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Identification of UHRF1/2 as new *N*-methylpurine DNA glycosylase-interacting proteins

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ABSTRACT

N-methylpurine DNA glycosylase (MPG), a DNA repair enzyme, functions in the DNA base excision repair (BER) pathway. Aberrant over-expression of MPG in various cancers suggests an important role of MPG in carcinogenesis. Identification of MPG-interacting proteins will help to dissect the molecular link between MPG and cancer development. In the present study, using immunoprecipitation coupled with mass spectrometry (IP/MS), we screened ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1), an essential protein required for the maintenance of DNA methylation, as a MPG-interacting protein. Endogenous co-immunoprecipitation assay in cancer cells confirmed that UHRF1 interacted with MPG in a p53 status-independent manner. Confocal microscopy showed that endogenous MPG and UHRF1 were co-localized in the nucleoplasm. Furthermore, co-immunoprecipitation assay indicated that UHRF2, the homolog of UHRF1, could also interact with MPG. These results show that MPG and the UHRF family of proteins interact, thus providing a functional linkage between MPG and UHRF1/2.

1. Introduction

Base excision repair (BER) is the major DNA-repair process responsible for handling the DNA damage induced by alkylating and oxidative agents and by spontaneous depurination, thereby maintaining genomic integrity [1,2]. *N*-methylpurine DNA glycosylase (MPG), the first identified enzyme in BER pathway, can recognize and excise the damaged bases and generate an apurinic/apyrimidinic (AP) site, which is subsequently repaired by downstream proteins of the BER pathway [3].

Cells lacking MPG show increased levels of DNA base damage, elevated mutation rates, and hypersensitivity to specific DNA damaging agents [4,5]. Interestingly, elevated levels of MPG can also cause frameshift mutagenesis and microsatellite instability [6,7]. High activity of MPG leads to the imbalance of BER pathway and the accumulation of cytotoxic and mutagenic base excision repair intermediates (e.g., abasic sites) [8]. Over-expression of MPG is observed in various cancers including breast, lung and colon cancers [9]. Collectively, MPG may display a bimodal behavior, whereby both low and high activities of which are associated with increased cancer risk [10]. We hypothesize that there are other molecules participating in the process which modulates the binary phenotypes of MPG.

\UHRF1 and UHRF2 are two members of the UHRF family which participate in DNA methylation maintenance and its inheritance during cell division [11,12]. UHRF1 is regulated by tumor suppressor genes and also exerts a feedback control on these genes [13,14]. Reports have found that UHRF1 acts as an oncogene protein to promote G1/S transition [15] and shows significant over-expression in lung, breast and prostate cancers [16]. However, UHRF2, a protein highly homologous to UHRF1 in sequence and structure, plays a different role by inducing an increased cell population percentage in G1 phase and exerts inhibitory effect regarding G1/S transition, indicating that UHRF2 may behave as a tumor suppressor protein [17].

Immunoprecipitation coupled with mass spectrometry (IP/MS) is one of the most practical approaches for identifying protein–protein interactions [18]. Here, using this technology, we identify UHRF1 as the specific MPG-interacting protein. Furthermore, UHRF2, the homolog of UHRF1, also interacts with MPG. These

Abbreviations: MPG, *N*-methylpurine DNA glycosylase; UHRF1/2, ubiquitin-like containing PHD and RING finger domains 1/2; IP/MS, immunoprecipitation coupled with mass spectrometry; BER, base excision repair.

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findings will establish novel functional linkage between MPG and UHRF1/2 and set a new direction for future cancer research.

2. Materials and methods

2.1. Plasmid constructs

Myc-MPG was constructed by inserting PCR amplified fragment into the related vectors. Detailed construct information of MPG was previously described [9]. Flag-UHRF1/2 was a kind gift from Dr. Jiemin Wong (East China Normal University, Shanghai, China).

2.2. Cell culture and transfection

Human breast cancer MCF7 cells and BT474 cells, human embryonic kidney HEK293T cells, human colon cancer HCT116 ($p53^{+/+}$ and $p53^{-/-}$, a kind gift from Dr. Qimin Zhan), human cervical cancer Hela cells and human lung adenocarcinoma H1299 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone) containing 10% fetal bovine serum (FBS; Hyclone). Cells were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols.

2.3. Antibodies

Anti-Myc antibody was purchased from Clontech. Anti-FLAG and anti-FLAG-HRP antibodies were from Sigma. Anti-MPG and anti-Myc-HRP antibodies were from Santa Cruz. Anti-UHRF1, anti-PKM2 and anti-SRSF1 were from Abcam.

2.4. Immunoprecipitation and immunoblotting

Cells were harvested at 48 h post-transfection and lysed in HEPES lysis buffer (20 mM HEPES, pH 7.2, 50 mM NaCl, 0.5% Triton X-100, 1 mM NaF and 1 mM DTT) supplemented with protease inhibitor cocktail (Roche). Immunoprecipitations were performed using the indicated primary antibody and protein A/G-agarose beads (Santa Cruz) at 4 °C. Lysates and immunoprecipitates were examined using the indicated primary antibodies followed by detection with the related secondary antibody and the SuperSignal chemiluminescence kit (Pierce).

2.5. Fluorescence analysis

For detection of colocalization by immunofluorescence, cells were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 (PBS). Proteins were stained using the indicated antibodies and detected with a TRITC-conjugated or FITC-conjugated secondary antibody. The nuclei were stained with DAPI (Sigma), and images were visualized with a Zeiss LSM 510 Meta inverted confocal microscope.

2.6. Immunoprecipitation coupled with mass spectrometry (IP/MS)

Nuclear proteins were extracted from cells and performed immunoprecipitation using the indicated primary antibody and protein A/G-agarose beads (Santa Cruz) at 4 °C. Immunoprecipitates were isolated by electrophoresis on denaturing SDS–polyacrylamide gels. After coomassie blue staining, protein bands

Table 1
The list of 37 potential MPG-interacting proteins identified by mass spectrometry analysis.

No.	Protein name
1	Serine/arginine-rich splicing factor 1 isoform 1 [Homo sapiens]
2	Serpin A12 precursor [Homo sapiens]
3	E3 ubiquitin–protein ligase UHRF1 isoform 1 [Homo sapiens]
4	Replication factor C subunit 4 [Homo sapiens]
5	Hypothetical protein LOC64897 [Homo sapiens]
6	Pyruvate kinase isozymes M1/M2 isoform M2 [Homo sapiens]
7	Pre-mRNA-splicing factor 38B [Homo sapiens]
8	Peroxiredoxin-1 [Homo sapiens]
9	Nuclear cap-binding protein subunit 1 [Homo sapiens]
10	Fragile X mental retardation syndrome-related protein 2 [Homo sapiens]
11	Filaggrin [Homo sapiens]
12	Putative ATP-dependent RNA helicase DHX33 isoform 1 [Homo sapiens]
13	Corneodesmosin precursor [Homo sapiens]
14	Far upstream element-binding protein 2 [Homo sapiens]
15	Rac GTPase-activating protein 1 [Homo sapiens]
16	Gelsolin isoform b [Homo sapiens]
17	F-actin-capping protein subunit alpha-1 [Homo sapiens]
18	Lutropin–choriogonadotropic hormone receptor precursor [Homo sapiens]
19	Tuftelin-interacting protein 11 [Homo sapiens]
20	Nucleolar complex protein 2 homolog [Homo sapiens]
21	Cystatin-M precursor [Homo sapiens]
22	Mortality factor 4-like protein 1 isoform 1 [Homo sapiens]
23	CLIP-associating protein 2 [Homo sapiens]
24	U4/U6.U5 tri-snRNP-associated protein 1 [Homo sapiens]
25	Serine/threonine–protein phosphatase 1 regulatory subunit 10 [Homo sapiens]
26	Regulation of nuclear pre-mRNA domain-containing protein 2 [Homo sapiens]
27	Obscurin isoform a [Homo sapiens]
28	Guanine nucleotide-binding protein-like 3 isoform 2 [Homo sapiens]
29	DNA-directed RNA polymerase I subunit RPA43 [Homo sapiens]
30	Cleavage stimulation factor subunit 1 [Homo sapiens]
31	Lysine-specific demethylase 2A [Homo sapiens]
32	Ribosomal RNA processing protein 1 homolog B [Homo sapiens]
33	Ribosomal RNA-processing protein 7 homolog A [Homo sapiens]
34	Nucleolar GTP-binding protein 2 [Homo sapiens]
35	La-related protein 4 isoform e [Homo sapiens]
36	Keratinocyte proline-rich protein [Homo sapiens]
37	Exosome complex exonuclease RRP45 isoform 2 [Homo sapiens]

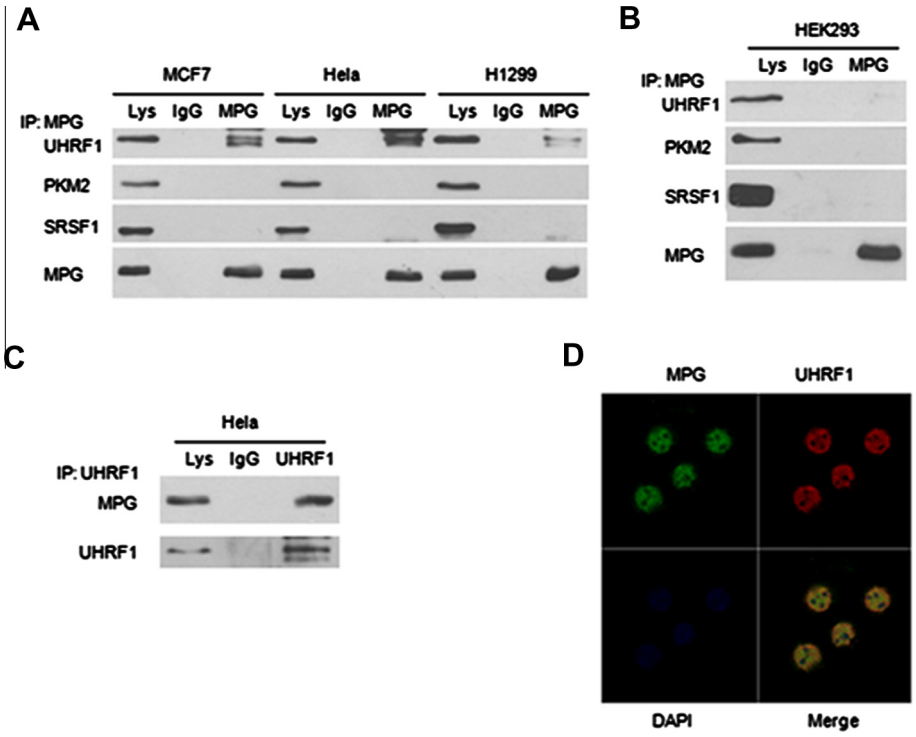

Fig. 1. Confirmation the interactions between MPG and SRSF1, PKM2, UHRF1, respectively. (A) Co-immunoprecipitations of endogenous MPG and SRSF1, PKM2, UHRF1 by MPG antibody or negative control IgG in MCF7, Hela, H1299, respectively. (B) Co-immunoprecipitations of endogenous MPG and SRSF1, PKM2, UHRF1 by MPG antibody or IgG in HEK293, respectively. (C) Co-immunoprecipitation of endogenous MPG and UHRF1 by UHRF1 antibody or IgG in Hela cells. Cell lysates and immunoprecipitates were detected by western blot analysis with the corresponding antibodies. IP, immunoprecipitation; IB, immunoblotting; Lys, lysate. (D) Co-localization of MPG and UHRF1 in the nucleoplasm of Hela cells. Indirect immunofluorescence analysis was performed. The cells were visualized by confocal microscopy and nuclei were stained with DAPI.

were excised and sent to Beijing Proteome Research Center (BPRC) for mass spectrometry analysis.

3. Results

3.1. Screening potential MPG-interacting proteins by IP/MS

MPG has been found to be abundantly expressed in nucleoplasm of human breast cancer MCF7 cells [19]. To find new binding proteins of MPG, we extracted nuclear proteins of MCF7 cells to perform immunoprecipitation by MPG antibody. Immunoprecipitates were isolated by SDS–polyacrylamide electrophoresis and stained with coomassie blue. Finally, mass spectrometry analysis of the corresponding protein bands predicted a total of 37 potential MPG-interacting proteins (Table 1).

3.2. UHRF1 interacts with MPG in cancer cells in vivo and displays a co-localization with MPG in the nucleoplasm

Among the list of 37 potential MPG-interacting proteins, SRSF1 (serine/arginine-rich splicing factor 1), PKM2 (pyruvate kinase isozymes M1/M2) and UHRF1 (ubiquitin-like, containing PHD and RING finger domains 1) attracted our attention based on their range on the list and biological functions. SRSF1 plays a central role in constitutive and alternative mRNA splicing. Depletion of SRSF1 triggers genomic instability, cell-cycle arrest and apoptosis [20]. PKM2 is an enzyme which catalyzes the later step of glycolysis [21]. UHRF1 is required for the maintenance of DNA methylation and histone modifications [22]. All these proteins play important roles in cancer development [16,20,21].

To confirm the interactions between MPG and SRSF1, PKM2, UHRF1, respectively, we performed endogenous immunoprecipitations by MPG antibody in various cancer cells including human breast cancer MCF7, human cervical cancer Hela, human lung adenocarcinoma H1299 and normal human embryonic kidney HEK293T cells. In three cancer cells, we found that endogenous UHRF1 was co-immunoprecipitated with endogenous MPG, but not by the control IgG. Meanwhile, no SRSF1 or PKM2 was detected in MPG antibody or control IgG immunoprecipitates (Fig. 1A). However, in normal human embryonic kidney HEK293 cells, none of UHRF1, SRSF1 and PKM2 could be detected in MPG antibody or control IgG immunoprecipitates (Fig. 1B). To further confirm the interaction between UHRF1 and MPG, we conducted reciprocal immunoprecipitation and indirect immunofluorescence assay in Hela cells. Endogenous MPG was co-immunoprecipitated with endogenous UHRF1, but not by the control IgG (Fig. 1C). Indirect immunofluorescence assay revealed that MPG and UHRF1 were predominantly co-localized in nucleoplasm of Hela cells (Fig. 1D).

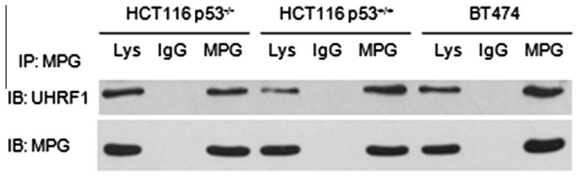

Fig. 2. Different p53 status has no influence on the interaction between MPG and UHRF1. Cell lysates from $p53^{+/+}$ HCT116, $p53^{-/-}$ HCT116 and *p53 mutant* BT474 cells were immunoprecipitated with anti-MPG antibody, followed by western blot analysis with anti-UHRF1 or anti-MPG antibodies.

3.3. UHRF1 interacts with MPG in a p53 status-independent manner

Both MPG and UHRF1 are regulated directly or indirectly by p53 and participate in p53-mediated biological activity [9,13]. We therefore tested whether different p53 status would affect the interaction between MPG and UHRF1. Endogenous immunoprecipitations by

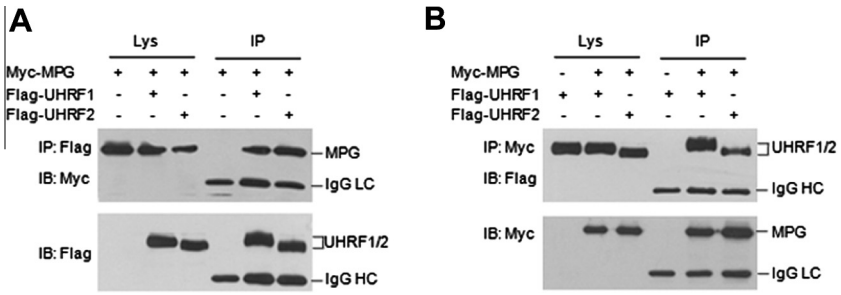

Fig. 3. UHRF2, the homolog of UHRF1, also interacts with MPG. HEK293 cells were transfected with Myc-tagged MPG and Flag-tagged UHRF1/2. After 48 h, cell lysates were immunoprecipitated with anti-Myc (A) or anti-Flag antibodies (B). The cell lysates and immunoprecipitates were detected by western blot analysis with anti-Myc or anti-Flag HRP antibodies.

MPG antibody were performed in human colon cancer $p53^{+/+}$ HCT116, $p53^{-/-}$ HCT116 and human breast cancer *p53 mutant* BT474 cells. In all three cell lines, endogenous UHRF1 was co-immunoprecipitated easily with endogenous MPG, but not by control IgG (Fig. 2). This result indicated that different p53 status could not affect the interaction between MPG and UHRF1.

3.4. UHRF2, the homolog of UHRF1, can also interact with MPG

UHRF2 is highly similar to UHRF1 in terms of sequence and structure, raising question as whether it can also interact with MPG. Myc-tagged MPG with Flag-tagged UHRF1 or UHRF2 was co-expressed in HEK293 cells followed by co-immunoprecipitation assays. Indeed, MPG was co-immunoprecipitated with both ectopic UHRF1 and UHRF2 (Fig. 3A). Reciprocal assays showed that UHRF1 or UHRF2 was also co-immunoprecipitated with the ectopic MPG (Fig. 3B). For negative control, there was no MPG or UHRF1 detected in anti-Myc or anti-Flag antibody immunoprecipitates from cells transfected with Myc-MPG or Flag-UHRF1 alone, respectively (Fig. 3A and B).

4. Discussion

In recent years, MPG has been shown to play an important role in carcinogenesis depending on its glycosylase activity to produce AP sites. However, the mechanism of overexpressed MPG in cancers remains poorly understood. We hypothesize that there are other proteins participating in the regulation of MPG activity. In our experiment, using IP/MS, we screened 37 potential MPG-interacting proteins (Table 1). We chose SRSF1, PKM2 and UHRF1 for further identification according to their biological significance and ranking. Co-immunoprecipitation and indirect immunofluorescence assay showed that endogenous UHRF1 interacted with endogenous MPG in cancer cells. However, interestingly, in normal HEK293 cells, co-immunoprecipitation assay showed that none of these proteins bound to MPG (Fig. 1). We hypothesize that UHRF1 interacts with MPG exclusively in cancer cells, which needs to be further tested.

In our previous study, we have found that MPG directly binds to the tumor suppressor p53 and regulates the expression level of pro-arrest gene downstream of p53 including p21, 14-3-3σ and Gadd45. The p53 status coordinates with MPG to play a pivotal role in alkylating agent induced base damage [9]. Other reports have demonstrated that UHRF1 is a direct target of E2F1 and promotes G1/S transition. P53, which is deficient in 50% of cancer, down-regulates UHRF1 through up-regulation of p21/WAF1 and subsequent deactivation of E2F1. The expression of UHRF1 is up-regulated in cancers, probably partially because of absence of wild type p53 [13,14,22]. Collectively, both MPG and UHRF1 are involved in p53-mediated biological activities. So we wondered whether the different p53 status would influence the interaction between MPG and UHRF1. Surprisingly, our results showed that UHRF1 could interact with MPG in a p53 status-independent manner, suggesting a stable interaction between UHRF1 and MPG (Fig. 2).

UHRF1 and UHRF2 are two members of UHRF family. Both of them possess an ubiquitin-like domain (UBL), a PHD domain, an SRA domain, and a RING domain. Homology of each domain between UHRF1 and UHRF2 is 65%, 60%, 76% and 79%, respectively [22]. Both of them can interact with DNA methyltransferase including DNMT1, DNMT3a, DNMT3b and G9a to maintain DNA methylation [23]. In our experiment, we firstly found that MPG, a DNA glycosylase other than a DNA methyltransferase, can also interact with both UHRF1 and UHRF2 (Fig. 3). This finding will help to reveal the unknown functional linkage between MPG-mediated DNA damage repair and UHRF1/2-mediated DNA methylation. Furthermore, MPG together with UHRF1 or UHRF2 is likely to be the combined targets of pivotal interest for developing new anticancer drugs.

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