Materials and Method

Note: To our best knowledge, this should represent an accurate description of the materials and methods required to reproduce our work. If any of the content on this page is difficult to interpret or should you have trouble repeating our work, do not hesitate to contact us as soon as possible in order for us to provide additional information and advice.

Entry Clone Source: Site-directed mutagenesis

Entry Clone Accession: n/a

SGC Construct ID: PHF8A-c380

GenBank GI number: gi|32698700

Vector: pNH-TrxT

Coding DNA sequence:

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CATATGCACCATCATCATCATCATTCTTC
TGGTATGAGCGATAAAATTATTCACCTGA
CTGACGACAGTTTTGACACGGATGTACTC
AAAGCGGACGGGGCGATCCTCGTCGATTT
CTGGGCAGAGTGGTGCGGTCCGTGCAAAA
TGATCGCCCCGATTCTGGATGAAATCGCT
GACGAATATCAGGGCAAACTGACCGTTGC
AAAACTGAACATCGATCAAAACCCTGGCA
CTGCGCCGAAATATGGCATCCGTGGTATC
CCGACTCTGCTGCTGTTCAAAAACGGTGA
AGTGGCGGCAACCAAAGTGGGCGCACTGT
CTAAAGGTCAGTTGAAAGAGTTCCTCGAC
GCTAACCTGGCCGGTACCGAGAACTTGTA
CTTCCAATCCATGCCAGTGAAGACCGGGA
GCCCTACGTTCGTCAGAGAGCTCCGGAGT
AGGACTTTTGACAGCTCAGATGAAGTGAT
TCTGAAGCCCACTGGAAATCAACTGACCG
TGGAATTCCTGGAAGAAAATAGCTTCAGT
GTGCCCATCCTGGTCCTGAAGAAGGATGG
GTTGGGCATGACGCTGCCCTCGCCATCAT
TCACTGTGAGGGATGTTGAACACTATGTT
GGTTCTGACAAAGAGATTGATGTGATTGA
TGTGACCCGCCAGGCTGACTGCAAGATGA
AGCTTGGTGATTTTGTGAAATACTATTAC
AGCGGGAAGAGGGAGAAAGTCCTCAATGT
CATTAGTTTGGAATTCTCTGATACCAGAC
TTTCTAACCTTGTGGAGACACCGAAGATT
GTTCGAAAGCTGTCATGGGTCGAAAACTT
GTGGCCAGAGGAATGTGTCTTTGAGAGAC
CCAATGTACAGAAGTACTGCCTCATGAGT
GTGCGAGATAGCTATACAGACTTTCACAT
TGACTTTGGTGGCACCTCTGTCTGGTACC
ATGTACTCAAGGGTGAAAAGATCTTCTAC
CTGATCCGCCCAACAAATGCCAATCTGAC
TCTCTTTGAGTGCTGGAGCAGTTCCTCTA
ATCAGAATGAGATGTTCTTTGGGGACCAG
GTGGACAAGTGCTACAAGTGTTCCGTGAA
GCAAGGACAGACACTTTTCATTCCCACAG
GGTGGATCCATGCTGTGCTGACGCCTGTG
GACTGCCTTGCCTTTGGAGGGAACTTCTT
ACACAGCCTTAACATCGAGATGCAGCTCA
AAGCCTATGAGATTGAGAAGCGGCTGAGC
ACAGCAGACCTCTTCAGATTCCCCAACTT
TGAGACCATCTGTTGGTATGTGGGAAAGC
ACATCCTGGACATCTTTCGCGGTTTGCGA
GAGAACAGGAGACACCCTGCCTCCTACCT
GGTCCATGGTGGCAAAGCCTTGAACTTGG
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CCTTTAGAGCCTGGACAAGGAAAGAAGCT
CTGCCAGACCATGAGGATGAGATCCCGGA
GACAGTGCGAACCGTACAGCTCATTAAAG
ATCTGGCCAGGGAGATCCGCCTGGTGGAA
GACATCTTCCAACAGAACTGACAGTAAAG
GTGGATACGGATCCGAATTCGAGCTCCGT
CGACAAGCTT

Tags and additions: N-terminal His6/Thioredoxin -tags with TEV protease cleavage site

Protein sequence (Tag sequence in lowercase):

mhhhhhhssgmsdkiihltddsfdtdvlk
adgailvdfwaewcgpckmiapildeiad
eyqgkltvaklnidqnpgtapkygirgip
tlllfkngevaatkvgalskgqlkeflda
nlagtenlyfqSMPVKTGSPTFVRELRSR
TFDSSDEVILKPTGNQLTVEFLEENSFSV
PILVLKKDGLGMTLPSPSFTVRDVEHYVG
SDKEIDVIDVTRQADCKMKLGDFVKYYYS
GKREKVLNVISLEFSDTRLSNLVETPKIV
RKLSWVENLWPEECVFERPNVQKYCLMSV
RDSYTDFHIDFGGTSVWYHVLKGEKIFYL
IRPTNANLTLFECWSSSSNQNEMFFGDQV
DKCYKCSVKQGQTLFIPTGWIHAVLTPVD
CLAFGGNFLHSLNIEMQLKAYEIEKRLST
ADLFRFPNFETICWYVGKHILDIFRGLRE
NRRHPASYLVHGGKALNLAFRAWTRKEAL
PDHEDEIPETVRTVQLIKDLAREIRLVED
IFQQN

Host: BL21(DE3)-R3-pRARE2

Expression protocol: Glycerol stock was used to inoculate 120 ml of LB medium (Luria Broth) supplemented with 50 µg/ml kanamycin and 35 µg/ml chloramphenicol. This starter culture was grown overnight at 37°C and used to inoculate 12 x 1 liter LB culture supplemented with 50 µg/ml kanamycin only. The cells were cultured at 37°C with vigorous shaking (160 rpm) until an OD_{600} of 0.5-0.6. At that point cells were induced with 0.1 mM IPTG and cultured o/n at 18°C. Cells were harvested at 9000 x g for 10 minutes and the cell pellet of 12L was resuspended in 240 ml of lysis buffer and stored at -80°C until further use. **Lysis buffer:** 500 mM NaCl, 5% glycerol, 50 mM HEPES pH 7.5, 20 mM imidazole, Complete EDTA-free protease inhibitor (Roche, 1tablet / 50ml).

Extraction method: Cell pellets from 12 liter previously re-suspended, were thawed and lysed by sonication, followed by centrifugation at 4°C for 45 minutes at 48 000 x g.

The supernatant was further clarified by filtration (0.45 mm) before loaded on the 5 ml HisTrap FF, column (GE heathcare).

Column 1: Ni-affinity, HisTrap FF, 5 ml (GE heathcare)

Buffers:
Lysis buffer: 500 mM NaCl, 5% glycerol, 50 mM HEPES pH 7.5, 20 mM imidazole, 0.5 mM TECP
Wash Buffer: 500 mM NaCl, 5% glycerol, 50 mM HEPES pH 7.5, 40 mM imidazole, 0.5 mM TECP
Elution Buffer: 500 mM NaCl, 5% glycerol, 50 mM HEPES pH 7.5, 250 mM imidazole, 0.5 mM TECP

Procedure: The centrifuged cell extract was loaded on the column at 5 ml/min on an AKTA-Xpress system (GE heathcare). The column was then washed with 10 column volumes of lysis buffer, 10 column volumes of wash buffer, and then eluted with 3 column volume of elution buffer at 5 ml/min. . The eluted protein was collected and analyzed by SDS-PAGE.

TEV cleavage: To the fraction containing PHF8A (in total 48.5 mg) 240 µl of TEV protease (6 mg/ml) was added. Cleavage was performed overnight at 4°C and examined by SDS-PAGE.

Column 2: Ni-NTA

Procedure: TEV-cleaved protein was exchanged into GF buffer, before being passed through 250 µl of Ni-NTA resin, to remove the TEV protease. The flow through was concentrated to 5 ml using Amicon Ultra 30k, Millipore concentrator and loaded on the Hiload 16/60 Superdex 200 column pre-equilibrated with GF buffer.
Column 3: Size Exclusion Chromatography (SEC) Hiload 16/60 Superdex 200 prep grade 120 ml (GE/Amersham Biosciences)
Procedure: Eluted proteins were collected in 1.8 ml fractions and analyzed on SDS-PAGE.
Buffers: **Gelfiltration bufffer:** 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5mM TCEP
Column 4: Ion exchange chromatography, HiTrap 5 ml QP
Procedure: Fraction containing PHF8A were pooled, diluted to 50 mM NaCl and loaded on the HiTrap 5ml QP pre-equilibrated with buffer A.
Buffers: **Ion exchange buffer:** **Buffer A:** 50mM NaCl, 20mM Tris/HCl pH 8, 0.5 mM TECP **Buffer B:** 1M NaCl,20mM Tris/HCl pH 8, 0.5 mM TECP Protein was eluted with a NaCl gradient from 50 mM-1 M.
Concentration: To the fractions containing PHF8A 5% glycerol was added and the protein was concentrated using centricon with 30 kDa cut off (Amicon Ultra 30k, Millipore) to 12.1 mg/ml.
Mass spectrometry characterization: LC-ESI-MS TOF confirmed the expected mass of 42.909 Da.
Crystallization: Prior to crystallization, protein was pre-incubated with 2mM N-oxalylglycine at 4oC. Crystals were grown at 4oC by vapour diffusion in sitting drops by mixing protein (12 mg/ml + 2 mM N-oxalylglycine) and well solution containing 1.5 M (NH4)2SO4 and 0.1 M sodium acetate pH 4.25 in a 2:1 ratio. Crystals were cryo-protected using 30% (v/v) glucose and flash-cooled in liquid nitrogen. **Data Collection:** Diffraction data to 2.15 Å resolution were collected at the Diamond Light Source beamline IO2.