Bacterial-Type Long-Chain Polyphosphates Bind Human Proteins in the Phosphatidylinositol Signaling Pathway

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Introduction

Inorganic polyphosphates are linear polymers of monophosphate residues (P_i) that exist as short chains (P_i30–120) in platelet δ-granules and as long chains in bacteria.[1] A higher chain length increases the activity of these anionic polymers to accelerate factor XIIa-mediated factor XI activation, thrombin generation, block tissue factor pathway inhibitor activity, and strengthen fibrin clots by enhancing their mechanical stability and resistance to fibrinolysis.[2,3] In bacteria, polyphosphates are associated with energy and phosphate storage, stress resistance, chelation of metal ions, and escaping host immunity.[4] During severe infections, long-chain polyphosphates from bacteria contribute to coagulopathy, neutrophil extracellular trap formation, and vascular-endothelial dysfunction.[3,5,6] However, the mechanisms that convey the pleiotropic activities of polyphosphates in living cells remain understudied.

Results and Discussion

Here, we report a screen using a human proteome microarray to identify novel polyphosphate-binding proteins. This HuProt™ microarray contains a large number of eukaryote-expressed proteins individually printed on a single array slide. A total of 19,394 unique proteins representing approximately 75% of the human proteome were evaluated. In total, 14,906 proteins could be assigned to cell physiological processes (►**Fig. 1A**). The microarray slides were probed with biotinylated long-chain polyphosphates followed by fluorescence detection (►**Fig. 1B**; full dataset: https://doi.org/10.5281/zenodo.5748254).

The best binding partner for polyphosphates was the protein DAB1 (Disabled-1; ►**Fig. 1C**). While DAB1 is a key player of the Reelin–DAB1 signaling pathway, regulating mammalian brain development by orchestrating cell migration, additional roles in nonneuronal tissues have become more evident in recent years.[7] Intracellular phosphorylation of DAB1 by Src family tyrosine kinases (Fyn/Src) facilitates downstream effector recruitment like PI3Ks (phosphatidylinositol 3-kinases), resulting in activation of multiple cellular signaling cascades. DAB1 interacts with PI3Ks in response to Reelin signaling.[8] DAB1 is phosphorylated after binding of activated protein C and PAR (protease-activated receptor) activation upstream of PI3Ks.[9] Reelin is also expressed by platelets and it was described that Reelin–platelet interactions can enhance platelet spreading on fibrinogen.[7,10]

We confirmed the interaction of polyphosphates with human DAB1 using isothermal titration calorimetry (ITC) at 25°C. As shown in ►**Fig. 1D**, polyphosphates interacted with recombinant DAB1 in an exothermic process with a heat release in the order of magnitude, which is usually observed for protein–protein interactions. However, it was not possible to fit the obtained data due to the low number of titration measurements performed with the limited amount of protein available. Since DAB1 does not contain a PASK domain for covalent attachment of polyphosphates,[11,12] we speculate that the binding could be due to electrostatic interactions. We used titration of polyphosphates to human serum albumin (HSA) as a negative control without signs of binding as no significant

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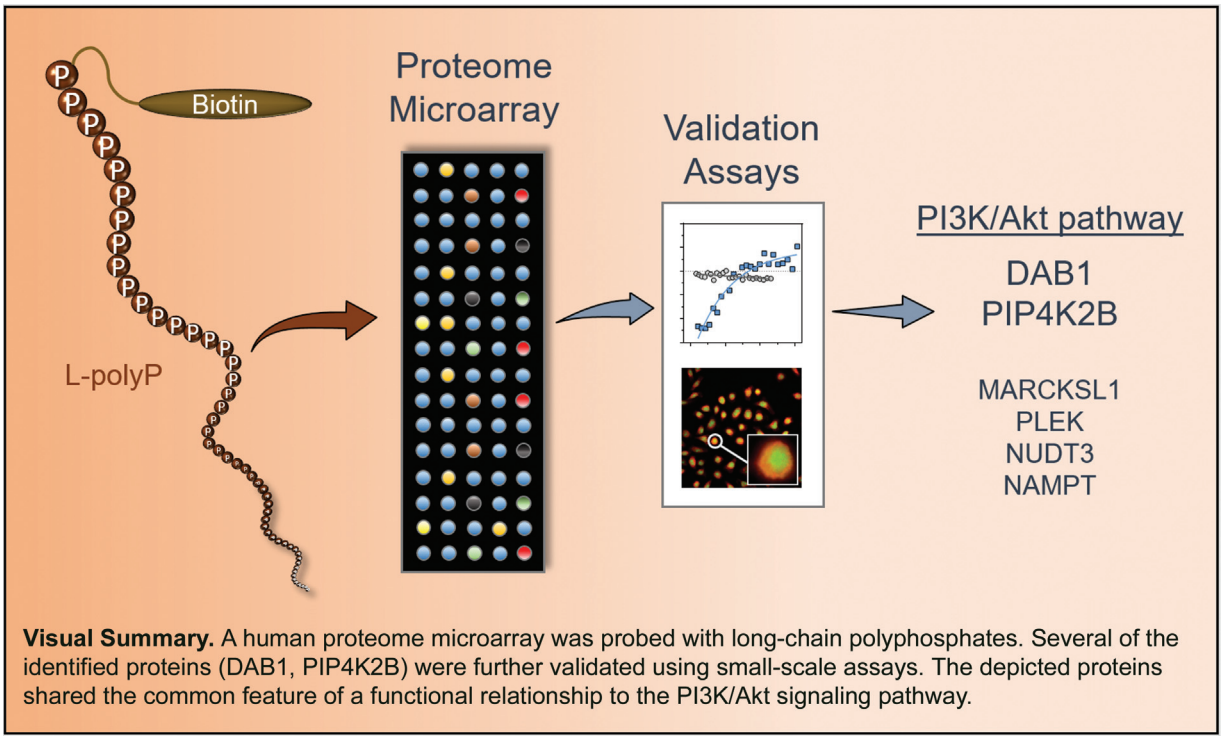

Visual Summary. A human proteome microarray was probed with long-chain polyphosphates. Several of the identified proteins (DAB1, PIP4K2B) were further validated using small-scale assays. The depicted proteins shared the common feature of a functional relationship to the PI3K/Akt signaling pathway.

heat evolvement was detectable (►**Fig. 1D**). DAB1 is an intracellular protein and not detectable in human plasma (►**Fig. 1E**). DAB1 gene expression was enriched in the brain among other tissues (►**Fig. 1F**).[13] Functionally, DAB1 signaling in the endothelial layer of the brain orchestrates neuro-glia-vessel communication.[14]

The proteome microarray screen also indicated the binding of polyphosphates to PIP4K2B (phosphatidylinositol-5-phosphate 4-kinase 2B; ►**Fig. 1C**). PIP4K2B is ubiquitously expressed in many tissues including endothelial cells (►**Fig. 1F**). PIP4K2B is a stress-regulated lipid kinase that catalyzes the phosphorylation of phosphatidylinositol-5-phosphate to form phosphatidylinositol-5,4-bisphosphate (PIP_2), which is a substrate for the phosphatidylinositol signal transduction pathway. Loss of PIP4K2B is associated with slower tumor growth in p53-deficient mice.[15,16]

Fluorescence microscopy revealed co-localization of antibody staining for DAB1 and PIP4K2B in HeLa cells probed with polyphosphates (►**Fig. 1G, H**).

Several other binding proteins were observed in the microarray with detectable affinity, albeit with lower scores than the threshold recommended by the manufacturer:

MARCKSL1 (myristoylated alanine-rich C-kinase substrate-like 1, ►**Fig. 1C**) can sequester PIP_2 within "lipid rafts" of the cell membrane for participation in later signal transduction events.[17] MARCKSL1 plays a critical role in the regulation of apoptosis and promotes antiangiogenic effects by reducing VEGF (vascular endothelial growth factor) and HIF-1α (hypoxia-inducible factor 1-alpha).[18]

PLEK (pleckstrin) is highly expressed in blood cells, especially platelets, bone marrow, and lymphoid tissue, and present in blood plasma (►**Fig. 1C, E**).[13] In platelets, PLEK is a major substrate of protein kinase C. Upon binding to PIP_2 it is involved in the regulation of multiple platelet processes, e.g., aggregation, degranulation, platelet activation, and secretion.[19,20]

The NUDT3 (nucleoside diphosphate-linked moiety X-type motif hydrolase-3, ►**Fig. 1C**) gene encodes for the diphosphoinositol polyphosphate phosphohydrolase-1. It belongs to the MutT, or Nudix, protein family and cleaves a β-phosphate from the diphosphate groups in diphosphoinositol pentakisphosphate and bisdiphosphoinositol tetrakisphosphate, suggesting that it may play a role in signal transduction.[21] NUDT3 was identified in a gain-of-function screen as a PI3K inhibitor resistance gene suggesting a role in this pathway.[22] Interestingly, NUDT3 was recently reported to show polyphosphatase activity with a requirement of Zn^{2+} as a cofactor.[23]

NAMPT/visfatin (nicotinamide phosphoribosyltransferase) induces endothelial angiogenesis through activation of the PI3K pathway.[24] Among the top 10 polyphosphate-binding proteins, VCX3A (variable charge, X-linked 3A; unknown function), AKAP8L (A-kinase anchoring protein 8-like), GRHPR (glyoxylate reductase/hydroxypyruvate reductase; metabolism), and GPD1 (glycerol-3-phosphate dehydrogenase; metabolism; ►**Fig. 1C**) have no documented connection to the phosphatidylinositol pathway. NAMPT, GRHPR, GPD1, and NACA (nascent-polypeptide-associated complex alpha polypeptide), but none of the other proteins shown in ►**Fig. 1C**, were also identified by another HuProt™ microarray screen probed with ultra-short polyphosphate (P_i8).[25] Likewise, GPD1 from *Trypanosoma* spp. is a binding partner of long-chain polyphosphates.[26]

In summary, the majority of the 10 proteins with highest polyphosphate binding were associated with the PI3K pathway. Interestingly, polyphosphates directly modulate PI3K/Akt signaling including in primary human vascular

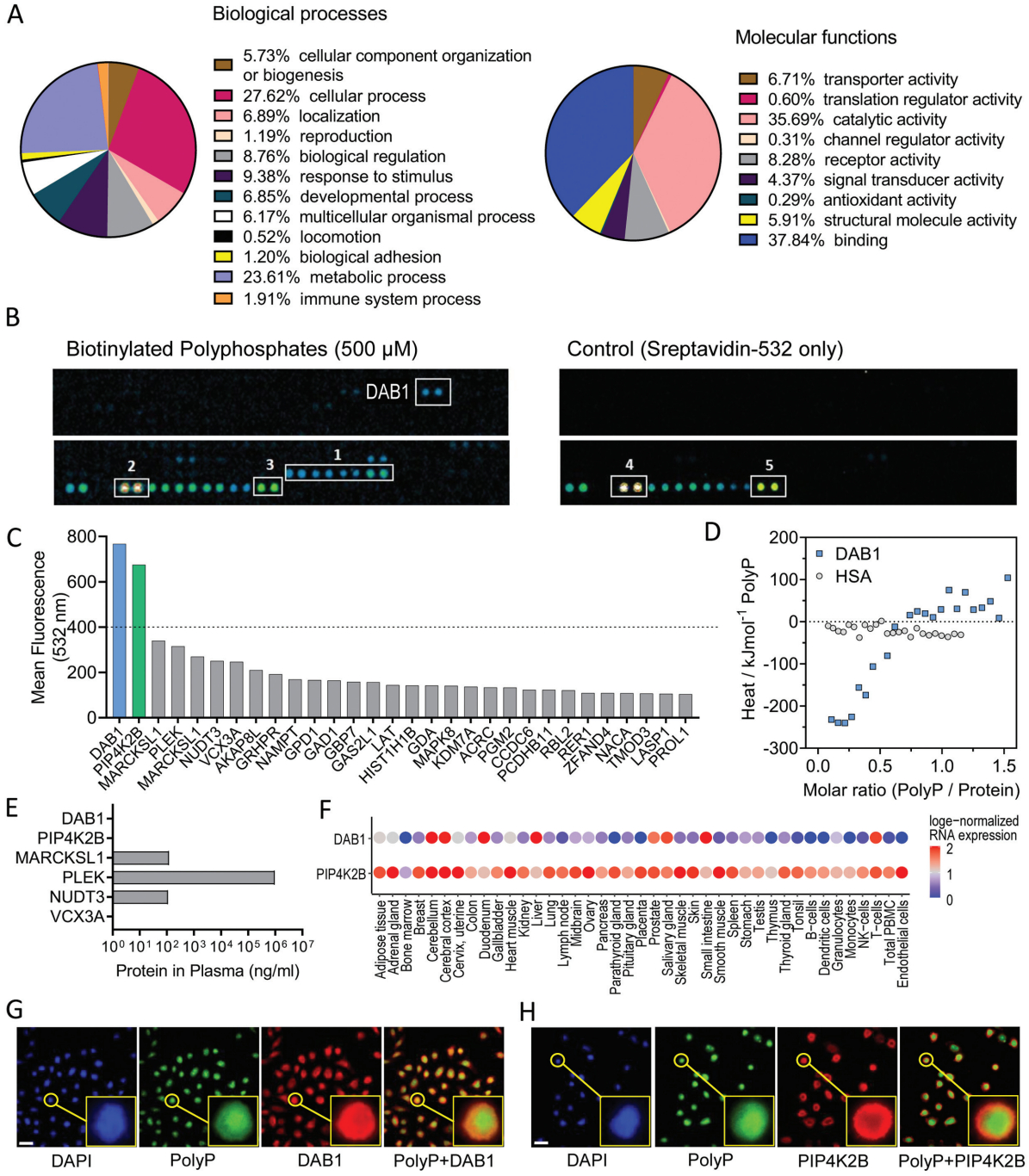

Fig. 1 A human proteome microarray for the identification of polyphosphate-binding proteins. (**A**) Overview of the proteins in the HuProt™ human proteome microarray. In total 19,394 human proteins were assessed and 14,906 (multiple mapping possible) were assigned to cell physiological processes using the PANTHER software. (**B**) Representative microarray slides probed with polyphosphates and controls showing the best hit DAB1. The left array shows the subarray with polyphosphates and the right panel depicts a control array. The bottom rows of the subarrays contain control spots; box 1: histones; boxes 2 and 4: biotinylated bovine serum albumin; boxes 3 and 5: directly fluorophore-labeled IgG. No specific protein interactions were observed for the subarray on the control slides (*right panel*). (**C**) Mean fluorescence intensities of polyphosphate-binding signals for the top 30 proteins in the microarray. The arithmetic mean of technical duplicates is shown and the horizontal dashed line indicates the assigned threshold. (**D**) Isothermal titration calorimetry of long-chain polyphosphates titrated to either recombinant human DAB1 or human serum albumin (HSA). (**E**) Concentrations of selected proteins in human plasma. The data were retrieved from the open-access Peptide Atlas (plasma non-glyco 2017) acquired through mass spectrometry-based proteomics. (**F**) Dot plot showing mRNA expression of DAB1 and PIP4K2B in human tissues and selected cell types. Data were retrieved from the Human Protein Atlas. (**G**) Images of fixed, permeabilized HeLa cells stained with antibodies for DAB1, biotinylated polyphosphates, merged image, and DAPI for nuclei. (**H**) PIP4K2B and polyphosphate staining of HeLa cells; scale bars: 25 µm.

endothelial cells.[5,27] The siRNA (small-interfering RNA) knockdown of PI3K/Akt/mTOR pathway components abrogates the endothelial barrier-disruptive effects of polyphosphates.[5] Short chains (P_i15) are sufficient to activate mTOR.[27] Hence, our studies expand the previous knowledge by uncovering several new polyphosphate-binding proteins with a relation to the PI3K/Akt pathway.

In contrast, our microarray screen could not confirm binding of polyphosphates to their putative receptors, RAGE and P2Y1,[28,29] which is in line with their nonsignificant role for mediating polyphosphate effects in macrophages.[4] However, a negative result in the microarray does not exclude that a protein binds polyphosphates under physiologic conditions, as binding characteristics are affected by protein folding and posttranslational modifications. Notably, anionic polyphosphates partner with cations (e.g., Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}), which affects the overall charge and binding characteristics of polyphosphates to proteins. Our current study did not investigate the influence of different cations on polyphosphate–protein interactions; or if short chain, platelet-derived polyphosphates can also bind the identified factors. Instead, we provide a comprehensive list of long-chain polyphosphate-binding human proteins to facilitate mechanistic studies on the molecular targets of polyphosphates in future.

Methods

Reagents: Long-chain polyphosphates (P_i700) were a gift from James H. Morrissey and Stephanie A. Smith (see Kerafast #EUI006 for further specifications). Human recombinant DAB1 was produced in *Escherichia coli* by Aviva Systems Biology (San Diego, California, United States).

Human Proteome Microarray: The HuProt™ v3 microarray was performed by Cambridge Protein Arrays Ltd., Babraham Research Campus, Cambridge CB22 3 AT, United Kingdom. Slides were probed with biotinylated long-chain polyphosphates (P_i700; 500 µM in 2% BSA/PBS [pH 7.45]-Tween 0.1%) followed by fluorescence detection using streptavidin-532.

ITC: The calorimetric measurements were performed using a NanoITC Low Volume (TA Instruments, Eschborn, Germany) with an effective cell volume of 170 µL. Briefly, 50 µL of a long-chain polyphosphate solution (20.8 µM in OPCA or PBS buffer) was titrated to a suspension of DAB1 (4 µM in OPCA buffer) or HSA (3 µM in PBS, negative control). The experimental temperature was kept constant at 25°C. Additionally, the same amount of polyphosphate solution was titrated into pure OPCA and PBS buffers to determine the heat of dilution for reference. The number and injected volume of the titration steps were the same for all measurements (25×2 µL). The spacing between injections was set to 300 seconds. The integrated reference heats were then subtracted from the integrated heats of the adsorption experiments. Due to the high costs and low availability of the DAB1 protein, the results are shown for one single series of measurements only. Thus, no fitting was performed. For calculating molar ratios, a length of 700 phosphate residues per chain was applied. Nano Analyze Data Analysis software from TA Instruments was used for the data evaluation of the ITC measurements (Software version 2.5.0) from TA Instruments.

Immunofluorescence microscopy: HeLa cells were fixed in 4% paraformaldehyde for 15 minutes, washed twice with PBS, incubated for 10 minutes with 0.25% Triton X-100, washed three times with PBS, blocked with 1% BSA in PBST for 30 minutes, and incubated with primary antibodies (1:100 dilution, rabbit anti-human DAB1, rabbit anti-human PIP4K2B; MyBioSource, San Diego, California, United States) and biotinylated polyphosphates (100 µM) at 4°C overnight. Next, slides were washed with PBS three times, incubated with secondary antibodies (1:200 dilution, goat anti-rabbit IgG-AF594; MyBioSource) and streptavidin-AF488 (ThermoFisher Scientific, Waltham, Massachusetts, United States) at room temperature in the dark for 1 hour, washed three times, and mounted with DAPI. Secondary antibody-only incubations served as negative controls and showed no unspecific staining. Images were acquired with a Nikon deconvolution wide-field epifluorescence system.

Bioinformatics: The consensus-normalized expressions in ►**Fig. 1F** were retrieved from the Human Protein Atlas (HPA) and are based on combined bulk transcriptome data from the HPA, Genotype-Tissue Expression (GTEx), and Functional Annotation of Mammalian Genomes 5 (FANTOM5) projects. Data for endothelial cells were retrieved from HPA from single cell transcriptomes. The colors represent the natural log ($\log_e$)-normalized RNA expression values plus one.[13]

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Conflict of Interest
M.B. is a consultant for and receives research support from ARCA Biopharma.

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References

1 Smith SA, Morrissey JH. 2013 scientific sessions Sol Sherry distinguished lecture in thrombosis: polyphosphate: a novel modulator of hemostasis and thrombosis. Arterioscler Thromb Vasc Biol 2015;35(06):1298–1305

2 Baker CJ, Smith SA, Morrissey JH. Polyphosphate in thrombosis, hemostasis, and inflammation. Res Pract Thromb Haemost 2018; 3(01):18–25
3 Zilberman-Rudenko J, Reitsma SE, Puy C, et al. Factor XII activation promotes platelet consumption in the presence of bacterial-type long-chain polyphosphate in vitro and in vivo. Arterioscler Thromb Vasc Biol 2018;38(08):1748–1760
4 Roewe J, Stavrides G, Strueve M, et al. Bacterial polyphosphates interfere with the innate host defense to infection. Nat Commun 2020;11(01):4035
5 Hassanian SM, Dinarvand P, Smith SA, Rezaie AR. Inorganic polyphosphate elicits pro-inflammatory responses through activation of the mammalian target of rapamycin complexes 1 and 2 in vascular endothelial cells. J Thromb Haemost 2015;13(05): 860–871
6 McDonald B, Davis RP, Kim SJ, et al. Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. Blood 2017;129(10):1357–1367
7 Khialeeva E, Carpenter EM. Nonneuronal roles for the reelin signaling pathway. Dev Dyn 2017;246(04):217–226
8 Bock HH, Jossin Y, Liu P, et al. Phosphatidylinositol 3-kinase interacts with the adaptor protein Dab1 in response to Reelin signaling and is required for normal cortical lamination. J Biol Chem 2003;278(40):38772–38779
9 Yang XV, Banerjee Y, Fernández JA, et al. Activated protein C ligation of ApoER2 (LRP8) causes Dab1-dependent signaling in U937 cells. Proc Natl Acad Sci U S A 2009;106(01):274–279
10 Tseng WL, Huang CL, Chong KY, et al. Reelin is a platelet protein and functions as a positive regulator of platelet spreading on fibrinogen. Cell Mol Life Sci 2010;67(04):641–653
11 Azevedo C, Livermore T, Saiardi A. Protein polyphosphorylation of lysine residues by inorganic polyphosphate. Mol Cell 2015;58 (01):71–82
12 Bentley-DeSousa A, Holinier C, Moteshareie H, et al. A screen for candidate targets of lysine polyphosphorylation uncovers a conserved network implicated in ribosome biogenesis. Cell Rep 2018; 22(13):3427–3439
13 Uhlén M, Fagerberg L, Hallström BM, et al. Proteomics. Tissue-based map of the human proteome. Science 2015;347 (6220):1260419
14 Segarra M, Aburto MR, Cop F, et al. Endothelial Dab1 signaling orchestrates neuro-glia-vessel communication in the central nervous system. Science 2018;361(6404):eaao2861
15 Fiume R, Stijf-Bultsma Y, Shah ZH, et al. PIP4K and the role of nuclear phosphoinositides in tumour suppression. Biochim Biophys Acta 2015;1851(06):898–910
16 Emerling BM, Hurov JB, Poulogiannis G, et al. Depletion of a putatively druggable class of phosphatidylinositol kinases inhibits its growth of p53-null tumors. Cell 2013;155(04):844–857
17 El Amri M, Fitzgerald U, Schlosser G. MARCKS and MARCKS-like proteins in development and regeneration. J Biomed Sci 2018;25 (01):43
18 Kim BR, Lee SH, Park MS, et al. MARCKSL1 exhibits anti-angiogenic effects through suppression of VEGFR-2-dependent Akt/PDK-1/mTOR phosphorylation. Oncol Rep 2016;35(02): 1041–1048
19 Yoon HS, Hajduk PJ, Petros AM, Olejniczak ET, Meadows RP, Fesik SW. Solution structure of a pleckstrin-homology domain. Nature 1994;369(6482):672–675
20 Harlan JE, Hajduk PJ, Yoon HS, Fesik SW. Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. Nature 1994;371(6493):168–170
21 Safrany ST, Caffrey JJ, Yang X, et al. A novel context for the 'MutT' module, a guardian of cell integrity, in a diphosphoinositol polyphosphate phosphohydrolase. EMBO J 1998;17(22):6599–6607
22 Le X, Antony R, Razavi P, et al. Systematic functional characterization of resistance to PI3K inhibition in breast cancer. Cancer Discov 2016;6(10):1134–1147
23 Samper-Martín B, Sarrias A, Lázaro B, et al. Polyphosphate degradation by Nudt3-Zn^{2+} mediates oxidative stress response. Cell Rep 2021;37(07):110004
24 Adya R, Tan BK, Punn A, Chen J, Randeva HS. Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis. Cardiovasc Res 2008;78(02):356–365
25 Azevedo C, Singh J, Steck N, et al. Screening a protein array with synthetic biotinylated inorganic polyphosphate to define the human PolyP-ome. ACS Chem Biol 2018;13(08):1958–1963
26 Negreiros RS, Lander N, Huang G, et al. Inorganic polyphosphate interacts with nucleolar and glycosomal proteins in trypanosomatids. Mol Microbiol 2018;110(06):973–994
27 Wang L, Fraley CD, Faridi J, Kornberg A, Roth RA. Inorganic polyphosphate stimulates mammalian TOR, a kinase involved in the proliferation of mammary cancer cells. Proc Natl Acad Sci U S A 2003;100(20):11249–11254
28 Dinarvand P, Hassanian SM, Qureshi SH, et al. Polyphosphate amplifies proinflammatory responses of nuclear proteins through interaction with receptor for advanced glycation end products and P2Y1 purinergic receptor. Blood 2014;123(06):935–945
29 Holmström KM, Marina N, Baev AY, Wood NW, Gourine AV, Abramov AY. Signalling properties of inorganic polyphosphate in the mammalian brain. Nat Commun 2013;4:1362