Molecular Biology

Entry Clone Accession: clone 27 (T615S mutation)
Entry Clone Source: Collaborator
SGC Construct ID: AASSA-c013
Protein Region: L455-P926
Vector: pFB-LIC-Bse
Tag: N-6HIS;N-TEV
Host: DH10Bac

Sequence (with tag(s)):
MGHHHHHHSSGVDLGTENLYFQSMALPDKYKYIQTLRESRERAQSLSMGTRRKVLVLG
SGYISEPVLEYLSRDGNIEITVGSDMKNQIEQLGKKYNINPVSMDICKQEEKLGFLVAKQD
LVISLLPYVLHPLVAKACITNKVNMVTASYITPALKELEKSVEDAGITIIGELGLDPGLDHM
LAMESIDKAKEVGATIESYISYCGGLPAPEHSNNPLRYKFSWSPVGVLMNVMQSATYLLD
GKVVNVAGGISFLDAVTSMDFFPGLNLEGYPNRDSTKYAEIYGISSAHTLLRGTLRYKGY
MKALNGFVKLGLINREALPAFRPEANPLTWKQLLCDLVGISPSSEHDVLKEAVLKKLGGD
NTQLEAAEWLGLLGDEQVPQAESILDALSKHLVMKLSYGPEEKDMIVMRDSFGIRHPSG
HLEHKTIDLVAYGDINGFSAMAKTVGLPTAMAAKMLLDGEIGAKGLMGPFSKEIYGPILE
RIKAEGIIYTTQSTIKP
Sequence after tag cleavage:
SMALPDKYKYIQTLRESRERAQSLSMGTRRKVLVLGSGYISEPVLEYLSRDGNIEITVGSD
MKNQIEQLGKKYNINPVSMDICKQEEKLGFLVAKQDLVISLLPYVLHPLVAKACITNKVN
MVTASYITPALKELEKSVEDAGITIIGELGLDPGLDHMLAMESIDKAKEVGATIESYISYCG
GLPAPEHSNNPLRYKFSWSPVGVLMNVMQSATYLLDGKVVNVAGGISFLDAVTSMDFFP
GLNLEGYPNRDSTKYAEIYGISSAHTLLRGTLRYKGYMKALNGFVKLGLINREALPAFRP
EANPLTWKQLLCDLVGISPSSEHDVLKEAVLKKLGGDNTQLEAAEWLGLLGDEQVPQAE
SILDALSKHLVMKLSYGPEEKDMIVMRDSFGIRHPSGHLEHKTIDLVAYGDINGFSAMAK
TVGLPTAMAAKMLLDGEIGAKGLMGPFSKEIYGPILERIKAEGIIYTTQSTIKP
DNA Sequence:
CCATGGGCCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTG
TACTTCCAATCCATGGCATTACCTGATAAATATAAATATATCCAGACACTCCGGGAGAG
CAGGGAACGTGCTCAGTCACTTTCAATGGGCACCAGGAGAAAGGTTTTGGTTCTTGG
ATCTGGCTACATATCTGAGCCTGTATTAGAATATTTATCAAGAGATGGCAATATAGAAAT
AACAGTAGGATCTGACATGAAGAATCAAATTGAACAGTTAGGCAAGAAATATAATATT
AATCCTGTTAGCATGGACATTTGTAAACAAGAAGAGAAGCTGGGCTTCTTGGTGGCA
AAACAGGATCTTGTCATCAGCTTGTTGCCTTATGTATTGCACCCTCTTGTGGCCAAGGC
CTGCATCACAAACAAAGTTAACATGGTCACTGCAAGCTACATCACACCAGCACTAAA
AGAATTGGAAAAGAGTGTGGAAGATGCTGGCATCACAATCATTGGTGAATTGGGATTG
GACCCTGGTCTGGATCACATGTTAGCAATGGAATCAATAGATAAAGCCAAGGAAGTGG
GAGCCACGATTGAATCATATATTTCCTACTGTGGTGGGCTTCCAGCCCCTGAACATTCA
AACAATCCATTGAGATATAAATTTAGCTGGAGTCCAGTGGGAGTTTTGATGAATGTAAT
GCAGTCTGCCACCTATCTGCTCGATGGAAAGGTTGTGAATGTTGCAGGAGGCATCTCC
TTTCTTGATGCCGTTACGTCCATGGATTTTTTTCCAGGATTAAATTTGGAAGGCTATCCT
AACAGAGACAGTACGAAATATGCTGAGATTTATGGCATTTCTTCTGCTCACACTTTGTT
GCGGGGGACACTGAGATATAAGGGATATATGAAAGCTTTGAATGGATTTGTAAAATTA
GGTCTTATAAACAGAGAAGCGCTTCCTGCCTTTAGACCTGAGGCCAACCCTCTCACCT
GGAAACAACTCCTCTGTGACCTAGTTGGGATTTCACCCTCCTCTGAGCATGATGTGTT
GAAGGAAGCTGTTCTTAAGAAACTAGGAGGAGACAATACCCAGTTGGAGGCTGCTGA
ATGGTTGGGCTTACTTGGGGATGAACAAGTTCCTCAGGCAGAGTCCATTCTGGATGCC
CTCTCCAAGCATTTGGTCATGAAGCTTTCCTATGGTCCTGAAGAAAAAGATATGATTGT
GATGAGAGACAGCTTTGGAATCAGACATCCTTCTGGACATTTAGAACATAAAACGATT

GATCTTGTGGCTTATGGGGACATCAATGGCTTTTCAGCCATGGCTAAAACCGTGGGGT
TACCCACCGCCATGGCAGCCAAAATGTTGCTTGATGGTGAAATTGGAGCCAAAGGCC
TAATGGGGCCCTTTTCAAAGGAGATCTATGGACCAATATTGGAGCGAATTAAAGCAGA
AGGCATTATATATACTACACAGAGTACAATTAAACCATGACAGTAAAGGTGGATACGGA
TCCGAATTCGAGCTCCGTCGACAAGCTT

Protein Purification

Buffers: Binding Buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole pH 7.4, 0.5 mM TCEP
Wash Buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole pH 7.4, 0.5 mM TCEP
Elution Buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.4, 0.5 mM TCEP

Procedure: Harvested cells were resuspended in lysis buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole pH 7.4, 0.5 mM TCEP, 1 µL per 1 mL protease inhibitor cocktail EDTA-free).
Cell pellet was dissolved in approximately 200 mL lysis buffer and broken by homogenization by 2 passes at 12,000 psi. The cell debris was pelleted at 35000 x g, 1h and the supernatant used for purification on a gravity flow Ni-NTA column (5 mL).

The clarified cell extract was added to 5 ml of Ni-NTA resin pre-equilibrated with lysis buffer and passed through a glass column. The column was then washed with Binding Buffer (2 x 50 mL) and Wash Buffer (2 x 50 mL). The protein was eluted with Elution Buffer in 5 x 5 mL fractions. The eluted fractions from column 1 were pooled and concentrated to 5 mL with a 30 kDa MWCO spin concentrator and injected into an S200 16/60 column (pre-equilibrated in GF Buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 0.5 mM TCEP, 5% Glycerol)) at 1.0 mL/min. 1.5 mL-fractions were collected. The eluted protein was cleaved overnight at 4 °C by TEV protease (1/20 (w/w)).
The following day protein sample was loaded onto 0.5ml Ni-sepharose column pre-equilibrated with GF buffer to remove uncleaved protein. Pooled protein fractions were concentrated to 13 mg/mL using a 30 kDa mwco concentrator.

Concentration: 85.0 mg/ml
Mass-spec Verification: Yes

Structure Determination

Crystallization: For the fragment screening campaign, crystals were soaked with compounds (10/50/500 mM) in the crystallization solution supplemented with 8% butanediol for 5-30 min, and frozen in liquid nitrogen.

Data Collection: *Beamline:* Dmnd I04-1; *Resolution:* 2.67 Å
Data Processing: For the fragment screening campaign, ligands were identified by DIMPLE [https://github.com/ccp4/dimple] using difference density maps. Weaker binders with low occupancy were evaluated using PANDDA [https://pandda.bitbucket.io/], based on statistical models to find ligand density present in a given dataset that is not present in majority of datasets.