Entry Clone Source: MGC

Entry Clone Accession: IMAGE:6141834

SGC Construct ID: TATDN1A-c104

GenBank GI number: gi|14042943

Vector: pNIC-CTHF. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

CTTAAGAAGGAGATATACTATGAAGTTTAT
CGATATTGGTATCAACTTGACTGACCCTAT
GTTCAGAGGAATTTATAGGGGGGTTCAAAA
GCATCAAGATGACTTACAGGATGTAATAGG
GAGAGCTGTCGAGATTGGTGTTAAAAAGTT
TATGATTACAGGTGGAAATCTACAAGACAG
TAAAGATGCACTGCATTTGGCACAAACAAA
TGGTATGTTTTTCAGTACAGTTGGATGTCA
TCCTACAAGATGTGGTGAATTTGAAAAGAA
TAACCCTGATCTTTACTTAAAGGAGTTGCT
AAATCTTGCTGAAAACAATAAAGGGAAAGT
TGTGGCAATAGGAGAATGCGGACTTGATTT
TGACCGACTGCAGTTTTGTCCCAAAGATAC
TCAACTCAAATATTTTGAAAAACAGTTTGA
ACTGTCAGAACAAACAAAATTACCAATGTT
TCTTCATTGTCGAAACTCACATGCTGAATT
TTTGGACATAACGAAAAGAAATAGAGATCG
GTGTGTAGGGGGAGTGGTGCATTCATTTGA
TGGTACCAAGGAAGCAGCAGCTGCTTTGAT
TGACTTGGATCTTTATATAGGATTTAATGG
TTGCTCACTGAAAACTGAAGCTAATTTGGA
AGTTTTGAAGTCAATTCCTAGTGAAAAATT
AATGATTGAGACAGATGCACCTTGGTGTGG
AGTCAAAAGTACACATGCTGGATCAAAATA
TATAAGAACTGCATTTCCTACCAAAAAGAA
GTGGGAAAGTGGGCACTGCTTAAAAGACAG
AAATGAACCCTGCCATATAATTCAAATATT
GGAGATAATGTCAGCAGTGAGAGATGAGGA
TCCACTGGAATTAGCCAATACACTATATAA
CAATACTATTAAAGTATTTTTTCCTGGAAT
AGCAGAGAACCTCTACTTCCAATCGCACCA
TCATCACCACCATGATTACAAGGATGACGA
CGATAAGTGAGGATCC

Final protein sequence (tag sequence in lowercase)

MKFIDIGINLTDPMFRGIYRGVQKHQDDLQ
DVIGRAVEIGVKKFMITGGNLQDSKDALHL
AQTNGMFFSTVGCHPTRCGEFEKNNPDLYL
KELLNLAENNKGKVVAIGECGLDFDRLQFC
PKDTQLKYFEKQFELSEQTKLPMFLHCRNS
HAEFLDITKRNRDRCVGGVVHSFDGTKEAA
AALIDLDLYIGFNGCSLKTEANLEVLKSIP
SEKLMIETDAPWCGVKSTHAGSKYIRTAFP

TKKKWESGHCLKDRNEPCHIIQILEIMSAV
RDEDPLELANTLYNNTIKVFFPGIaenlyf
qshhhhhhdykddddk

Tags and additions: C-terminal His6 tag and FLAG tag, preceded by a TEV protease cleavage site: aenlyfq*shhhhhhdykddddk (* - TEV cleavage site)
Tag removed: yes

Host: BL21 (DE3)-R3-pRARE2

Expression protocol: The glycerol stock of host strain BL21 (DE3)-R3-pRARE2 was used to inoculate 10 ml of TB (terrific 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 a 1 litre culture in TB supplemented with 50 µg/ml kanamycin only. The culture was grown at 37°C until the OD_{600} reached ~3.0. After that the temperature was lowered to 18°C. Protein production was induced with 0.1 mM IPTG and recombinant TATDN1 was expressed at that temperature overnight. The next day cells were harvested by centrifugation at 5000 rpm for 20 minutes then the supernatant was discarded and pellets re-suspended in 70ml of 2x lysis buffer. Stored at -80°C.

Cell Extraction: 2X Lysis buffer: 100 mM K-phosphate, pH 7.5, 1 M NaCl, 20% glycerol 1 mM TCEP, 1x Protease Inhibitors Cocktail Set VII (Calbiochem, 1/1000 dilution), and 15 units/ml Benzonase.
Procedure:
Frozen cells, previously re-suspended, were thawed, and supplemented with: TCEP, Benzonase and protease inhibitors. Cells were lysed by sonication.
Nucleic acids and cell debris were removed by adding 0.15% PEI (polyethyleneimine), stirring for 30 minutes, then centrifugation for 30 minutes at 17,000RPM. The supernatant was then further clarified by filtration (Acrodisc filters, 0.2 µm).

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

Buffers:2X Lysis buffer: 100 mM K-phosphate, pH 7.5, 1 M NaCl, 10% glycerol, 0.5 mM TCEP; **Wash buffer:** 50 mM K-phosphate, pH 7.5, 500 mM NaCl, 30 mM imidazole, 10% glycerol, 0.5 mM TCEP; **Elution buffer:** 50 mM K-phosphate, pH 7.5, 500 mM NaCl, 300 mM imidazole, 10% glycerol, 0.5 mM TCEP.

Procedure: The cell extract was loaded on the column at 4 ml/min on an ÄKTA-express system (GE Healthcare). The column was washed with 10 volumes of 1X lysis buffer, 10 volumes of wash buffer, and then eluted with elution buffer at 4 ml/min. The eluted peak of A280 was automatically collected

Column 2: Gel filtration, Hiload 16/60 Superdex S75 prep grade, 120 ml (GE Healthcare)

Gel Filtration Buffer: 50 mM HEPES pH7.5, 300 mM NaCl, 5% glycerol and 0.5 mM TCEP

Procedure: The eluted fraction from the Ni-affinity Histrap column was loaded on the gel filtration column pre-equilibrated in GF buffer at 0.80 ml/min. Eluted proteins were collected in 1.8-ml fractions and analyzed on SDS-PAGE

Column 3: Ni-NTA (Qiagen)

Enzymatic treatment: Fractions containing TATDN1 were pooled, and supplemented with TEV at an enzyme-to-protein ration of 1:20. The mixture was left incubating overnight at 4°C, and next day was applied through 1ml Ni-NTA slurry in a 10mm gravity column pre-equilibrated with wash buffer. The flow-through and wash fractions (3x 1ml wash buffer)

containing TEV-cleaved protein were collected and buffer-exchanged in GF buffer using a PD-10 desalting column.
Wash buffer: 50 mM K-phosphate, pH 7.5, 500 mM NaCl, 30 mM imidazole, 10% glycerol 0.5 mM TCEP.
Elution buffer: 50 mM K-phosphate, pH 7.5, 500 mM NaCl, 300 mM imidazole, 10% glycerol 0.5 mM TCEP.

Protein concentration: Protein was stored in 50 mM HEPES pH7.5, 300 mM NaCl and 5% Glycerol at -80°C. The protein was concentrated to 52.4 mg/ml using a Centricon centrifugal device with a 10 kDa cut off. The protein concentration was determined spectrophotometrically using ε^{280}= 21430.

Mass spectrometry characterization: ESI-MS revealed that the protein had a mass of 34088.4 Da (Expected mass 34047.2). There is a mass discrepancy of +42 Da, identified as Gly-to-Val substitution at position 292.

Crystallisation: Prior to crystallization protein was supplemented with 1.5 mM $NiCl_2$.
Crystals were grown at 4°C by vapour diffusion in sitting drops mixing protein (52.39 mg/ml) and well solution containing 0.2M NaCl, 0.1M BIS-TRIS pH 6.5 and 25% PEG 3350 at a protein to precipitant ratio of 1:1.
Crystals were cryo-protected using 25% (v/v) ethylene glycol and flash cooled in liquid nitrogen.

Data collection: Resolution: 1.3 Å; **X-ray source:** Diamond Light Source beamline IO3