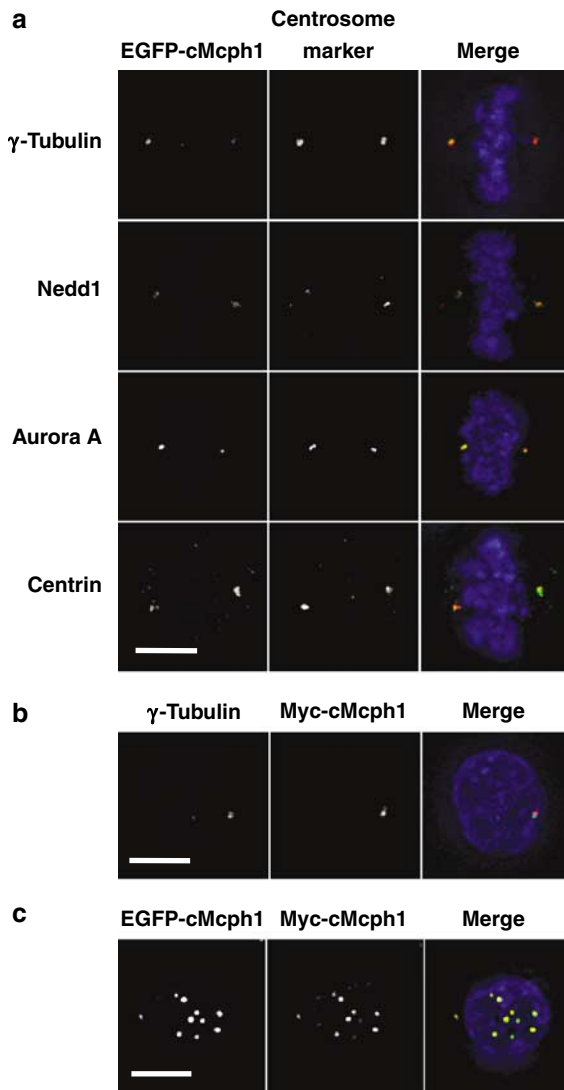


Figure 1 Centrosomal localization of cMcp1. Cloning and primary antibodies are detailed in Supplementary Information. Culture of wild-type, *Brcal*^{-/-} cells (R Franklin and K Hiom, in preparation), *Chk1*^{-/-} (Zachos *et al.*, 2003) and *H2AX* mutant (Sonoda *et al.*, 2007) DT40 cells and stable transfections by electroporation were performed as described previously (Dodson *et al.*, 2004). For transient transfections, 15 μ g of endotoxin-free DNA (Qiagen, Crawley, UK) was introduced into 5×10^6 cells using nucleofection (Amaxa, Cologne, Germany, programme B-23). Cells were fixed and prepared for microscopy and imaging performed using an Olympus BX51 microscope as described (Dodson *et al.*, 2004). Cells were counterstained with DAPI (blue) before fluorescence microscopy. Scale bars, 5 μ m. (a) EGFP-cMcp1 (green)-expressing DT40 cells stained with antibodies to the centrosomal or pericentrosomal proteins (red) indicated at left. (b) Myc-cMcp1-expressing (green) DT40 cell stained for γ -tubulin (red). (c) EGFP-cMcp1 (green)-expressing DT40 cell transiently transfected with a myc-cMcp1 expression construct and then stained with anti-myc antibodies (red) after 10 Gy irradiation (IR).

shown in Figure 1a, N-terminally, EGFP-tagged cMcp1 localized to γ -tubulin-containing foci, which we confirmed as centrosomes by immunofluorescence microscopy with antibodies against the centrosome markers centrin, Aurora-A and the pericentriolar

marker, Nedd1 (Haren *et al.*, 2006). C-terminally tagged cMcp1-EGFP and myc-cMcp1 also localized to centrosomes (unpublished data and Figure 1b), and we saw a complete colocalization of EGFP-cMcp1 and myc-cMcp1 (Figure 1c), demonstrating that the observed localization was not a tagging artefact.

To confirm our observations on cMcp1 localization, we raised an antiserum (11989) to the N-terminal part of cMcp1. In immunofluorescence microscopy experiments, this antiserum recognized overexpressed myc-tagged cMcp1 (Figure 2a), demonstrating its specificity for cMcp1. Using these anti-cMcp1 antibodies, we observed endogenous cMcp1 in IRIF that colocalized with those formed by γ -H2AX (Figure 2b) and the centrosomal localization of cMcp1 throughout the cell cycle (Figure 2c). While a centrosomal signal was consistently observed with antiserum 11989, we saw IRIF in only a small fraction of irradiated cells. As 11989 was raised and affinity-purified against an epitope-tagged fragment of cMcp1, it is likely that reactivity to this epitope reduces the anti-cMcp1-specific titre of the antiserum. We are generating additional antisera to explore this issue further. Together with published data on Mcp1 localization (Xu *et al.*, 2004; Lin *et al.*, 2005), these observations indicate that the localization of tagged, overexpressed cMcp1 reflects the behaviour of the endogenous protein.

Central to the signalling cascades that control cell cycle arrests after DNA damage are the ATM and ATR kinases, members of a family of large, phosphatidylinositol 3-OH kinase-related serine-threonine kinases (PIKKs; Shiloh, 2003) that activate the Chk1 and Chk2 effector kinases to impose cell cycle checkpoints (Bartek and Lukas, 2003). We tested whether the localization of cMcp1 to centrosomes was dependent on ATM or Brcal by examining EGFP-cMcp1 localization in *Atm*^{-/-} and *Brcal*^{-/-} DT40 cells. As shown in Figure 3a, we observed EGFP-cMcp1 at the centrosome in both knockout lines, demonstrating that its centrosomal localization is independent of ATM and Brcal. We also observed EGFP-cMcp1 at the centrosome in *Chk1*^{-/-} DT40 cells (unpublished data), but the further definition of the localization was impaired by an apparent reduction in H2AX phosphorylation in Chk1-deficient cells. Centrosomal localization of cMcp1 was unaffected by ionizing radiation, was independent of cell cycle stage, and occurred at multiple centrosomes that were induced by DNA damage (unpublished data), suggesting that cMcp1 is a constitutive component of the centrosome, similar to the products of the three other identified genes that are mutated in primary microcephaly, CDK5RAP2 (*MCPH3*), ASPM (*MCPH5*) and CENPJ (*MCPH6*; Bond *et al.*, 2005).

Next, we tested for the formation of IRIF by cMcp1. Myc-tagged cMcp1 colocalized with EGFP-labelled cMcp1 (Figure 1c), which in turn localized to γ -H2AX foci (Figure 3a). The initial, rapid induction of γ -H2AX foci after irradiation (IR) is followed by a reduction in the number of foci over several hours, presumably reflecting the loss of a DNA damage signal following repair (Paull *et al.*, 2000). cMcp1 also localized to

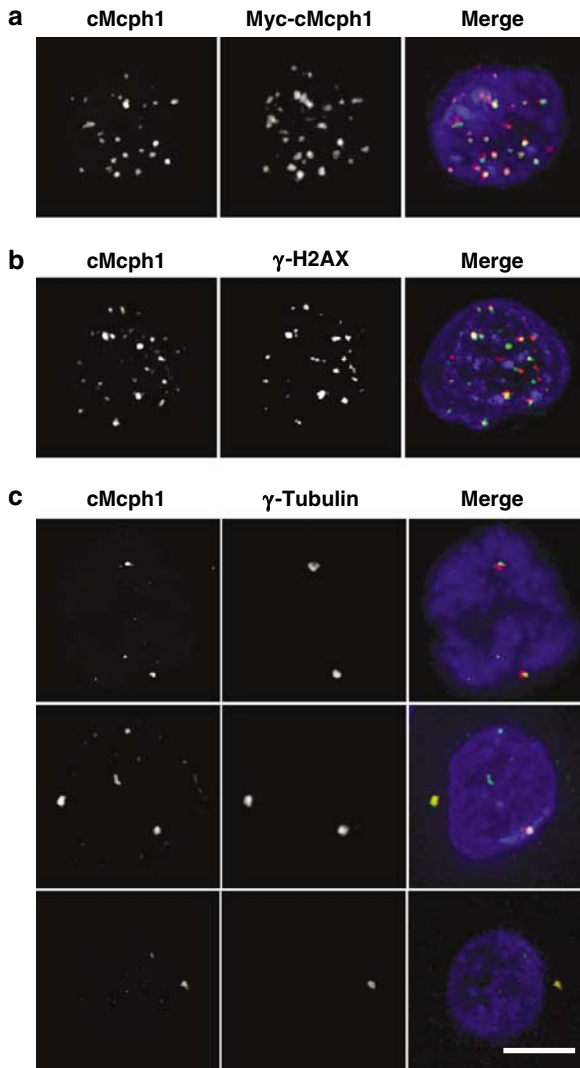


Figure 2 Immunofluorescence microscopy of cMcp1. The N-terminal 248 amino acids of cMcp1 were bacterially expressed as a His-tagged fusion protein by cloning into pET30a (Novagen, Madison, WI, USA), then purified over a HIS-Select nickel affinity gel (Sigma, Dublin, Ireland) and used as an immunogen to generate rabbit polyclonal anti-cMcp1 serum 11989 (Harlan Seralabs, Loughborough, UK). (a) Myc-cMcp1 (red)-expressing DT40 cells were stained with affinity-purified anti-cMcp1 11989 (green). Micrograph shows recognition by the anti-cMcp1 antiserum of the myc-cMcp1 IRIF after 10 Gy irradiation (IR). (b) Immunofluorescence micrograph of a wild-type DT40 cell showing colocalization of cMcp1 (green) with γ -H2AX foci (red) induced by 10 Gy IR. (c) Immunofluorescence micrograph of a wild-type DT40 cells showing centrosomal colocalization of cMcp1 (green) with γ -tubulin (red) in mitotic, S/G₂ and G₁-phase cells (upper, middle and lower panels, respectively). Cells were counterstained with DAPI (blue) before fluorescence microscopy. Scale bars, 5 μ m.

homologous recombinational repair foci formed by the Rad51 recombinase after IR (Figure 3b; Paull *et al.*, 2000), showing that cMcp1 persists at sites of DNA damage beyond the initial recognition phase. The kinetics of EGFP-cMcp1 focus formation and disappearance paralleled the behaviour of γ -H2AX in wild-type and in *Atm*^{-/-} and *Brcal*^{-/-} DT40 cells (Figure 3c), consistent with an upstream role in the DNA damage

response for Mcph1 (Rai *et al.*, 2006). Notably, the delay in the disappearance of IRIF in *Brcal*^{-/-} cells that reflects the repair deficiency in this cell line (Vandenberg *et al.*, 2003) was mirrored by a delay in the resolution of Mcph1 foci. These data demonstrate that cMcp1 foci are controlled in a DNA damage responsive manner similar to radiation-induced γ -H2AX foci.

We then tested whether the formation of cMcp1 IRIF is dependent on H2AX phosphorylation. As shown in Figure 3d, *H2AX*^{-/-} DT40 cells showed no cMcp1 IRIF, 2 h after 10 Gy IR. While transgenic expression of wild-type H2AX protein restored cMcp1 IRIF formation, expression of H2AX in which the conserved Ser-139 was mutated to Ala (S139A) did not support IRIF formation by cMcp1 (Figure 3d). These data show clearly that γ -H2AX is required for cMcp1 IRIF formation.

We next investigated the functions of the different domains of cMcp1 in responding to DNA damage by transfection of a deletion series of EGFP-tagged cMcp1 expression constructs (Figures 4a and b). As shown in Figure 4c, the N-terminal BRCT1 domain was required for centrosomal localization of cMcp1 in irradiated cells, but not IRIF formation. Interestingly, the loss of the BRCT1 domain still allowed centrosomal localization in unirradiated cells. This may suggest that the tandem BRCT2, 3 repeat has a limited affinity for the centrosome that is weaker than the IRIF interaction. The constitutive centrosome localization suggests the recognition of a centrosome component by the BRCT1 domain, although the extent to which single BRCT domains can interact with phosphoproteins is unclear (Manke *et al.*, 2003; Yu *et al.*, 2003).

The C-terminal BRCT3 domain was necessary for the formation of cMcp1 IRIF, but not for centrosomal targeting (Figure 4c). Loss of part of the region between BRCT1 and BRCT2 did not abrogate IRIF formation or centrosome localization, demonstrating that the BRCT domains are the key determinants directing cMcp1 localization. Importantly, we saw a similar localization pattern with both the N- and C-terminally tagged forms of cMcp1, consistent with the observed localization being independent of tagging. Expression of each of the EGFP-tagged cMcp1 proteins was confirmed by immunoblot analysis (unpublished data). Tandem C-terminal BRCT domains, which can act as phosphoserine/phosphothreonine-binding modules, are common motifs in IRIF-forming proteins (Manke *et al.*, 2003; Yu *et al.*, 2003). The C-terminal BRCT domains of MDC1/NFBD1 bind directly to γ -H2AX to form MDC1/NFBD1 IRIF (Stucki *et al.*, 2005). The C-terminal BRCT domains of PTIP (Manke *et al.*, 2003) and BRCA1 (Au and Henderson, 2005) are required for IRIF formation, and H2AX is also necessary for IRIF formation by 53BP1 and BRCA1 (Celeste *et al.*, 2002, 2003). However, pull-down experiments in HeLa nuclear extracts with a H2AX phosphopeptide found MDC1/NFBD1 to be the principal γ -H2AX-binding protein in human cells (Stucki *et al.*, 2005), so that any other potential BRCT- γ -H2AX interactions, such as Mcph1- γ -H2AX, in IRIF are likely to be indirect.

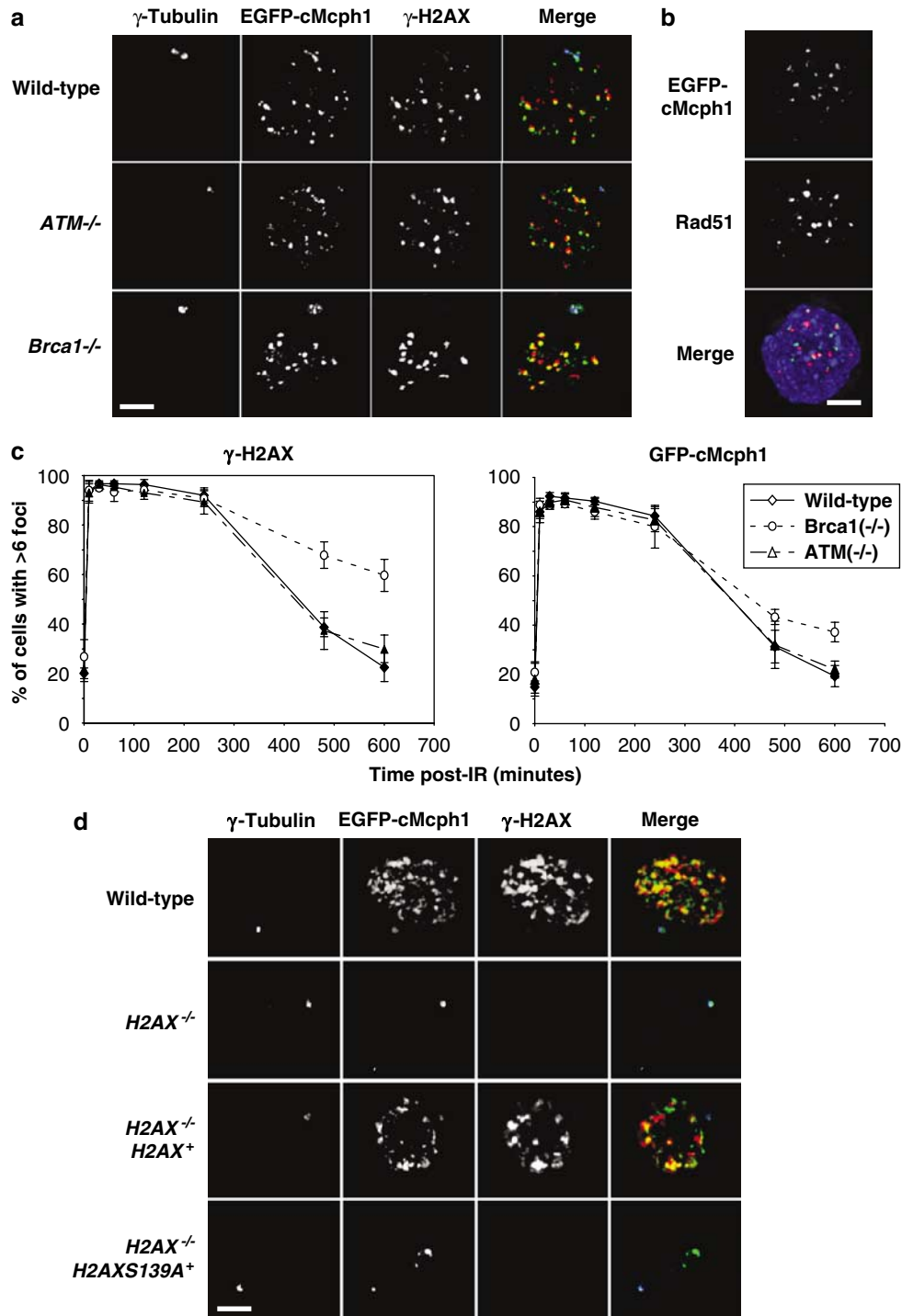


Figure 3 Genetic dependencies of cMcp1 IRIF formation. **(a)** Localization of EGFP-cMcp1 in *ATM*^{-/-} and *Brca1*^{-/-} DT40 cells. Micrographs show colocalization of EGFP-tagged cMcp1 (green) with γ -tubulin (blue) and γ -H2AX foci (red) induced by 10 Gy IR. **(b)** Micrograph showing colocalization of EGFP-tagged cMcp1 (green) with Rad51 foci (red) induced by 10 Gy IR. DNA is shown in blue. **(c)** Genetic dependencies of cMcp1 localization to damage foci. Wild-type, *ATM*^{-/-} and *Brca1*^{-/-} DT40 cells were transfected with pEGFP-cMcp1. Twelve hours post transfection, cells were subjected to 5 Gy IR, then fixed at different times and stained for γ -H2AX and γ -tubulin. Transfected cells were identified by centrosomal EGFP-cMcp1 signals and γ -H2AX foci were quantitated by eye in the transfected cells. Results shown are the means \pm s.d. of three separate experiments in which at least 300 cells were counted at each time point. **(d)** Localization of EGFP-cMcp1 in *H2AX* mutant DT40 cells. Micrographs show colocalization of EGFP-tagged cMcp1 (green) with γ -tubulin (blue) and γ -H2AX foci (red) induced by 10 Gy IR. Scale bars, 5 μ m.

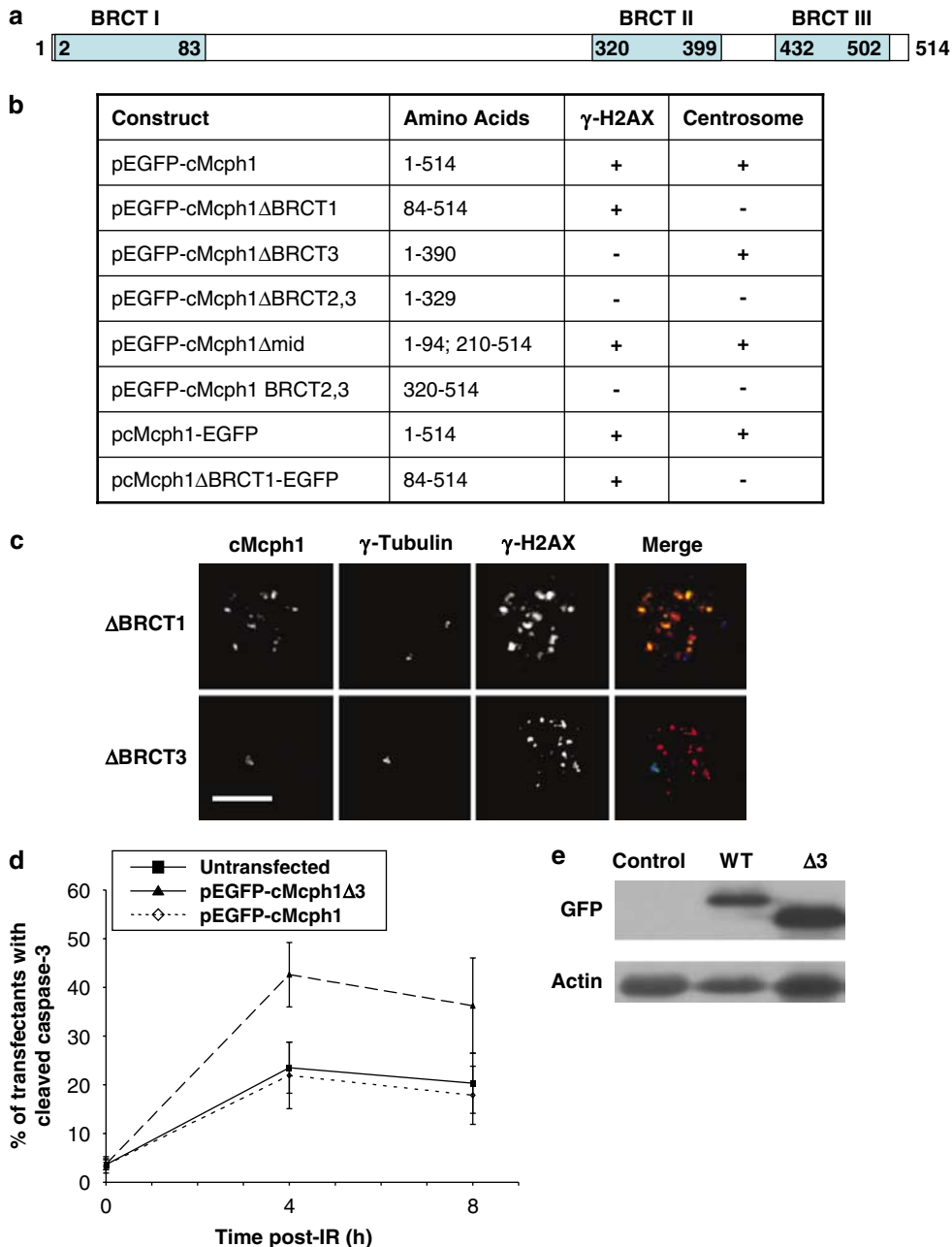


Figure 4 Analysis of deletion mutants of cMcp1. (a) Diagrammatic representation of cMcp1 protein showing BRCT domains. (b) Table summarizing the expression constructs analysed and localization patterns 2 h after 10 Gy irradiation (IR) determined for the recombinant proteins indicated. (c) Micrographs showing localization of EGFP-tagged cMcp1 deletion mutants (green), γ -H2AX (red) and γ -tubulin (blue) in DT40 cells 2 h after 10 Gy IR. Scale bars, 5 μ m. (d) DT40 cells transiently transfected with EGFP-tagged full-length cMcp1 or cMcp1 Δ BRCT3 were subjected to 10 Gy IR and analysed by immunofluorescence microscopy for cleaved caspase-3 at the indicated time points post treatment. Transfectants were identified by EGFP signal. Data points are the mean \pm s.d. of six separate experiments in which 300 cells were counted per time point. (e) Immunoblot of expression levels of the EGFP-cMcp1 transgenes used for the experiment in (d). 'Control', 'WT', ' Δ 3', untransfected, EGFP-tagged full-length cMcp1 and cMcp1 Δ BRCT3, respectively.

To determine whether the inability to form cMcp1 IRIF impeded the cellular response to DNA damage, we quantitated the induction of apoptosis in wild-type and cMcp1-overexpressing DT40 cells following IR treatment. As shown in Figures 4d and e, the same level of apoptosis was induced by

IR in cells that express EGFP-cMcp1 as in wild-type DT40 cells. However, expression of EGFP-tagged cMcp1 Δ BRCT3 caused a higher level of apoptosis after IR, suggesting an elevated radiosensitivity resulting from an inability to form cMcp1 IRIF.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).