RESEARCH ARTICLE

Phosphorylation-dependent signaling controls degradation of DNA mismatch repair protein PMS2†

Inga Hinrichsen, Isabel M. Weßbecher, Meik Huhn, Sandra Passmann, Stefan Zeuzem, Guido Plotz, Ricardo M. Biondi, Angela Brieger*

Medical Clinic I, Biomedical Research Laboratory, University Clinic Frankfurt, 60590 Frankfurt a.M., Germany

*Correspondent footnote:

Angela Brieger, PhD

Medical Clinic I,

Biomedical Research Laboratory

University Clinic Frankfurt

Theodor-Stern-Kai 7

D-60590 Frankfurt a.M.

Germany

Phone: +49 - 69 - 6301 - 6218

Fax:+49 - 69 - 6301 - 84441

Email:a.brieger@em.uni-frankfurt.de

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/mc.22709]

Additional Supporting Information may be found in the online version of this article.

Received 18 April 2017; Accepted 28 July 2017

Molecular Carcinogenesis

DOI 10.1002/mc.22709

Acknowledgments

This work was supported by grant 109651 of the Deutsche Krebsstiftung, grant 2015.161.1 of the Wilhelm Sander-Stiftung and institutional funds of the Goethe-University Frankfurt.

Abbreviations

DNA mismatch repair (MMR); colorectal cancer (CRC), wild type (wt)

Abbreviated title

Degradation of PMS2

Abstract

MutLα, a heterodimer consisting of MLH1 and PMS2, plays an important role in DNA mismatch repair and has been shown to be additionally involved in several other important cellular mechanisms. Previous work indicated that AKT could modulate PMS2 stability by phosphorylation. Still, the mechanisms of regulation of MutLα remain unclear.

The stability of MutLα subunits was investigated by transiently overexpression of wild type and mutant forms of MLH1 and PMS2 using immunoblotting for measuring the protein levels after treatment. We found that treatment with the cell-permeable serine/threonine phosphatase inhibitor, Calyculin, leads to degradation of PMS2 when MLH1 or its C-terminal domain is missing or if amino acids of MLH1 essential for PMS2 interaction are mutated. In addition, we discovered that the C-terminal tail of PMS2 is relevant for this Calyculin-dependent degradation. A direct involvement of AKT, which was previously described to be responsible for PMS2 degradation, could not be detected. The multi-kinase inhibitor Sorafenib, in contrast, was able to avoid the degradation of PMS2 which postulates that cellular phosphorylation is involved in this process.

Together, we show that pharmacologically induced phosphorylation by Calyculin can induce the selective proteasome-dependent degradation of PMS2 but not of MLH1 and that the PMS2 degradation could be blocked by Sorafenib treatment. Curiously, the C-terminal Lynch Syndrome-variants $MLH1^{L749P}$ and $MLH1^{Y750X}$ make PMS2 prone to Calyculin induced degradation. Therefore, we conclude that the specific degradation of PMS2 may represent a new mechanism to regulate MutLα.

Key words: Lynch Syndrome; colorectal cancer; MLH1; MutLα; posttranslational modification

Introduction

The DNA Mismatch repair (MMR) is essential for genetic stability by repairing DNA replication errors. MutLα, consisting of MLH1 and PMS2, is of great importance here [1]. After mismatch-recognition by MutSα/β the ATP-dependent recruitment of MutLα is catalyzed by the N-terminus of MLH1 while the C-terminal domain of MLH1 interacts with and stabilizes PMS2 [2-4]. PMS2 in turn encodes an endonuclease activity which is important for 3'-strand repair [5]. Single missense mutations in MLH1 or PMS2 are described to be able to inhibit proteins' dimerization [6]. Besides MMR, MLH1 also takes part in the regulation of cell cycle checkpoints and apoptosis [7], in meiotic reciprocal recombination and meiotic mismatch repair [8] and cytoskeletal organization [9] while several MLH1 interacting proteins have been published [10]. However, the regulation of MutLα, which might be an essential step to enable the interaction of MLH1 or PMS2 with other proteins, is not yet understood. Protein modification by phosphorylation might play a role here. Little is known about phosphorylation of MMR proteins. Christmann *et al*. described phosphorylation of the MMR initiation complex MutSα and showed that phosphorylation resulted in increased mismatch binding while dephosphorylation induced nuclear translocation [11]. Romeo *et al.* postulated that MLH1 stability and phosphorylation is BRCA1-dependent and attributed to ATM/ATR activity [12]. Jia *et al.* supposed a direct role of PI3K/AKT signaling pathway in the regulation of PMS2 by phosphorylation [13].

However, neither Romeo *et al.* [12] nor Jia *et al.* [13] analyzed the whole MutLα complex.

In the current study, we analyzed the effect of phosphorylation on the stability of MLH1 and PMS2.

Material and Methods

Cell line

MLH1-deficient HEK293T [14], obtained from Dr. Kurt Ballmer (Paul Scherer Institute, Villingen, Switzerland) were grown in DMEM with 10% FCS.

Antibodies and Plasmids

Anti-MLH1 (G168-728) and anti-PMS2 (E19) were from Pharmingen (BD Biosciences, Heidelberg, Germany), anti-MLH1 (N-20) was from Santa Cruz (Texas, USA), anti-beta Actin (Clone AC-15) was from Sigma-Aldrich (Munich, Germany) and anti-phospho-AKT (Ser473) (Clone 587F11) was from Cell Signaling (New England Biolabs, Frankfurt, Germany). Fluorescence labeled goat anti-rabbit IRDye800CW and goat anti-mouse IRDye680LT were from LI-COR (LI-COR Biosciences, Bad Homburg, Germany).

The pcDNA3.1+/MLH1 and pcDNA3.1+/PMS2 expression plasmids were described previously [15]. In addition, several MLH1- and PMS2-fragments or -variants were generated: $MLH1^{1-505}$, $MLH1^{342-756}$, $PMS2^{1-649}$, $PMS2^{362-862}$, $PMS2^{1-699}$, $PMS2^{1-774}$, $PMS2^{1-784}$, $PMS2^{1-795}$, $PMS2^{1-822}$, $PMS2^{T156A}$, $PMS2^{S791A}$, $PMS2^{S793A}$, $PMS2^{S801A}$, $PMS2^{S809A}$, $PMS2^{S815A}$, $PMS2^{T820A}$, $PMS2^{T824A}$, $PMS2^{S825A}$, $PMS2^{T832A}$, $PMS2^{T849A}$, $PMS2^{S860A}$, $PMS2^{K804R}$, $PMS2^{K814R}$, $PMS2^{K828R}$, $PMS2^{K829R}$, $PMS2^{K804R/K814K}$, $PMS2^{K828R+K829R}$, $PMS2^{K804R/K814R/K828R}$ and $PMS2^{K804R/K814R/K828R/K829R}$ (for detailed primer information see supplementary table 1). $MLH1^{L749P}$ and $MLH1^{Y750X}$ were previously described [6].

Transient transfection and drug treatment

Transiently transfection of HEK293T was carried out as described previously [9]. In brief, HEK293T were transfected at 50-70% confluence with: pcDNA3.1+/MLH1 wild type (wt), pcDNA3.1+/MLH1-fragments or pcDNA3.1+/MLH1-variants and/or pcDNA3.1+/PMS2 wt, pcDNA3.1+/PMS2-fragments or pcDNA3.1+/PMS2-variants (0.5µg/ml, respectively) using 2µg/ml of the cationic polymer polyethylenimine (Polysciences, Warrington, PA). 48h post transfection cells were incubated for 8h in Opti-MEM (Gibco) and treated as indicated for additional i) 16h with 50nM of the serine/threonine phosphatase inhibitor Calyculin (LC laboratories, Woburn, USA), ii) 4h with 50µM of the tyrosine phosphatase inhibitor Pervanadate, iii) 16h with 50nM Calyculin followed by 1h with 10U/µg Alkaline Phosphatase (Calf Intestinal (CIP), New England Biolabs, Frankfurt, Germany), iv) 16h with 10µM of the proteasome inhibitor Bortezomib (LC laboratories, Woburn, USA), v) 16h with 50nM Calyculin and 10µM Bortezomib, vi) 16h with 1µM of the allosteric AKT inhibitor MK2206 (Selleckchem, Houston, USA) or vii) 16h with DMSO (control).

For combination treatments with Calyculin and kinase inhibitors cells were incubated 48h post transfection for 1h in Opti-MEM (Gibco) and treated for 8h with a) 1µM of the protein kinase B inhibitor MK2206 (Calbiochem, Darmstadt, Germany), b) 1µM of the protein kinase C inhibitor Bisindolylmaleimide (BIM) (Sigma Aldrich, St. Louis, USA) , c) 50µM of the protein kinase CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) (Calbiochem, Darmstadt, Germany), d) 20µM of the calmodulin-dependent protein kinase (CaMK) II inhibitor KN-93 (Santa Cruz, Dallas, USA), e) 10µM of the mitogen-activated protein (MAP) kinase inhibitor SB 203580 (Selleckchem, Houston, USA), f) 1µM of the DNA Protein Kinase catalytic subunit (DNA-PKcs) and phosphoinositide 3-kinase inhibitor KU60648 (Axon Medchem, Groningen, Netherlands), g) 1µM of the multi-kinase inhibitor Staurosporin (Sigma Aldrich, St. Louis, USA) or h) 10µM of the

multi-kinase inhibitor Sorafenib (Biomol GmbH, Hamburg, Germany) combined with 200nm Calyculin (Calbiochem, Darmstadt, Germany), respectively.

Finally, cells were harvested and protein extracts were analyzed by Western blotting.

Immunoprecipitation

Immunoprecipitations were carried out using 300 µg of whole cell extract in a total volume of 500 µl precipitation buffer (50 mM HEPES-KOH (pH 7.6), 100 mM NaCl, 0.5 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 1% Triton X-100) with 1 µg of anti-MLH1 N-20. After one hour of agitated incubation at 4°C, protein G sepharose (20 µl) were added and incubation continued for 3 h. Precipitates were extensively washed in cold precipitation buffer. The sepharose was boiled in SDS-PAGE sample buffer and proteins were separated on Phos-tag-PAGEs followed by wet Western blotting on PVDF membranes and antibody detection using standard procedures.

Phos-tag-PAGE

For mobility shift detection of MLH1 and PMS2 phosphoprotein isotypes immunoprecipitated samples were separated on Phos-tag-PAGEs as described by Kinoshita *et al.* [16], followed by wet Western blotting on PVDF membrane and antibody detection using standard procedures. Experiments were performed at least three times.

Western blotting

Proteins were separated on 10% polyacrylamide gels, followed by Western blotting on nitrocellulose membrane and antibody detection using standard procedures.

Band intensity of protein expression was quantified using Multi Gauge V3.2 program (Fujifilm, Tokyo, Japan). Experiments were performed at least three times.

Results

MLH1 and PMS2 are phosphorylatable at serine/threonine residues.

To investigate the effect of phosphorylations on the stability of MutLα we treated MutLα subunits transfected HEK293T with the serine/threonine phosphatase inhibitor Calyculin, the tyrosine phosphatase inhibitor Pervanadate or a combination of Calyculin and CIP. Consistent with MutLα being phosphorylated at serine or threonine position(s) Calyculin treatment resulted in a mobility shift of MLH1 and PMS2 using a special Phos-tag-PAGE while Pervanadate had no influence (Fig.1A). The mobility shift of MLH1 and PMS2 after Calyculin treatment could be removed by treatment with CIP (Fig.1A). In addition, a clearly visible up-shift smear of MLH1 and PMS2 using common SDS-PAGE could be detected (Fig.1B). The induction of phosphorylation had no effect on the stability of cotransfected MutLα wt subunits.

C-terminal interaction of MLH1 and PMS2 prevents phosphorylation-dependent degradation of PMS2

To encircle distinct areas of MLH1 important for phosphorylation we coexpressed $MLH1^{1-505}$ (without C-terminus) and $MLH1^{342-756}$ (without N-terminus) with PMS2. As shown in Figure 2A, PMS2 was expressed (albeit weakly) when coexpressed with $MLH1^{1-505}$ in untreated cells but lost after Calyculin treatment. In contrast, PMS2 was normally expressed, showing the up-shift smear when coexpressed with MLH1 or $MLH1^{342-756}$ after treatment (Fig.2A). Therefore, we conclude a phosphorylation-dependent degradation of PMS2 which is prevented by the C-terminus of MLH1.

Furthermore, we tested the Calyculin effect on PMS2 after coexpression with two MLH1-variants diminished in PMS2 interaction, $MLH1^{L749P}$ and $MLH1^{Y750X}$ [6]. Both variants were detected in patients with Lynch Syndrome. $MLH1^{L749P}$ carries a missense

mutation at position 749 and MLH1^{Y750X} is C-terminally truncated. PMS2 was expressed in untreated cells after coexpression with MLH1^{L749P} and MLH1^{Y750X} but almost disappeared after Calyculin treatment (Fig.2B).

The C-terminus of PMS2 is essential for its phosphorylation-dependent degradation

To find out which parts of PMS2 are essential for its Calyculin-dependent degradation we generated PMS2$^{1-649}$ (without C-terminus) and PMS2$^{364-862}$ (without N-terminus).

As shown in Figure 2C+D untreated PMS2, PMS2$^{1-649}$ and PMS2$^{364-862}$ were well expressed both if coexpressed with MLH1 and if single expressed. After Calyculin treatment, MLH1-coexpressed PMS2 constructs (Fig.2C) and single expressed PMS2$^{1-649}$ (Fig.2D) were well detectable showing an up-shift smear. In contrast, single expressed PMS2 and PMS2$^{364-862}$ were undetectable after Calyculin treatment (Fig.2D). We therefore postulated that the C-terminus of PMS2 is essential for Calyculin-dependent degradation of PMS2.

In parallel, the effect of Calyculin on single expressed MLH1, MLH1$^{1-505}$ or MLH1$^{342-756}$ was analyzed (Fig.2E). All MLH1 constructs showed the typical up-shift smear indicating phosphorylation but were not degraded after Calyculin treatment (Fig.2E).

Inhibition of the proteasome avoids phosphorylation-dependent PMS2 degradation

To determine whether proteasomal degradation was responsible for the reduction of PMS2 we cotreated PMS2 transfected HEK293T with Calyculin and Bortezomib. As shown in Figure 3A inhibition of the proteasome with Bortezomib rescues PMS2 from phosphorylation-dependent degradation. The PMS2 expression in Calyculin and Bortezomib treated cells was equal to the expression in the untreated controls.

The C-terminal tail of PMS2 mediates its Calyculin-dependent degradation

To localize the exact area within the C-terminus of PMS2 relevant for its phosphorylation-dependent degradation we generated (in addition to $PMS2^{1-649}$ which is missing the C-terminus and stably expressed during Calyculin treatment) four PMS2 fragments with varying C-terminal tail lengths: $PMS2^{1-699}$, $PMS2^{1-774}$, $PMS2^{1-795}$ and $PMS2^{1-822}$. These PMS2 fragments were designed by taking into consideration the model of the three-dimensional PMS2 structure [17] (supplementary Fig.1).

We then analyzed the stability of all PMS2 constructs after Calyculin treatment (Fig.3B) and amounts of PMS2 were assessed by measuring the signal intensities of protein bands with Multi Gauge V3.2 software setting the related untreated approaches as 100% (Fig.3C).

While $PMS2^{1-649}$ was very strongly expressed in untreated and Calyculin-treated cells, the expression of $PMS2^{1-699}$ and $PMS2^{1-774}$ was much weaker and decreased to 45% or 33% after Calyculin treatment. The expression of $PMS2^{1-795}$, PMS2 wt and $PMS2^{1-822}$ even dropped down to 20%, 11% and 4% after treatment (Fig.3B+C).

Thus, the increased cellular phosphorylation triggered by Calyculin induced PMS2-degradation mediated by the C-terminal region of PMS2.

Mutations of C-terminal serine, threonine or lysine residues could not inhibit PMS2 degradation

In an attempt to localize amino acids in the PMS2 tail relevant for its phosphorylation-dependent PMS2 degradation we focused on the C-terminal region from amino acid position 790 to 862 (supplementary Fig.2A+C).

Altogether, seven serine and four threonine residues exist in the C-terminal tail of PMS2 (GenBank accession: NP_000526.1). We mutated each of these amino acids to

alanine, single expressed all PMS2-variants and analyzed the influence of Calyculin treatment on PMS2's stability (supplementary Fig.2A+C). $PMS2^{1-785}$ which is missing the C-terminal tail and is stable after Calyculin treatment, served as a control. All PMS2-variants were well expressed in untreated cells but none of the PMS2-variants were detectable after Calyculin treatment.

During proteasomal degradation lysine residues of corresponding proteins are tagged with ubiquitin by special E3 ligases followed by protein disassembling and degradation. Hence, we mutated lysine residues K804, K814, K828 and K829 (supplementary Fig. 2B+C) to arginine residues and determined the effect of this mutations or combinations of them on PMS2 stability after Calyculin treatment.

As shown in supplementary Figure 2B, neither single mutations of these terminal lysine residues of PMS2 nor a combination of four lysine residue mutations were sufficient to abrogate the degradation of PMS2. All variants were degraded after Calyculin treatment.

AKT is not involved in the C-terminal dependent degradation of PMS2

Previous work indicated that AKT is responsible for the regulation of PMS2 [13]. To investigate the role of AKT in the observed process, we used HEK293T cells which had been described to contain constitutively active AKT [18]. HEK293T were transiently transfected with MLH1 and/or PMS2 and treated with the allosteric AKT inhibitor MK2206. As shown in supplementary Figure 3 the treatment with MK2206 significantly reduced AKT activity (monitored by the detection of the amount of phosphorylated AKT) but the expression level of MLH1 or PMS2 did not show any difference compared to the controls after treatment.

Sorafenib treatment can rescue PMS2 from degradation

In order to filter out corresponding kinases or signaling pathways which are involved in the degradation process of PMS2 we medicated PMS2 transfected HEK293T cells with several specific and unspecific kinase inhibitors before Calyculin treatment.

While MK2206, BIM, TBB, SB203580, KN-93, KU60648 and Staurosporin had no effect, Sorafenib was able to prevent PMS2 from the degradation process induced by Calyculin (Fig.4). Since Sorafenib is targeting several important signaling pathways [19] we therefore postulate that phosphorylation-dependent signaling is involved in the specific degradation of PMS2.

Discussion

The great importance of MLH1 to stabilize PMS2 is well known for several years [20]. In this study, we could show for the first time that Calyculin-induced increase of the cellular serine/threonine phosphorylation by inhibition of protein phosphatases triggers the degradation of PMS2. Moreover, we could demonstrate that the degradation of PMS2 under those conditions is directed by its C-terminal end and is mediated by the proteasome system. MLH1 wt but not C-terminally truncated MLH1 fragments or $MLH1^{L749P}$ and $MLH1^{Y750X}$ variants (which harbor reduced PMS2 binding capacity [20]) could prevent PMS2 from Calyculin-dependent degradation. We therefore speculate that freely accessible or poorly attached PMS2 C-terminus enables phosphorylation-related degradation of PMS2. In contrast, MLH1 is well expressed and remains stable after treatment with Calyculin and one might assume that increased cellular phosphorylation could enable MLH1 to interact with partner proteins others than PMS2 a hypothesis which has to be determined in detail by further experiments.

However, C-terminal amino acids responsible for phosphorylation or degradation of PMS2, could not be identified by us so far. It could be anticipated that more than one amino acid in the C-terminal domain of PMS2 has to be phosphorylated. Moreover, amino acids not located directly in the C-terminus of PMS2 might be responsible to target PMS2 for proteasomal-mediated degradation as it has been described in other proteins [21].

Although, AKT has been previously assumed to modulate PMS2 stability [13] we could not show that inhibition of AKT by MK2206 influences the expression and degradation of MutLα, MLH1 or PMS2. Thus, AKT seems to play no role in the observed process. In contrast, using different specific and unspecific kinase inhibitors we found that Sorafenib was able to avoid the Calyculin-dependent degradation of PMS2. Therefore, we complement the effect of phosphatase treatment with the effect of a protein kinase inhibitor, enforcing the main concept that cellular phosphorylation is involved in the regulation process of PMS2 degradation.

In summary, our results highlight that the amount of PMS2 can be regulated by intracellular signaling events mediated by phosphorylation and the proteasome system which might enable the human system to control the interplay of PMS2 with MLH1 specifically.

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Figure legends

Figure 1

Mobility shift detection of MLH1 and PMS2 phosphoprotein isotypes

HEK293T were transfected with MLH1 and PMS2, treated with Calyculin, Pervanadate, Calyculin and CIP or DMSO (control). After separation of **(A)** immunoprecipitated proteins on a special Phos-tag-PAGE or **(B)** whole cell extracts on a common SDS-PAGE, Western blotting was performed.

Calyculin treatment resulted in a mobility shift or a clearly visible up-shift smear of MLH1 as well as PMS2 after Calyculin but not after Pervanadate treatment. The Calyculin effect was neutralized by CIP treatment.

Figure 2

The C-terminus of MLH1 protects PMS2 from Calyculin-dependent degradation while its C-terminus is essential for the process

HEK293T were transfected with **(A)** MLH1 wt, $MLH1^{1-505}$ or $MLH1^{342-756}$ and PMS2 wt, **(B)** MLH1 wt, $MLH1^{L749P}$ or $MLH1^{Y750X}$ and PMS2 wt, **(C)** MLH1 wt and PMS2 wt, $PMS2^{1-649}$ or $PMS2^{362-862}$, **(D)** PMS2 wt, $PMS2^{1-649}$ or $PMS2^{362-862}$ and **(E)** MLH1 wt, $MLH1^{1-505}$ or $MLH1^{342-756}$ and treated with Calyculin or DMSO (control). Expression of MLH1 and PMS2 was analyzed by Western blotting, controlled by beta Actin.

Figure 3

The proteasomal degradation of PMS2 is based on its C-terminal tail

HEK293T were transfected with **(A)** PMS2 and treated with Calyculin or Bortezomib or Calyculin and Bortezomib and **(B)** PMS2 wt, $PMS2^{1-649}$, $PMS2^{1-699}$, $PMS2^{1-774}$, $PMS2^{1-}$

^{795} or PMS2$^{1-822}$ and treated with Calyculin or DMSO (control). Expression of PMS2 or PMS2 fragments was determined by Western blotting, controlled by beta Actin. **(C)** Amounts of the degradation of PMS2 fragments were assessed by measuring the signal intensities of protein bands with Multi Gauge V3.2 software. Comparability was facilitated by setting the PMS2 levels of untreated cells as 100%.

Figure 4

Kinase inhibition rescues PMS2 from degradation

PMS2 overexpressing HEK293T were treated with the kinase inhibitors MK2206, BIM, TBB, SB203580, KN-93, KU60648, Staurosporin and Sorafenib, respectively, in parallel to Calyculin treatment. Mono treatment with Calyculin served as positive control and treatment with DMSO as negative control. The PMS2 expression was analyzed by Western blotting, controlled by beta Actin. Sorafenib was able to block the Calyculin-dependent degradation process of PMS2.

Supplementary Figure 1

C-terminal PMS2 fragments. PMS2 constructs harboring varying C-terminal tail lengths were created based on the model of the three-dimensional structure of PMS2 [17]. Only C-terminal domains are shown; deleted areas are colored in red.

Picture below, amino acid 749 (red) and Δ750 tail (orange) of MLH1 are shown in the dimerized C-termini of MLH1 (green) and PMS2 (blue).

Supplementary Figure 2

C-terminal serine, threonine or lysine residues could not inhibit PMS2 degradation

HEK293T were transfected with **(A)** PMS2 wt, $PMS2^{S791A}$, $PMS2^{S793A}$, $PMS2^{S801A}$, $PMS2^{S809A}$, $PMS2^{S815A}$, $PMS2^{T820A}$, $PMS2^{T824A}$, $PMS2^{S825A}$, $PMS2^{T832A}$, $PMS2^{T849A}$ and $PMS2^{S860A}$ or **(B)** with PMS2 wt, $PMS2^{K804R}$, $PMS2^{K814R}$, $PMS2^{K828R}$, $PMS2^{K829R}$, $PMS2^{K804R/K814R}$, $PMS2^{K828R/K829R}$, $PMS2^{K804R/K814R/K828R}$ or $PMS2^{K804R/K814R/K828R/K829R}$ and treated with Calyculin or DMSO (control). Expression of PMS2 or PMS2-variants was determined by Western blotting, controlled by beta Actin. **(C)** Localization of all tested C-terminal serine, threonine and lysine residues of PMS2 are shown schematically.

Supplementary Figure 3

Inhibition of AKT did not affect the expression of MutLα

HEK293T were cotransfected with MLH1 and/or PMS2 and treated with MK2206. The efficiency of AKT inhibition was monitored using anti-phospho-AKT. MLH1 and PMS2 expression was determined by Western blotting, controlled by beta Actin.

Figure 1

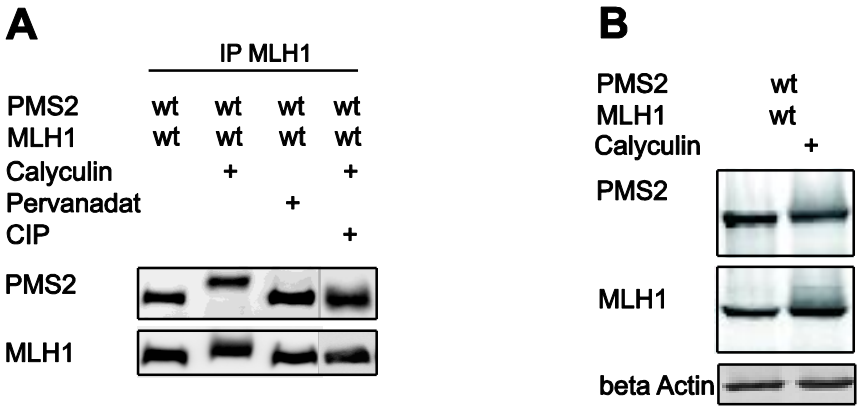

Figure 2

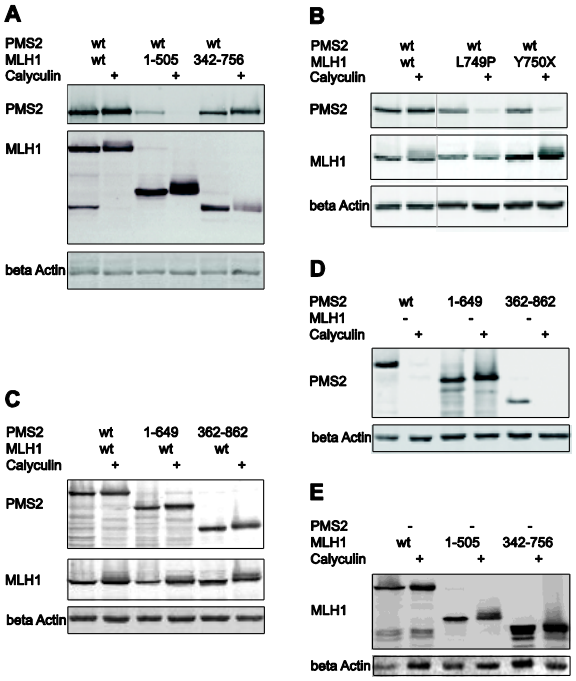

Figure 3

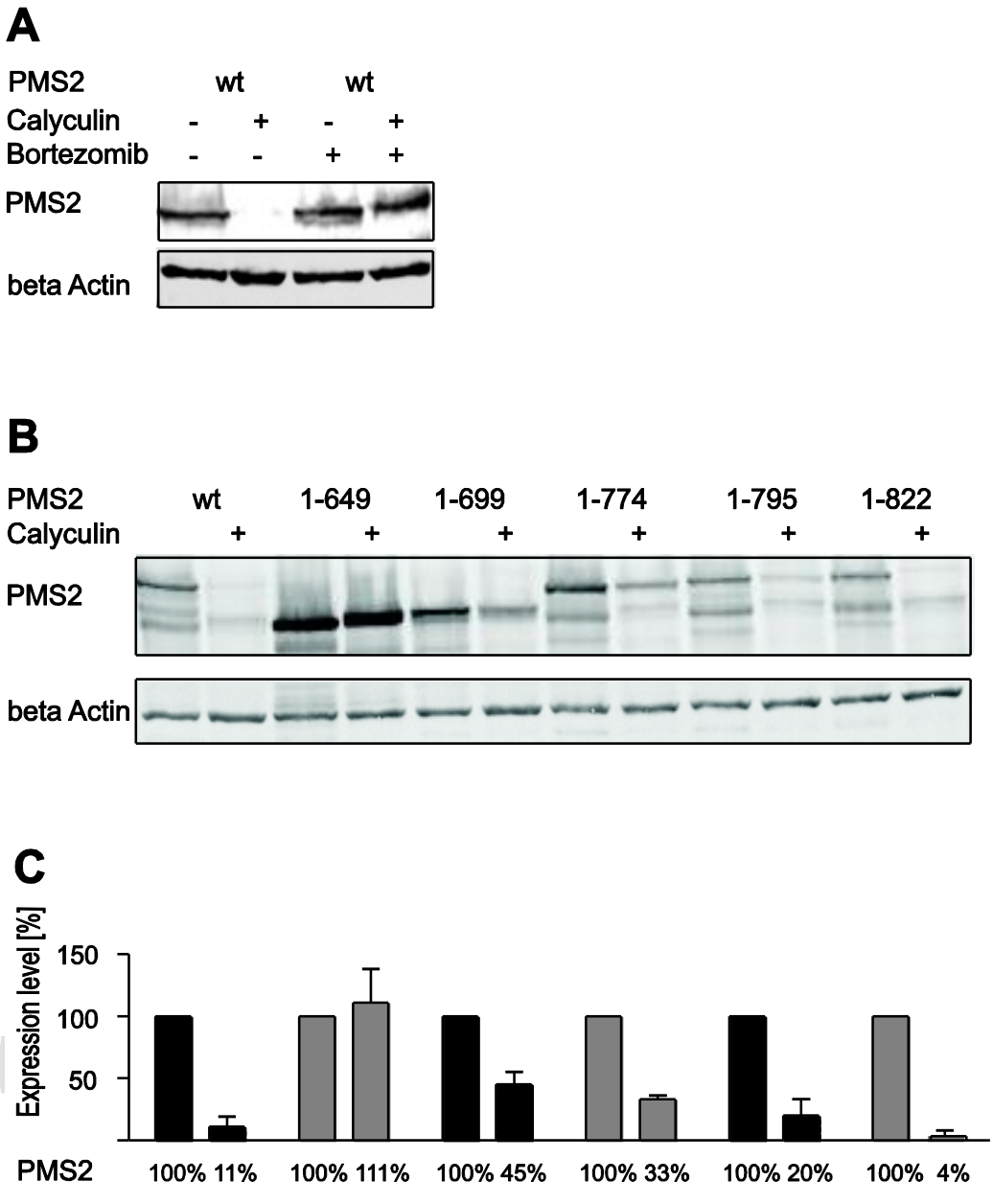

Figure 4