J. Integrative Natural Sci.
Vol.17, No.4 (2024) pp.123-132
https://doi.org/10.13160/ricns.2024.17.4.123

Biochemical Characterization of Protein Phosphatase 4 Inhibitory Protein

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Abstract

Protein phosphatase 4 (PP4) plays a crucial role in various cellular pathways, and its proper regulation is essential for optimal phosphorylation dynamics and supporting critical cellular pathways, including DNA double-strand break repair. A previously identified PP4 regulator, termed PP4 inhibitory protein (PP4IP), has been implicated in modulating PP4 activity. However, the biochemical properties of PP4IP and the details of its relationship with the PP4 complex remain poorly understood. In this study, we provide insights into the biochemical characteristics of PP4IP and investigate its physical interaction with PP4. Contrary to a previous report, we were not able to detect any ribonuclease activity in PP4IP purified from *E. coli*, insect, or mammalian cells. On the other hand, our findings reveal that PP4IP dimerization is required for its interaction with the PP4 complex, and that PP4R3α is a key mediator of this interaction. This study provides a clear understanding of the mechanism underlying PP4-PP4IP complex formation.

Keywords : Protein Phosphatase 4 Inhibitory Protein, Dimerization, Proximity Labeling, Protein-Protein Interaction

(**Received** November 19, 2024; **Revised** November 26, 2024; **Accepted** December 1, 2024)

1. Introduction

Protein phosphatase 4 (PP4) has significant roles in various cellular pathways including the DNA damage response, energy metabolism, immune response, and neural development[1]. The PP4 complex consists of a single catalytic subunit PP4C and five regulatory subunits (PP4R1, PP4R2, PP4R3α, PP4R3β, PP4R4), forming either heterodimer (PP4C-PP4R1 and PP4C-PP4R4) or heterotrimer (PP4C-PP4R2-PP4R3α and PP4C-PP4R2-PP4

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R3β) depending on its composition[1]. Timel y regulation of PP4 is crucial for an appro priate DNA damage response and cell survi val[2]. Various studies have identified mecha nisms of PP4 regulation through post-transl ational modifications (PTMs) and endogeno us inhibitors[1].

Previously, we identified an uncharacterized protein, chromosome 19 open reading frame 43 (C19ORF43), as a novel regulator of PP4, naming it PP4 inhibitory protein (PP4IP)[3]. PP4IP interacts with PP4C-PP4R2-PP4R3α complex and inhibits enzymatic activity of PP4 by regulating the integrity of the PP4 complex. Both depletion and overexpression of PP4IP alter the DNA damage-dependent phosphorylation kinetics of PP4 substrates, such as γH2AX, RPA2, and KAP1, leading to impaired homologous recombination and non-homologous end joining.

In addition to our study, two independent research group revealed the different functions of C19ORF43. One group identified it as human telomerase RNA-interacting ribonuclease (hTRIR) and showed its exoribonuclease activity[4]. On the other hand, the other group elucidated that C19ORF43 represses the formation of telomeric repeat-containing RNA (TERRA) R-loops at telomere and persistent telomeric cohesion[5]. The inhibitory function of C19ORF43 requires its interaction with tankyrase, and C19ORF43 deficiency in aged cells reduces DNA damage and delays replicative senescence.

Elucidating the protein-protein interaction (PPI) helps understand the biochemical characteristics of protein, which can be utilized for developing drugs[6]. Among various strategies for studying PPI, affinity purification (AP) is one of the most commonly used techniques for screening interacting candidates[7]. By utilizing antibodies, this method allows the capture and precipitation of a protein of interest (POI) along with its interacting partners. This approach can detect both direct and indirect interactions within large protein complexes. However, AP often falls short in detecting weak or transient interactions[7]. To overcome this limitation, proximity labeling techniques have emerged as powerful tools. In these methods, a biotin ligase fused to POI enzymatically adds biotin molecules to nearby proteins. Proximity-dependent biotin identification (BioID) and engineered ascorbate peroxidase (APEX) represent two prominent variations of this strategy[8].

BioID method uses BirA biotin ligase, originally sourced from *Escherichia coli*[9]. Wild type BirA targets specific lysine residues on the protein surface. However, a variant with R118G mutation (BirA*) exhibits promiscuous biotinylation activity, and codon optimization increases its soluble and active expression in human cells[10-11]. Its capacity to gradually label targets over time proves particularly valuable in detecting transient interactions, with a labeling radius extending to approximately 10 nm[12]. Alternatively, APEX functions by oxidizing biotin-phenols to phenoxyl radicals, which then biotinylate adjacent proteins in the presence of hydrogen peroxide (H_2O_2)[13]. The biotinylated proteins via both methods

are isolated using streptavidin-bead conjugates and analyzed through mass spectrometry[14-15]. This integrated approach has significantly contributed to the elucidation of protein interactomes across diverse biological contexts.

While we revealed the contribution of PP4IP to PP4 enzymatic activity, the precise biochemical relationship between the PP4 complex and PP4IP remains unclear. Here, we elucidated the biochemical characteristics of PP4IP using various expression systems. Contrary to the previous report, recombinant PP4IP proteins obtained from bacterial, insect, and mammalian cells do not exhibit ribonuclease activity. Additionally, PP4IP forms dimers through disulfide bonds between cysteine residues, and this dimerization is required for its interaction with the PP4 complex. Furthermore, we identify PP4R3α as the key mediator of the interaction between PP4IP and the PP4 complex by using BioID proximity labeling.

2. Materials and Methods

2.1. Cell culture and chemicals

293T17, HeLa, and RPE-1 cells were cultured in Dulbecco's-Modified Eagle Media (DMEM) media containing 10% (v/v) fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin.

Sf-9 insect cell line was cultured in Sf-900 III SFM (serum-free medium; Thermo Fisher Scientific, Waltham, MA, USA) including 25 U/ml penicillin-streptomycin at 27℃ in non-CO_2 incubator with agitation at 130 rpm.

Biotin and β-mercaptoethanol were purchased from Merck KGaA (Darmstat, Germany). Isopropyl β-D-1-thiogalactopyrano-side (IPTG) and N-ethylmaleimide (NEM) were obtained from Duchefa Biochemie (Haarlem, Netherlands) and Thermo Fisher Scientific, respectively.

2.2. Plasmids and siRNAs transfection

Genjet (SignaGen Laboratories, Frederick, MD, USA) and was used for plasmid trans fection. Small interfering RNAs (siRNAs) were transfected with Lipofectamine 3000 (Thermo Fisher Scientific). Sequences of si RNAs used are as follows: PP4C sense 5'-CGCUAAGGCCAGAGAGAUCUUGGUA-3', antisense 5'-UACCAAGAUCUCUCUGG CCUUAG-CG-3'; PP4R2 sense 5'-CCAAG CUAUACUGAGA-GGUCUAAUA, antisense 5'-CCAGGCCACUUAA-UCGACCAAAGG U-3'; PP4R3α_1 sense 5'-UGAA-UUAAGU CGCCUUGAAUU-3', antisense 5'-UUC-AA GGCGGACUUAAUUCAUU-3'; PP4R3α_2 sense 5'-AGAAGACAAACCUAGUAAAUU -3', antisense 5'-UUUACUAGGUUUGUCU UCUUU-3'. For an efficient knockdown of PP4R3α, a 1:1 mixed stock of two types o f PP4R3α siRNA was used[16-17]. Control si RNA was purchased from Bioneer (Daejeo n, Republic of Korea).

2.3. Immunoblotting

Cells were lysed in a lysis buffer containing 50 mM Tris HCl pH 7.5, 150 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1% IGEPAL CA-630 (Merck

KGaA), 20 U/ml Benzonase (Enzynomics, Daejeon, Republic of Korea), and a protease inhibitor cocktail (Quartett, Potsdam, Germany). Protein concentration in the supernatant was determined using a BCA assay (Thermo Fisher Scientific). Cell lysates were resuspended in 2X SDS sample loading buffer, heated, and loaded onto an acrylamide gel for gel electrophoresis.

2.4. Recombinant protein expression and purification

To express PP4IP in E. coli, XL1-Blue strain was transformed with pQE30-PP4IP and grown in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin at 220 rpm at 37℃. Upon reaching an OD600 of 0.6 to 0.8, 0.2 mM IPTG was added to induce expression of the recombinant PP4IP. Cells were harvested 3 hours post-IPTG induction.

For protein purification, cell pellet was lysed in a buffer composed of 20 mM sodium phosphate pH 7.4, 500 mM NaCl, 20 mM imidazole, 5 mM MgCl2, 0.3 mg/ml lysozyme, and 10 U/ml Benzonase. After centrifugation, the supernatant was transferred to an Econo-Pac chromatography column (Bio-Rad, Hercules, CA, USA) containing 1 ml Ni-NTA resin (Thermo Fisher Scientific) that had been pre-equilibrated with a binding buffer (20 mM sodium phosphate pH 7.4, 500 mM NaCl, and 20 mM imidazole). Once all the lysate had flown through, the resin was washed with a wash buffer (20 mM sodium phosphate pH 7.4, 500 mM NaCl, and 40 mM imidazole), followed by eluting the protein with an elution buffer (20 mM sodium phosphate pH 7.4, 500 mM NaCl, and 250 mM imidazole).

To purify PP4IP using baculovirus expression system, Sf-9 cells were infected with the baculovirus encoding PP4IP at a multiplicity of infection (MOI) of 0.1 and incubated for approximately 66 hours at 27℃ incubator with agitation. After harvesting, cells were lysed in a buffer including 20 mM sodium phosphate pH 7.4, 500 mM NaCl, 20 mM imidazole, and 0.5% IGEPAL CA-630, and cell lysate was subjected to protein purification as described above.

2.5. Immunoprecipitation

Cells were lysed in IP buffer including 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% IGEPAL CA-630, 10% glycerol and a protease inhibitor cocktail. Supernatant obtained after centrifugation was incubated with ANTI-FLAG-magnetic bead (Merck KGaA) at 4℃ overnight. The immunoprecipitate was washed with IP buffer three times, followed by immunoblotting.

2.6. Ribonuclease activity assay

The assay was performed under the identical experimental conditions described in a previous publication, with the exception of the source of total cellular RNA[4]. In brief, 1 μg of PP4IP proteins purified from XL1-blue and Sf-9 cells, and the immunoprecipitates from 293T17 cells were incubated with 2 μg of total RNA extracted from HeLa cells for 30 minutes

at 37℃ in a buffer containing 0.5 mM Tris-HCl, 25 mM NaCl, and 2% glycerol (pH 7.4). Ribonuclease A (RNase A) was used as a positive control. After the reaction, RNA was cleaned up by using RNA Clean and Concentrator kit (Zymo Research, Irvine, CA, USA) and analyzed by agarose gel electrophoresis.

2.7. Proximity labeling and affinity enrichment of biotinylated proteins

PP4IP gene was cloned into pcDNA3.1-mycBioID vector (#35700) obtained from Addgene (Watertown, MA, USA), and 293T17 cells were transfected with the plasmid encoding BirA*-PP4IP. The next day, cells were incubated with 50 μM biotin overnight. Subsequently, the cells were lysed in BioID buffer comprising 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.4% SDS, 1% IGEPAL CA-630, 1.5 mM MgCl2, 1 mM EGTA, 25 U/ml Benzonase, and a protease inhibitor cocktail. After centrifugation, the supernatant was incubated overnight at 4℃ with streptavidin-magnetic beads (NEB, Ipswich, MA, USA). After washing the beads three times with 1 ml of BioID buffer without Benzonase and protease inhibitor cocktail, the beads were resuspended with 3X SDS sample loading buffer supplemented with 1 mM biotin for subsequent immunoblotting.

3. Results

3.1. PP4IP does not have ribonuclease activity *in vitro*

To understand the biochemical characteristics of PP4IP, we purified a recombinant PP4IP using three expression systems: bacterial, insect, and mammalian expression systems. For bacterial expression system, we transformed E. coli XL1-Blue strain with pQE-30-PP4IP plasmid, followed by purification using Ni-NTA (**Fig. 1**(A)). For baculovirus expression system, Sf-9 insect cells were infected with the baculovirus encoding PP4IP and were subjected to purification (**Fig. 1**(B)). Furthermore, PP4IP overexpressed in 293T17 cells was purified by immunoprecipitation (**Fig. 1**(C)).

Previous research indicated that TRIR (another name of PP4IP) had ribonuclease (RNase) activity[4]. To validate this, we tested the RNase activity of PP4IP purified from three different expression systems. However, contrary to the previous finding, PP4IP did not degrade RNA (**Fig. 1**(D)).

3.2. PP4IP homo-dimerization is necessary for the interaction with the PP4 complex

Unexpectedly, we found that purified PP4IP formed a homodimer in vitro through a disulfide bond, despite having only one cysteine in its overall protein sequence (**Fig. 2**(A), (B)). To assess whether this dimerization is also observed in human cells, we expressed PP4IP proteins with different affinity tags. We confirmed that FH-PP4IP interacts with Myc-PP4IP (**Fig. 2**(C)).

To investigate the importance of PP4IP homo-dimerization, we examined the interaction between PP4IP and PP4R2 in the

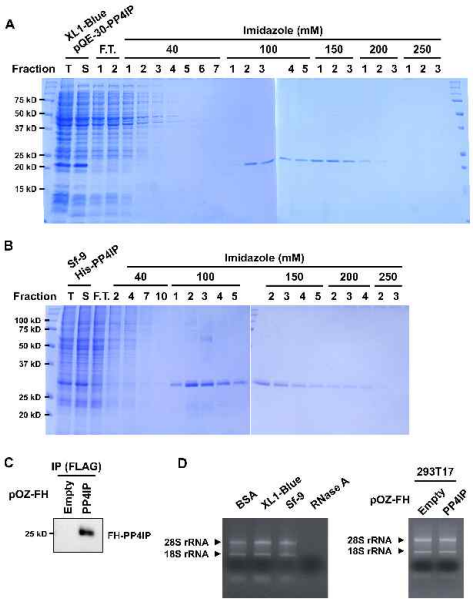

Fig. 1. Purified PP4IP does not have RNase activity. (A) Purification of PP4IP from XL1-Blue containing pQE-30-PP4IP. (B) Purification of PP4IP expressed in Sf-9 insect cells. (C) Immunoprecipitation of FH-PP4IP from 293T17 cells. (D) In vitro RNase activity assay with recombinant PP4IP purified from E. coli, insect cells, and mammalian cells.

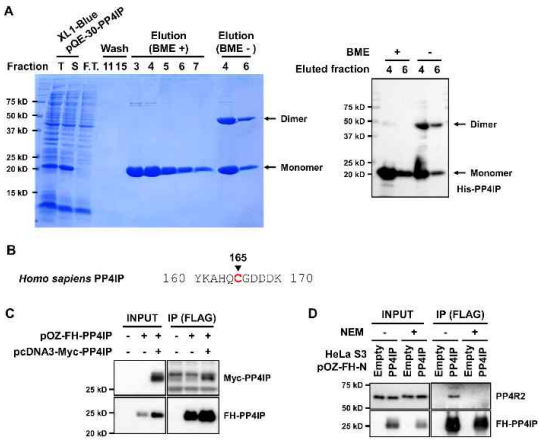

Fig. 2. PP4IP forms homodimer via disulfide bond. (A) Dimerization of purified PP4IP. (B) A sole cysteine residue on PP4IP amino acid sequence. (C) PP4IP dimer is also formed in mammalian cells. (D) PP4IP dimerization is required for interaction with PP4R2.

presence of N-ethylmaleimide (NEM), which irreversibly forms a thioester bond with cysteine and is used to block disulfide bonds[18]. In the presence of NEM, PP4IP did not interact with PP4R2, suggesting that PP4IP dimerization is required for the interaction with the PP4 complex (**Fig. 2**(D)).

3.3. PP4R3α mediates the interaction between PP4IP and the PP4 complex

We wondered which PP4 subunits mediate the interaction between the PP4 complex and PP4IP. However, we were not able to compare the interaction of PP4IP with the PP4 complex deficient each subunit, because depleting a single subunit resulted in downregulation of the other subunits (**Fig. 3**(A)). To address this problem, we utilized BioID technology by expressing PP4IP fused to BirA mutant (BirA*) (**Fig. 3**(B)). As a result, only PP4R3α, but not PP4C and PP4R2, was found in the biotinylated precipitate (**Fig. 3**(C)). Given that the biotinylation efficiency of BirA* decreases with distance from the enzyme, this result indicates that PP4IP interacts with the PP4 complex through PP4R3α.

4. Discussion

The first report on C19ORF43 described it as having RNase activity and named it human telomeric RNA interacting RNase (hTRIR)[4]. However, our results are not consistent with this conclusion. The discrepancy might result from the difference

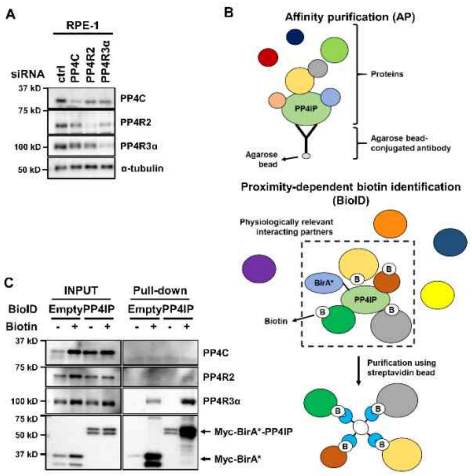

Fig. 3. PP4R3α is a mediator of the interaction between the PP4 complex and PP4IP. (A) Simultaneous downregulation of PP4 subunits after depleting each subunit. (B) Comparison of affinity purification and proximity-dependent biotin identification. (C) Confirmation of the interaction between PP4IP and PP4 subunits through proximity labeling.

in host used for expressing PP4IP. We used E. coli XL1-Blue strain, insect cells (Sf-9), and human cancer cells (293T17), whereas the other group used only the E. coli BL21(DE3) strain. We also attempted to express PP4IP in BL21(DE3) with pQE30-PP4IP vector, but were not able to get any colonies (data now shown). These data suggest that PP4IP expressed from BL21(DE3) might be toxic or exhibit host-specific RNase activity. To investigate the cause of this discrepancy, it would be helpful to compare the protein structures of recombinant PP4IP purified from BL21(DE3) and XL1-Blue. These structures can then be compared to a predictive structure generated by AlphaFold AI program to determine which structure is closer to the human PP4IP structure[19]. Several strategies have been proposed for expressing toxic proteins in E. coli, and recombinant PP4IP might be successfully obtained from BL21(DE3) strain using these approaches[20].

A recently published study referring C19ORF43 has shown that C19ORF43 is necessary to resolve telomere cohesion formed by R-loop structures between sister chromatids[5]. Additionally, telomere cohesion was resolved by treatment with RNase H1 and S1 nuclease in vitro, which catalyze the cleavage of RNA in RNA/DNA duplex and ssDNA, respectively. However, incubation with either recombinant RNase A, which specifically targets ssRNA but not RNA/DNA hybrid, or C19ORF43 did not resolve it. These results suggest two possibilities: 1) C19ORF43 does not have RNase activity, or 2) C19ORF43 does not degrade RNA in RNA/DNA duplex, at least R-loop.

PP4IP can form a homodimer through a disulfide bond with its sole cysteine residue. Blocking additional formation of PP4IP dimers by adding NEM inhibited the interaction between PP4R2 and PP4IP, indicating that PP4IP dimer, not monomer, is required for interaction with the PP4 complex. This observation suggests that the monomer-to-dimer transition of PP4IP could fine-tune its interaction with PP4 and serve as a novel regulatory mechanism for controlling PP4 complex activity. To test this hypothesis, it would be useful to express PP4IP in either a monomer or dimer. However, we found that the PP4IP mutant, where a sole cysteine residue is replaced with alanine, is expressed at significantly lower levels compared to the wild-type

PP4IP. This makes it difficult to compare the effects of the wild type and the mutant (data not shown). Meanwhile, a construct where two PP4IP proteins are linked by a linker could readily form PP4IP dimers.

Additionally, proximity labeling data suggest that PP4IP interacts with the PP4 complex through PP4R3α. However, to fully confirm this suggestion, additional evidence is required, such as in vitro binding assay. Unfortunately, we failed to express human PP4C in E. coli and insect cells, limiting our ability to perform these assays (data not shown). Moreover, depletion of either PP4R2 or PP4R3α downregulates other PP4 subunits, which makes it hard to isolate PP4R2- or PP4R3α-free PP4 complex. On the other hand, PP4IP does not have a consensus binding motif for PP4R3α[21]. Identifying their physical relationship is helpful to understand its regulation mechanism.

Acknowledgements

This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1I1A3059916).

References

[1] J. Park and D.-H. Lee, “Functional roles of protein phosphatase 4 in multiple aspects of cellular physiology: a friend and a foe.”, BMB Rep., Vol. 53, No. 4, pp. 181-190, 2020.

[2] D.H. Lee and D. Chowdhury, “What goes on must come off: phosphatases gate-crash the DNA damage response, Trends.”, Biochem. Sci., Vol. 36, No. 11, pp. 569-577, 2011.

[3] J. Park, J. Lee and D.H. Lee, “Identification of Protein Phosphatase 4 Inhibitory Protein That Plays an Indispensable Role in DNA Damage Response.”, Mol. Cells, Vol. 42, No. 7, pp. 546-556, 2019.

[4] J. Xie, Z. Chen, X. Zhang, H. Chen and W. Guan, “Identification of an RNase that preferentially cleaves A/G nucleotides.”, Sci. Rep., Vol. 7, p. 45207, 2017.

[5] S. Sze, A. Bhardwaj, P. Fnu, K. Azarm, R. Mund, K. Ring and S. Smith, “TERRA R-loops connect and protect sister telomeres in mitosis.”, Cell Rep., Vol. 42, No. 10, p. 113235, 2023.

[6] H. Lu, Q. Zhou, J. He, Z. Jiang, C. Peng, R. Tong and J. Shi, “Recent advances in the development of protein–protein interactions modulators: mechanisms and clinical trials.”, Signal Transduct. Target Ther., Vol. 5, No. 1, p. 213, 2020.

[7] J.H. Morris, G.M. Knudsen, E. Verschueren, J.R. Johnson, P. Cimermancic, A.L. Greninger and A.R. Pico, “Affinity purification–mass spectrometry and network analysis to understand protein-protein interactions.”, Nat. Protoc., Vol. 9, No. 11, pp. 2539-2554, 2014.

[8] C.-L. Chen and N. Perrimon, “Proximity-dependent labeling methods for proteomic profiling in living cells.”, Wiley Interdiscip. Rev. Dev. Biol., Vol. 6, No. 4, p. e272, 2017.

[9] A. Chapman-Smith and J.E. Cronan, “The enzymatic biotinylation of proteins: a post-translational modification of exceptional specificity.”, Trends Biochem. Sci., Vol. 24, No. 9, pp.359-363, 1999.

[10] K. Kwon and D. Beckett, “Function of a conserved sequence motif in biotin holoenzyme synthetases.”, Prot. Sci., Vol. 9, No. 8, pp. 1530-1539, 2000.

[11] U. Mechold, C. Gilbert and V. Ogryzko, “Codon optimization of the BirA enzyme gene leads to higher expression and an improved efficiency of biotinylation of target proteins in mammalian cells.”, J. Biotechnol., Vol. 116, No. 3, pp. 245-249, 2005.

[12] D.I. Kim, B. Kc, W. Zhu, K. Motamedchaboki, V. Doye and K.J. Roux, “Probing nuclear pore complex architecture with proximity-dependent biotinylation.”, Proc. Nati. Acad. Sci. U.S.A., Vol. 111, No. 24, pp. E2453-E2461, 2014.

[13] T.M.T. Nguyen, J. Kim, T.T. Doan, M.-W. Lee and M. Lee, “APEX Proximity Labeling as a Versatile Tool for Biological Research.”, Biochemistry, Vol. 59, No. 3, pp. 260-269, 2020.

[14] H.-W. Rhee, P. Zou, N.D. Udeshi, J.D. Martell, V.K. Mootha, S.A. Carr and A.Y. Ting, “Proteomic Mapping of Mitochondria in Living Cells via Spatially Restricted Enzymatic Tagging.”, Science, Vol. 339, No. 6125, pp. 1328-1331, 2013.

[15] R. Gupta, K. Somyajit, T. Narita, E. Maskey, A. Stanlie, M. Kremer, D. Typas, M. Lammers, N. Mailand, A. Nussenzweig, J. Lukas and C. Choudhary, “DNA Repair Network Analysis Reveals Shieldin as a Key Regulator of NHEJ and PARP Inhibitor Sensitivity”, Cell, Vol. 173, No. 4, pp. 972-988.e923, 2018.

[16] D. Chowdhury, X. Xu, X. Zhong, F. Ahmed, J. Zhong, J. Liao, D.M. Dykxhoorn, D.M. Weinstock, G.P. Pfeifer and J. Lieberman, “A PP4-phosphatase complex dephosphorylates gamma-H2AX generated during DNA replication.”, Mol. Cell., Vol. 31, No. 1, pp. 33-46, 2008.

[17] J. Lee, G. Adelmant, J.A. Marto and D.H. Lee, “Dephosphorylation of DBC1 by Protein Phosphatase 4 Is Important for p53-Mediated Cellular Functions.”, Mol. Cells., Vol. 38, No. 8, pp. 697-704, 2015.

[18] M. Herscovitch, W. Comb, T. Ennis, K. Coleman, S. Yong, B. Armstead, D. Kalaitzidis, S. Chandani and T.D. Gilmore, “Intermolecular disulfide bond formation in the NEMO dimer requires Cys54 and Cys347.”, Biochem. Biophy. Res. Commun., Vol. 367, No. 1, pp. 103-108, 2008.

[19] K. Tunyasuvunakool, J. Adler, Z. Wu, T. Green, M. Zielinski, A. Žídek, A. Bridgland, A. Cowie, C. Meyer, A. Laydon, S. Velankar, G.J. Kleywegt, A.

Bateman, R. Evans, A. Pritzel, M. Figurnov, O. Ronneberger, R. Bates, S.A.A. Kohl, A. Potapenko, A.J. Ballard, B. Romera-Paredes, S. Nikolov, R. Jain, E. Clancy, D. Reiman, S. Petersen, A.W. Senior, K. Kavukcuoglu, E. Birney, P. Kohli, J. Jumper and D. Hassabis, "Highly accurate protein structure prediction for the human proteome." Nature, Vol. 596, pp. 590-596, 2021.

[20] R. Jiang, S. Yuan, Y. Zhou, Y. Wei, F. Li, M. Wang, B. Chen and H. Yu, "Strategies to overcome the challenges of low or no expression of heterologous proteins in *Escherichia coli*.", Biotechnol. Adv., Vol. 75, p. 108417, 2024.

[21] Y. Ueki, T. Kruse, M.B. Weisser, G.N. Sundell, M.S.Y. Larsen, B.L. Mendez, N.P. Jenkins, D.H. Garvanska, L. Cressey, G. Zhang, N. Davey, G. Montoya, Y. Ivarsson, A.N. Kettenbach and J. Nilsson, "A Consensus Binding Motif for the PP4 Protein Phosphatase.", Mol. Cell, Vol. 76, No. 6, pp. 953-964. e956, 2019.