**Entry Clone Source:** MGC

**Entry Clone Accession:** IMAGE:7939740

**SGC Construct ID:** LCN15A-c008

**GenBank GI number:** gi|42714611

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

**Amplified construct sequence:**
MHHHHHHSSGVDLGTENLYFQSMQAEVLLQ
PDFNAEKFSGLWYVVSMASDCRVFLGKKDH
LSMSTRAIRPTEEGGLHVHMEFPGADGCNQ
VDAEYLKVGSEGHFRVPALGYLDVRIVDTD
YSSFAVLYIYKELEGALSTMVQLYSRTQDV
SPQALKSFQDFYPTLGLPKDMMVMLPQSDA
CN

**Final protein sequence:**
MHHHHHHSSGVDLGTENLYFQ^SMDHYKAK
SMQAEVLLQPDFNAEKFSGLWYVVSMASDC
RVFLGKKDHLSMSTRAIRPTEEGGLHVHME
FPGADGCNQVDAEYLKVGSEGHFRVPALGY
LDVRIVDTDYSSFAVLYIYKELEGALSTMV
QLYSRTQDVSPQALKSFQDFYPTLGLPKDM
MVMLPQSDACN

^ TEV cleave site

**Tags and additions:** N-terminal, TEV cleavable hexahistidine tag.

**Expression strain:** BL-21(DE3)-R3-Rosetta (A homemade phage resistant version of BL21(DE3) containing the pRARE2 plasmid from Rosetta II (DE3) cells).

**Transformation:** The construct DNA was transformed into competent cells of the expression strain by a standard heat shock procedure.

**Glycerol stock preparation:** A number of colonies were used to inoculate 1 ml of LB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was incubated in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture.

**Expression:** 10 ml of a glycerol stock was used to inoculate 120 ml of LB containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was incubated at 37°C overnight.1 5ml starter culture was used per litre TB, containing 50 µg/ml kanamycin. The culture was incubated at 37°C until OD~1.5, when the temperature of the incubator was reduced to 18°C. At 18°C, expression was induced with 0.1 mM IPTG and the culture continued o/n.

**Cell harvest:** Cells were pelleted at 6238x g for 15 min at 4°C, and stored at -80°C. The yield was 9.6g cells / litre culture.

**Cell Lysis: Lysis Buffer:** 50mM HEPES pH 7.4, 500mM NaCl, 5% glycerol, 10 mM Imidazole pH 7, 1mM PMSF, 0.5mM TCEP; The pellets were resuspended in lysis buffer. They were passed 4 times through an Emulsiflex C5 high-pressure homogeniser, collecting a final volume of approximately 35 ml/ litre culture. Cell debris and DNA were spun down at

45000x g, 60 min ( Beckman JA 18 17500 rpm). The supernatant was collected to which Benzonase was added with a 60 min incubation on ice.

**Purification: Wash Buffer:** 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 30 mM Imidazole, 0.5mM TCEP pH 7.4; **Elution Buffer:** 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole, 0.5mM TCEP pH 7.4; **Gel Filtration buffer:** 50 mM Hepes pH 7.4, 500 mM NaCl, 5% glycerol, 0.5mM TCEP; Purification was performed in an Akta Express system (GE Healthcare) with an automated program for Ni-affinity (HisTrap FF) and gel filtration (HiLoad 16/60 S75) chromatography steps.

**Column 2 Procedure:** The Ni-sepharose column was serially connected to the DAE-52 column and was loaded by gravity flow. The column was then washed with 30 ml wash buffer at gravity flow. The protein was eluted by gravity flow by applying 5-ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 200 and 250 mM); fractions were collected until essentially all protein was eluted.

**Step 1: Ni-affinity, HisTrap Crude FF, 1 ml** The clarified cell extract was loaded on the column at 0.8 ml/min on the AKTA-express system. The column was washed with 40 cv of binding buffer, 10 cv of wash buffer and eluted with elution buffer at 0.8 ml/min. The eluted peak of $A_{280}$ was automatically collected.

**Step 2: Gel filtration, Hiload 16/60 Superdex S200 prep grade, 120 ml** The eluted fractions from the Ni-affinity column was loaded on the gel filtration column pre-equilibrated in Gel Filtration buffer at 1.2 ml/min. Eluted proteins were collected in 1.8 ml fractions and analyzed on SDS-PAGE.

**TEV protease digestion:** Peak fractions from the gel filtration containing LCN15A were pooled and TEV protease was added at a molar ratio of 1:44. The digestion was left overnight at 4 °C. SDS-PAGE and Mass Spec confirmed TEV digestion. His-TEV and contaminating proteins were removed by binding to Ni resin, pre-equilibrated in GF buffer. The flow through containing TEV-cleaved protein, was collected and concentrated using a Amicon centrifugal filter with a 10kDa MW cut off. To remove any precipitation, the concentrated protein was centrifuged 20 min/ 14000rpm/ 4°C and the supernatant was collected. The final concentration was 16.6 mg protein /ml and yield 10 mg/ litre culture. The protein was flash frozen and stored at -80°C in 70ml aliquots.

**Mass spec. characterisation:** Measured: 18002.8; Expected: 18002.6

**Crystallisation:** Crystals were grown at 4°C by vapour diffusion in sitting drops mixing protein (16.6mg/ml) and precipitant solution 1.4 M Sodium Citrate, 0.1M HEPES pH 8.2 in a 2:1 ratio. Crystals were cryo-protected in 25% ethylene glycol and flash frozen in liquid nitrogen.

**Data Collection:** Data was collected to a resolution of 1.63 Å at Diamond Light Source beamline IO2.