Modification of promyelocytic leukemia zinc finger protein (PLZF) by SUMO-1 conjugation regulates its transcriptional repressor activity

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Running title: Sumoylation of PLZF

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SUMMARY

Promyelocytic leukemia zinc finger (PLZF) protein is a sequence-specific DNA-binding protein that represses the transcriptional activity of target genes such as those for cyclin A and the interleukin-3 receptor α chain. The PLZF gene becomes fused to the retinoic acid receptor α gene as a result of the t(11;17)(q23;q21) chromosomal translocation that is associated with acute promyelocytic leukemia. We now show that endogenous PLZF in human promyelocytic leukemia HL-60 cells is modified by conjugation with SUMO-1 (small ubiquitin-related modifier-1) and that PLZF colocalized with SUMO-1 in the nucleus of transfected human kidney 293T cells. Site-directed mutagenesis identified lysine-242 in the RD2 domain of human PLZF as the sumoylation site. A luciferase reporter gene assay suggested that SUMO-1 modification of this residue is required for transcriptional repression by PLZF, and an electrophoretic mobility-shift assay showed that this modification increases the DNA-binding activity of PLZF. PLZF-mediated regulation of the cell cycle and transcriptional repression of the cyclin A2 gene were also dependent on sumoylation of PLZF on lysine-242. These results demonstrate that PLZF is modified by SUMO-1 conjugation, and that this modification regulates the biological functions of PLZF.

INTRODUCTION

Promyelocytic leukemia zinc finger protein (PLZF)\(^1\) is a sequence-specific DNA-
binding transcriptional regulator. It comprises 673 amino acids and contains nine
krüppel-like C2H2 zinc finger domains and an NH2-terminal POZ (Pox virus and zinc
finger) or BTB (broad complex, tramtrack, bric-a-brac) domain. (1,2) PLZF is
expressed in human CD34+ myeloid progenitor cells and in primitive multipotent cell
lines, and its gene is fused to that of retinoic acid receptor α (RARα) in a variant of
acute promyelocytic leukemia (APL) associated with the t(11;17)(q23;q21)
chromosomal translocation (2). Whereas RARα activates key genes required for normal
myelopoiesis, the PLZF-RARα fusion protein represses the expression of some of these
genes in a dominant negative manner. PLZF colocalizes with the PML (promyelocytic
leukemia) protein, suggesting a possible link with the pathogenesis of the more common
t(15;17)-associated form of APL (3-5).

In its role as a transcriptional repressor, PLZF binds to the promoter of target genes,
including those for cyclin A2 and the interleukin-3 receptor α chain (IL-3Rα), and
interacts with the corepressors N-CoR, SMRT, and Sin3A as well as histone
deacetylases. The POZ/BTB domain overlaps with a transcriptional repression domain
(RD1) and mediates PLZF self-association (2,4) and interaction with histone
deacetylases. A second transcriptional repression domain (RD2) is located downstream
of the POZ domain and interacts with the ETO-AML1 protein in t(8;21)-associated
acute myelocytic leukemia; this protein may function as a PLZF corepressor (6,7).

During development, PLZF contributes to patterning of the limb and axial skeleton by
acting as an upstream regulator of Hox gene expression (8-10). PLZF is also implicated in the proliferation and survival of hematopoietic cells as a result of its repression of transcription of the cyclin A2 gene (11). Expression of PLZF gene is regulated by Evi-1 oncoprotein and novel guanine-rich site binding protein (8).

Like ubiquitin, small ubiquitin-related modifier (SUMO) is covalently conjugated to target proteins. Three SUMO isoforms, SUMO-1 to-3, have been identified in mammals (12,13). The SUMO-1–interacting motif comprises the sequence ÈKXE, where È is a large hydrophobic residue, K is the lysine to which SUMO-1 is conjugated, X is any amino acid, and E is glutamic acid. The pathway of sumoylation is similar to that of ubiquitination, involving a cascade of three enzymes (E1, E2, and E3) and resulting in the formation of a bond between the COOH-terminal carboxyl group of SUMO and a lysine side chain of the target protein (14,15). The SUMO-activating enzyme (E1) is a heterodimer of AOS-1 and UBA2; the SUMO-conjugating enzyme (E2) is UBC9; and the protein-SUMO ligases (E3) include members of the PIAS family and RanBP2 (14,16).

In contrast to ubiquitination, sumoylation does not appear to promote protein degradation but rather contributes to the regulation of protein-protein interaction, to subcellular compartmentalization, and to protein stability. The sumoylation of diverse proteins, including c-Jun, c-Myb, IκB, p53, PML, and histone deacetylases, influences the function of these proteins in transcriptional repression or activation or in cell cycle regulation as well as their nuclear localization or ubiquitination (15,17). SUMO-1
modification of transcription factors has thus been shown to affect the organization of specific protein complexes at sites in the nuclear matrix and thereby to regulate the activities of these factors either negatively or positively (16,18-20).

We have now investigated whether PLZF is modified by sumoylation. PLZF has indeed found to be modified by SUMO-1 conjugation in vivo. We identified Lys$^{242}$ in the RD2 domain of PLZF as the target for sumoylation and found that such modification modulates the transcriptional activity of PLZF as well as PLZF function in cell cycle regulation.

**EXPERIMENTAL PROCEDURES**

**Plasmids and mutagenesis**

Various expression vectors for wild-type human PLZF were kindly provided by J. D. Licht (Mount Sinai School of Medicine, New York, NY) (7,21,22). The PLZF mutant K242R was generated with the use of a QuikChange site-directed mutagenesis kit (Stratagene) and was verified by DNA sequencing. For expression of Flag epitope-tagged wild-type PLZF or PLZF(K242R) in 293T cells, the corresponding cDNAs were cloned into pFlag-CMV-5 (Sigma). For expression of glutathione S-transferase (GST) fusion proteins of wild-type PLZF or PLZF(K242R) in bacteria, the corresponding
cDNAs were cloned into pGEX 5X-1 (Amersham Biosciences). Human SUMO-1 vector was kindly provided by R.T. Hay (University of St. Andrews, Fife, United Kingdom) (23).

Cell culture and transfection

The human kidney cell line 293T was maintained in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL). The human APL cell line HL-60 was maintained in RPMI 1640 (Gibco-BRL) supplemented with 15% FBS and glutamine (2 mg/ml). All tissue culture media were also supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco-BRL). For transient transfection, 293T cells were grown in 60-mm culture dishes to 50 to 80% confluence and then transfected with expression vectors (total of 2–10 µg) by the calcium phosphate method (24). Forty-eight hours after transfection, the cells were collected for immunoprecipitation.

Immunoprecipitation

HL-60 cells were incubated for 40 min in 1 ml of methionine- and cysteine-free medium, metabolically labeled for 45 min with 100 µCi of Trans [35S] Label (NEN), washed, and then lysed in an ice-cold lysis buffer [phosphate-buffered saline (PBS) containing 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 µg/ml),
aprotinin (10 µg/ml), 10 mM N-ethylmaleimide, and 200 µM iodoacetamide]. The cell lysates were subjected to immunoprecipitation with mouse monoclonal antibodies to PLZF (Oncogene), and the resulting precipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and with a Fuji BAS 2500 Imager.

Transfected 293T cells were lysed in lysis buffer and subjected to immunoprecipitation by incubation for 3 h at 4°C with mouse monoclonal antibodies to Flag (Sigma). Immune complexes were collected by centrifugation after the addition of protein A-Sepharose (Amersham Biosciences). The beads were washed five times with lysis buffer, and the immunoprecipitates were then subjected to immunoblot analysis with the antibodies to Flag or with mouse monoclonal antibodies to SUMO-1 (anti-Xpress; Invitrogen) (25).

**Immunofluorescence analysis**

Cells were transferred to six-well glass chamber slides and, after 12 h, transfected with expression vectors for green fluorescent protein (GFP)-tagged SUMO-1 (1 µg) and Flag-PLZF (1 µg). After 24 h, the cells were washed twice with PBS, fixed for 20 min with 3.7% formaldehyde in PBS, and incubated at room temperature first in blocking solution (2% bovine serum albumin in PBS) twice for 10 min (or overnight) and then with antibodies to Flag (1:200 dilution in blocking solution) for 1 h. The cells were washed twice for 10 min with PBS, incubated for 1 h with Texas red-conjugated goat antibodies to mouse immunoglobulin G (1:300 in blocking solution) (Jackson
Immuno Research), and washed twice for 10 min. Cover slips were applied with the use of antifade reagent (FluoroGuard, Bio Rad), and the stained cells were examined with a Nikon confocal microscope equipped with a 100× objective (26, 27).

**Luciferase reporter gene assay**

293T cells were transfected in six-well dishes with 400 ng of an expression vector for His–SUMO-1, 400 ng of a vector for Flag-PLZF, and 200 ng of a firefly luciferase reporter plasmid (p4×IL3R-tk-luc) that harbors four copies of a PLZF binding site upstream of the thymidine kinase gene promoter (kindly provided by J. D. Licht) (4). For normalization of transfection efficiency, cells were also transfected with 100 ng of a β-galactosidase expression plasmid (pCMV-β-gal). After incubation for 36 h, the cells were assayed for luciferase activity with a luciferase assay system (Promega) as well as for β-galactosidase activity. Expression of PLZF protein was evaluated by immunoprecipitation and immunoblot analysis of cell extracts with antibodies to Flag (19, 28).

**EMSA analysis**

In vitro protein translation was performed with the TNT-coupled rabbit reticulocyte lysate system (Promega). GST-PLZF and GST-PLZF(K242R) fusion proteins (5 µg) purified from bacteria were incubated for 30 min at 37°C in a reaction mixture (30 µl) containing in vitro-translated SUMO-1 (0.5 µg), HeLa cell extracts, purified UBC9 (1
µg) (kindly provided by R.T. Hay), and an ATP-regenerating system. The PLZF proteins were then isolated with glutathione-agarose beads and subjected to electrophoretic mobility-shift assay (EMSA) by incubation for 1 h on ice in 30 µl of a reaction mixture containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 0.1 ng of 32P end-labeled DNA probe (7), 0.5 µg of poly(dI-dC), and 0.1 mg/ml bovine serum albumin. The binding mixture was then subjected to electrophoresis on a native 4% polyacrylamide gel in 0.5× Tris-borate-EDTA, and PLZF-DNA complexes were detected by autoradiography (20).

Cell cycle analysis

HL-60 cells stably expressing wild-type PLZF or PLZF(K242R) were fixed with ice-cold 70% ethanol, centrifuged for 5 min at 1000 × g, and resuspended in PBS containing 5 mM EDTA and RNaseA (50 µg/ml). After incubation for 30 min at 37°C, the cells were stained with propidium iodide (50 µg/ml) and analyzed by flow cytometry with a FACS Calibur instrument (Becton Dickinson) (11).
**PLZF is modified by SUMO-1 in vivo**

Immunoprecipitation of endogenous PLZF from HL-60 cells that had been metabolically labeled with $[^{35}\text{S}]$methionine and $[^{35}\text{S}]$cysteine yielded two labeled bands on analysis by SDS-PAGE and autoradiography (Fig. 1A). The major band migrated at a position corresponding to a molecular mass of 81 kDa, consistent with the size of wild-type PLZF, whereas the minor band migrated at a position corresponding to a molecular size of 96 kDa. This observation suggested that the 96 kDa protein is a modified form of PLZF, and the difference in size between the two proteins was consistent with the addition of one molecule of SUMO-1. To determine whether PLZF is indeed modified by sumoylation, we transiently transfected 293T cells, which do not express endogenous PLZF at a detectable level, with an expression vector for Flag-tagged PLZF in the absence or presence of a vector for His-tagged SUMO-1. Flag-PLZF was then immunoprecipitated from the transfected cells and subjected to immunoblot analysis with antibodies to Flag or to SUMO-1 (Fig. 1B). Immunoblot analysis with the antibodies to Flag of the Flag-PLZF precipitates (or of the original lysates) prepared from cells coexpressing His-SUMO-1 revealed the presence of a prominent band corresponding to Flag-PLZF. This additional band was not detected in the immunoprecipitates (or lysates) prepared from cells expressing Flag-PLZF in the absence of His-SUMO-1, indicating that it corresponded to a SUMO-1-modified form of PLZF.
We next determined whether PLZF colocalized with SUMO-1 in 293T cells cotransfected with plasmids encoding Flag-PLZF and GFP-tagged SUMO-1. Immunofluorescence analysis with antibodies to Flag and confocal microscopy revealed that Flag-PLZF immunofluorescence and GFP-SUMO-1 fluorescence were colocalized in nuclear granules (Fig. 1C), again indicating that PLZF is modified by conjugation with SUMO-1 \textit{in vivo}.

**Lysine-242 is the target residue of PLZF for SUMO-1 modification**

To identify the residue of PLZF that is modified by SUMO-1, we examined the amino acid sequence of the human protein for the presence of the consensus sequence (ÈKXE) for such modification. The RD2 domain of PLZF indeed contains the sequence VK242TE, which matches the consensus sumoylation motif perfectly (Fig. 2A). We mutated Lys$^{242}$ of PLZF to arginine and then tested the ability of the PLZF(K242R) mutant to undergo SUMO-1 conjugation in transfected 293T cells.

Immunoprecipitation and immunoblot analysis revealed that mutation of Lys$^{242}$ in the sumoylation consensus sequence prevented conjugation of Flag-PZLF with His-SUMO-1 (Fig. 2B), indicating that this residue is indeed the target for modification by SUMO-1.

**SUMO-1 modification regulates transcriptional repression by PLZF**
To examine whether SUMO-1 modification affects the transcriptional regulatory activity of PLZF, we compared the activity of wild-type PLZF with that of the sumoylation-resistant mutant PLZF(K242R) with the use of a luciferase reporter assay. The reporter plasmid contained four copies of a high-affinity binding site for PLZF derived from the IL-3Rα gene promoter (Fig. 3A). 293T cells were transfected with this reporter plasmid as well as with expression vectors for His-SUMO-1 and either wild-type Flag-PLZF or the mutant Flag-PLZF(K242R). The marked repression of luciferase gene transcription apparent in cells expressing wild-type PLZF was almost completely abolished by the K242R mutation (Fig. 3B). The expression of the corresponding luciferase reporter plasmid lacking the PLZF binding sites was not affected by either wild-type or mutant PLZF (data not shown). These results thus suggested that sumoylation of Lys242 is required for transcriptional repression by PLZF.

To determine whether sumoylation regulates transcriptional repression by PLZF through an effect on the interaction of PLZF with its DNA binding site, we performed EMSA analysis. Whereas the binding of wild-type GST-PLZF to the specific DNA probe was markedly increased by incubation with SUMO-1 and a sumoylation reaction mixture, the GST-PLZF(K242R) mutant did not bind substantially to the probe in the absence or presence of these components (Fig. 3C). These data thus indicated that SUMO-1 modification of PLZF regulates transcriptional repression by this protein.
through an effect on its binding affinity for DNA.

**SUMO-1 modification regulates PLZF function in the cell cycle**

To investigate the possible effect of SUMO-1 modification on the function of PLZF in regulation of the cell cycle, we generated HL-60 cell lines stably expressing wild-type PLZF or PLZF(K242R). These cells were synchronized by incubation for 72 h in serum-deficient medium (0.5% FBS) and were then reexposed to complete medium for 24 h. The DNA content of the cells was then determined by flow cytometry. Compared with HL-60 cells stably transfected with the empty vector, cells stably expressing wild-type PLZF exhibited a marked increase in the proportion of cells in S phase and a decrease in the percentage of cells in G₀-G₁ (Fig. 4A). In contrast, stable expression of PLZF(K242R) induced a small decrease in the proportion of cells in S phase.

PLZF represses transcription of the gene for cyclin A2. We therefore finally determined the effect of PLZF sumoylation on cyclin A expression in stably transfected HL-60 cells. Immunoblot analysis revealed that stable expression of wild-type PLZF resulted in a decrease in the abundance of cyclin A compared with that in cells transfected with the empty vector (Fig. 4B). In contrast, stable expression of PLZF(K242R) had no such effect.
DISCUSSION

APL accounts for 10 to 15% of adult acute myeloid leukemia. One of the causes of this condition is the t(11;17)(q23;q21) translocation that disrupts the PLZF gene, resulting in the generation of two new transcripts, PLZF-RARα and RARα-PLZF.

PLZF is localized to the nucleus of cells, where it functions as a transcriptional repressor through its interaction with various corepressors, including histone deacetylases, and regulates cell cycle progression by inhibiting transcription of the cyclin A2 gene (11,24,29).

We have now shown that PLZF is modified by SUMO-1 conjugation at Lys242, and that mutation of this residue to prevent its sumoylation greatly inhibited the biological activity of PLZF.

We thus found that sumoylation of PLZF appeared to localize in nucleus and to be required for transcriptional repression by this protein in a luciferase reporter gene assay (Fig. 3B). Furthermore, EMSA analysis revealed that this requirement for modification of PLZF by SUMO-1 conjugation in transcriptional repression by PLZF was due, at least in part, to a sumoylation-induced increase in the affinity of PLZF for its DNA binding site (Fig. 3C). It has been reported that the expression of heat shock protein (hsp) genes are regulated by heat shock transcription factors (HSFs), which bind to the heat shock element (HSE) in the promoter of heat shock genes and stimulate their transcription. Constitutive or stress-induced sumoylation of the heat shock factors...
HSF1 and HSF2 stimulate also their DNA binding activity, and can regulate the function of HSFs (20,30).

PLZF belongs to the POK (POZ domain and krüppel zinc finger) family of proteins, all of which interact directly with the PML protein (31). POK family proteins localize to nuclear subdomains as a result of self-interaction or association with transcriptional corepressors (32). PLZF shares many functional properties with LAZ3 (BCL6), which also concentrates in nuclear subdomains and acts as a sequence-specific transcriptional repressor to inhibit cell growth (31,33).

Although PLZF has been shown to associate with other POK proteins involved in cell cycle progression, little has been known of the role of SUMO-1 in the control of cell growth, differentiation, and survival (33). We have now shown that sumoylation of PLZF appears to be required for the transcriptional repression of the cyclin A2 gene by PLZF and for the effects of this protein on cell cycle distribution. Comparing with stably expressing PLZF (K242R), wild type PLZF increased in the proportion of cells in S phase and decreased in the percentage of cells in G0-G1 whereas PLZF (K242R) induced a small decrease in the proportion of cells in S phase (Fig. 4).

Since the number of SUMO targets has increased rapidly, the function and regulation of SUMO conjugation will apparently vary depending on the substrates.

The enzymatic pathway responsible for the sumoylation of PLZF remains to be characterized, although E3s such as RanBP2 and PIAS may play a role. The formation
of a thioester between SUMO and E1 is required for SUMO activation. UBC9 physically interacts with most sumoylation substrates, suggesting that it might be sufficient for substrate recognition (15).

In summary, we have demonstrated posttranslational modification of the transcriptional repressor PLZF. Dynamic posttranslational modifications, such as phosphorylation and acetylation, regulate the biological functions of many proteins. Modification of PLZF by SUMO-1 conjugation increased the DNA binding affinity, transcriptional repressor activity, and cell cycle regulatory function of this protein.

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FOOTNOTES

1 Abbreviations: PLZF, promyelocytic leukemia zinc finger protein; RARα, retinoic acid receptor α; APL, acute promyelocytic leukemia; PML, promyelocytic leukemia; IL-3Rα, interleukin-3 receptor α chain; SUMO, small ubiquitin-related modifier; GST, glutathione S-transferase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; EMSA, electrophoretic mobility-shift assay.

FIGURE LEGENDS

Figure 1. Conjugation of PLZF with SUMO-1 in vivo. (A) HL-60 cells were
metabolically labeled with $[^{35}\text{S}]$methionine and $[^{35}\text{S}]$cysteine, washed, and lysed. The cell lysate was then subjected to immunoprecipitation with antibodies to PLZF, and the resulting precipitate was analyzed by SDS-PAGE and autoradiography. (B) 293T cells were transfected with vectors for Flag-PLZF (5 µg) and His-SUMO-1 (5 µg), or with the corresponding empty vector, as indicated. Cell lysates were then subjected to immunoprecipitation (IP) with antibodies to Flag ($\alpha_{\text{Flag}}$), and the resulting precipitates were subjected to immunoblot analysis (IB) with antibodies to Flag or to SUMO-1.

Cell lysates (10% of the input for immunoprecipitation) were also directly subjected to immunoblot analysis with antibodies to Flag. (C) 293T cells were cotransfected with expression plasmids encoding Flag-PLZF and GFP–SUMO-1. The cells were then subjected to immunofluorescence analysis with antibodies to Flag and Texas red-conjugated secondary antibodies (red fluorescence). The GFP–SUMO-1 fusion protein was visualized by GFP fluorescence (green). Colocalization of the two types of fluorescence is indicated in yellow in the merged image.

**Figure 2.** Identification of Lys$^{242}$ of PLZF as the target residue for sumoylation. (A) Schematic representation of the domain organization of PLZF and comparison of the consensus sumoylation motif of human PLZF with those of the indicated proteins. (B) 293T cells were transfected with vectors for wild-type Flag-PLZF or the Flag-PLZF(K242R) mutant, in the absence or presence of a vector for His-SUMO-1, as
indicated. Cell lysates were then subjected to immunoprecipitation and immunoblot analysis as in Figure 1B.

**Figure 3.** Modulation of the transcriptional regulatory activity of PLZF by sumoylation. (A) Schematic representation of the luciferase reporter plasmid containing four PLZF binding sites (BS) from the mouse IL-3Rα gene promoter upstream of the thymidine kinase (TK) gene promoter from herpes simplex virus (HSV). (B) 293T cells were cotransfected with the luciferase reporter plasmid (IL3R-tk-Luc) and expression vectors for His-SUMO-1 and either wild-type Flag-PLZF or Flag-PLZF(K242R). The luciferase activity of cell lysates was subsequently determined and normalized as described in Experimental Procedures. Data are expressed in arbitrary units and are means ± SD of values from five independent experiments. Expression of the wild-type and mutant PLZF proteins was also evaluated by immunoprecipitation and immunoblot analysis with antibodies to Flag. (C) Purified wild-type GST-PLZF or GST-PLZF(K242R) was incubated with *in vitro*-translated SUMO-1 and a sumoylation reaction mixture containing HeLa cell extract (E1), purified UBC9 (E2), and an ATP-regenerating system before analysis by EMSA with a 32P-labeled oligonucleotide containing a PLZF binding site.

**Figure 4.** Modulation of the cell cycle regulatory function of PLZF by sumoylation. (A)
HL-60 cells were subjected to electroporation with expression vectors for wild-type PLZF or PLZF(K242R), or with the empty vector, and were then subjected to selection in the presence of G418 (1 mg/ml) for 2 weeks. The resulting stable cell lines were deprived of serum for 72 h, reexposed to complete medium for 24 h, and then analyzed for DNA content by propidium iodide staining and flow cytometry. Data correspond to the percentages of cells in G0-G1, S, and G2-M phases of the cell cycle and are means ± SD of values from three independent experiments. (B) HL-60 cells stably transfected as in (A) were subjected to immunoblot analysis with antibodies to PLZF and to cyclin A (sigma).
Figure 1
A

<table>
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<tr>
<th>PLZF</th>
<th>POZ</th>
<th>RD2</th>
<th>Zn finger</th>
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<tr>
<td>VK^{242}TE*</td>
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p53  | L   | M   | F   | K   | T   | E   | G   | P   | D   |
Ixβα | D   | G   | L   | K   | K   | E   | R   | L   | L   |
c-Jun| Q   | A   | L   | K   | E   | E   | P   | Q   | T   |
PLZF (K242) | A   | E   | V   | K   | T   | E   | M   | M   | Q   |

B

Flag-PLZF : - + + - -
Flag-PLZF (K242R) : - - - + +
His-SUMO-1 : - - + - +

Figure 2
Figure 3
Figure 4
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