Mutation in PHC1 implicates chromatin remodeling in primary microcephaly pathogenesis

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Primary microcephaly (PM) is a developmental disorder of early neuroprogenitors that results in reduction of the brain mass, particularly the cortex. To gain fresh insight into the pathogenesis of PM, we describe a consanguineous family with a novel genetic variant responsible for the disease. We performed autozygosity mapping followed by exome sequencing to detect the causal genetic variant. Several functional assays in cells expressing the wild-type or mutant gene were performed to understand the pathogenesis of the identified mutation. We identify a novel mutation in PHC1, a human orthologue of the Drosophila polyhomeotic member of polycomb group (PcG), which significantly decreases PHC1 protein expression, increases Geminin protein level and markedly abolishes the capacity to ubiquitinate histone H2A in patient cells. PHC1 depletion in control cells similarly enhances Geminin expression and decreases histone H2A ubiquitination. The ubiquitination defect and accumulation of Geminin with consequent defect in cell cycle are rescued by over-expression of PHC1 in patient cells. Although patients with the PHC1 mutation exhibit PM with no overt progression of the disease, patient cells also show aberrant DNA damage repair, which is rescued by PHC1 overexpression. These findings reveal several cellular defects in cells carrying the PHC1 mutation and highlight the role of chromatin remodeling in the pathogenesis of PM.

INTRODUCTION

Primary microcephaly (PM) is a phenotype characterized by significant reduction of brain size, particularly the cortex, in the absence of gross-structural defects, and variable degree of intellectual disability (1). When present in isolation, i.e. non-syndromic, PM is usually inherited as an autosomal-recessive trait with an expansive list of genes identified to date (2). These genes include MCPH1 (microcephalin), WDR62, CDK5RAP2, CEP152, CEP135, ASPM, CENPJ and STIL, and encode proteins that are almost universally involved in the biology of the centrioles, cellular organelles that play critical roles in cell division and cell cycle checkpoints (1,3–5). The conventional view of PM pathogenesis is that abnormal mitosis dynamics in early neuroprogenitors lead to a reduction in the final number of cells that populate the cortex. Specifically, these stem cell-like progenitors that possess the capacity of symmetric and asymmetric cell division lose important regulatory mitotic cues that are required to proliferate and maintain an adequate pool of differentiated cortical neuronal cells (6,7). This model is strongly supported by empirical evidence and provides a framework for viewing PM in the context of a generalized biological defect. Indeed, in syndromes that are characterized by severe growth retardation and microcephaly (commonly known as primordial dwarfism with microcephaly), the role of centriolar biology is being increasingly recognized (8). Conversely, the recent finding that mutation in CENPJ, an established disease gene

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in PM, can also cause primordial dwarfism with microcephaly.

Another cause of microcephaly increasingly considered
clinically is an underlying defect in DNA damage repair
(10–13). Fanconi anemia, Nijmegen breakage syndrome, Nij-
megen breakage syndrome-like and Bloom syndrome are
examples of disorders in which PM ranges from being occa-
sional to a more important clinical feature (13). BRIT1/
MCPH1, a major gene responsible for PM, is believed to
cause PM through its role in mitotic spindle alignment and
division plane of neuroprogenitors, and also has an established
role in DNA damage response (3,6). These observations to-
gether with the overlap in the pathogenesis of syndromic
and nonsyndromic PM suggest that, in addition to the well-
established aberrations in cell cycle and abnormal spindle
formation seen in cells from patients with PM, DNA damage
repair may be an additional abnormal feature. However,
whether all cellular defects observed in patient cells are an
actual cause of isolated nonsyndromic PM remain unclear.

Polycomb group (PcG) is a term used to describe genes,
mutations of which result in abnormal body segmentation in
Drosophila similar to that observed in the Polycomb (Pc)
mutants, hence their name. PcG proteins associate to form dis-
tinct classes of multimeric polycomb repressive complexes
(PRCs), such as PRC1 and PRC2. These proteins function as
transcriptional repressors that silence specific genes via chro-
matin remodeling (14). Specifically, the enzymatic subunits
of PRC2 EZH1 and EZH2 are responsible for the di- and tri-
methylation of Lys27 of histone H3. On the other hand, two
subunits of PRC1 Bmi1 and Ring1 act as chromatin remodelers
through mono-ubiquitination of histone H2A (15,16). PHC1,
another component of PRC1, plays a central role in gene regu-
lation but its precise role in the E3 ubiquitin ligase complex-
mediating H2A ubiquitination remains unknown.

Here, we describe a family with nonsyndromic PM in whom
we identify a novel genetic lesion linked to abnormal chroma-
tin remodeling. We discover a novel mutation in PHC1 that
markedly abrogates the capacity of patient cells to ubiquitinate
H2A, with concomitant increased Geminin expression and
several cellular defects, which link for the first time a PcG
gene to PM. Mutation in a gene encoding an important chro-
matin remodeling protein provides fresh insights into the
pathogenesis of PM and reveals a new regulatory process con-
trolling brain size.

RESULTS

Identification of a family that defines a novel PM locus

The family was a consanguineous Saudi family consisting of
healthy parents, two children (brother and sister) with PM
and four healthy siblings. The head circumference of the
13-year-old sister was ≏5.8SD below the mean, her height
was 3.6SD below the mean and the corresponding measure-
ments for her 6-year-old brother were 4.3SD and 2.3SD
below the mean, respectively. They both had normal brain
MRI results except for a small brain size (Fig. 1A) and func-
tioned in the low-normal range for their chronological age
at school. Formal IQ testing revealed a score of 80 in the
sister. Their past medical history and physical examination
were unremarkable otherwise.

Linkage analysis highlighted two linkage regions neither of
which overlaps with known PM genes (Fig. 1B). The first
region on 5q14.1-14.3 (10 MB) only spanned 58 genes, some
of which were excluded by direct sequencing (LOC644936,
SPZ1, SSBP2). The other region on 12p13.33-12.3 (15 MB)
was much more gene-dense spanning 293 genes, including
RAD52, RAD51AP1, CDC43, RAB6IP2, ENO2, PHB2,
COPS7A, NDUFA9, LTBR, VAMP1, PTMS, FGF23, EMG1,
C12orf34, and CLECL1. These genes were considered good
candidates but later excluded by direct sequencing. The two
linkage regions in the family were consistent with homozygos-
ity mapping (Supplementary Material, Fig. S1A).

Exome sequencing identifies a novel genetic lesion in PM

Given the number of genes in the loci highlighted by autozy-
gosity mapping and our failure to identify mutations in apparent-
ly likely candidate genes by direct sequencing, we performed
exome sequencing on one affected member. We considered
only variants that are homozygous, coding/splicing (excluding
synonymous changes), not present in dbSNP, EVS or 1000
Genomes, located within the linkage interval(s), not found in
199 Saudi exomes, and segregated with the phenotype within
the family. This approach allowed us to identify one single
variant NM_004426.2:c.2974C>T, p.(Leu992Phe) in PHC1
in the 12p13.33-13.1 locus whereas no candidate variants
were identified in the other locus (GRCh37/hg19:chr12:
9,092,014) (Fig. 1C). In addition to being the only novel
coding or splicing variant within the candidate runs of homozy-
gosity, the mutation met the following candidacy criteria: con-
servation across species (Supplementary Material, Fig. S2),
high pathogenicity score, absence in ethnically matched
control chromosomes (554 Saudi individuals were screened
for PHC1 mutation) by direct sequencing and absence in the
Exome Variant Server.

PHC1 mutation promotes rapid PHC1 degradation

To gain insights into the effect of PHC1 missense mutation,
we measured PHC1 protein expression in EBV-transformed
lymphoblast cell lines established from three normal individu-
als and from two patients diagnosed with PM. The relative
abundance of PHC1 mRNA was similar in control and patient
cells (Fig. 2A). In contrast, we observed a marked reduction
(~72%) in PHC1 protein level in patient cells compared
with control cells (Fig. 2B). The significant decrease in
PHC1 expression in patient cells was confirmed by performing
immunoblotting with a second PHC1 antibody from another
commercial source (Supplementary Material, Fig. S3A) and
by indirect immunofluorescence (Supplementary Material,
Fig. S3B). The observation that patient cells express signifi-
cantly less PHC1 protein than control cells suggested that
the reduction of PHC1 may be due to enhanced protein de-
gradation. To test this hypothesis, control and patient cells
were treated with cycloheximide to inhibit de novo protein
synthesis and the rate of PHC1 protein degradation was mea-
sured over time. In patient cells, PHC1 protein half-life was
about half that of control cells, where 50% of PHC1 was
degraded after 6 h in patient cells whereas the same amount of PHC1 was degraded after 12 h in control cells (Fig. 2C). Next we investigated whether the proteasome pathway is responsible for PHC1 protein breakdown. For this, control and patient cells were treated with the specific proteasome inhibitor MG-132 for 12 and 24 h, and the PHC1 protein level was analyzed by immunoblotting. Treatment of control and patient cells with MG-132 resulted in stabilization of PHC1 protein (Fig. 2D), as well as co-treatment of the cells with cycloheximide and MG-132 (Supplementary Material, Fig. S4). Therefore, PHC1 mutation enhances the proteasome-mediated degradation of PHC1.
PHC1 mutation leads to Geminin accumulation and impairs H2A ubiquitination

PHC1 is part of the PRC1, which maintains several genes in a repressive state. This complex includes PHC2 and BMI1 that function as E3 ligase to promote H2A ubiquitination (Ub H2A) (17). Selected members of the PcG complex are also reported to act as E3 ubiquitin ligase for Geminin (18). Based on these observations, we examined Geminin protein expression and Ub H2A in control and patient cells. We observed increased Geminin expression in patient cells (Fig. 2E). Next, we asked whether PHC1 normally interacts with H2A and is implicated in H2A ubiquitination. Endogenous PHC1 was immunoprecipitated from control lymphocytes of three normal individuals and from Patient 1 and 2 lymphocytes was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) on 4–12% NuPAGE gel, and subjected to immunoblotting with PHC1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. (C) Control and patient cells were incubated with cycloheximide (50 μg/ml) to inhibit general protein synthesis and total cell lysates were analyzed by SDS–PAGE. After transfer of the proteins, PHC1 protein expression was analyzed by immunoblotting using a specific PHC1 antibody. GAPDH was analyzed in parallel as a loading control (n = 3). The half-life based on these experiments is shown in the adjacent graph. (D) Recovery of PHC1 level after treatment with 40 μm of MG-132 proteasome inhibitor. Control 1 and 2 and Patient 1 and 2 cells were treated with MG-132 proteasome inhibitor for the indicated times and were analyzed by immunoblotting using anti-PHC1 antibody. (E) Increased Geminin protein expression in patient cells carrying PHC1 mutation. Thirty micrograms of total cells lysate from control and patient cells were examined by western blot using Geminin and GAPDH-specific antibodies.
protein complex, which is impaired in patient cells due to quantitative defect in PHC1. Next, we asked whether PHC1 mutation affects Ub-H2A. For this, we measured Ub-H2A in control and patient cells by western blot using a specific anti-Ub-H2A antibody recognizing ubiquitinated-Lys119. The reduced level of Ub-H2A was detected in patient cells compared with control cells, whereas the total H2A level was identical in control and patients cells (Fig. 3B). This result suggests that the reduction of PHC1 in mutant cells alters Geminin expression and impairs H2A ubiquitination.

The results described before suggest that PHC1 mutation may regulate cellular levels of Geminin and that reduction of Ub-H2A in patient cells may be causally linked to the PHC1 mutation. To address this, we specifically silenced PHC1 in control lymphoblasts using RNA interference and measured the impact on Geminin expression and Ub-H2A. We used three different siRNA against PHC1, which all resulted in loss of PHC1, whereas control siRNA had no effect. Specific elimination of PHC1 resulted in increased Geminin expression whereas Ub-H2A was severely reduced.

Figure 3. PHC1 mutation increases Geminin expression and H2A ubiquitination. (A) Association between PHC1 and H2A. PHC1 was immunoprecipitated from control and patient cells and the immunoprecipitated material was analyzed by SDS–PAGE. After transfer, membranes were probed with anti-PHC1 or Ub-H2A. Immunoglobulin G antibody was used as negative control in the immunoprecipitation reaction. This experiment was repeated three times. Relative quantification is shown. (B) Reduced H2A ubiquitination in PHC1 mutant cells. Western analysis of total cell lysates from control and patient cells was analyzed using a specific anti-Ub-H2A recognizing Lys 119. GAPDH is shown as a loading control. Relative quantification is shown. (C) Accumulation of Geminin and loss of H2A ubiquitination is a direct consequence of PHC1 mutation. PHC1 was silenced in control cell 1 and 2 using 800 pMol of three different siRNA designed against PHC1. Whole cell proteins lysates were prepared and analyzed by immunoblotting to examine levels of PHC1, Ub-H2A, Geminin and GAPDH were used as internal control. The blot is representative of three independent experiments.
(Fig. 3C). Together, these results show that PHC1 is required for proper Geminin expression. They also indicate that PHC1 which normally forms a complex with H2A, is necessary for H2A ubiquitination, and that the missense mutation we identified in patient cells impairs this novel function.

**PHC1 mutation causes defect in DNA repair at baseline and after irradiation**

Previous studies have shown that PHC1 primarily localizes in the cell nucleus (19–21) and that proteins of the PcG in general and PRC1 in particular play a role in DNA repair (22). In addition, MCPH1 deficiency causes PM and is associated with cellular defects in DNA damage repair (23–25). H2A ubiquitination is also essential for DNA repair (26). Based on these observations, we tested the hypothesis that mutation in PHC1 has effects on cellular functions of the patient cells such as impaired DNA damage response secondary to Ub-H2A reduction. To this end, we used Comet assay that detects both single-strand and double-strand breaks by measuring the amount of DNA that leaks from single nucleus after electrophoresis, at baseline and after treatment with ionizing radiation (IR). Low-level DNA damage was observed in both control and patient cells at baseline (Fig. 4A) although patient cells had significantly higher damaged DNA compared with control cells ($P < 0.02$) (Fig. 4B). Four and 8 h post-IR treatment, patient cells showed a progressive increase in ‘comet tail’ length indicative of DNA damage, whereas control cells had significantly smaller ‘tails’ (Fig. 4A and C). Eight hours post-IR, the average ‘tail DNA length’ of patient cells was 200 μm whereas ‘tail length’ in control cells was only 50 μm (Fig. 4C and D). Patient cells defective in PHC1 also showed a progressive increase in ‘comet tail’ length indicative of a defective DNA damage repair after treatment with another DNA damaging agent H$_2$O$_2$ (Supplementary Material, Fig. S5). These results indicate impaired DNA damage repair in cells carrying the PHC1 mutation.

**PHC1 mutation causes abnormal cell cycle activity**

PM is characterized by abnormalities in cell cycle progression (27,28). Since we observed accumulation of Geminin in patient cells and that Geminin has been shown to play a role in DNA content higher than 4N in patient but not control cells treated with IR, indicating polyploidy in these cells (Fig. 5B). Mitotic index at baseline was also significantly lower in patient cells compared with control cells. After IR, control cells displayed a reduced mitotic index, whereas mitotic index in patient cells remained mostly unchanged (Fig. 5C). These observations indicate a reduced proliferative capacity of patient cells compared with control cells.

To understand the effect of the PHC1 mutation on global gene expression, we analyzed gene expression profiling in control and patient cells using microarray analysis. Strikingly, most significantly dysregulated genes in patient cells were involved in cellular growth and proliferation, and cell cycle ($P < 10^{-18}$ and $P < 10^{-17}$, respectively). Genes related to cell death, DNA replication, recombination and DNA repair were also significantly altered in patient cells compared with control cells ($P < 10^{-13}$ and $P < 10^{-7}$, respectively) (Supplementary Material, Fig. S6). These data indicate defects in cellular proliferation, cell cycle and DNA repair in patient cells and substantiate our data described before.

The DNA damage defect observed in patient cells suggests that it may be due to impaired recruitment of PHC1 to damaged chromatin. To test this possibility, we performed cell fractionation and measured PHC1 and Ub-H2A expression in the chromatin and nuclear fraction of control and patient cells under basal condition and after IR by western analysis. Under normal conditions, PHC1 was found in the chromatin and soluble nuclear fraction in both control and patient cells. As expected, a lower amount of PHC1 was detected in both fractions in patient cells compared with control cells (Fig. 5D). IR treatment resulted in a substantial increase in PHC1 binding to chromatin in control cells, whereas the same amount of PHC1 remained bound to chromatin in patient cells (Fig. 5D). Ub-H2A also increased in control cells after IR exposure, whereas Ub-H2A remained the same in patient cells after IR treatment. These results show that a large fraction of PHC1 is recruited to chromatin regions in control cells in response to DNA damage, which correlates with H2A ubiquitination. This response is significantly reduced in patient cells, suggesting that PHC1 mutation qualitatively impairs the DNA repair response following DNA damage. These results suggest a novel role of PHC1 in DNA damage repair through ubiquitination of H2A. They also show that PHC1 mutation impairs proper expression of PHC1 protein, which results in H2A ubiquitination defect and higher sensitivity to DNA damage. Taken together, these results suggest that mutation in PHC1 induces defects in DNA repair and cell cycle progression, elicited by abnormalities in chromatin regulation.

**Over-expression of PHC1 in patient cells rescues cellular defects in DNA damage repair and cell cycle**

To further explore the role of PHC1 in the functional defects of patient cells, we restored adequate levels of PHC1 in Patient 1 and 2 cells by overexpressing wild-type PHC1, and measured the impact on H2A ubiquitination and Geminin expression. Increasing PHC1 protein level in patient cells resulted in a progressive increase in Ub-H2A with concomitant decrease in Geminin expression (Fig. 6A). Next, we tested whether elevated levels of PHC1 can rescue the DNA.
damage sensitivity and cell cycle abnormalities in patient cells. Patient cells transfected with a shuttle vector showed damaged DNA after IR treatment as indicated by the presence of ‘tails’ measured by Comet assay. On the contrary, Patient 1 and 2 cells expressing high levels of PHC1 showed minimal DNA damage after IR treatment (Fig. 6B). The average ‘tail’ length was $\sim 50 \mu m$ in patient cells overexpressing PHC1 compared with 200 $\mu m$ in patient cells transfected with shuttle vector control (Fig. 6C). Overexpression of PHC1 in patient cells also reversed cell cycle abnormalities.

**Figure 4.** PHC1 mutation increases DNA damage. (A) Increased DNA damage in patient lymphocytes compared with control cells at baseline and 4–8 h following a single dose of IR at 5 Gy. Images represent individual cell nuclei with tail DNA derived from the damaged DNA. Patient cells show progressively more damage compared with control cells. (B) Assessment of ‘comet tail’ lengths for the long-tailed nuclei using unified scale (upper panel) showing and close up view (lower panel) showing that patient cells show low level but significant impairment in their ability to repair DNA after a single IR dose. (C) Measurement of ‘comet tail’ lengths for the long-tailed nuclei 4 and 8 h post-IR treatment showing that lymphocytes from patient cells have impaired DNA repair whereas cells from unaffected individuals were able to repair DNA more effectively. (D) Quantification of the relative ‘comet tail’ length in control and patient cells. The data shown represent the mean $\pm$ SD of three independent experiments.
**Figure 5.** PHC1 mutation leads to cell cycle abnormalities and impaired DNA damage response. (A) Cell cycle defects in patient cells at baseline and after IR treatment. Representative histograms of flow cytometry analysis for control and patient lymphoblastoid cells are shown. Cells were treated with IR (10 Gy) and at the indicated time points the DNA content of 30,000 counts from each sample was analyzed using propidium iodide staining. The table represents the percentage of cells in the G1 and G2/M phase of the cell cycle from three independent experiments. (B) Polyploidy detected in PHC1 mutant cells. Values represent percentages of cells with polyploidy (DNA content >4N) obtained from the flow cytometry analysis. Data are representative of three independent experiments. (C) Reduced mitotic index of PHC1 mutant cells. Mitotic index (MI) was determined as the product of the G1/G2 ratio. Data shown represent mean ± SD of three independent experiments. (D) PHC1 mutation impairs DNA damage response by reducing H2A ubiquitination and recruitment of PHC1 to chromatin regions. Chromatin and nuclear fractions were isolated from patient and control cells before and after IR exposure (5 Gy), and 30 μg of proteins were subjected to immunoblotting using anti-PHC1 and anti-Ub-H2A antibodies. Total H2A and GATA-4 were used as loading controls in the chromatin and nuclear fraction, respectively. Quantification of this data is shown in (E).
Figure 6. Exogenous expression of PHC1 rescues H2A ubiquitination and prevents DNA damage and cell cycle defects. (A) Elevated PHC1 expression in patient cells restores H2A ubiquitination. Patient 1 and 2 cells were transfected with increasing amount of PHC1 expression vector. Forty-eight hours after transfection, cell lysates were prepared and subjected to western blotting using anti-PHC1 and anti-Ub H2A-specific antibodies. GAPDH was used as loading control. These blots are representatives of three independent experiments. (B) Increased PHC1 expression inhibits DNA damage in patient lymphoblasts. Patient 1 and 2 cells were transfected with increasing amount of PHC1 expression vector. Forty-eight hours after transfection, cells were prepared and subjected to DNA damage analysis using comet assay. Relative quantification is shown. (C) Quantification of comet assay measured as relative comet length in 50 cells. (D) PHC1 overexpression restores normal cycle profile in patient cells. Representative histograms of flow cytometry analysis. Transformed lymphoblasts of the two patients were transfected with shuttle- or PHC1-plasmid. 48 h post transfection, cells were treated with IR (10 Gy). Then, at the indicated time points, the distribution of cell cycle was determined by propidium iodide staining followed by flow cytometry. Values represent percentages of cell cycle phase-specific populations. Data are representative of three independent experiments.
Consistent with our previous results, transfection of patient cells with a shuttle vector showed ~50% of the cells with G2/M block under basal condition and a similar percentage of cells in G2/M after IR. Overexpression of PHC1 in Patient 1 and 2 cells reversed the G2/M block at baseline as the majority of the cells (~56%) were in G1 phase and only 18% were in G2/M. Patient cells expressing adequate levels of PHC1 responded properly to IR treatment since a higher fraction of the cells (~32%) were arrested in G2/M after 24 h (Fig. 6D). Thus, overexpression of PHC1 can rescue aberrant cell cycle progression. Together, these results indicate that adequate levels of PHC1 are sufficient to ubiquitinate H2A and downregulate Geminin. Normal PHC1 expression is also required for proper progression through the cell cycle and for adequate DNA damage response.

**DISCUSSION**

PcG proteins consist of two major complexes PRC1 and PRC2, and were originally identified in *Drosophila* where they form large protein complexes in chromatin to repress homeotic HOX genes during development (29,30). Polyhomeotic proteins are core components of the PRC1 and act in concert with Trithorax (Thx) to maintain a tightly regulated spatial domain of expression of HOX genes. (31) Three mammalian orthologs of Ph have been identified, namely PHC1, PHC2 and PHC3, whose role in repressing HOX genes is highly conserved (21). Despite functional redundancy, individual Ph proteins have unique biochemical properties and are expressed differently in various tissues (14,32). Our study now shows that chromatin remodeling complexes are implicated in the pathogenesis of PM. PM has been associated with defects in a number of genes that play a role in centriole biology and mitosis plane (1). In this study, we identify a family with PM carrying a novel mutation in PHC1, which links, for the first time, PM to abnormal chromatin modifications (Fig. 7).

A prominent effect of the PHC1 mutation is to reduce PHC1 protein abundance secondary to increased degradation by the proteasome. Since PM has been linked to cell cycle abnormalities previously, we performed detailed cell cycle analysis in control and patient cells. Cell cycle profiles revealed that the mutation is pathogenic as a striking cell cycle block was observed in patient cells at baseline that was rescued by PHC1 overexpression. Our microarray analysis revealed that a large number of genes involved in cell cycle regulation are dysregulated in patient cells. Therefore, the PHC1 mutation recapitulates an important cellular feature of cells isolated from PM patients. Cells carrying the PHC1 mutation have also increased Geminin expression, which was recapitulated in RNAi experiments in control cells and in patient cells where PHC1 was ectopically expressed. Since Geminin has an established role in cell cycle control (33,34), PM pathogenesis could be due in part to cell cycle abnormalities as a consequence of Geminin accumulation. This possibility is currently under investigation.

Several proteins involved in PM and members of the PRC play critical roles in DNA repair pathway (3,23,25). Based on these observations, we tested the possibility that cells isolated from affected individuals display functional abnormalities linked to DNA repair defects. Our results show reduced H2A ubiquitination, impaired recruitment of PHC1 to chromatin regions in response to DNA damage and reduced repair of DNA lesions in patient cells. Gene expression profiling also revealed that among the top 10 dysregulated networks in patient cells were genes involved in DNA repair. Therefore, one likely functional consequence of the PHC1 mutation is to prevent adequate recruitment of PHC1 to DNA lesions through altered chromatin conformation, which in turn impairs the recruitment of DNA repair proteins for efficient DNA repair. Consistent with these, a syndromic form of microcephaly has recently been described where microcephaly is congenital and the underlying mutation in PNKP adversely affects DNA damage repair (35). It has been hypothesized that...
neurons are especially sensitive to DNA damage and that a defect in DNA damage repair will preferentially affect neurons perhaps through activation of DNA damage checkpoints and apoptosis (11). This study together with our data show clear cellular defects in DNA damage repair in patient cells, thus supporting a link between impaired DNA damage response and PM. However, whether the abnormal DNA damage response plays a prominent role in the disease remains elusive. Indeed, patients in our study were born with PM that is nonprogressive in nature. One possibility is that PHC1 mutation leads to changes in chromatin remodeling with two separate consequences: primarily alteration in cell cycle progression and in rare conditions where DNA damage occurs, impairment in DNA repair. Therefore, it appears that the connection between the cellular defects observed in mutant cells and microcephaly is more complex than previously thought.

Two other members of the PRC1, Ring1 and Bmi1, have been shown to be involved in H2A ubiquitination (17). Our results now point toward PHC1 as another member of PRC1 that plays a role in this process. Interestingly, the mutation we report here (Leu992Phe) in PHC1 lies within the SAM domain (Supplementary Material, Fig. S7), which has been classified as an E2 ubiquitin-conjugating enzyme-binding domain responsible for target protein binding (36). Thus, it will be important to evaluate in the future whether the PHC1 mutation disrupts interaction with other components of PcG complex, such as Ring1/Bmi/Mel18 and Scom1.

Mouse Phc1 was originally described as Rae28 and human PHC1 as RAED28 (37,38). Rae28/Phc1-deficient mice display cephalic neural crest defect, abnormal facies, parathyroid and thymic hypoplasia together with skeletal and cardiac abnormalities (38–41). It is worth highlighting that the absence of the equivalent manifestations in our human patients with PHC1 mutation may be explained on the basis of the different nature of the two mutations, i.e. hypomorphic PHC1 mutation versus nullimorphic Phc1 mutation. Another explanation for the apparent differences in phenotypes between patients carrying the PHC1 mutation and Phc1-deficient mice could be due to the young age of the patients, thus not showing yet the various characteristics seen in Phc1−/− mice. Also, although microcephaly was not specifically reported, the available images suggest that Phc1−/− mice did have microcephaly (40).

Overall, the data we present in this study suggest that PM may be a final common pathway of several cellular perturbations including centriolar/mitotic defects, cell cycle abnormalities and DNA damage response defects due to abnormalities in chromatin remodeling. It remains to be seen whether and how these processes also converge in the molecular pathogenesis of PM.

**MATERIALS AND METHODS**

**Human subjects**

All patients were evaluated by board-certified clinical geneticists and pediatric neurologists. An informed written consent was used to recruit the patients and their relatives (KFSHRC IRB Protocol #208006).

**DNA extraction**

A 5–10 ml venous blood sample was collected in ethylenediaminetetraacetic acid (EDTA) and Na-heparin tubes from each subject, parent and sibling. DNA extraction was carried out using Puregene Blood Core Kit C (Qiagen, Germantown, MD, USA) following manufacturer’s instructions and quantified using Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

**Genotyping, linkage analysis and homozygosity mapping**

DNA samples were processed on the Affymetrix Axiom™ Genome-Wide H Array Plate (Affymetrix, Santa Clara CA, USA) following the manufacturer’s instruction. Genotypic data were used for linkage analysis and homozygosity mapping using EasyLinkage (V5.08) (42) and AutoSNPa (43), respectively.

**PCR and direct sequencing**

Mutation-harboring exons (as per exome sequencing) of PHC1 and INO80 were amplified in a Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 μl, containing 20 ng DNA and the recommended amounts of dNTPs (Epicentre Biotechnologies, Madison, WI, USA), primers (Metabion, Martinsried, Germany) and of Hotstar Taq DNA polymerase (Qiagen, Germantown, MD, USA). For polymerase chain reaction (PCR), an initial denaturation step at 95°C for 10 min was followed by 35 cycles of denaturation at 94°C for 40 s, annealing for 40 s and extension at 72°C for 45 s, followed by a final extension step of 72°C for 10 min. Primer sequences are available upon request. The PCR amplicons were sequenced by dye termination sequencing using BigDye Terminator Cycle Sequencing V3.1 Kit and Prism 370XL Genetic Analyzer (Applied Biosystems). DNA sequences were analyzed using the Seqman program of the DNASTAR analysis package (Lasergene, Madison, WI, USA).

**Exome sequencing**

Exome capture was performed using TruSeq Exome Enrichment kit (Illumina) following the manufacturer’s protocol. Samples were prepared as an Illumina sequencing library and, in the second step, the sequencing libraries were enriched for the desired target using the Illumina Exome Enrichment protocol. The captured libraries were sequenced using Illumina HiSeq 2000 Sequencer. The reads are mapped against UCSC hg19 (http://genome.ucsc.edu/) by BWA (http://bio-bwa.sourceforge.net/). The SNPs and Indels are detected by SAMTOOLS (http://samtools.sourceforge.net/).

**Transformation of control and patient cells with EBV and culture condition**

Lymphoblastoid cell lines from healthy donors and patients with PM were established by Epstein–Barr virus (EBV) transformation of peripheral blood mononuclear cells (PBMC). The PBMC were isolated from whole blood by Ficoll-Hypaque gradient and then transformed using EBV-shedding marmoset
Cells were plated on coverslides in six-well dishes at a density of 0.5 × 10^6 cells/well. After fixation in formaldehyde, cells were permeabilized and stained with anti-PHC1 antibody followed by fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G (Sigma, dilution 1:100). The cover slides were mounted with Vectashield mounting media containing 4’,6-diamidino-2-phenylindole (Vector Laboratories). Signals were observed by fluorescence microscopy using a Nikon Eclipse Ti-E microscope.

**siRNA gene silencing**

Control lymphoblasts isolated from two normal individuals were transfected with three different siRNAs (Invitrogen) against PHC1 using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instruction. Scrambled control siRNA was used in parallel. Briefly, 800 pm of siRNA and Lipofectamine were diluted in Opti-MEM media (Invitrogen) and the mixture was added to the cells and incubated for 24 h. Total cell lysates were analyzed by immunoblotting as described before.

**Immunoprecipitation**

Total cell protein extracts were incubated with Dynal protein A magnetic beads (Invitrogen) coupled to anti-PHC1 (Abnova, USA) polyclonal antibody or anti-ubiquitinated H2A (lys 119) (Millipore) in a pulldown buffer (50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [pH 7.5], 1 mM EDTA, 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml pepstatin A). Cell extracts were incubated with the beads overnight at 4°C while mixing on a rotating wheel. Beads not conjugated with antibody were used as negative control. Supernatants were collected, and the beads were then washed with pulldown buffer and left as a 50% slurry. Bead fractions were analyzed on 4–12% SDS-PAGE, and the presence of PHC1 and ubiquitinated-H2A was detected by immunoblotting using the corresponding antibodies.

**Comet assay**

The comet assay (alkali method) was modified from that described earlier (44). Roughened microscope slides were dipped briefly into 1.5% hot (60°C) normal melting agarose (NMA) (Denville Scientific, USA) prepared in phosphate-buffered saline (PBS). The slides were dried and coated with 300 ml of 1.0% NMA in PBS and the agarose was allowed to solidify. Subsequently, 1000 lymphoblasts (20 μl) cells from normal control and patient cells subjected to ± 5 Gy of IR were mixed with 95 ml of 0.75% low melting point agarose (LMA) (Sigma). The mixture was spread on the slide using a cover slip and then allowed to solidify at 4°C. After removal of the cover slip, the slides were immersed in freshly prepared cold (4°C) lysing solution (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris; pH 10–10.5; 1% Triton X-100 and 10% DMSO added just before use) for at least 2 h. The alkaline unwinding, electrophoresis and neutralization steps were performed by removing the slides from the lysis solution and placing them in the electrophoresis chamber, which was then filled with freshly made alkaline buffer (300 mM NaOH...
and 1 mM EDTA, pH 12.6). The cells were exposed to alkali for 30 min to allow for DNA unwinding and the expression of alkali-labile sites. Subsequently, the DNA was electrophoresed for 30 min at 300 mA and 25 V. All of the above steps (preparation of slides, lysis and electrophoresis) were conducted under red light or without direct light in order to prevent additional DNA damage. After electrophoresis, the slides were placed in a horizontal position and washed three times (5 min each) with 0.4 M Tris buffer, pH 7.5, to neutralize the excess alkali. Finally, 70 ml of ethidium bromide (2 μg/ml) was added to each slide, which was then covered with a cover slip, stored in a humidified box at 4°C and analyzed using a Nikon Eclipse Ti-E fluorescence microscope under ×40 objective with a calibrated ×10 eyepiece. Images of 50 randomly selected cells were analyzed from each sample. Tail lengths (nuclear region + tail) were measured at ×40 magnification in which one pixel unit was ~0.085 μm. The fluorescence microscope was equipped with a BP546/12-nm excitation filter and a 590-nm barrier filter. Cells were also scored visually into two classes, according to tail size (from undamaged to damaged). The final overall rating for the DNA damage score was represented as a value of the average of comet length in 50 cells and error bars represents standard deviation.

**Cell cycle analysis**

For cell cycle analysis by flow cytometry, cells were seeded in a six-well plate at density of 5 × 10^4 cells per well in 2 ml media. After 18 h, the cells were exposed to 10 Gy of IR and harvested at 0, 24 and 48 h after IR. The cell cycle distribution was then analyzed using propidium iodide (PI) staining followed by flow cytometry using a FACS Calibur flow cytometer (BD Biosciences). For PI staining cells were harvested, washed with PBS and stained for 1 h in a hypotonic solution containing PI (40 μg/ml), 0.1% sodium citrate and 0.1% triton X-100 as described in (45).

**IR Irradiation and chromatin fractionation**

Cells were irradiated with 5 Gy IR by XRAD 320 (Precision X-Ray, Inc., USA). Typically, 10^6 cells were used for each extraction. Cells were harvested by centrifugation and were lysed in ice-cold Buffer A [10 mm piperazine-N-N′-bis [2-ethane sulfonic acid] (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.5% Triton X-100 supplemented with freshly added protease inhibitors]. Supernatants were recovered and constituted the soluble fraction. Pellets were washed once in buffer A, lysed in buffer B (350 mM Tris-Cl, pH 8.1, 10 mM EDTA, 1% SDS, 0.2 mM EGTA, 1 mM DTT, protease inhibitors cocktail), incubated on ice for 10 min, and then sonicated on ice for 30 s. Micrococcal nuclease (MNase I) was then added to the homogenate and the chromatin fraction was collected by centrifugation at 14 000 rpm for 10 min at 4°C. Proteins from the supernatant and chromatin fraction were analyzed by SDS–PAGE and immunoblotting with the indicated antibodies. Signals were visualized by chemiluminescence as described before.

**Gene expression profiling**

Lymphoblasts from the two patients in Family 1 and unrelated normal controls (performed in triplicates) were used for global expression profiling using Affymetrix’s GeneChip® Human Genome U133 Plus 2.0 Arrays. Sample handling, cDNA synthesis, cRNA labeling and synthesis, hybridization, washing and scanning of chips and all related quality controls were performed according to manufacturer’s instructions. Significantly modulated genes were defined as those with absolute fold change (FC) > 2 and adjusted P-value of <0.05. We used Benjamini–Hochberg step-up procedure to control the false discovery rate (FDR) at 5%. Functional pathway, gene ontology and network analyses were executed using DAVID Bioinformatics Resources, Expression Analysis Systematic Explorer (EASE) and Ingenuity Pathways Analysis (IPA) 6.3 (Ingenuity Systems, Mountain View, CA). A right-tailed Fisher’s exact test was used to calculate the P-value determining the probability that the biological function (or pathway) assigned to that dataset is explained by chance alone. Statistical analyses were performed with the MATLAB software packages (Mathworks, Natick, MA), and PARTEK Genomics Suite (Partek, Inc., St. Louis, MO, USA).

**Over-expression of PHC1**

**PHC1** expression vector was from Origene, USA. Control or patient lymphoblasts were transfected with empty shuttle vector or **PHC1** expression vector expressing GFP using lipofectamine 2000. Twenty-four hours after transfection, cells were washed and maintained in growing media for 48 h. Subsequently, cells were processed for western analysis, comet assay or cell cycle analysis as described before.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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