Published in final edited form as:

DNA Repair (Amst). 2018 May ; 65: 20–25. doi:10.1016/j.dnarep.2018.03.002.

The MLH1 ATPase Domain is needed for Suppressing Aberrant Formation of Interstitial Telomeric Sequences

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Abstract

Genome instability gives rise to cancer. MLH1, commonly known for its important role in mismatch repair (MMR), DNA damage signaling and double-strand break (DSB) repair, safeguards genome stability. Recently we have reported a novel role of MLH1 in preventing aberrant formation of interstitial telomeric sequences (ITSs) at intra-chromosomal regions. Deficiency in MLH1, in particular its N-terminus, leads to an increase of ITSs. Here, we identify that the ATPase activity in the MLH1 N-terminal domain is important for suppressing the formation of ITSs. The ATPase activity is also needed for recruiting MLH1 to DSBs. Moreover, defective ATPase activity of MLH1 causes an increase in micronuclei formation. Our results highlight the crucial role of MLH1's ATPase domain in preventing the aberrant formation of telomeric sequences at the intra-chromosomal regions and preserving genome stability.

Keywords

MLH1; ATPase; interstitial telomere sequences

Introduction

The MMR system keeps the fidelity of replication by repairing replication errors such as base-base mispairs and insertions/deletions. It plays a substantial role in restoring the fidelity of DNA and contributing to genome integrity [1]. The components of MMR are conserved in a broad range of species including *E.coli*, yeast and human [2]. The main components of MMR in human cells are MutS homologs (MSH2, MSH3, MSH6), MutL homologs (MLH1, MLH3, PMS1, PMS2), the PCNA sliding clamp. RPA. EXO1, HMGB1, RFC, DNA polymerase δ and ligase 1 also play a crucial part in MMR [3, 4]. The MutS homologs form

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Author contributions

PJ performed experiments and collected all the data. WC directed the study. PJ and WC wrote the paper.

Conflict of interests

None.

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the heterodimers MSH2-MSH6 (known as MutS α) and MSH2-MSH3 (MutS β), which play a critical role in mismatch recognition and initiation of repair. MLH1 heterodimerizes with PMS2, PMS1, or MLH3 to form MutL α (MLH1-PMS2), MutL β (MLH1-PMS1), or MutL γ (MLH1-MLH3), respectively [5]. During MMR, DNA mismatches are recognized by MutS which recruits MutL to the mismatch sites. EXO1 assists in removing the daughter-strand DNA containing mismatches, while the parental strand DNA is stabilized by RPA. RPA and HMGB1 stimulate MutS α -activated, EXO1-catalyzed excision. The gap is then filled by Pol δ and sealed by DNA ligase I [4]. Mutations in MSH2, MSH6, MLH1 or PMS2 that cause constitutional MMR defects lead to the development of Lynch Syndrome (also known as hereditary non-polyposis colorectal cancer), which is associated with high risk of colorectal and other cancers [6, 7].

Cumulative evidence supports that MMR proteins are also involved in MMR-independent pathways including homologous recombination, DSB repair, and DNA damage response [8, 9]. Deficiency in MMR genes has been associated with increased resistance to apoptosis after DNA damage and the elevated frequency of drug resistance [10–12]. It has been reported that MSH2 and MSH6 are recruited to DNA damage sites in the G1 phase in XPA cells after UV irradiation [13], and the MutL α -defective 293T cells are deficient in UVA-induced DNA damage response, and expressing recombinant MutL α rescues such deficiency [14].

The human MLH1 is a 756 amino-acids protein that can be roughly divided into two domains: the N-terminal domain (NTD) and the C-terminal domain (CTD) [15]. The N-terminus harbors an ATPase domain and the MSH2-binding motif, whereas the C-terminal domain enables MLH1 interaction with its binding partners and also possesses the binding site for PMS2 that is important for the endonucleolytic function of PMS2. Numerous studies have demonstrated the importance of the ATPase activity in MLH1 function. Mutations in the ATPase domain of hMutL α abolish the ATP hydrolytic activity of hMutL α [16]. ATP hydrolysis is indispensable for hMutL α activity in MMR, and mutant hMutL α lacks the MMR activity, indicating the important function of hMutL α ATP hydrolysis in MMR, which is consistent with studies of MutL in *E.coli* [17–19]. In Lynch syndrome, missense mutations in or near the ATP binding site confer increased mutagenesis, perhaps due to diminished ATP hydrolysis [17]. Previous studies also report that the ATPase-deficient MLH1 knockin mice exhibit a dramatic reduction in class-switch recombination but not somatic hypermutation, as well as decreased accumulation of 53BP1, suggesting that MLH1 is involved in DNA end processing through its ATPase activity [20]. Finally, loss of MLH1 N-terminus also abrogates its recruitment to DNA damage sites induced by laser-irradiated or etoposide [21, 22]. Studies have also shown that MMR proteins also participant in signaling dysfunctional telomeres [23, 24].

Telomeres are repetitive TTAGGG sequences present at chromosomal ends that are pivotal for the maintenance of genome stability [25]. However, in some vertebrate species, short stretches of (TTAGGG)_n tandem repeats can also be found at intra-chromosome loci, the so-called interstitial telomeric sequences (ITSs). ITSs contribute to chromosome breakage, recombination and rearrangement. Therefore, ITSs may be an important source of genome instability [22, 26–31]. We have previously identified that the N-terminus of MLH1

(residues 1–389) is required for recruiting MLH1 to DNA damage sites and also suppresses aberrant formation of ITSs in telomerase-positive cells. The aberrant formation of ITSs appears to be associated with DSB formation, and such formation may induce chromosome instability such as chromosome loss and micronuclei formation [22].

Given the important role of the ATPase domain in MLH1 function and DSBs repair, we hypothesize that the ATPase activity may be involved in suppressing ITS formation. In this report, we dissect the role of the ATPase domain and the MSH2-binding domain of MLH1 in ITS suppression by analyzing mutants in the ATPase domain and in the MSH2-binding domain. We find that mutations with the loss of ATPase activity abolish MLH1 accumulation at DSBs, fail to suppress ITS formation, and induce micronuclei formation, while mutations in MSH2-binding motif show no such defects. Our results suggest that the ATPase activity of MLH1 is vital for preventing the formation of aberrant ITSs and guarding genome stability.

Materials and Methods

Plasmids

pBabe-hygro containing FLAG-tagged full-length MLH1 or its N-terminal truncation containing 2–389 amino acids (NTD) were described previously [22]. QuikChange Site-directed Mutagenesis kit (Agilent) was used to generate E34A, G67R, H112D, G244D and H329P mutations in NTD. All constructs were sequenced to ensure sequence accuracy. pGEX-2T-FLAG-MLH1-N-E34A, pGEX-2T-FLAG-MLH1-N-G67R, pGEX-2T-FLAG-MLH1-N-H112D, pGEX-2T-FLAG-MLH1-N-G244D and pGEX-2T-FLAG-MLH1-N-H329P were constructed by digesting corresponding pBabe-hygro-FLAG-NTD mutations and then subcloned into the pGEX-2T vector.

Cell culture

MLH1-deficient human colorectal tumor cell line HCT116 and its isogenic cell line HCT116^{MLH1} (HCT116 complemented with chromosome 3 carrying the wild-type hMLH1 gene) [32] were cultured in McCoy's media supplemented with 10% fetal bovine serum at 37°C containing 5% CO₂. HCT116^{MLH1} was cultured in the presence of G418 (0.3 mg/ml) to maintain stable expression of MLH1. HCT116 MLH1 N-terminal mutants (E34A, G67R, H112D, G244D, H329P) cells were generated by retroviral transduction followed by hygromycin selection.

Chemicals and Antibodies

Etoposide (Sigma) was dissolved in DMSO and stored under sterile conditions at –20°C in dark and was used to generate DNA damage. Primary antibodies used were rabbit polyclonal anti-FLAG (Cell Signaling), monoclonal mouse anti-actin (BD Biosciences), rabbit polyclonal anti-53BP1 (Novus). Secondary antibodies were horseradish peroxidase conjugated anti-mouse IgG and anti-rabbit IgG (BD Biosciences) for western blotting, Dylight 488 anti-mouse IgG (ThermoFisher) and Dylight 549 anti-rabbit IgG (ThermoFisher) for immunofluorescence.

Immunofluorescence (IF)

Cells were grown in chamber slides, fixed with 4% paraformaldehyde and permeabilized with 0.15% Triton X-100, blocked with 3% BSA at 37°C for 1 hr in humidified chamber, incubated with primary antibodies for overnight at 4°C, washed with PBS for three times, and then incubated with secondary antibody at 37°C for 1 hr. Slides were then washed with PBS for three times, dried in ethanol series, and mounted with DAPI-containing mounting media (Vector Lab). Z-stack images were taken at a 0.275 μm thickness per slice under Zeiss AxioImager M2 epi-fluorescence microscope with a 100 $\times$ oil objective.

Protein purification

A single colony from *E. coli* containing recombinant pGEX-2T-FLAG-MLH1-NTD mutations was inoculated to 2 ml of 2X LB (Luria-Bertani) medium, incubated for 12–15 hrs at 37°C with vigorous shaking, diluted 1:100 into 100 ml fresh pre-warmed 2X LB medium, and incubated at 16–20°C with shaking until OD600 reaches 0.5–1. IPTG was added to a final concentration of 0.2 mM and incubation continued for additional 4–6 hrs. After sedimenting cells at $7,700 \times g$ for 10 min at 4°C, pellets were resuspended in ice-cold 1X PBS, and lysed with Microfluidizer Processor (M-110L, Microfluidics). Triton X-100 was then added into the lysate to a final concentration of 1% and mixed gently for 30 min to aid in solubilization of the fusion protein. Following centrifugation at $12,000 \times g$ for 15 min at 4°C, supernatant was transferred to a fresh container, and then incubated with a 50% slurry of Glutathione Sepharose 4B (ThermoFisher) with gentle agitation at room temperature for 30 min. The mixture was then transferred to a disposable column, washed with 1X PBS three times, and proteins were eluted with glutathione elution buffer [10 mM reduced glutathione, 50 mM Tris-HCl (pH 8.0)] and analyzed by SDS-PAGE.

ATPase activity measurement

The ATPase activity was measured with the colorimetric ATPase assay kit (Innova Biosciences) following instructions provided by the manufacturer. The color reached maximum at 30 min after incubation and the absorbance at 600 nm was measured with Synergy H1 microplate reader (BioTek).

FISH

Metaphase cell collection and FISH were performed as previously described [33]. HCT116 and HCT116^{MLH1} cells were treated with 3 μM etoposide for 2 hrs, recovered for 24 hrs, treated with colcemid (0.1 $\mu\text{g}/\text{ml}$). The metaphase cells were collected and prepared for metaphase spreads. Images were acquired with a Zeiss AxioImager M2 epifluorescence microscope with a 100 $\times$ oil objective. During image analysis, unprocessed monochrome images were examined to locate telomeric sequences.

Statistical analysis

Means were compared using a Student's *t*-test. Proportions were compared using a binomial *Z*-statistic. A Holm–Bonferroni correction was used on all multiple pairwise comparisons to control for familywise error.

Results

Expression of MLH1 N-terminal mutants in HCT116 cells

Previously we have observed that suppression of aberrant ITS formation requires the NTD of MLH1, and the treatment with DSB inducer appears to increase the frequency of ITS in telomerase-expressing cells [22]. The NTD consists of two functional domains: the ATPase domain and the MSH2 binding domain [15, 34, 35]. To determine which domain is important for ITS suppression, we used the separation-of-function mutants in the NTD, including E34A and G67R (both in the ATPase activity domain), and H112D, G244D, H329P (all in the MSH2-interacting domain) (Fig. 1A). Previous reports have shown that the E34A mutation has no detectable ATPase activity while retains interaction with MutS α [16]. The G67R mutation abolishes ATP binding and hydrolysis [36]. In contrast, the H112D mutation retains the ATPase activity comparable to wild type, but is defective in binding to MutS α -DNA [16]. Mutations G244D and H329P are identified in Lynch syndrome patients [37, 38], and both mutations reside outside the ATPase domain (Fig. 1A). Neither H112D, G244D, nor H329P is expected to affect the ATPase activity of MLH1. Using retroviral transduction, we constructed E34A, G67R, H112D, G244D and H329P mutants in MLH1 N-terminus and then established stable cell lines in human colorectal adenocarcinoma cell line HCT116. HCT116 was chosen because the MLH1 gene is silent due to a homozygous mutation in MLH1 that results in a truncated, non-functional protein [39]. Immunoblotting was performed to validate the expression of all exogenous expressed mutants (Fig. 1B).

Effects of MLH1 mutants on the ATPase activity

To determine whether the ATPase activity of MLH1 was affected by N-terminal mutations, we expressed and purified recombinant glutathione S-transferase (GST)-fused full-length MLH1, NTD, along with GST-fused NTD mutants from *E. coli* (Fig 2A), and then measured ATPase activities of purified proteins. As expected, GST alone did not possess any ATPase activity (Fig. 2B). The ATPase activity was maximal in wild-type full-length MLH1, and the NTD showed similar ATPase activity (Fig. 2B). The N-E34A and N-G67R mutations greatly abolished the ATPase activity (Fig. 2B), consistent with previous reports [16, 36]. In contrast, mutations in the MSH2-interacting domain (N-H112D, N-G244D and N-H329P) had no defect in the ATPase activity (Fig. 2B).

The ATPase activity in MLH1 N-terminus is responsible for MLH1 localization to the sites of DNA damage

Although MLH1 is the MMR core protein modulating the timing and spatial relationships of the activities of the MMR complex [40], it also plays an important role in other DNA metabolism pathways such as class switch recombination, single strand annealing, triplet repeat expansion, DSB repair, and DNA damage response [21, 41]. In response to laser-induced DNA damage, MLH1 is accumulated at the damage sites [21]. We and others have shown that the NTD of MLH1 is responsible for recruiting MLH1 to DNA damage sites, while deletion of NTD abolishes MLH1 recruitment [21, 22], suggesting that the NTD is required for MLH1 function in DNA damage response. Since the ATPase activity of MMR proteins is implicated in DNA damage signaling [42], we investigated whether the ATPase activity of MLH1 is needed for MLH1 accumulation at damage sites. We stably expressed

wild type FLAG-tagged NTD and NTD mutants (E34A, G67R, H112D, G244D, H329P) in HCT116 cells. Immunofluorescent staining showed that all of them predominantly localized in nuclei (Fig. 3A). Cells were then analyzed for FLAG-MLH1 accumulation at the 53BP1-marked DSBs following etoposide treatment (Fig. 3B). To eliminate random overlaps of numerous FLAG-MLH1 foci with 53BP1, all images were taken in Z-stacks and only colocalizations present in multiple Z-stacks were counted during image analysis. In agreement with our previous observations, a portion of wild-type NTD accumulated at 53BP1 foci (Fig. 3B), suggesting that a subset of wild-type NTD are recruited to DSBs. Furthermore, N-H112D, N-G244D, N-H329P exhibited normal accumulation at break sites similar to wild-type NTD, while N-E34A and N-G67R significantly diminished MLH1 accumulation at 53BP1-marked DSBs (Figs. 3B, 3C). Our results suggest that the ATPase activity of NTD is required for MLH1 recruitment to DSBs.

ITS suppression relies on the ATPase activity of MLH1

Due to the importance of the ATPase activity of MLH1 in colocalization with DNA damage, we then tested whether this ATPase activity was required for ITS suppression. We cultured HCT116 cells stably expressing NTD, N-E34A, N-G67R, N-H112D, N-G244D and N-329P, and then performed telomere FISH on metaphase cells. The HCT116^{MLH1} cell line is the isogenic cell line of HCT116 that has been corrected with transfer of chromosome 3 containing wild-type MLH1 [32] and therefore was used as the positive control. As shown in Figs. 4A and 4B, mutants that abolished the ATPase activity, including E34A and G67R, failed to suppress ITS formed in MLH1-deficient cells, while H112D, G244D, H329P and wild type MLH1-NTD suppressed the formation of ITSs. This result suggests that the ATPase activity is required for suppressing aberrant formation of ITSs.

In addition, we also confirmed our previous observation that the DSB inducer elevates ITS frequency, and such elevation depends on the ATM/ATR activity [22] (Fig. 4B). In all cells analyzed, etoposide treatment increased ITS formation compared to untreated samples (Fig. 4B). Similar to untreated cells, E34A and G67R failed to suppress ITS formation while H112D, G244D, H329P and wild type MLH1-NTD all suppressed the formation of ITS (Fig. 4B).

The ATPase activity of MLH1 acts as a suppressor of micronuclei formation

Next, we investigated the effect of inactive ATPase on the formation of micronuclei, which is a marker of genome instability. We analyzed wild-type NTD and all five mutants used in this study by DAPI staining (Fig. 5A). As shown in Fig. 5B, N-E34A and N-G67R, which abolished the ATPase activity, dramatically increased the frequency of micronuclei. In contrast, mutations in the MSH2-binding domain (N-H112D, N-G244D and N-H329P) did not induce micronuclei formation. Taken together, our results show that loss of ATPase activity increases the numbers of micronuclei, implying that the ATPase activity in MLH1 N-terminus plays an important role in inhibiting ITS-induced genome instability.

Discussion

Human MutL-family complexes (MutL α : MLH1-PMS2, MutL β :MLH1-PMS1, MutL γ :MLH1-MLH3) exhibit multiple molecular activities including DNA binding, DNA cleavage, ATP binding and hydrolysis [43]. In our recent study, we have uncovered an important function of MLH1 in safeguarding the integrity of genomic DNA. MLH1 represses the aberrant formation of ITSs in telomerase-expressing cells. Our data also suggest that the DNA damage response function of hMLH1 may be involved in repressing ITS formation [22]. Given the importance of the ATPase activity of MutL in DNA damage response, here, we selected five separation-of-function mutants in hMLH1 to determine the role of the ATPase in ITS suppression. Results in the present study reveal that the ATPase-deficient mutants (E34A and G67R) fail to suppress ITS formation, while mutants retaining the ATPase activity (H112D, G244D, H329P) preserve MLH1's ability to suppress ITS formation (Fig. 4). Our results therefore suggest that the ATPase activity of MLH1 is required for ITS suppression. Furthermore, we also find that the ATPase activity of MLH1 is involved in suppressing the formation of micronuclei, which is one of the characteristics indicative of genome instability.

Telomeres cap chromosome ends and prevent them from degradation and recombination [44]. However, if canonical telomeric repeats sequences are formed at intra-chromosomal regions, these sequences may contribute to chromosome breakage, recombination and rearrangement [22, 26–31]. In *Saccharomyces cerevisiae*, the Pif1 helicase inhibits *de novo* telomerase addition of telomeric sequences at DSBs [45]. It has been reported that yeast Pif1 reduces the level of telomerase binding to telomeres by its ATPase activity, suggesting that the ATPase activity of Pif1 is essential for inhibiting the addition of telomeric sequences at DSBs [45, 46]. Our result that the ATPase activity of hMLH1 is required for preventing aberrant ITS formation is reminiscent to the function of Pif in yeast.

It is noticeable that etoposide-induced MLH1 colocalization with 53BP1 foci is partial (Fig. 3B) [22], in agreement with the previous study showing that only a subset of MLH1 molecules are recruited to DSB stripes induced by laser microbeam irradiation [21]. The same is also true for MSH2 and MSH3 [21]. In addition, the number of MLH1 foci markedly outnumbers 53BP1 foci (Fig. 3B), suggesting that MLH1 molecules are abundant and not all MLH1 molecules are needed at DSBs. The interactome of MLH1 has been shown to contain proteins involved not only in MMR and DSB repair, but also in other functions including protein import/export, ubiquitin pathway, cell cycle, cellular signaling, and others [41]. Thus, it is highly possible that the DSB-independent MLH1 molecules function in other biological pathways.

Two ATPase-defective mutations were utilized in our work, albeit they confer different activities, with one of them retaining ATP binding and the other possessing the ATP hydrolysis ability. Therefore, our results do not address which activity is required for suppressing of ITS and micronuclei formation. Since we cannot formally exclude the possibility that ATP binding and hydrolysis are implicated in those process, it is likely that both ATP binding and hydrolysis activities are essential for ITS suppression, and loss of either activity abolishes the response of MLH1 to DNA damage and other important cellular

functions. Further studies will be required to determine whether protein-protein interactions or specific steps in ATP binding or hydrolysis are involved in MLH1-mediated ITS suppression and genome instability.

Acknowledgments

We are grateful to Chai Lab members for technical help and helpful discussions. This work was supported in part by National Institutes of Health R03CA223625 to WC.

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Fig. 1. Expression of MLH1 N-terminal mutants in HCT116
(A) Schematic structure of functional domains in full-length MLH1 and its N-terminus. Orange box: ATP-binding domain. Purple box: MutS homolog interacting domain. Blue box: EXO1/PMS2/MLH3/PMS1-interacting domain.
(B) Immunoblotting shows the expression of Flag-tagged MLH1 N-terminal mutants in HCT116 cells. FL-WT: Full length of wide-type MLH1. NTD: N-terminus of MLH.

Fig. 2. Measurement of ATPase activities in MLH1 N-terminal mutants

(A) Analysis of the purified FL GST-MLH1 fusion protein and variants GST-NTD fusion proteins by coomassie blue staining. M: molecular weight marker. Invalid: Sample that was mistakenly loaded into the lane.

(B) Relative ATPase activity of in vitro purified GST fusion proteins. Three independent experiments were performed. Two tailed t-tests were used to calculate statistical significance and error bars are SEM.

Fig. 3. The ATPase activity of MLH1 N-terminus is required for MLH1 localization to DNA damage sites

(A) NTD mutant proteins localize in nucleus in untreated cells. FL-WT, WT NTD and mutant NTDs were stably expressed with retroviral transduction. FLAG antibody (green) was used to detect the localization of FLAG-tagged proteins.

(B) Recruitment of NTD mutants to DNA damage sites induced by etoposide treatment (0.3 μ M, 2 hrs). After etoposide treatment, IF was performed with anti-53BP1 (red) and anti-FLAG (green). Amplified inserts show MLH1 accumulation at 53BP1 foci after treatment. Numbers correspond to the positions in the original images. ATPase defective mutants (E34A and G67R) showed few accumulation at 53BP1 foci.

(C) Percentage of cells with MLH1/53BP1 colocalization (>5 colocalizations per cell).

Results were from three independent experiments. Two tailed t-tests were used to calculate statistical significance. Error bars: SEM.

Fig. 4. The ATPase activity of MLH1 is needed for suppressing ITS formation

(A) Representative metaphase chromosome images with ITS formation. White arrows indicate ITSs. For easy visualization, the images with strong ITS signals were picked. The actual ITS signals on the majority of metaphase chromosomes are weaker than the displayed ones.

(B) Frequency of ITS formation in HCT116 cells stably expressing wild-type MLH1 and MLH1 mutants. All p values are compared to the full-length wild-type MLH1 (FL) in the same group (i.e. same colored bars). * $p < 0.02$, ** $p < 0.01$, *** $p < 0.001$. Each experiment was repeated with three replicates. Two-tailed t-tests were used to calculate statistical significance. Error bars: SEM.

Fig. 5. The ATPase activity of MLH1 is important for suppressing micronuclei (MN) formation
 (A) Defective ATPase activity in MLH1 N-terminal mutants leads to MN formation. White arrows point to MN.
 (B) Frequency of MN formation. Results were from three independent experiments. Two tailed t-tests were used to calculate statistical significance and error bars are SEM.