Entry Clone Source: MGC

Entry Clone Accession: IMAGE:6141834

SGC Construct ID: TATDN1A-c104

GenBank GI number: gi|14042943

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

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CTTAAGAAGGAGATATACTATGAAGT
TTATCGATATTGGTATCAACTTGACT
GACCCTATGTTCAGAGGAATTTATAG
GGGGGTTCAAAAGCATCAAGATGACT
TACAGGATGTAATAGGGAGAGCTGTC
GAGATTGGTGTTAAAAAGTTTATGAT
TACAGGTGGAAATCTACAAGACAGTA
AAGATGCACTGCATTTGGCACAAACA
AATGGTATGTTTTTCAGTACAGTTGG
ATGTCATCCTACAAGATGTGGTGAAT
TTGAAAAGAATAACCCTGATCTTTAC
TTAAAGGAGTTGCTAAATCTTGCTGA
AAACAATAAAGGGAAAGTTGTGGCAA
TAGGAGAATGCGGACTTGATTTTGAC
CGACTGCAGTTTTGTCCCAAAGATAC
TCAACTCAAATATTTTGAAAAACAGT
TTGAACTGTCAGAACAAACAAAATTA
CCAATGTTTCTTCATTGTCGAAACTC
ACATGCTGAATTTTTGGACATAACGA
AAAGAAATAGAGATCGGTGTGTAGGG
GGAGTGGTGCATTCATTTGATGGTAC
CAAGGAAGCAGCAGCTGCTTTGATTG
ACTTGGATCTTTATATAGGATTTAAT
GGTTGCTCACTGAAAACTGAAGCTAA
TTTGGAAGTTTTGAAGTCAATTCCTA
GTGAAAAATTAATGATTGAGACAGAT
GCACCTTGGTGTGGAGTCAAAAGTAC
ACATGCTGGATCAAAATATATAAGAA
CTGCATTTCCTACCAAAAAGAAGTGG
GAAAGTGGGCACTGCTTAAAAGACAG
AAATGAACCCTGCCATATAATTCAAA
TATTGGAGATAATGTCAGCAGTGAGA
GATGAGGATCCACTGGAATTAGCCAA
TACACTATATAACAATACTATTAAAG
TATTTTTTCCTGGAATAGCAGAGAAC
CTCTACTTCCAATCGCACCATCATCA
CCACCATGATTACAAGGATGACGACG
ATAAGTGAGGATCC
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Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smGV
GLVDCHCHLSAPDFDRDLDDVLEKAK
KANVVALVAVAEHSGEFEKIMQLSER
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YNGFVLPCLGVHPVQGLPPEDQRSVT
LKDLDVALPIIENYKDRLLAIGEVGL
DFSPRFAGTGEQKEEQRQVLIRQIQL
AKRLNLPVNVHSRSAGRPTINLLQEQ
GAEKVLLHAFDGRPSVAMEGVRAGYF
FSIPPSIIRSGQKQKLVKQLPLTSIC
LETDSPALGPEKQVRNEPWNISISAE
YIAQVKGISVEEVIEVTTQNALKLFP
KLRHLLQK

^ TEV cleavage site

Tags and additions: Cleavable N-terminal His_6 tag.

Host: BL21 (DE3)R3-pRARE2 (Phage resistant strain).

Growth medium, induction protocol: The glycerol stock of host strain BL21 (DE3)-R3-pRARE2 was used to inoculate 10ml of TB (terrific Broth) supplemented with 50 µg/ml kanamycin and 34µg/ml chloramphenicol. This starter culture was grown overnight at 37°C and used to inoculate a 1L culture in TB supplemented with 100µg/ml kanamycin only. The culture was grown at 37° until the OD_{600} reached ~3.0. After that the temperature was lowered to 18°C. Protein production was induced with 1 mM IPTG and recombinant TATDN3 was expressed at that temperature overnight. The next day cells were harvested by centrifugation at 5000rpm for 20 minutes then the supernatant was discarded and pelletes re-suspended in 70ml of 2x lysis buffer. Stored at -80°C.
Lysis buffer: 100 mM K-phosphate, pH7.5; 1M NaCl; 20% glycerol; 1 mM TCEP; 1x Protease Inhibitors Cocktail Set VII (Calbiochem, 1/1000 dilution); 15 units/ml Benzonase.
Extraction buffer, extraction method: 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), then centrifugatioin for 30 minutes at 17,000rpm.

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

Column 1 Buffer:
2x Lysis buffer: 100 mM K-phosphate, pH 7.5; 1 M NaCl; 20% 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.

Column 1 Procedure: The cell extract was loaded on the column at 4ml/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 4ml/min. The eluted peak of A_{280} was automatically collected.

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

Column 2 Buffers: 50 mM HEPES, pH 7.5; 300 mM NaCl; 5% glycerol and 0.5 mM TCEP.

Column 2 Procedure: The eluted fraction from the Ni-affinity HisTrap column was loaded on the gel filtration column in buffer at 0.8ml/min. Eluted proteins were collected in 1.8ml fractions and analysed on SDS-PAGE.

Enzymatic treatment: Fractions containing TATDN3 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 containing TEV-cleaved protein was collected.

Mass spectrometry characterization: ESI-MS revealed the presence of two peaks showing mass of 30124.45Da and 30190.32Da (expected mass 30121.8Da). As the mass difference could not be traced to exact post translational modifcation, using MS-MS the presence of the right protein was confirmed.

Protein concentration: Protein was stored in 50 mM HEPES pH 7.5, 300 mM NaCl and 5% glyercol at -80°C. The protein was concentrated to 8mg/ml using a Centricon centrifugal device with a 10kDa cut off. The protein concentration was determined spectrophotometrically.

Crystallisation: Crystals were grown at 4°C by vapour diffusion in sitting drops mixing protein (8mg/ml) and well solution containing 0.02M $ZnCl_2$, 20% w/v PEG 6000 and 10% v/v ethylene glycol. Crystals were cryo-protected using 25% v/v ethylene glycol and flash cooled in liquid nitrogen.

Data Collection:
Resolution: 2.75Å.
X-ray source: Diamond Light Source beamline IO2.