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Citation for published version:

O'Driscoll, M, Jackson, AP & Jeggo, PA 2006, 'Microcephalin: a causal link between impaired damage response signalling and microcephaly', *Cell Cycle*, vol. 5, no. 20, pp. 2339-44.
<<https://www.landesbioscience.com/journals/cc/article/3358/>>

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Cell Cycle

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Review

Microcephalin

A Causal Link Between Impaired Damage Response Signalling and Microcephaly

Mark O'Driscoll¹,

Andrew P. Jackson²

Penny A. Jeggo^{1,*}

¹Genome Damage and Stability Centre; University of Sussex; East Sussex, UK

²MRC Human Genetics Unit; Western General Hospital, Edinburgh, UK

*Correspondence to: Penny Jeggo; Genome Damage and Stability Centre; University of Sussex; East Sussex BN1 9RQ UK; Tel.: +0044.1273.678.482; Fax: +0044.1273.678.121; Email: p.a.jeggo@sussex.ac.uk

Original manuscript submitted: 08/10/06

Manuscript accepted: 09/04/06

Previously published online as a *Cell Cycle* E-publication:

<http://www.landesbioscience.com/journals/cc/abstract.php?id=3358>

KEY WORDS

ATR-signalling, DNA damage response, Seckel Syndrome, Primary Microcephaly, cell cycle checkpoints

ACKNOWLEDGEMENTS

We thank the Leukaemia Research Fund for supporting M.O'D. The P.A.J. laboratory is supported by the Medical Research Council, the Human Frontiers Science Programme, EU grant RadRisc and DNA repair and the International Association for Cancer Research. A.P.J.'s laboratory is supported by the Medical Research Council.

ABSTRACT

Seckel Syndrome (SS) and Primary Microcephaly (MCPH) are disorders exhibiting marked microcephaly with a head circumference less than three standard deviations below the mean. ATR-Seckel Syndrome is conferred by mutations in ataxia and telangiectasia and Rad3 related (ATR), a kinase that activates a DNA damage signalling response. Cell lines from additional SS patients, who are normal for ATR, show defective ATR signalling, suggesting that they carry mutations in other components of the ATR pathway. Primary Microcephaly is distinct from SS since patients display solely microcephaly without accompanying marked growth delay. *MCPH1*, the first Primary Microcephaly causative gene identified, encodes three BRCT domains, similar to other damage response proteins. Recent studies employing *MCPH1* siRNA or exploiting cell lines from *MCPH1* patients have shown that *MCPH1* functions in the ATR-dependent DNA damage response pathway. Additionally, *MCPH1* has a function in the regulation of mitotic entry that is ATR-independent and confers a characteristic phenotype of premature chromosome condensation. Recent studies will be reviewed and their relationship to the aetiology of microcephaly discussed.

BRAIN DEVELOPMENT AND MICROCEPHALY

The human brain develops from a swelling at the rostral end of the neural tube. Rapid and sustained cellular proliferation during fetal development is required to generate the brain, and especially the cerebral cortex. Cerebral cortical surface area has increased over a thousand fold during mammalian evolution, and consequently in higher mammals to accommodate the brain within the skull, the cerebral cortex must fold to assume an invaginated, convoluted appearance with gyri and sulci.

Microcephaly is a clinical term denoting reduced head size, defined as reduced head circumference greater than 3 standard deviations below the mean (-3 s.d.). Reduced head size reflects an underlying reduction in brain volume (Fig. 1A), and not surprisingly has multiple aetiologies, both genetic (nonsyndromic, syndromic or chromosomal) and environmental (eg. perinatal asphyxia, and intrauterine infection). Autosomal recessive Primary Microcephaly (MCPH, OMIM251200) is a form of microcephaly in which patients exhibit solely a reduced cerebral cortex without other developmental abnormalities or neurological deficits (aside from mental retardation).¹ MCPH brains, although very small (typically -4 to -12 s.d.), exhibit normal cortical architecture suggesting that microcephaly is a consequence of reduced cell number either as a result of reduced neuroprogenitor division or increased apoptosis during neurogenesis (Table 2).

Microcephaly is also a common feature observed in genetic disorders that additionally confer growth delay (e.g., Phenylketonuria, Down's syndrome, DiGeorge syndrome). A striking example is Seckel Syndrome (SS) (OMIM210600), where microcephaly and growth delay is characteristic and marked. Dysmorphic facial features (sloping forehead, prominent nose, small chin) are also observed in such patients explaining the original description of 'Bird-headed dwarfism' (Fig. 1A).² To date, the only causative genetic defect identified in SS is a hypomorphic mutation in Ataxia telangiectasia and Rad3-related (*ATR*), which encodes a protein that plays a central role in the response to DNA damage.³ Furthermore, cell lines from additional non-ATR mutated SS patients display defects in ATR-dependent damage responses.⁴ Nijmegen Breakage Syndrome (NBS) (OMIM251260) patients also display Seckel-like features including microcephaly, growth delay and similar facial features, and Nbs1, the defective protein, functions in the response to DNA damage (Table 2). Together these findings provide evidence for a link between defective DNA damage response signalling and microcephaly.

Currently six MCPH loci and four genes conferring Primary Microcephaly have been identified (Table 1).⁵ *Microcephalin* (*MCPH1*) was the first causative Primary Microcephaly gene identified.^{5,6} It was subsequently independently identified as “*Brit1*” (BRCT-inhibitor of hTERT expression) in a screen for genes influencing telomerase expression.⁷ Although the MCPH1 disorder is classified as Primary Microcephaly, patients can display growth retardation, feature also seen in SS and NBS patients. MCPH1 encompasses three BRCT motifs, with N-terminal and tandem C-terminal locations (Fig. 1B). BRCT motifs are commonly found in DNA damage response proteins, particularly those functioning as mediators in the signalling response providing provocative although circumstantial evidence that MCPH1 might function in a DNA damage response pathway.⁶

DNA DAMAGE RESPONSE SIGNALLING

Two DNA damage response signalling pathways have been described, both of which are regulated by related phosphoinositol 3-kinase-like kinases (PIKKs).⁸ Ataxia telangiectasia mutated (*ATM*), the kinase defective in the disorder ataxia telangiectasia (A-T), is activated by DNA double strand breaks (DSBs), whereas *ATR* responds to single stranded DNA regions that can arise at stalled replication forks or during the processing of bulky lesions such as a UV photodimer. Both kinases have partner proteins which facilitate lesion targeting; Nbs1 promotes *ATM* activation and *ATR* interacting protein (*ATRIP*) is required for *ATR* recruitment to damage sites.⁹⁻¹⁰ In the present context, it is relevant that Nbs1 also functions in the *ATR* signalling response.¹¹ Kinase activation promotes phosphorylation of the histone variant, H2AX, at the site of damage, which facilitates the recruitment of mediator proteins, including 53BP1, MDC1 and *Brc1*, giving rise to characteristic ionising radiation (IR)-induced foci (IRIF).¹² *ATM* and *ATR* phosphorylate many of the same substrates, thereby initiating overlapping damage responses although recent evidence suggests that there is specificity as well as overlap in substrate phosphorylation. Of significance, *ATM* appears to preferentially target the transducer kinase, *Chk2*, whereas *ATR* has a preference for *Chk1*.¹⁰ Finally, via phosphorylation of effector proteins either directly or indirectly by *ATM/ATR*, DNA damage response signalling leads to cell cycle checkpoint arrest and/or the onset of apoptosis. Interestingly, whilst *ATR* is essential, *ATM* is nonessential.¹³ Moreover, in distinction to *ATR-SS*, A-T patients display progressive ataxia, immunodeficiency, pronounced cancer predisposition and do not display microcephaly (Tables 1 and 2).

STUDIES ON MCPH1 FUNCTION

Three routes have been traversed to examine MCPH1 involvement in the DNA damage response. Firstly, using cell biology, HA tagged- and endogenous MCPH1 was shown to colocalise with MDC1 and γ -H2AX IRIF in response to IR, suggesting that MCPH1 localises to the sites of DNA damage.¹⁴⁻¹⁶ Secondly, siRNA knock down of MCPH1 has been employed to examine the impact of reduced MCPH1 function on the response to DNA damage. Strikingly, knock down of MCPH1 was accompanied by co-knock down at the transcriptional level of *Brc1* and *Chk1* via an unknown mechanism.^{14,15} MCPH1 knock down also impacted upon MDC1, 53BP1 and *ATM* foci formation although it did not impair *ATM* activation, monitored by *ATM*-S1981 phosphorylation, nor *Chk2* phosphorylation or protein levels.^{14,16} MCPH1 siRNA also caused radio-

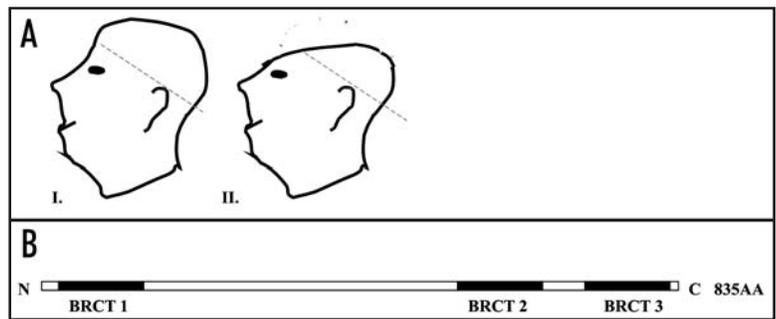


Figure 1. (A) A pictorial representation of microcephaly. The line drawings show a normal individual (i) alongside a microcephalic individual (ii). Note that the reduced occipitofrontal circumference (depicted by the dashed line) of the microcephalic individual results in a sloping forehead and apparent protrusion of the face, as originally noted by Seckel.² (B). Schematic representation of MCPH1 protein showing the relative locations of the three BRCT domains.

resistant DNA synthesis, a characteristic phenotype of A-T cells due to impaired IR-induced intra-S-phase checkpoint arrest, reduced G₂/M checkpoint arrest after IR and increased sensitivity to IR. Despite the fact that *Chk2* phosphorylation was not affected by MCPH1 siRNA, Nbs1 phosphorylation after IR was reduced.^{14,15} Taken together, it was concluded that MCPH1 is required for the formation of damage response foci and additionally functions to transcriptionally regulate *Chk1* and *Brc1*, hence acting as a crucial DNA damage regulator. Interestingly, changes in MCPH1 expression were found in a number of tumour cell lines and in primary tumour material, suggesting that MCPH1 functions as a tumour suppressor gene.¹⁶

The third, distinct approach to examine MCPH1 function involved an analysis of cell lines derived from MCPH1 patients. Initial studies demonstrated that MCPH1 patient lymphocytes and derived cell lines display a characteristic phenotype of elevated numbers of cells with prematurely condensed chromosomes (PCCs) (see below for further details).¹⁷ No evidence of a damage response defect to IR was observed, however.¹⁷ More recently, we focused on an analysis of the *ATR* signalling response in MCPH1 defective cells due to the overlapping clinical features of MCPH1 deficiency and *ATR-SS*.⁴ These studies revealed a downstream role for MCPH1 in the *ATR*-signalling response that is distinct to the findings described using siRNA. Activation of *ATR* and phosphorylation of key *ATR*-substrates, including Nbs1, following replication fork stalling were normal. Additionally, MCPH1 cells recover from replication fork stalling normally in contrast to an *ATR-SS* cell line. Strikingly, however, MCPH1 cells were unable to effect *ATR*-dependent G₂/M checkpoint arrest. Additionally, elevated nuclear fragmentation after replication fork stalling was observed as well as the appearance of supernumerary mitotic centrosomes, two further features common with *ATR-SS* cells. In contrast to studies employing siRNA, MCPH1 patient cells expressed normal protein levels of *Chk1* and *Brc1*, and *Chk1* was phosphorylated normally after DNA damage. Indeed, two independent *Chk1* functions occur normally in MCPH1 cell lines (53BP1 foci formation following replication stalling and replication fork stability). These findings, therefore argue that MCPH1 functions in the *ATR*-dependent DNA damage response pathway in a manner that is independent of *Chk1* or *Brc1* regulation. MCPH1 cells showed impaired degradation of *Cdc25A*, identical to that observed in *ATR*-Seckel cells, both in unperturbed cell growth as well as following UV irradiation. These findings suggest that MCPH1 acts to regulate *Cdc25A*. Interestingly, MCPH1 was also found to

Table 1 **An overview of Primary Microcephaly and several known DNA damage response disorders**

Condition	Clinical Features	Gene	Functional Domains	Cellular features & proposed/demonstrated function.
MCPH1	Microcephaly Growth retardation	<i>Microcephalin (BRIT1)</i>	3 BRCT domains (C-terminal tandem).	Defective DNA damage response and cell cycle regulation. Patient cells exhibit premature chromosome condensation (PCC). Supernumerary mitotic centrosomes.
MCPH2	Primary microcephaly	Unknown		
MCPH3	Primary microcephaly	<i>CDK5RAP2</i>	Spindle association domain.	Centrosome organization, specifically in neurons. Homolog of fly centrosomin.
MCPH4	Primary microcephaly	Unknown		
MCPH5	Primary microcephaly	<i>ASPM</i>	2 Calponin homology domains. 81 IQ repeats. ASH domain.	Spindle microtubule nucleation at centrosome.
MCPH6	Primary microcephaly	<i>CENPJ</i>	Tcp 10 domain.	Centrosome association via γ -tubulin. Ortholog of fly and worm SAS4, a protein required for centriole replication.
ATR-Seckel	Severe microcephaly. Pre and postnatal growth retardation.	<i>ATR</i>	PI3-kinase, FAT, FATC.	Defective ATR signalling, G2/M checkpoint defective, supernumerary mitotic centrosomes.
Non-ATR-Seckel	Microcephaly. Growth retardation.	Unknown		Cells exhibit defective ATR pathway function.
NBS	Microcephaly. Pre and postnatal growth retardation. Lymphoid malignancy.	<i>NBS1</i>	BRCT, FHA.	Defective DSB-induced damage response. Radiosensitivity. Cells also exhibit defective ATR pathway function.
A-T	Modest growth delay. Progressive cerebellar degeneration. Ataxia. Lymphoid malignancy.	<i>ATM</i>	PI3-kinase, FAT, FATC.	Defective DSB-induced damage response.

interact with Chk1.⁴ The regulation of Cdc25A activity and stability is still poorly understood. However, there is clear evidence that Chk1 phosphorylates Cdc25A at multiple sites that can regulate both its activity and ubiquitin-dependent degradation.¹⁸

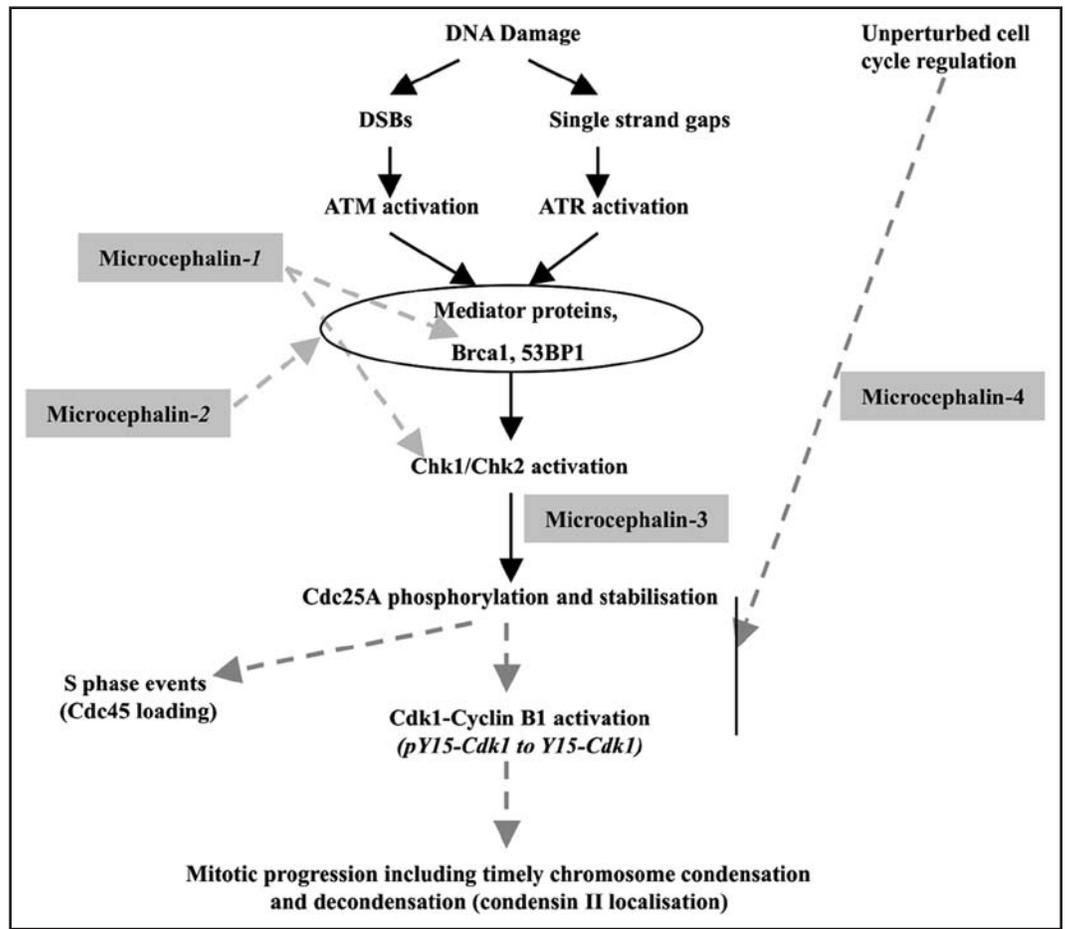
Importantly, the analysis of MCPH1 cell lines has revealed an impact of MCPH1 deficiency that has proved to be distinct to its impact on the DNA damage response. As mentioned above, MCPH1 cells show a striking elevation in prophase-like PCC cells. This phenotype is due to MCPH1 deficient cells condensing their chromosomes within an intact nuclear envelope during G₂ and also delaying decondensation post mitosis, strongly suggesting that loss of MCPH1 causes aberrant regulation of chromosome condensation.^{17,19} The PCC phenotype is not observed in A-T nor in ATR-SS cells suggesting that it is not a consequence of a defective DNA damage response.⁴ Strikingly, we observed that the levels of inhibitory Tyr15 phosphorylated Cdk1 (pY15-Cdk1) observed in cells released following synchronisation at the G₁/S boundary decreased rapidly in MCPH1 cell lines compared to control cells in parallel to elevated PCC formation.⁴ Moreover, normal G₂ cells were not observed in MCPH1 cell lines but instead cells with PCCs were G₂-like, since they did not express phospho-Ser10-histone H3, retained an intact nuclear envelope but lacked detectable pY15-Cdk1. Taken together, these findings suggest that MCPH1 has an ATR-independent role in regulating mitotic entry by maintaining inhibitory Cdk1 phosphorylation. Interestingly, another recent study using MCPH1 patient cell lines, showed that condensin II was localised to the nucleus in PCC cells and in some cases was bound to

the central chromosomal axis, even though condensin I was still in the cytoplasm.²⁰ In contrast, condensin II is cytoplasmically localised in normal G₂ cells. Although nuclear localisation in some cells can be observed, its enrichment in the chromatid axis is rare. This suggests that a consequence of MCPH1 deficiency is the premature binding of condensin II to chromatin. Furthermore, siRNA depletion of condensin II subunits was able to alleviate the PCC phenotype as well as the delayed post-mitotic decondensation phenotype. The PCC phenotype was also observed in HeLa, lymphoblastoid cell lines and U20S cells after MCPH1 siRNA, and in HeLa cells this was prevented by simultaneous depletion of condensin II. Knock down of condensin I did not impact upon the PCC phenotype.

RECONCILING THE MULTIPLE IMPACTS OF MCPH1 DEFICIENCY

Taken together, these findings suggest that MCPH1 deficiency has two manifestations: one results in a perturbed DNA damage response whilst the second impacts upon the regulation of mitotic entry. A consideration of the impact of MCPH1 deficiency on the DNA damage response is complicated by the finding that different results are obtained by MCPH1 siRNA compared to the analysis of patient cells. In evaluating findings from patient cell lines, it is important to consider whether the mutations are hypomorphic or null. As evident from the analysis on NBS cell lines, it can be difficult to establish whether an apparently null mutation might confer residual function.²¹ Indeed, there may well be strong selection for hypomorphic mutations that permit patient viability. Downstream

Figure 2. Impacts of MCPH1 deficiency. One function of MCPH1 (depicted by microcephalin-1 box) suggested by siRNA studies is the transcriptional regulation of Brca1 and Chk1. A second function of MCPH1 (depicted by microcephalin-2 box) suggested by siRNA studies is the formation of MDC1, 53BP1, p-ATM and NBS1 foci. γ -H2AX foci form normally, however. Studies on MCPH1 cell lines have exposed an MCPH1 function that cannot be attributed to an impact on Brca1 or Chk1 expression in the DNA damage response that is downstream of Chk1 activation but impacts upon Cdc25A stabilization (depicted by microcephalin-3 box). This could represent a role in facilitating Chk1 phosphorylation of Cdc25A. MCPH1 can interact with Chk1. Finally, MCPH1 has a function that does not overlap with the DNA damage response in regulating entry into mitosis via the regulation of Y15-Cdk1 phosphorylation (depicted by microcephalin - 4 box), revealed by studies on patient cell lines.



reinitiation can occur with N-terminal truncating mutations, and C-terminal deletions can leave expressed N-terminal fragments. Of the two cell lines examined for DNA damage responses, one was clearly hypomorphic exhibiting potential for read through of a stop codon.⁴ Since Chk1 and Brca1 are essential, MCPH1 would be expected to be essential if it transcriptionally regulates either gene. Thus, one possible explanation for the different findings is that hypomorphic MCPH1 mutations in the patients confer separation of function phenotypes that impact upon some but not all MCPH1 functions. However, siRNA knock down has the potential to provide artefactual results. Distinct MCPH1-specific siRNA oligonucleotides gave identical results and transfection with siRNA resistant *MCPH1* cDNA corrected the reduced Chk1/Brca1 expression, rendering it

unlikely that the impact on Chk1/Brca1 expression can be attributable to off-target siRNA effects. However, significant knock down of Chk1/Brca1 following MCPH1 siRNA has not been universally observed although elevated PCC formation appears a reproducible finding.^{4,19} Cell line specificity may provide a possible explanation for the differing impacts. Impacts on cell cycle progression also need to be considered since Brca1 is cell cycle regulated, and MCPH1 siRNA may perturb cell growth.

Any model for MCPH1 function needs to reconcile the conclusions from these different approaches. A scheme showing the differing impacts of MCPH1 deficiency is presented in Figure 2. siRNA studies point to an upstream role of MCPH1 in transcriptionally regulating Chk1 and Brca1 (Box 1, Fig. 2). siRNA studies

Figure 3. Spindle pole orientation is fundamental for normal brain development. Symmetric cell division of a shaded progenitor stem cell (A) results from cytokinesis along a cleavage plane with an apical-basal orientation. This generates two identical progenitor daughter cells and laterally expands the neuroepithelium. Asymmetric division (B) occurs upon reorientation of the spindle pole, and consequently the cleavage plane, generating two cells of different developmental fates, a progenitor stem cell and a neuron. The neuron then migrates away from the ventricular zone, along glial tracks, ultimately forming the six-layered laminar neocortex.

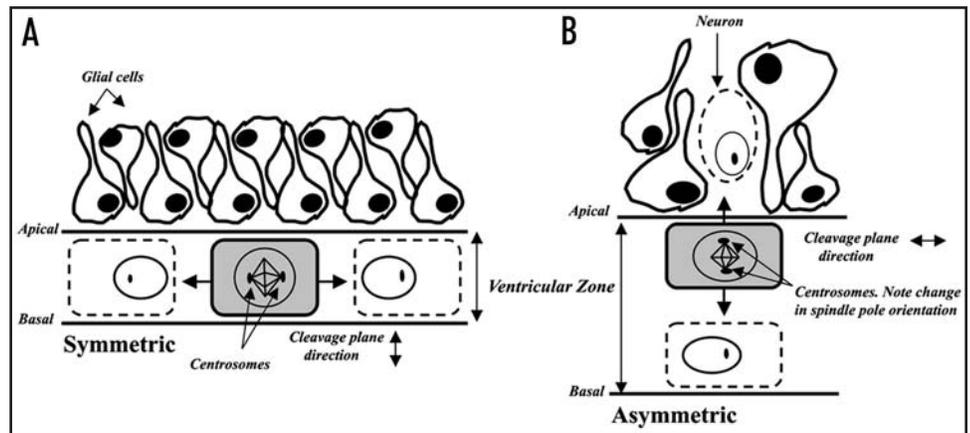


Table 2 **A clinical comparison between Primary Microcephaly to known DNA damage response disorders**

	Structural Brain Development	Growth	Neurological
ATR-Seckel	Severe microcephaly (-12s.d).	Pre and postnatally retarded (mean -5s.d).	Mental retardation.
NBS	Microcephaly (-4 to -8s.d)	Pre and postnatally retarded.	Normal to moderate mental retardation.
MCPH1	Microcephaly (-3 to -10s.d) with simplified gyri.	Postnatal growth retardation (mean -3s.d).	Mild mental retardation.
MCPH2-6	Microcephaly (-4 to -12s.d).	Apparently normal.	Mild to moderate mental retardation.
A-T	Grossly normal with selective progressive Purkinje neuronal loss.	Mild growth delay.	Ataxia with normal oculomotor abnormalities

also suggest a distinct function for MCPH1 in regulating mediator protein foci formation (Fig. 2, Box 2). The studies with MCPH1 cell lines demonstrate a further impact on ATR signalling which cannot be attributed to any upstream impact on foci formation or Chk1 and Brca1 regulation. These latter studies point to a clinically relevant role of MCPH1 in the DNA damage response pathway that is downstream of Chk1 activation but impinges upon Cdc25A regulation (Fig. 2, Box 3). Since Cdc25A also impacts upon targets of the ATM pathway, such as the regulation of Cdc45 following DSBs in S phase cells which regulates the initiation of replication, it is possible that MCPH1 cell lines will show impaired ATM signalling, although normal G₂/M checkpoint arrest was observed in one study.¹⁷ MCPH1 could impact upon Cdc25A regulation by acting as a mediator protein to facilitate its phosphorylation by Chk1 and/or Chk2. Alternatively, MCPH1 could regulate proteins that themselves regulate Cdc25A phosphorylation including the Wee1-Myt1 kinases. It is also possible that MCPH1 could impart an impact via regulation of ubiquitin-mediated degradation of Cdc25A. Currently, it is not known whether MCPH1 also impacts upon the phosphorylation of Cdc25B and C, which function cooperatively with Cdc25A to control cell cycle regulation although details of the interplay between these three phosphatases is still poorly understood.¹⁸ Finally, the cellular studies have also identified an MCPH1 function in unperturbed cell cycle regulation that is distinct from any role in ATR signalling (Fig. 2, Box 4), which represents a highly important finding. Indeed, the PCC phenotype is a characteristic feature of all MCPH1 cell lines, which is recapitulated by siRNA studies. The evidence suggests that MCPH1 regulates the levels of pY15-Cdk1, either by promoting phosphorylation or enhancing dephosphorylation. A consequence of reduced pY15-Cdk1 appears to be premature chromosome condensation caused in part by untimely localisation of condensin II. Recent studies have argued that ATR-dependent signalling regulates unperturbed cell cycle progression as well as after DNA damage.²² It is important to realise, however, that neither ATR-Seckel nor A-T cell lines display the PCC phenotype characteristic of MCPH1 cell lines, although MCPH1 cell lines show similar defects in ATR-signalling responses to ATR-Seckel cells.⁴ Thus, the evidence suggests that MCPH1 has an important role in regulating mitotic entry that is distinct to its role in the DNA damage response.

CENTROSOMES, ATR AND MICROCEPHALY

The analysis of patient cell lines has the advantage that it allows the cellular analysis to be linked to clinical features. MCPH1 is, to date, the only characterised protein defective in Primary Microcephaly patients that has a role in the DNA damage response. MCPH1 patients, in contrast to other Primary Microcephaly patients, display reduced stature, a characteristic shared with conditions exhibiting defective ATR-signalling (ATR-Seckel and NBS). This, the growth delay seen in MCPH1 patients may be a consequence of ATR-signalling defects. In contrast, microcephaly is a feature observed in ATR-SS as well as all Primary Microcephaly patients. Another feature observed in MCPH1 and ATR-SS cell lines is the presence of mitotic cells with supernumerary centrosomes, which can represent up to 30 % of the cells within a population, suggesting that ATR via MCPH1 may regulate centrosome stability.⁴ Strikingly, defects in coordinating microtubule nucleation at centrosomes or other defects affecting centrosomal biogenesis have been observed in other Primary Microcephaly patients or cell lines from them.²³ Cerebrocortical development is achieved via a highly regulated sequence of neural progenitor cell division, migration and differentiation (Fig. 3). Neuroprogenitor cells form in the neuroepithelium of the ventricular zone around the ventricles of the developing brain where they symmetrically divide generating more progenitor cells in a lateral expansion (Fig. 3A). Each of these progenitor cells undergoes asymmetric cell division by altering the cleavage plane from the apical to basal orientation by repositioning the spindle pole, creating a daughter progenitor stem cell and a neuron (Fig. 3B). The neuronal cells migrate from the ventricles along radial glia tracks undergoing terminal differentiation ultimately forming a six-layered laminar neocortex. Therefore, spindle pole orientation is a critical determinant of normal brain development. It is anticipated, therefore, that brain development might be highly sensitive to any perturbation in the maintenance of centrosome stability and function.²⁴ Recently, Abnormal Spindle in mammalian (Aspm, the MCPH5 locus, Table 1) has been shown to be essential in maintaining cleavage plan orientation that allows symmetric cell division.²⁵ Therefore, centrosome stability and correct spindle pole orientation potentially represent an 'Achilles heel' in brain growth during development.

CONCLUSIONS

In conclusion, recent studies have provided important insight into the function of MCPH1, a protein that is critical for normal brain development. Although data from the analysis of patient cell lines and siRNA approaches are somewhat confusing and apparently discrepant, there is mounting evidence that MCPH1 is a damage response protein that functions to ensure efficient cell cycle checkpoint arrest after DNA damage. In distinction to this response, and probably as importantly, MCPH1 functions to regulate timely mitotic entry. As a consequence, cell lines from MCPH1 patients have abnormal G₂-like cells which have inappropriately commenced premature condensation yet not progressed normally into mitosis. MCPH1 thus appears to be a protein that functions at the gateway of cell cycle regulation and its interface with the DNA damage response, and we await further studies to reveal the precise point(s) at which this fascinating protein functions.

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