

## XX06CUL3A (4AP2) Materials & Methods

### KLHL11: Kelch-like protein 11, BTB/BACK domains Material & Methods

**Entry Clone Source:** IMAGE

**SGC Construct ID:** KLHL11A-c003

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

**DNA sequence:**

```
CATATGCACCATCATCATCATCATTCTTC
TGGTAGATCTGGGTACCGAGAACCTGT
ACTTCCAATCCATGGAAGCCGAGGATTTC
GAGTGCAGCTCTCACTGCTCAGAGCTGTC
CTGGCGGCAGAACCGAGCAGCGGCCAGG
GCCTCTCTGCGACATTACCCCTGTGCTTC
GGCGGGGCTGGAGGCCGCGAGTTCCGGGC
CCACCGCTCGGTACTGGCTGCCGCCACCG
AGTACTTCACGCCCTGCTCTGGGCCAG
TTTCCGAGTCCCGCTCGGACGGGTGGA
GATGCGCAAGTGGAGCTCCGAGCCGGGC
CCGAACCCGACACAGTGGAAAGCCGTAATC
GAGTACATGTACACCAGCGCATCCCGT
CAGCACGGGCAGCGTGCACGAGGTGCTGG
AGTGGCCGACAGGTTCTACTCATCGT
TTAAAAGAATTTGTGGAGAATTCTCAA
GAAAAAAACTTCATCTCTCAAATTGTGTGG
CAATTCTAGCTTAGCACACATGTACACC
CTGAGCCAACCTGCTCTGAAGGCTGCTGA
TATGATACGGAGAAATTCCACAAAGTGA
TTCAGGATGAAGAATTTATACGTTACCT
TTCCATCTCATTAGAGACTGGCTTCAGA
TTTGGAAATTACAGTTGATTCTGAAGAGG
TTCTCTTGAAACCGTTTGAAATGGTT
CAGAGAAATGCTGAAGAGAGAGAGAGATA
CTTGAGAAACTTTAAATTGCTCAGGT
TGTCCCAGATGAAACCTACCTACCTACT
CGACATGTCAAACCAAGAGAGAGCTGGTAGC
CAATAATGAAGTTGTCAAGTTGGTCG
CTGACGCAGTGGAGAGACATGCTCTGAGA
GCTGAGAATATAACATCTGGCACATGACA
GTAAAGGTGGATACGGATCCGAA
```

**Final protein sequence (Tag sequence in lowercase):**

```
mhhhhhhssgvdlgtenlyfq^sMEA
EDFECSSHCELSWRQNEQRQGLFC
DITLCFGGAGGREFRAHRSVLAAATE
YFTPLLSQFSESRSGRVEMRKWSSE
PGPEPDTVEAVIEYMYTGRIRVSTGS
VHEVLELADRFLLIRLKEFCGEFLKK
KLHLSNCVAIHSLAHMYTLSLALKA
```

ADMIRRNFKVIQDEEFYTLPFHLIR  
DWLSDLEITVDSEEVLFETVLKWVQR  
NAEERERYFEELFKLLRLSQMKPTYL  
TRHVKPERLVANNEVCVKLVADAVER  
HALRAENIQSGT

^ TEV protease recognition site

**Tags and additions:** Cleavable N-terminal His6 tag

**Host:** BL21(DE3)-R3-pRARE2.

**Growth Medium & Induction Protocol:** A glycerol stock was used to inoculate a 10ml starter culture containing LB media with 50 $\mu$ g/ml kanamycin and 34  $\mu$ g/ml chloramphenicol. The starter culture was grown overnight at 37°C with shaking at 200 rpm. The following morning, four 2L baffled flasks containing 1 L Terrific Broth (TB) media/kanamycin were each inoculated with 2 ml of the starter culture. Cultures were incubated at 37°C with shaking at 180 rpm until an OD<sub>600nm</sub> = 1.5 was reached. The flasks were then cooled down to 18°C. Protein expression was induced by addition of IPTG to a final concentration of 0.5mM and expression carried out overnight. Cells were harvested by centrifugation at 6000 rpm at 4°C for 15 min. Cell pellets from each flask were resuspended in 30ml binding buffer (50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole), transferred to 50 ml tubes, and stored at -20°C.

**Extraction buffer, extraction method:** The frozen cells were thawed and 0.5mM TCEP, 1mM PMSF added to the cell suspension. The cells were lysed using an Emulsiflex C5 cell homogeniser. The cell lysate was clarified by centrifugation at 20000 rpm at 4°C for 1 h. The supernatant was recovered for purification and filtered through a syringe filter with a 1.2 $\mu$ m pore size.

**Column 1:** Ni-Affinity Chromatography  2.5ml Ni-sepharose slurry applied to a 1.5 x 10 cm column.

**Column 1 Buffers:**

**Binding buffer:** 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole

**Wash buffer:** 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 30mM Imidazole

**Elution buffer:** 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 50 to 250mM Imidazole

**Column 1 Procedure:** 2.5ml of 50 % Ni-sepharose slurry (Amersham) was added to the filtered lysate, which was incubated with the Ni-sepharose for 1 hour at 4°C with slow rotation to maximize binding. The lysate was then applied to a 1.5 x 10 cm column by gravity flow and allowed to drip through. The remaining resin was then washed with 2x50ml binding buffer and 1x30ml wash buffer to remove nonspecifically binding proteins. The bound target protein was eluted by applying a step gradient of imidazole (5 ml fractions of elution buffer supplemented with 50mM, 100mM, 150mM and 250mM imidazole). The protein content of collected fractions was visualized using SDS-PAGE and fractions containing KLHL11 were pooled.

**CUL3: Cullin 3 N-terminal domain Material & Methods**

**Entry Clone Source:** Synthetic

**SGC Construct ID:** CUL3A-c012

**Vector:** pNIC-CTHF. Details [[PDF](#)]; Sequence [[FASTA](#)] or [[GenBank](#)].

**DNA sequence:**

CTTAAGAAGGAGATATACTATGAGCAACC  
TGTCCAAGGGTACCGGCAGTCGTAAGGAT  
ACCAAAATGCGTATCCGCGCCTTCCGAT  
GACTATGGACGAAAATACGTCAATTGCA  
TTTGGGATCTGCTGAAGAACGCTATCCAG  
GAGATTCAAGCGTAAAACAATTCTGGTCT  
GTCATTGAAGAGCTGTACCGCAACGCCT  
ATACTATGGTGCACAAAGCATGGTGA  
AAACTGTATAACGGCCTGCGTGAAGTGGT  
CACCGAGCACCTGATCAATAAGGTGCGCG  
AGGATGTCTGAACAGCCTGAATAATAAC  
TTCCTGCAGACCCCTGAATCAGGCCTGGAA  
CGACCATCAGACCGCTATGGTATGATCC  
GTGACATTCTGATGTACATGGATCGCGTT  
TATGTGCAGCAGAATAACGTCGAAAATGT  
TTACAACCTGGGCTGATCATTTTCGTG  
ACCAGGTTGTGCGCTATGGCTGCATCCGT  
GACCACCTGCGCCAGACCCCTGCTGGATAT  
GATTGCCCGTGAACGCAAGGGCGAGGTGCG  
TTGATCGTGGCGCCATCCGCAACGCTTGT  
CAGATGCTGATGATTCTGGGCTGGAGG  
CCGTTCTGTCTACGAAGAGGACTTCGAGG  
CGCCCTTCTGAAATGAGTGCAGAGTTC  
TTTCAGATGGAATCGCAGAAATTCCCTGGC  
GGAAAACAGCGCATCAGTTATATCAAGA  
AAAGTGAAGCTCGTATCAACGAAGAGATT  
GAGCGCGTTATGCATTGCCTGGATAAGTC  
AACCGAAGAGCCAATTGTGAAAGTGGTGC  
AACCGCGAGCTGATCTCAAGCACATGAAA  
ACCATTGTCGAAATGGAAAACAGCGGCCT  
GGTTCATATGCTGAAGAACGGTAAACCG  
AAGACCTGGGCTGTATGTACAAGCTGTTC  
AGCCGTGTGCCGAATGGCTGAAAACCAT  
GTGCGAATGTATGAGCAGCTATCTCGTG  
AGCAGGGCAAGGCCTGGTGTCCGAAGAG  
GGTGAAGGCAAAACCCCTGTGGACTACCG  
CCAGGGTCTGGACGATCTGAAGAGCGTT  
TCGACCGCTTCTGCTGGAGTCCTCAAT  
AACGATCGTCTGTTAACAGACCATTGC  
AGGCGACTTCGAATACTTCTGAATCTGA  
ACAGTCGCTGCCCGAGTATCTGGCAGAG  
AACCTCTACTTCCAATCGCACCATCATCA  
CCACCATGATTACAAGGATGACGACGGATA  
AGTGAGGATCC

**Final protein sequence (Tag sequence in lowercase):**

MSNL SKGT GSRK DTKMRIRAFPMTMD  
EKYVNSIWDLLKNAIQEIQRKNNNSGL  
SFEELYRNAYTMVLKHGEKLYTGLR  
EVVTEHLINKVREDVLSLNNNFLQT  
LNQAWNDHQ TAMVMIR DIL MYMDRVY  
VQQNNVENVYNLGLIIFRDQVVRYGC

IRDHLRQTLLDMIARERKGEVVDRGA  
IRNACQMLMILGLEGRSVYEEDEAP  
FLEMSAEFFQMESQKFLAENSASVYI  
KKVEARINEEIERVMHCLDKSTEEPI  
VKVVERELISKHMKTIVEMENGLVH  
MLKNGKTEDLGCYKLFSRVPNGLKT  
MCECMSSYLREQGKALVSEEGERGKNP  
VDYRQGLDDLKSRFDRFLLESFNNDR  
LFKQTIAGDFEYFLNLNSRSPEYLa  
nlyfq^shhhhhdykdddk

^ TEV protease recognition site

**Tags and additions:** Cleavable C-terminal His6 and FLAG tag

**Host:** BL21(DE3)-R3-pRARE2.

**Growth Medium & Induction Protocol:** A glycerol stock was used to inoculate a 20ml starter culture containing LB media with 50 $\mu$ g/ml Kanamycin and 34  $\mu$ g/ml chloramphenicol. The starter culture was grown overnight at 37°C with shaking at 200 rpm. The following morning, six 2L baffled flasks containing 1 L Terrific Broth (TB) media/kanamycin were each inoculated with 2 ml of the starter culture. Cultures were incubated at 37°C with shaking at 180 rpm until an OD<sub>600nm</sub> = 1.5 was reached. The flasks were then cooled down to 18°C. Protein expression was induced by addition of IPTG to a final concentration of 0.5mM and expression carried out overnight. Cells were harvested by centrifugation at 6000 rpm at 4°C for 15 min. Cell pellets from each flask were resuspended in 30ml binding buffer (50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole), transferred to 50 ml tubes, and stored at -20°C.

**Extraction buffer, extraction method:** The frozen cells were thawed and 0.5mM TCEP, 1mM PMSF added to the cell suspension. The cells were lysed using an Emulsiflex C5 homogeniser. The cell lysate was clarified by centrifugation at 20000 rpm at 4°C for 1 h. The supernatant was recovered for purification and filtered through a syringe filter with 1.2 $\mu$ m pore size.

**Column 1:** Ni-Affinity Chromatography  2.5ml Ni-sepharose slurry applied to a 1.5 x 10 cm column.

**Column 1 Buffers:**

**Binding buffer:** 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole

**Wash buffer:** 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 30mM Imidazole

**Elution buffer:** 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 50 to 250mM Imidazole

**Column 1 Procedure:** 2.5ml of 50 % Ni-sepharose slurry (Amersham) was added to the filtered lysate, which was incubated with the Ni-sepharose for 1 hour at 4°C with slow rotation to maximize binding. The lysate was then applied to a 1.5 x 10 cm column by gravity flow and allowed to drip through. The remaining resin was then washed with 2x50ml binding buffer and 1x30ml wash buffer to remove nonspecifically binding proteins. The bound target protein was eluted by applying a step gradient of imidazole (5 ml fractions of elution buffer supplemented with 50mM, 100mM, 150mM and 250mM imidazole). The protein content of collected fractions was visualized using SDS-PAGE and fractions containing CUL3 were pooled.

**Complex Material & Methods**

**Column 1:** Size Exclusion Chromatography □ S200 HiLoad 16/60 Superdex run on ÄKTAXpress.

**Column 1 Buffers:**

**Gel Filtration buffer:** 10mM HEPES pH7.5, 150mM NaCl, 1mM TCEP

**Column 1 Procedure:** Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. The two partially purified proteins were combined in an equimolar ratio and concentrated to a volume of 2ml using an Amicon Ultra-15 filter with a 10kDa cut-off. The concentrated protein complex was applied to the equilibrated S200 16/60 column, and run at a flow-rate of 1 ml/min. Fractions were visualized using SDS-PAGE and those containing the complex were pooled.

**Column 2:** Ion Exchange Chromatography □ HiTrap Q HP 5ml run on Äkta Express

**Column 2 Buffers:**

**IEX buffer A (binding):** 50mM HEPES pH7.5, 50mM NaCl, 0.5mM TCEP

**IEX buffer B (elution):** 50mM HEPES pH7.5, 500mM NaCl, 0.5mM TCEP

**IEX sample buffer:** 50mM HEPES pH7.5, 0.5mM TCEP

**Column 2 Procedure:** Prior to applying the protein, the HiTrap Q HP 5ml column was washed with buffer B and equilibrated in buffer A. Eluted protein from the gel filtration column was diluted in sample buffer 10x to give a final buffer concentration of 50mM NaCl. The diluted protein was applied onto the equilibrated HiTrap Q column and the column washed with 4 column volumes of buffer A. Bound proteins were eluted with a gradient up to 50% buffer B over 100ml at 4 ml/minute and 1ml fractions were collected by peak detection. Fractions were visualized using SDS-PAGE and those containing the KLHL11/CUL3 complex were pooled. After concentration, some of the protein was immediately used for crystallization experiments and the rest was divided into 100 $\mu$ l aliquots and stored at -80°C.

**Mass spec characterization:** The purified native complex was homogeneous. KLHL11 had an experimental mass of 34473.3, consistent with the calculated mass of the protein (calculated MW of this species = 34474.3). CUL3 had an experimental mass of 47992.97, consistent with loss of the N-terminal initiator methionine (calculated MW of this species = 48125.1). 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.

**Concentration and complex formulation:** The purified complex was buffer exchanged into gel filtration buffer and concentrated to a final concentration of 17mg/ml (measured by OD<sub>280</sub> based on extinction coefficient 70250) in an Amicon Ultra-15 filter with a 10 kDa cut-off.

**Crystallization:** Initial crystal screens were performed with the full complex at 17 mg/ml. For diffraction-quality crystals, the protein was diluted to 8.5 mg/ml and buffered in 10mM HEPES, pH 7.5, 150mM NaCl, 0.5 mM TCEP. Crystals were grown at 20°C in 300 nl sitting drops mixing 150 nl protein solution with 150 nl of a reservoir solution containing 0.1 M BisTrisPropane pH 7, 25% PEG3350, 0.15M sodium iodide and 8% EthGly. On mounting crystals were cryo-protected with an additional 25% ethylene glycol.

**Data Collection:**

**Resolution:** 2.8 $\text{\AA}$  **X-ray source:** Diamond IO3

Crystals of the KLHL11/CUL3 complex diffracted to a resolution of 2.8 Å (scaled resolution). A full dataset was collected at 100 K on Diamond Light Source beamline I03. Crystals belonged to the space group I121 with unit-cell parameters a= 147.5 Å b= 40.2 Å c= 234.7 Å,

$\alpha=90^\circ$   $\beta=107.3^\circ$   $\gamma=90^\circ$ . A single complex was present in the asymmetric unit. Data were indexed and integrated using XDS and scaled using SCALA.

Initially, crystals were grown of a complex containing a smaller CUL3 construct with a truncated N-terminus (see SGC deposition target XX01CUL3A). Data were collected to 3.1 Å and experimental phases calculated. Crystals of this KLHL11/CUL3 complex were soaked for 30mins in the crystallization buffer supplemented with 2mM Thiomersal. Data were collected at Diamond Light Source and experimental phases calculated by SIRAS using SHARP. The phased and density-improved map was used to build an initial model using BUCCANEER. This model was further built and refined using COOT and REFMAC5, including the calculated Hendrickson Lattman coefficients as a restraint during refinement. This model (PDB 4APF) was then used as a molecular replacement solution for the larger KLHL11 / CUL3 complex described here (PDB 4AP2), which was completed in COOT and refined with BUSTER.