Supporting Information

Biochemical and proteomic studies of human pyridoxal 5'-phosphate-binding protein (PLPBP)

Anja Fux[1] and Stephan A. Sieber[1,*]

[1]Department of Chemistry, Chair of Organic Chemistry II, Center for Integrated Protein Science (CIPSM), Technische Universität München, Lichtenbergstraße 4, 85748 Garching, Germany

[*]Correspondence: stephan.sieber@tum.de

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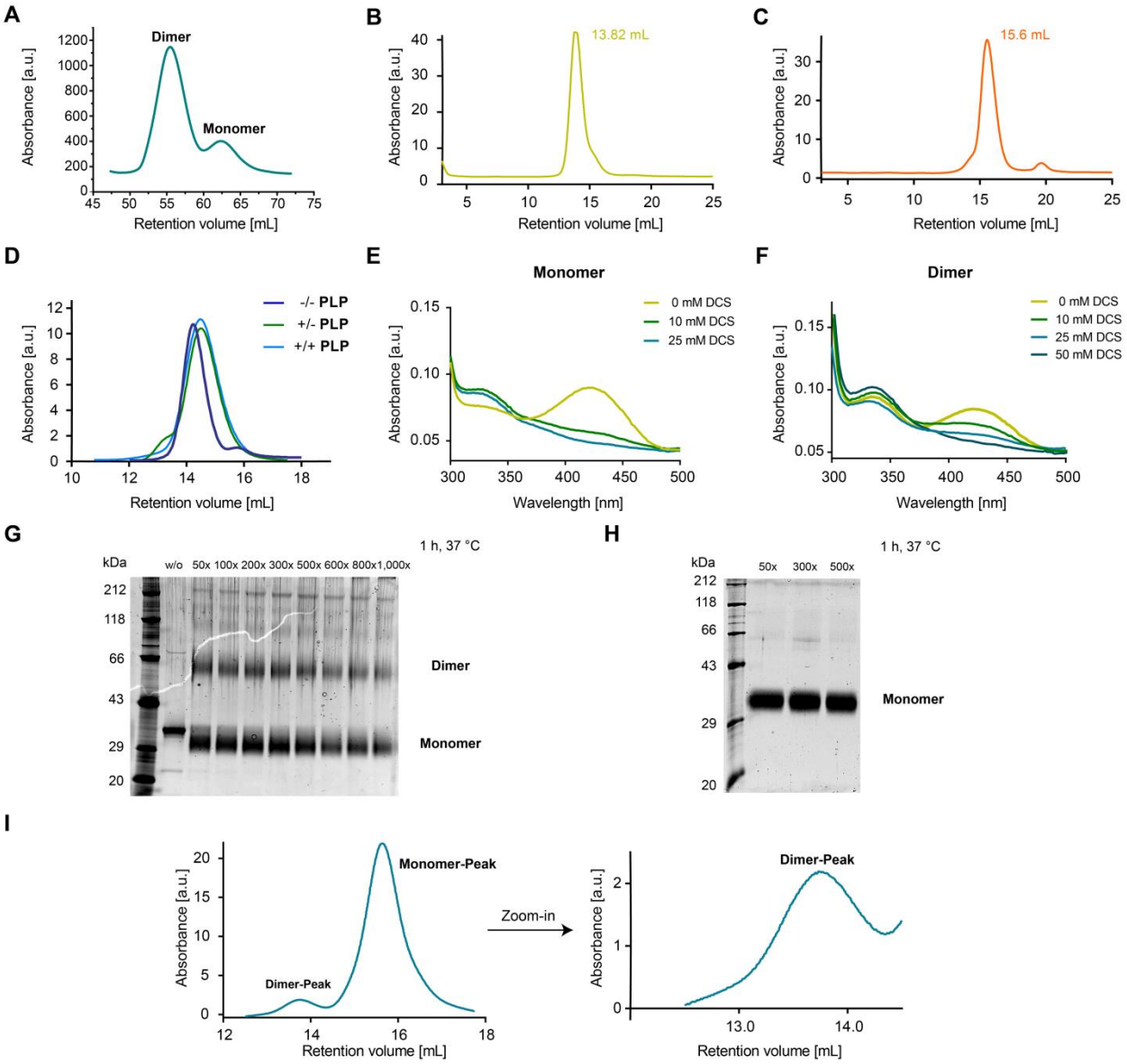
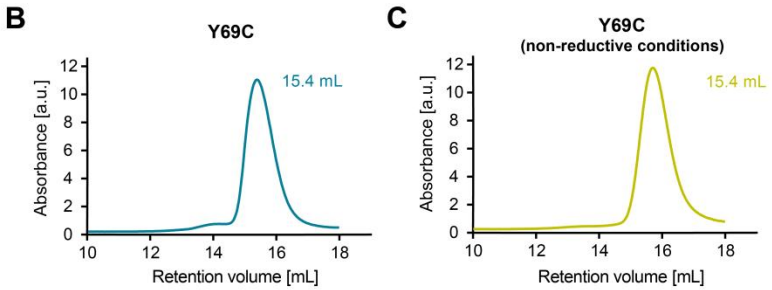


Figure S1 Properties of recombinant human PLPBP. (**A**) The main oligomeric state of Strep-tagged PLPBP is dimeric, with a small fraction of monomer according to size-exclusion chromatography (SEC; preparative scale). PLPBP dimer (**B**) and monomer (**C**) analyzed *via* SEC (analytical scale) after separate concentration and storage of proteins at -80 °C. (**D**) Pre-incubation of dimeric PLPBP with either excess of **PLP** and separation in buffer without **PLP** (+/-) or in buffer containing **PLP** (+/+) or pre-incubation and separation in the absence of **PLP** (-/-; analytical scale; retention volume of monomer: approx. 15.5 mL). Incubation with 10 mM of the **PLP**-binding antibiotic D-cycloserine (DCS) leads to almost complete displacement of **PLP** from monomeric PLPBP (**E**) whereas 50 mM DCS were required for the PLPBP

dimer (**F**, P = OPO_3^{2}).[1] (**G**) Chemical cross-linking utilizing the DSSO cross-linker revealed a strong covalent fixation of PLPBP dimer even with a 50-fold excess of linker and a reaction time of 1 h at 37 °C as shown by SDS-PAGE. (**H**) Chemical cross-linking of the PLPBP monomer with DSSO linker revealed no dimer formation upon SDS-PAGE analysis. (**I**) SEC after DSSO cross-linking of the PLPBP dimer (exemplarily presented for 50-fold excess) for 1 h at 37 °C. Although addition of the linker destabilized the PLPBP dimer, a sufficient amount was left (zoom-in) for MS sample preparation. The curve looked similar for incubation with 100-fold DSSO excess.

A

PLPBP variant	Yield [mg]
WT	3.82
Y69C	1.32
R41Q	0.14
R241Q	0.34
V45D	0.05



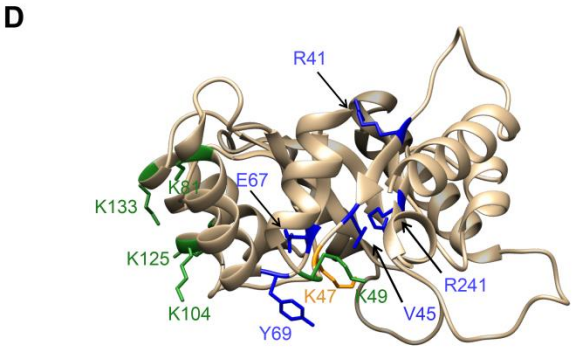


Figure S2 Impact of pathogenic mutations on PLPBP cofactor binding, stability, and oligomerization. (**A**) Protein yields of PLPBP wild-type (WT) and mutants from two liters of *Escherichia coli* Rosetta 2 (DE3) expression culture. Analytical size-exclusion chromatograms of Y69C mutant under reductive (**B**) and non-reductive conditions (**C**). (**D**) Position of pathogenic mutations (blue) with respect to binding site (orange), as well as lysine residues involved in cross-linking contacts (green) presented on the human WT PLPBP model structure.

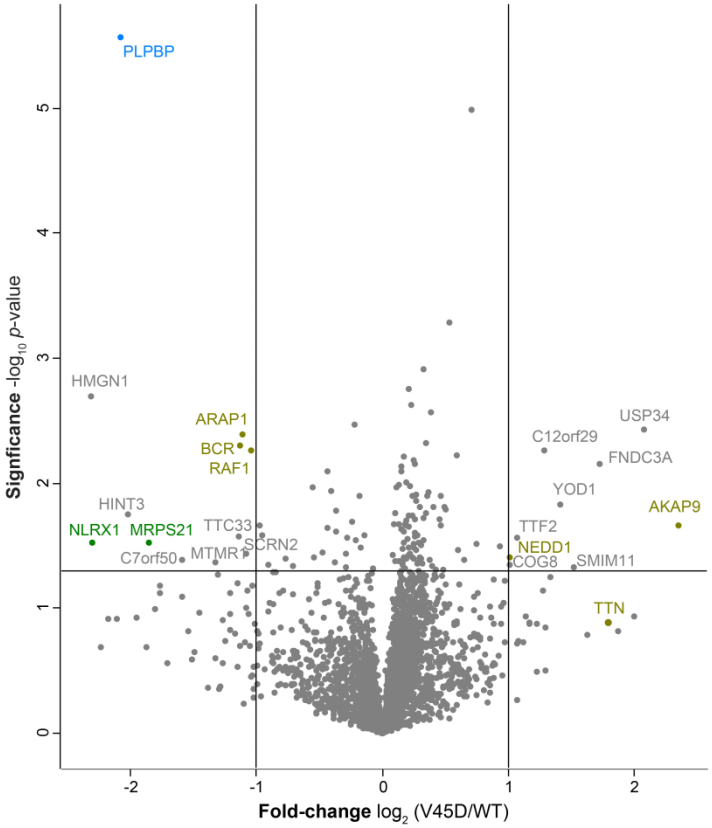


Figure S3 Proteomic changes upon V45D expression in HEK293. Volcano plot representing *t*-test results of V45D mutant overexpression compared to overexpression of the WT in PLPBP KO cells (*n*=4 biological replicates). Cut-off values were defined as enrichment factor of $\log_2 = 1$ (2-fold enrichment) or depletion factor of $\log_2 = -1$ (2-fold depletion), respectively, and $-\log_{10}$ (*p*-value) of 1.3 (solid lines). Dysregulated mitochondrial proteins are highlighted in green, **PLP**-DEs in blue, and proteins connected to the cytoskeleton in ocher.

2 Supporting Tables

Table S1 Cross-linking of PLPBP with MS-cleavable DSSO linker. Detected cross-links for PLPBP monomer and dimer (FDR<0.01). Cross-links of DSSO between corresponding peptides A and B are shown together with XlinkX scores and position in the sequence. For each cross-link we indicate, in which sample it was detected (excess of DSSO, fragmentation strategy, oligomeric state of PLPBP). The lysine in parentheses is the site of the detected cross-link.

1 1:50_EThcD (Monomer) **2** 1:50_MS3 (Monomer) **3** 1:100_EThcD (Monomer)

4 1:100_MS3 (Monomer) **5** 1:50_EThcD (Dimer) **6** 1:50_MS3 (Dimer)

7 1:100_EThcD (Dimer) **8** 1:100_MS3 (Dimer)

Peptide A	Peptide B	Position	Sample No.	XlinkX Score
LAD[K]VNSSWQR	[K]GSPER	125_133	1, 2, 4, 5, 6, 7, 8	55.06, 156.12, 75.67, 90.13, 198.45, 75.67, 187.45
ASNP[K]ILSLCPEIK	[K]GSPER	81_133	1, 2, 5, 6, 7, 8	81.88, 244.15, 100.09, 229.49, 65.29, 185.67
[K]PTPDK	CAADV[K]APLEVAQEH	255_266	2, 5, 6, 7, 8	100.11, 44.12, 148.22, 50.16, 151.74
T[K]PADMVIEAYGHGQR	[K]GSPER	49_133	7	35.78
T[K]PADMVIEAYGHGQR	ASNP[K]ILSLCPEIK	49_81	5, 6, 8	118.74, 216.22, 69.81
LVAVS[K]TKPADMVIEAYG HGQR	[K]GSPER	47_133	5, 6, 8	58.74, 124.37, 58.74
[K]PTPDK	ASNP[K]ILSLCPEIK	255_81	5, 6	65.29, 200.97
LVAVS[K]TKPADMVIEAYG HGQR	ASNP[K]ILSLCPEIK	47_81	5	41.48
T[K]PADMVIEAYGHGQR	LAD[K]VNSSWQR	49_125	5	39.15
[K]PTPDK	T[K]PADMVIEAYGHGQR	255_49	5, 6	36.47, 124.37
QNVN[K]LMAVPNLFMLETV DSVK	[K]GSPER	104_133	5	33.41

Table S4 Primer sequences of PLPBP wild-type (WT) and mutants.

Protein	forward primer (5'->3')	reverse primer (5'->3')
WT	ggggacaagtttgtacaaaaaagcaggctttgagaatcttt attttcagggctggagagctggcagcatgtcg	ggggaccactttgtacaagaaagctgggtgtcagtgctcct gtgccacctc
Y69C	agcagttcctgaacgcagttctcgccaaaagtgc	gcacttttggcgagaactgcgttcaggaactgct
R241Q	ccaaaaatcgtgcttcctatttggacatttgtagatcctactt	aagtaggatctacaaatgtccaaataggaagcacgattttt gg
R41Q	accgccactagctggggctggatgg	ccatccagccccagctagtggcggt
V45D	ggtttggttttgctgtccgccactagccgg	ccggctagtggcggacagcaaaaccaaacc
E67K	tcctgaacgtagttcttgccaaaagtgcgctgc	gcagcgcacttttggcaagaactacgttcagga

3 Experimental procedures

3.1 Biochemical and biological methods

3.1.1 Protein cloning, expression, and purification

Cloning and overexpression of recombinant proteins

N-terminally Strep-II tagged PLPBP was cloned using the primers listed in Table S4 and as template a PLPBP ORF clone, NM_007198.3 in a pcDNA3.1^{+}/C-(K)DYK vector (GeneScript, Cat# OHu09065) was applied.[2] Cloning was performed using the Invitrogen Gateway cloning system with pDONR201^{Kan} as the donor vector and pDest007^{Amp} as the destination vector. PLPBP was expressed in *E. coli* Rosetta 2 (DE3; Merck, Cat# 71400) carrying a chloramphenicol resistance plasmid. Bacteria were grown in lysogeny broth (LB)-media containing ampicillin (100 µg•mL^{-1}) and chloramphenicol (34 µg•mL^{-1}) at 37 °C to an OD_{600} of 0.6-0.8 and expression was induced by adding 0.2 µg•mL^{-1} anhydrotetracycline (ATET). Expression was carried out for 2 h at 37 °C. Bacteria were harvested and washed with PBS (6,000 x *g*, 4 °C) prior to cell lysis and protein purification.

PLPBP mutants were generated *via* Quick Change site-directed mutagenesis[3] using the primers listed in Table S4 and either the pDest007^{Amp}-PLPBP template for recombinant protein expression or the pcDNA3.1^{+}-PLPBP vector for transfection. Polymerase chain reaction (PCR) products were digested with DpnI (New England BioLabs, Cat# R0176S) to remove wild-type (WT) template DNA. Plasmids carrying point mutated PLPBP were transformed into *E. coli* XL1 Blue (Agilent, Cat# 200249) for nick repair, previous to transformation into the Rosetta 2 (DE3) strain for recombinant protein expression or into the Top10 strain (Thermo Fisher Scientific, Cat# C404010) for transfection. Expression of point mutants was carried out as described for WT PLPBP.

Cell lysis, purification, and analytics of recombinant proteins

The bacterial overexpression culture was resuspended in strep binding buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 1 mM EDTA) and lysed by sonication. The lysate was clarified by centrifugation (36,000 x *g*, 30 min, 4 °C). Supernatant was loaded onto a StrepTrap column (5 mL, GE Healthcare, Cat# 28-9075) equilibrated with binding buffer using an Äkta purification system (GE Healthcare). After extensive washing, proteins were eluted in binding buffer containing 2.5 mM desthiobiotin. Size exclusion chromatography (SEC) was applied for further

purification. WT PLPBP was loaded onto a preparative Superdex 75 column (16 x 600 mm, GE Healthcare) equilibrated with SEC buffer (20 mM HEPES pH 7.6, 100 mM KCl, 1 mM DTT). PLPBP mutants were purified using analytical SEC. Proteins were loaded onto an analytical Superdex 200 column (10 x 300 mm, GE Healthcare) equilibrated with SEC buffer (20 mM HEPES pH 7.6, 100 mM KCl, 1 mM DTT) or in addition with SEC buffer lacking DTT for the Y69C mutant. For Strep-tag removal, concentration of WT PLPBP was determined after Strep-purification and DTT was added to a final amount of 1 mM (from a 500 mM stock prepared fresh in ddH_2O). The digestion was initiated with the addition of a 40-fold molar excess of TEV-protease and incubation carried out for 16 h at 10 °C and 300 rpm. Afterwards, buffer was exchanged to His-loading buffer (20 mM sodium dihydrogene phosphate pH 8.0, 150 mM NaCl, and 10 mM imidazole) using a concentrator (10 K MWCO). A HisTrap column (5 mL, GE Healthcare, Cat# GE29-0510-21) was equilibrated with His-loading buffer previous to loading of PLPBP to remove His-tagged TEV protease. Finally, the protein was loaded onto the SEC column for purification as described above. Biochemical and biological experiments with WT PLPBP except those comparing it to the PLPBP mutants (comp. Figure 2) were performed with the tag-free protein. Oligomeric state of the proteins was determined according to calibration curves. Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Cat# 23225). Molecular weight of the proteins was confirmed by intact-protein mass spectrometry (MS, compare 3.1.2). Proteins were stored at –80 °C in small aliquots. In order to monitor the impact of **PLP** on the oligomerization behavior of PLPBP, 10 µM dimer or monomer were incubated either 10 min at 37 °C in SEC buffer alone or buffer containing 100 µM **PLP** and separated using the analytical Superdex 200 (10 x 300 mm, GE Healthcare) equilibrated with SEC buffer containing 100 µM **PLP** when indicated. UV/Vis spectra of protein samples (100 µM) were recorded in SEC buffer at 37 °C in duplicates on an InfiniteM200 PRO reader (TECAN, Cat# IN-MNANO; 300-600 nm, 2 nm increments).

3.1.2 Intact-protein MS

Sample preparation for intact-protein MS

10 µM protein samples (25 µL in SEC buffer) were either directly treated with 10 mM $NaBH_4$ (2 µL of 250 mM stock prepared fresh in 0.1 M NaOH) at r. t. for 30 min, or previously incubated with a 4-fold molar excess of **PLP** if indicated. Residual $NaBH_4$ was quenched by acidification to pH 5-6 with HCl (5-10 µL of 0.5% FA) and neutralized to pH 7 with NaOH (5-10 µL of 0.1 M NaOH). Samples were diluted to 50 µL with PBS (5 µM final enzyme concentration) and subjected to intact-protein MS.

Intact-protein MS measurement

Full-length protein measurements were performed as described previously.[4] Proteins were measured on a MassPREP On-Line Desalting Cartridge (Waters) on an Ultimate 3000 HPLC system (Dionex) coupled to a Finnigan LTQ-FT Ultra mass spectrometer (Thermo Fisher Scientific) with electrospray ionization (spray voltage 4.0 kV, tube lens 110 V, capillary voltage 48 V, sheath gas 60 arb, aux gas 10 arb, sweep gas 0.2 arb). Xcalibur Xtract Software (Thermo Fisher Scientific) was used for data analysis and deconvolution.

3.1.3 Thermal stability assay with recombinant proteins

2 µM of PLPBP WT and mutants in SEC buffer were incubated either with or without 20-fold molar excess of **PLP** (2 µL of a corresponding stock) and SYPRO orange protein gel stain (Thermo Fisher Scientific, Cat# S6650) was added to a final concentration of 1x from a 5,000x stock. Temperature was increased from 20 to 89.6 °C with a heating rate of 0.3 °C per min, monitoring fluorescence at 569 nm in a CFX96 Real-Time System (Bio-Rad, Cat# 20421). Three independent replicates were performed for each condition. Denaturation curves were fitted according to a sigmoidal trace. Melting temperatures were calculated as mean value with corresponding standard deviation (SEM).

3.1.4 PLPBP incubation with D-cycloserine (DCS)

130 µM of PLPBP monomer and dimer were incubated with 10, 25, or 50 mM DCS (Roth, Cat# CN37.1; added from 10x stocks in water, pH adjusted to neutral), respectively, for 20 min at 25 °C in a total volume of 10 µL. Afterwards, UV/Vis-spectra were recorded as described.

3.1.5 Western blot analysis

HEK293 PLPBP knock-out (KO) cells were grown as described under 3.2 and transfected with the PLPBP overexpression vectors in 6-well plates as described under 3.2.2. After transfection, cells were washed once with cold PBS and then scraped to detach. For inspection of KO HEK293 cells, KO and HEK293 WT cells were cultivated as described under 3.2, seeded in 6-well plates, grown until 80-90% confluence, afterwards scraped to detach, and washed with PBS. Cells were lysed by incubation for 15 min at 4 °C in lysis buffer (1% (v/v) NP-40 in PBS pH 7.4). Supernatant was clarified at 21,000 $x\ g$ for 20 min at 4 °C. Protein concentration was adjusted with a BCA-assay. Lysate was mixed with 2x gel loading buffer. Samples were separated on a 12.5% SDS-gel (80 µg of lysate per cell line) and plotted on a PVDF membrane (Roti-PVDF, 0.2 µm, Roth, Cat# 8989.1) using a semi-dry blotting station (Trans-Bot SD Semi-Dry Transfer Cell, Bio-Rad, Cat# 1703940). Blocking was performed using 3% BSA (w/v) in

PBS-T (PBS supplemented with 0.5% Tween-20) at r.t. for 1 h. Rabbit polyclonal anti-FLAG antibody (0.8 mg•mL^{-1}, Sigma Aldrich, Cat# F7425) was diluted 1:500 in 3% BSA in PBS-T and added to the membrane. For analysis of KO HEK293 cells compared to WT HEK293 cells, a rabbit polyclonal anti-PLPBP antibody (1.02 mg•mL^{-1}, Thermo Scientific, Cat# PA5-32036) was diluted 1:1,000 in 3% BSA in PBS-T and added to the membrane. Immunobinding was carried out overnight at 4 °C. After extensive washing the membrane was incubated with secondary antibody (goat anti-rabbit antibody conjugated to horseradish peroxidase (0.5 mg•mL^{-1}, Invitrogen, Cat# 32260) diluted 1:5,000 in 3% BSA in PBS-T for 1 h at r.t. The membrane was washed and chemo-luminescence was detected after incubation with freshly prepared ECI western blotting substrate solution (Pierce, Cat# PIER80196) in a Luminescent LAS 4000 image analyzer (Fujifilm, ordered *via* GE Healthcare, Cat# 28955810). The membrane was stained with ponceau S (Sigma Aldrich, Cat# P3504) to inspect for equal protein loading amounts.

3.2 Cell culture

HEK293 (female, Cat# 85120602) were obtained from ECACC *via* Sigma Aldrich. HEK293 PLPBP KO cells were obtained from Prof. Sander M. Houten (Icahn School of Medicine at Mount Sinai, NY, US).[5] Cells were cultivated in DMEM (HEK293, Sigma Aldrich, Cat# D5671) media supplemented with 10% L-glutamine and 10% FCS at 37 °C and humidified 5% CO_2 atmosphere. HEK293 cells carrying the PLPBP KO were cultivated in DMEM media in the presence of penicillin, streptomycin, and amphotericin B (1x final concentration diluted from a 100x stock, Sigma Aldrich, Cat# A5955). The cells were routinely tested for mycoplasma contamination.

3.2.1 Cell culture of HEK293 WT and KO for whole proteome analysis

For comparison of protein expression of WT and PLPBP KO HEK293, cells were cultured as described under 3.2 with the exception that the KO cells were maintained in the absence of antibiotic and antimyotic for six passages to avoid altered protein expression caused by the different cultivation compared to the WT cells. Cells were seeded in 20 cm² dishes per replicate and were grown until a confluence of 80-90%. Four biological replicates were prepared for WT HEK293 and PLPBP KO cells, respectively.

3.2.2 HEK293 transfection with PLPBP overexpression plasmids

HEK293 PLPBP KO cells were cultured as described under 3.2. For whole proteome analysis, two million knock-out cells were seeded in 5 mL media per 20 cm^2 dish per replicate and let

settle down overnight. 8 µg PLPBP WT-containing transfection vector (pcDNA3.1$^+$/C-(K)DYK; NM_007198 ORF clone, GenScript, Cat# OHu09065D) or 8 µg pcDNA3.1$^+$/C-(K)DYK vectors carrying PLPBP point mutants were diluted in 0.5 mL Opti-MEM (Thermo Fisher Scientifc, Cat# 31985062) and incubated for 5 min at r.t. 20 µg Lipofectamine 2000 (Thermo Fisher Scientific, Cat# 11668030) were diluted in 0.5 mL Opti-MEM and were incubated for 5 min at r.t. Afterwards both mixtures were unified and incubated another 20 min at r.t. Finally, 1 mL transfection mix was added directly to the cells covered with growth media per plate. Four biological replicates were prepared per WT and mutants transfection. For expression analysis of PLPBP WT and mutants *via* western blot, one million HEK293 PLPBP KO cells were seeded per well in a 6-well plate. For transfection, 4 µg DNA were diluted in 250 µL Opti-MEM and 10 µg Lipofectamine 2000 were diluted in 250 µL Opti-MEM and incubated for 5 min at r.t. Afterwards both mixtures were unified and incubated another 20 min at r.t. Finally, 0.5 mL transfection mix were added directly to the cells covered with growth media per plate. Transfection was carried out for 48 h at 37 °C for all conditions.

3.3 Binding site identification of PLPBP

3.3.1 Sample preparation and LC-MS/MS analysis

Recombinant PLPBP (4 µg, 50 µL) was incubated with 5 equivalents of **PLP** in SEC buffer for 30 min at r.t. and was subsequently reduced with 10 mM $NaBH_4$ (2 µL of 250 mM stock prepared fresh in 0.1 M NaOH) for 30 min at r.t. The protein was precipitated adding ice-cold acetone (4× volume) and incubated at –20°C overnight. Precipitated protein was pelleted by centrifugation (18,000 x *g*, 15 min, 4 °C) and washed with ice-cold MeOH (2 × 0.2 mL), using sonication to resuspend the pellets between washes. Protein was resuspended in 100 µL denaturation buffer (6 M urea, 2 M thiourea in 20 mM HEPES pH 7.5) and reduced with 5 mM TCEP (1 µL of 500 mM stock in 20 mM HEPES pH 7.5) for 1 h at 37 °C. Proteins were alkylated using 10 mM IAA (2 µL of 500 mM stock in 20 mM HEPES pH 7.5) for 30 min at 25 °C, followed by quenching with 10 mM DTT (2 µL of 500 mM stock in 20 mM HEPES pH 7.5) for 30 min at 25 °C. Samples were diluted with 300 µL 50 mM TEAB previous to addition of trypsin (0.4 µL from 0.5 mg•mL^{-1}stock, Sequencing Grade, Promega, Cat# V5111) and overnight digestion at 37 °C. Desalting with C18 cartridges (Waters, Cat# WAT054960) and preparation for LC-MS/MS analysis was performed as described previously.[4] LC-MS/MS analysis on Fusion mass spectrometer was performed as for proteomic samples (comp. 3.5.3) with the exception that the HPLC gradient was shortened to 62 min to account for reduced peptide amount. Samples were separated using a gradient raising buffer B from 5 to 28% in 37 min, followed by a buffer B

increase to 35% within 5 min. Buffer B content was further raised to 90% within the next 0.1 min and held another 10 min at 90%. Subsequently buffer B was decreased to 5% and held until the end of the run.

3.3.2 Statistical analysis of MS data

MS raw files were analyzed with MaxQuant software (version 1.6.0.1) as described in the proteomics section. For **PLP** binding site identification, the **PLP** moiety (+ 231.02966) at lysine was additionally set as a variable modification. As we expect one **PLP** modification site in PLPBP, we selected the site with the highest confidence based on best PEP, score, and manual evaluation of MS/MS spectra from MaxQuant.

3.4 Chemical cross-linking combined with mass spectrometry (XL-MS)

3.4.1 Chemical cross-linking of PLPBP

DSSO was synthesized as described previously.[6] PLPBP monomer and dimer (5 µM in 50 µL SEC buffer) were incubated with varying molar excess of DSSO cross-linker (stock solutions dissolved in DMSO) for 1 h at 37 °C with shaking (300 rpm). Samples were analyzed *via* SDS-PAGE. The gel was stained with SYPRO Ruby Protein Gel Stain according to the manufacturer's protocol (Thermo Fisher Scientific, Cat# S12000). For MS analysis, PLPBP dimer (20 µM in 200 µL SEC buffer) was incubated with either 50 or 100-fold excess DSSO for 1 h at 37 °C and 300 rpm and subsequently separated on an analytical Superdex 200 (10 x 300mm, GE Healthcare) connected to an Äkta system (GE Healthcare) in SEC buffer. Fractions corresponding to monomer and dimer were unified and evaporated *in vacuo*.

3.4.2 MS preparation of cross-linked proteins

MS preparation of cross-linked PLPBP was performed as described previously.[6] In brief, proteins were denatured, reduced, alkylated and predigested with Lys-C (Wako, Cat# 125-05061) followed by overnight incubation with trypsin (Sequencing Grade, Promega, Cat# V5111). Cross-linked peptides were enriched *via* cation-exchange chromatography (Empore Cation Extraction 47 mm Disks, SUPELCO, Cat# 66889-U) and desalted with double layer C18 stage-tips (Empore disk-C18, 47 mm, SUPELCO, Cat# 66883-U) previous to MS analysis.

3.4.3 MS analysis of cross-linked peptides

MS analysis of cross-linked PLPBP peptides was performed as described previously.[6] In brief, peptides were separated within 105 min on a UltiMate 3000 nano HPLC system (Thermo Fisher Scientific) equipped with an Acclaim C18 PepMap100 75 µm ID x 2 cm trap and an Acclaim

PepMap RSLC C18 separation column (75 µm ID x 50 cm) coupled to an EASY-source equipped Thermo Fisher LTQ Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). Each sample was measured twice, one time using the EThcD-CID and the other time using the MS^2-MS^3 fragmentation strategy for DSSO cross-linked peptides.

3.4.4 Computational analysis of cross-linking data

Computational analysis of cross-linked PLPBP peptides was performed as described previously.[6] In brief, MS raw data for DSSO cross-linked proteins were analyzed using Proteome Discoverer Version 2.2 and its XlinkX plug-in. The database contained the PLPBP sequence, as well as all common contaminants from the MaxQuant (version 1.6.0.1)[7] bin folder to receive statistically significant results. The detected cross-links resulting from the two fragmentation strategies, as well as DSSO excesses were combined. A PLPBP model crystal structure was generated based on the template crystal structure from yeast (PDB 1B54)[8] using SWISS-MODEL.[9–12] The PLPBP crystal structure served as input for dimer modeling using HADDOCK software.[13] Cross-links solely occurring in the dimer fraction of PLPBP were defined as active residues using the Easy prediction interface and default parameters. Cross-links were displayed onto the dimer model (cluster 1_1) using the XLinkAnalyzer[14] (version 1.1) plug-in into Chimera[15] (version 1.11) applying a distance threshold of 35 Å for DSSO.

3.5 Proteomic methods

3.5.1 Cross-link/co-IP

Cross-link/co-IP was performed using the DSSO cross-linker as described previously.[6] In brief, cells were cultivated as described under 3.2, seeded on separate plates (150 cm^2) per replicate and grown until a confluence of 80-90%. Afterwards, cells were harvested and cross-linked with 2 mM DSSO in PBS at 37 °C for 1 h. After quenching of the cross-linker cells were lysed, protein concentration was adjusted for all replicates and pull-down was performed applying a rabbit-polyclonal anti-PLPBP antibody (1.02 $mg \cdot mL^{-1}$, Thermo Scientific, Cat# PA5-32036) 1:100 for 3 h at 4 °C under rotation. Targeted pull-downs were compared to equal amounts of isotype control to account for non-specific binding to antibody constant regions. Four biological replicates were prepared for each the targeted pull-down against PLPBP and the isotype control, respectively. After extensive washing, proteins were digested on-bead at 37 °C and overnight using trypsin (Sequencing Grade, Promega, Cat# V5111). Desalting was performed using C18 stage-tips (Empore disk-C18, 47 mm, SUPELCO, Cat# 66883-U). Peptides were evacuated *in vacuo* and re-dissolved in water containing 1% formic acid (FA) previous to LC-MS/MS analysis.

3.5.2 Sample preparation for full-proteome analysis

Cells were washed once with cold PBS (4 °C) and were scraped to detach in 300 µL lysis buffer (PBS pH 7.4 supplemented with 1% (v/v) NP-40) per plate. Cells were incubated for 20 min on ice previous to centrifugation for 20 min at 21,000 x *g* and 4 °C. Protein concentration was adjusted to 200 µg per replicate in 300 µL lysis buffer using a BCA assay and precipitated adding 4x volume of ice-cold acetone (–80 °C) and incubation at –20 °C overnight. Acetone precipitated proteins were pelletized (4 °C, 10 min, 21,000 x *g*) and washed twice with ice-cold MeOH (–80 °C), using sonication to resuspend the pellets between washes. Proteins were dissolved in 200 µL denaturation buffer (6 M urea, 2 M thiourea in 20 mM HEPES pH 7.5) and reduced for 1 h at 37 °C with 1 mM DTT added from a 500 mM stock prepared freshly in 20 mM HEPES pH 7.5. Proteins were alkylated with 5 mM IAA added from a 500 mM stock prepared freshly in 20 mM HEPES pH 7.5 for 30 min at 25 °C. IAA was quenched by adding 5 mM DTT from a 500 mM stock prepared freshly in 20 mM HEPES pH 7.5 and incubating another 30 min at 25 °C. Pre-digestion took place by adding Lys-C (1:80 (w/w), Wako, Cat# 125-05061) for 4 h at 37 °C. Samples were diluted 4-fold with 50 mM TEAB and incubated with trypsin (1:80 (w/w), sequencing grade, modified; Promega, Cat# V5111) overnight at 37 °C. In order to quench the reaction, FA was added to a final amount of 1% (v/v). Samples were desalted using C18 columns (Waters, Cat# WAT054960) and prepared for MS analysis as described previously.[4]

3.5.3 LC-MS/MS analysis of proteomic samples

Samples were analyzed *via* LC-MS/MS using a UltiMate 3000 nano HPLC system (Thermo Fisher Scientific) equipped with an Acclaim C18 PepMap100 75 µm ID x 2 cm trap and an Acclaim PepMap RSLC C18 separation column (75 µm ID x 50 cm) coupled to an EASY-source equipped Thermo Fisher LTQ Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). Samples were loaded onto the trap column at a flow rate of 5 µL•min^{-1} with aqueous 0.1% trifluoroacetic acid (TFA) and then transferred onto the separation column at 0.3 µL•min^{-1}. Buffers for the nano-chromatography pump were aqueous 0.1% FA (buffer A) and 0.1% FA in acetonitrile (ACN, buffer B). Samples were separated using a gradient raising buffer B from 5 to 22% in 112 min, followed by a buffer B increase to 32% within 10 min. Buffer B content was further raised to 90% within the next 10 min and held another 10 min at 90%. Subsequently buffer B was decreased to 5% and held until end of the run (total: 152 min). During sample separation MS full scans were performed at 120,000 resolution in the orbitrap with quadrupole isolation. The MS instrument was operated in a 3 s top speed data dependent mode. The scan range was set from 300 to 1,500 m/z with 60% RF lens amplitude. The AGC target was set to 2.0e5, the maximum ion injection time was 50 ms (co-IP), or 35 ms (whole proteome analysis)

and internal calibration was performed using the lock mass option. Peptides with intensity higher than 5.0e3 and charge state 2-7 were fragmented with HCD (30%). MS^2 scans were recorded in the ion trap operating in rapid mode. The isolation window was set to 1.6 m/z and the AGC target to 1.0e4 with maximum injection time of 100 ms. Ions were injected for all available parallelizable time. Dynamic exclusion time was 60 s with 10 ppm low and high mass tolerance.

3.5.4 Statistical analysis of proteomics data

MS raw data were searched with MaxQuant software (version 1.6.0.1) and default settings (with the exceptions that label-free quantification and match between runs were activated). Searches were performed against a Uniprot database of *Homo sapiens* proteome (taxon identifier: 9606, reference reviewed and unreviewed, downloaded on 28.08.2019). Resulting data were further analyzed using Perseus software version 1.6.0.0[16]. The rows were filtered (only identified by site, potential contaminant, reverse) and $\log_2$ transformed. Biological replicates were grouped, filtered for 3 out of 4 valid values in at least one group and missing values were imputed for total matrix using default settings. A both sided, two-sample Student's t-test was performed and derived *p*-values were corrected for multiple testing by the method of Benjamini and Hochberg with a significance level of $p = 0.05$. Volcano plots were generated by plotting $\log_2$ (fold change of different conditions) against $-\log_{10}$ (*p*-value). **PLP**-dependent proteins (gene ontology (GO)-term: 0030170; **PLP**-binding), proteins involved in cytoskeleton organization (GO term: 0007010), and mitochondrial proteins (GO term: 0005739; mitochondrion) were identified with the help of a GO annotation file for *H. sapiens* submitted on 26.06.2019.[17]

4 References

(1) Amorim Franco, T. M.; Favrot, L.; Vergnolle, O.; Blanchard, J. S. Mechanism-Based Inhibition of the Mycobacterium Tuberculosis Branched-Chain Aminotransferase by d -And l -Cycloserine. *ACS Chem. Biol.* **2017**, *12*, 1235–1244.

(2) Fux, A.; Pfanzelt, M.; Kirsch, V. C.; Hoegl, A.; Sieber, S. A. Customizing Functionalized Cofactor Mimics to Study the Human Pyridoxal 5'-Phosphate-Binding Proteome. *Cell Chem. Biol.* **2019**, *26*, 1–8.

(3) Liu, H.; Naismith, J. H. An Efficient One-Step Site-Directed Deletion, Insertion, Single and Multiple-Site Plasmid Mutagenesis Protocol. *BMC Biotechnol.* **2008**, *8*, 91.

(4) Hoegl, A.; Nodwell, M. B.; Kirsch, V. C.; Bach, N. C.; Pfanzelt, M.; Stahl, M.; Schneider, S.; Sieber, S. A. Mining the Cellular Inventory of Pyridoxal Phosphate-Dependent Enzymes with Functionalized Cofactor Mimics. *Nature Chemistry* **2018**, *10*, 1234-1245.

(5) Johnstone, D. L.; Al-Shekaili, H. H.; Tarailo-Graovac, M.; Wolf, N. I.; Ivy, A. S.; Demarest, S.; Roussel, Y.; Ciapaite, J.; Van Roermund, C. W. T.; Kernohan, K. D.; Kosuta, C.; Ban, K.; Ito, Y.; McBride, S.; Al-Thihli, K.; Abdelrahim, R. A.; Koul, R.; Al Futaisi, A.; Haaxama, C. A.; Olson, H.; Sigurdardottir, L.; Arnold, G. L.; Gerkes, E. H.; Boon, M.; Heiner-Fokkema, M. R.; Noble, S; Bosma, M.; Jans, J.; Koolen, D. A.; Kamsteeg, E.; Drögemöller, B.; Ross, C. J.; Majewski, J.; Cho, M. T.; Begtrup, A.; Wasserman, W. W.; Bui, T.; Brimble, E.; Violante, S.; Houten, S. M.; Wevers, R. A.; van Faassen, M.; Kema, I. P.; Lepage, N.; Care4Rare Canada Consortium; Lines, M. A.; Dyment, D. A.; Wanders, R. J. A.; Verhoeven-Duif, N.; Ekker, M.; Boycott, K. M.; Friedman, J. M.; Pena, I. A.; van Karnebeek, C. D. M. PLPHP Deficiency: Clinical, Genetic, Biochemical, and Mechanistic Insights. *Brain* **2019**, *142*, 542–559.

(6) Fux, A.; Korotkov, V. S.; Schneider, M.; Antes, I.; Sieber, S. A. Chemical Cross-Linking Enables Drafting ClpXP Proximity Maps and Taking Snapshots of In Situ Interaction Networks. *Cell Chem. Biol.* **2018**, *26*.

(7) Cox, J.; Mann, M. MaxQuant Enables High Peptide Identification Rates, Individualized p.p.b.-Range Mass Accuracies and Proteome-Wide Protein Quantification. *Nat. Biotechnol.* **2008**, *26*, 1367–1372.

(8) Eswaramoorthy, S.; Gerchman, S.; Graziano, V.; Kycia, H.; Studier, F. W.; Swaminathan,

S. Structure of a Yeast Hypothetical Protein Selected by a Structural Genomics Approach. *Acta Crystallogr. - Sect. D Biol. Crystallogr.* **2003**, *59*, 127–135.

(9) Kiefer, F.; Arnold, K.; Künzli, M.; Bordoli, L.; Schwede, T. The SWISS-MODEL Repository and Associated Resources. *Nucleic Acids Res.* **2009**, *37*, 387-392.

(10) Biasini, M.; Bienert, S.; Waterhouse, A.; Arnold, K.; Studer, G.; Schmidt, T.; Kiefer, F.; Cassarino, T. G.; Bertoni, M.; Bordoli, L.; Schwede, T. SWISS-MODEL: Modelling Protein Tertiary and Quaternary Structure Using Evolutionary Information. *Nucleic Acids Res.* **2014**, *42*, 251-258.

(11) Arnold, K.; Bordoli, L.; Kopp, J.; Schwede, T. The SWISS-MODEL Workspace: A Web-Based Environment for Protein Structure Homology Modelling. *Bioinformatics* **2006**, *22*, 195–201.

(12) Guex, N.; Peitsch, M. C.; Schwede, T. Automated Comparative Protein Structure Modeling with SWISS-MODEL and Swiss-PdbViewer: A Historical Perspective. *Electrophoresis* **2009**, *30*, 162-173.

(13) Dominguez, C.; Boelens, R.; Bonvin, A. M. J. J. HADDOCK: A Protein-Protein Docking Approach Based on Biochemical or Biophysical Information. *J. Am. Chem. Soc.* **2003**, *125*, 1731–1737.

(14) Kosinski, J.; von Appen, A.; Ori, A.; Karius, K.; Mueller, C. W.; Beck, M. Xlink Analyzer: Software for Analysis and Visualization of Cross-Linking Data in the Context of Three-Dimensional Structures. *J. Struct. Biol.* **2015**, *189*, 177–183.

(15) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera—A Visualization System for Exploratory Research and Analysis. *J Comput Chem* **2004**, *25*, 1605–1612.

(16) Tyanova, S.; Temu, T.; Sinitcyn, P.; Carlson, A.; Hein, M. Y.; Geiger, T.; Mann, M.; Cox, J. The Perseus Computational Platform for Comprehensive Analysis of (Prote)Omics Data. *Nature Methods* **2016**, *13*, 731–740.

(17) Ashburner, M.; Ball, C. A.; Blake, J. A.; Botstein, D.; Butler, H.; Cherry, J. M.; Davis, A. P.; Dolinski, K.; Dwight, S. S.; Eppig, J. T.; Harris, M. A.; Hill, D. P.; Issel-Tarver, L.; Kasarskis, A.; Lewis, S.; Matese, J. C.; Richardson, J. E.; Ringwald, M.; Rubin, G. M.; Sherlock, G. Gene Ontology: Tool for the Unification of Biology. *Nature Genetics*. **2000**,

25, 25–29.