

ZMPSTE24A (2YPT) Materials & Methods

Entry Clone Source: MGC

Entry Clone Accession: IMAGE: 5269064

SGC Construct ID: ZMPSTE24A-c029

GI Number: 10269

Vector: pFB-CT10HF-LIC. Details [[PDF](#)]; Sequence [[FASTA](#)] or [[GenBank](#)]

DNA sequence:

CTTAAGAAGGAGATATACTATGGGA
TGTGGGCATCGCTGGACGCTTG
GAGATGCCGGCGAGAACGCTATCTT
CGGGGCCGTGCTGCTCTTCCTGGA
CAGTGTATCTTGGAGACCTTCCTA
GCACAGCAGCAGAGAACGATATATAA
AACAAACAACATGTACCACCGGAGT
TAGGACAGATCATGGATTCTGAAACA
TTTGAGAAATCTCGACTCTATCAACT
GGATAAAAGCACTTCAGCCTCTGGT
CAGGACTCTATTCAAGAGACTGAAGGC
ACTCTTATTCTCTCTTGAGGAAT
ACCTTATCTCTGGAGACTTCTGGAC
GGTTCTGTGGTTATGCTGGCTTGGA
CCAGAATATGAGATCACTCAGTCCCT
GGTGTTCAGCTGTTGGCTACACTTT
TCAGTGCATTGGCTGGTTGCCATGG
AGTCTTATAATACTTTGTGATAGA
AGAAAAACATGGCTTCAATCAACAGA
CTTGGGTTCTCATGAAAGATGCA
ATCAAGAAATTGTTGTGACTCAGTG
CATTGTTGCCTGTGCTTCACCTC
TACTTACATTATTAAAATTGGGGT
GACTATTTTATTATGCCTGGCT
GTTCACATTAGTTGTGCTCTGGTTC
TTGTCACAATCTATGCTGATTATATT
GCCCTTTATTGACAAATCACACC
TCTGCCTGAGGGAAAGCTAAAGAAG
AAATTGAAGTAATGGCAAAGAGTATT
GACTTCCATTGACGAAGGTGTATGT
TGTGGAAGGATCTAACGCTCTTCCC
ACAGCAATGCTATTATGGCTTC
TTCAAGAACAGCGAATAGTTGTT
TGACACTCTACTAGAAGAGTACTCTG
TACTAAACAAAGACATCCAGGAGGAT
TCTGGCATGGAACCCGCAATGAGGA
AGAAGGGAACAGTGAAGAAATAAG
CTAAAGTTAAAATAAGAAACAAGGA
TGTAAAAATGAGGAGGTACTCGCTGT
ACTAGGCCATGCACTGGGCACTGGGA

AGTTGGGACATACAGTCAAAAATATC
ATTATTAGCCAGATGAATTCTTCCT
GTGTTTTTTATTGCTGTATTAA
TTGGTCGAAAGGAGCTTTGCTGCA
TTGGTTTTATGATAGCCAACCCAC
TCTTATTGGACTATTGATCATCTC
AGTTTATTTCACCTTACAATGAG
GTTCTTCTTTGCCTAACAGTCCT
AAGCCGCAGATTGAGTTCAAGCTG
ATGCATTGCCAAGAAACTGGGAAG
GCTAAAGACTTATATTCTGCTTTAAT
CAAACCTAACAAAGATAACTGGGAT
TCCCTGTTCTGACTGGTTCTCA
ATGTGGCATTATTCTCATCCTCCACT
GCTAGAGAGACTCAAGCTTGAAAA
CTATGAAGCAACACGCAGAGAACCTC
TACTTCCAATCGCACCACATCACCA
TCACCATCACCACCATGATTACAAGG
ATGACGACGATAAGTGAGGATCC

Expressed sequence (small letters refer to tag sequence):

MGMWASLDALWEMPAEKrifGAVLfF
SWTVYLWETFLAQQRRIYKTTTHVP
PELGQIMDSETFEKSRLYQLDKSTFS
FWSGLYSETEGTLILLFGGIPYLWRL
SGRFCGYAGFGPEYEITQSLVFLLA
TLFSALAGLPWSLYNTFVIEEKHGFn
QQTLGFFMKDAIKKFVVTQCILLPVS
SLLYIIKIGGDYFFIYAWLFTLVVS
LVLVTIYADYIAPLFKFTPLPEGKL
KEEIEVMAKSIDFPLTKVYVVEGSKR
SSHSNAYFYGFFKNKRIVLFDTLLEE
YSVLNKDIQEDSGMEPRNEEGNSEE
IKAKVKNKKQGCKNEEVLAVLGHALG
HWKLGHFTVKNIIISQMNSFLCFFLFA
VLIGRKELFAAFGFYDSQPTLIGLLI
IFQFIFSPYNEVLSFCLTVLSRRFF
QADAFAKKLGKAKDLYSALIKLNKD
LGFPVSDWLFMSMWHYSHPPLLERLQA
LKTMKQHAENLYFQ^shhhhhhhhh
dykdddk

Tags and additions: C-terminal, TEV cleavable deahistidine / FLAG tag.

Host: Spodoptera frugiperda (SF9) insect cells

Growth Medium & Induction Protocol: Insect cells with a density of 2×10^6 per litre of cell culture in SF900 medium (Invitrogen) were infected with recombinant baculovirus (5ml P2 virus per litre cell culture) and incubated for 72 hours. Cells were harvested by centrifugation and the pellet was flash frozen in liquid N₂.

Extraction buffer, extraction method: Frozen pellets were thawed and re-suspended in extraction buffer supplemented with 1% OGNG/ 0.1% CHS and incubated at 4°C on a tube

rotator. The extracted protein was separated from insoluble membranes by centrifugation and collected for purification.

Extraction/wash buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl

Column 1: Co-affinity. Cobalt Talon (Clontech), 0.75 ml of 50 % slurry/ 1L of cells in 1.5 x 10 cm column, washed with extraction/ wash buffer.

Column 1 Buffers:

Extraction/wash buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl; 20 mM imidazole, detergent at 3x CMC.

Elution buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl; 250 mM imidazole, detergent at 3xCMC

Column 1 Procedure: The solubilised membrane protein was batch bound to Cobalt Talon resin equilibrated with extraction buffer at 4°C for one hour and subsequently poured into a gravity flow glass column. The column was then washed with 30CV of wash buffer followed by elution in 1CV fractions until all the protein was eluted.

Column 2: Size Exclusion Chromatography. Superdex200 (GE healthcare)

Column 2 Buffer:

Gel Filtration buffer: 20 mM HEPES pH 7.5, 200 mM NaCl, detergent at 1.2xCMC

Column 2 Procedure: The protein was concentrated and applied to a Superdex 200 gel filtration column equilibrated with gel filtration buffer using an AKTA purifier system.

Enzymatic Treatment: TEV protease (1:4, TEV:protein) was added overnight at 4°C to purified protein. The protease was removed and tag cleaved protein was collected by binding to Cobalt talon and collecting the flow-through.

Mass spec characterization:

The purified protein was homogeneous and had an experimental mass of 55460Da, as expected from the primary sequence with a post translational modification of loss of methionine. Masses were determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% methanol in water with 0.1% formic acid.

Protein Concentration: Protein was concentrated to ~20mg/ml using a 100kDa cut-off concentrator and back diluted to 9-11mg/ml. A three-fold excess of a farnesylated nonapeptide corresponding to the C-terminus of prelamin A (residues 656-664; QSPQNC(farn)SIM) was added prior to setting up crystallization plates

Crystallization: P1 crystals were obtained under similar conditions to the native enzyme at 20°C. Crystals grew from sitting drops (150nl) comprising 100nl of concentrated protein / peptide complex and 50nl of reservoir equilibrated against 20µl of reservoir solution (0.1M HEPES pH 7.0, 0.1M calcium chloride, 29% (v/v) PEG 400).

Data Collection:

Resolution: 3.8Å

Data were collected at 100K on the microfocus beamline I24 at Diamond Light Source using helical / straight line scans with a beamsize of 10µm x 10µm (wxh).

Structure Solution: Datasets collected from ZMPSTE24 / prelamin A peptide (Q656-M664) co-crystals showed that the peptide-binding site was empty. These □co-crystals□ were then

soaked with reservoir solution containing 5mM CSIM tetrapeptide and OGNG/CHS detergent for 24h at 6°C. The coordinates of the native P1 structure (4AW6) were used as a starting model for refinement with BUSTER. Clear difference $Fo-Fc$ electron density in each of the four molecules in the asymmetric unit was interpreted based on the known binding mode of peptides and inhibitors to metalloproteases. The peptide register, with Ser662 and Ile663 positioned in the S1 and S1' subsites respectively, was determined based on both the length of the electron density, and the definition of the C-terminal. The entire tetrapeptide was modeled in two cases (molecules B&D) whereas only C661-I663 was modeled in molecules A & E. In all cases, the quality of the electron density was not sufficient to resolve the CD1 atom of Ile663 and this atom was not included in the model. Refinement was performed with BUSTER with TLS, NCS and LSSR restraints to the native structure.