Project:
High resolution structural analysis of purified HTT proteins

Experiment:
Cloning and test expression of HTT fragment clones

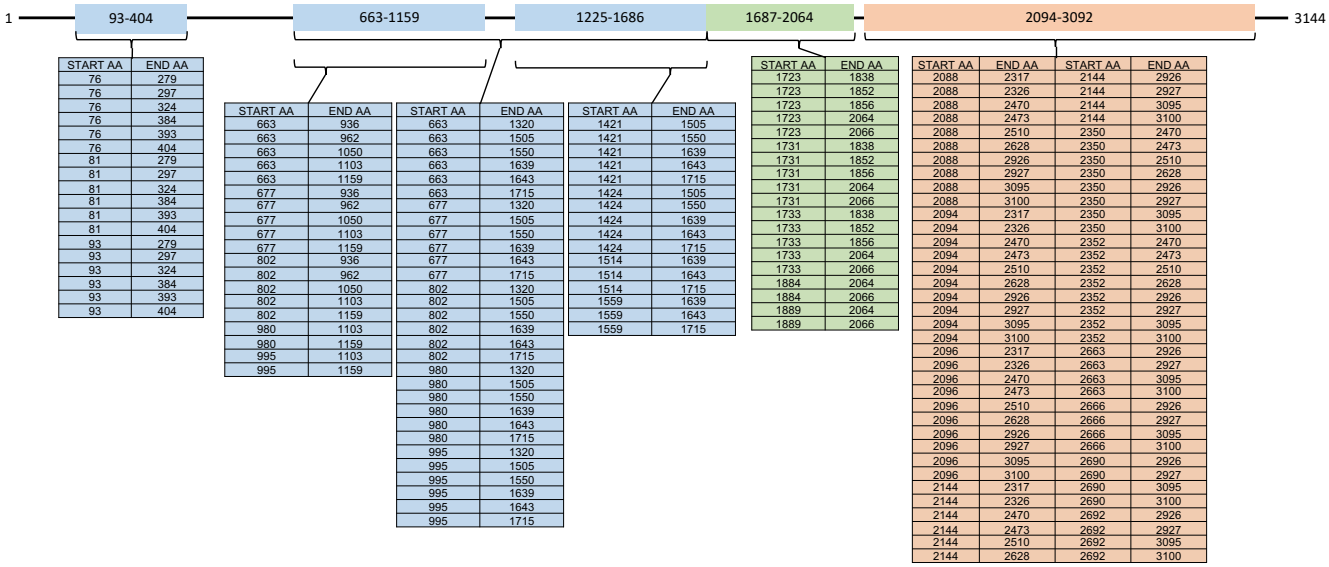
Date completed:
2019/02/28

Rationale:
I aim to complement our previous work on purifying stable regions of huntingtin by cloning further regions of huntingtin corresponding to discrete HEAT repeats for eukaryotic expression. Constructs will initially be screened by BVES in insect sf9 cells. High quality constructs will undergo extensive crystallization experiments and structure determination by X-ray crystallography using well established high-throughput and systematic protocols in place at the SGC. Any solved structures may allow generation of a pseudoatomic resolution (<4 Å) model of the HTT protein using the cryoEM model as a guide.

Experimental approach:

Materials and methods:

1. Design and cloning of HTT HEAT repeat domain constructs:



START AA	END AA
76	279
76	297
76	324
76	384
76	393
76	404
81	279
81	297
81	324
81	384
81	393
81	404
93	279
93	297
93	324
93	384
93	393
93	404

START AA	END AA
663	936
663	962
663	1050
663	1103
663	1159
677	936
677	962
677	1050
677	1103
677	1159
802	936
802	962
802	1050
802	1103
802	1159
980	1103
980	1159
995	1103
995	1159

START AA	END AA
663	1320
663	1505
663	1550
663	1639
663	1643
663	1715
677	1320
677	1505
677	1550
677	1639
677	1643
677	1715
802	1320
802	1505
802	1550
802	1639
802	1643
802	1715
980	1320
980	1505
980	1550
980	1639
980	1643
980	1715
995	1320
995	1505
995	1550
995	1639
995	1643
995	1715

START AA	END AA
1421	1505
1421	1550
1421	1639
1421	1643
1421	1715
1424	1505
1424	1550
1424	1639
1424	1643
1424	1715
1514	1639
1514	1643
1514	1715
1559	1639
1559	1643
1559	1715

START AA	END AA
1723	1838
1723	1852
1723	1856
1723	2064
1723	2066
1731	1838
1731	1852
1731	1856
1731	2064
1731	2066
1733	1838
1733	1852
1733	1856
1733	2064
1733	2066
1884	2064
1884	2066
1889	2064
1889	2066

START AA	END AA	START AA	END AA
2088	2317	2144	2926
2088	2326	2144	2927
2088	2470	2144	3095
2088	2473	2144	3100
2088	2510	2350	2470
2088	2628	2350	2473
2088	2926	2350	2510
2088	2927	2350	2628
2088	3095	2350	2926
2088	3100	2350	2927
2094	2317	2350	3095
2094	2326	2350	3100
2094	2470	2352	2470
2094	2473	2352	2473
2094	2510	2352	2510
2094	2628	2352	2628
2094	2926	2352	2926
2094	2927	2352	2927
2094	3095	2352	3095
2094	3100	2352	3100
2096	2317	2663	2926
2096	2326	2663	2927
2096	2470	2663	3095
2096	2473	2663	3100
2096	2510	2666	2926
2096	2628	2666	2927
2096	2926	2666	3095
2096	2927	2666	3100
2096	3095	2690	2926
2096	3100	2690	2927
2144	2317	2690	3095
2144	2326	2690	3100
2144	2470	2692	2926
2144	2473	2692	2927
2144	2510	2692	3095
2144	2628	2692	3100

1st round of HTT fragments cloned into pFBOH-MHL expression vector

Analysis of the published cryoEM HTT-HAP40 structure model reveals defined HEAT domains; N-HEAT 1 spans aa. 93-404, N-HEAT 2 consists of two sections spanning aa. 663-1159 and aa. 1225-1686, the BRIDGE spans aa. 1687-2064 and C-HEAT spans aa. 2094-3092. NB: all amino acid numbering in this report assumes a polyQ expansion of 23 glutamine residues. Informed by analysis of the structure, the

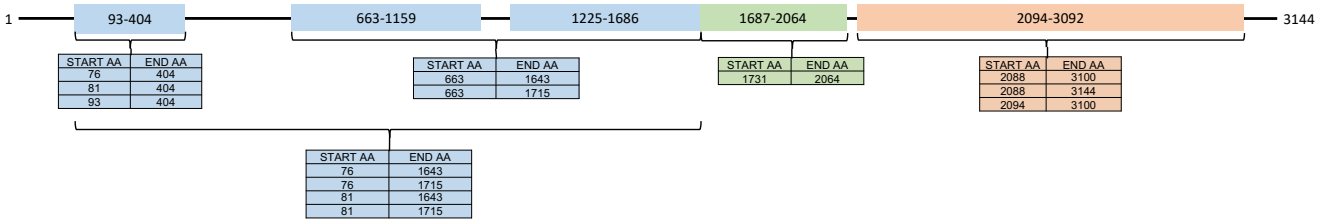
terminal residues comprising structured HEAT repeats and omitting large unstructured regions of the HTT molecule, constructs were designed for different HTT fragments for protein expression.

HTT fragments, with start and stop sites detailed, were cloned into the pFBOH-MHL expression vector using the ligase-independent cloning method. Baculovirus expression of these constructs yields proteins with N-terminal TEV-cleavable His-tag (MHHHHHHSSGRENLYFQG…).

In the second round of cloning, a more select number of HTT constructs comprising larger fragments or "complete" domains was cloned into the pBacMam2-DiEx-LIC expression vector, again using the ligase-independent cloning method. This expression vector permits protein expression through transient transfection of mammalian cells or viral transduction of insect or mammalian cells. Two constructs were made for each fragment, one with a simple C-terminal FLAG tag (…DYKDDDDK) and the other with a C-terminal, TEV cleavable dual His and FLAG tag (…AENLYFQSHHHHHHHHHHDYKDDDDK).

All constructs were sequence verified.

NB: all cloning was completed by Peter Loppnau.



2nd round of HTT fragments cloned into pBACMAM2-DiEx-LIC expression vector

2. Small-scale test expression in Sf9 cells:

For each construct, bacmids were generated and used to transfect Sf9 insect cells (Invitrogen). Passage 1 (P1) baculoviruses were harvested and used to set up P2 test expression cultures. 4 mL of culture was grown for each construct in deep-well 24-well blocks at 27 °C with 130 rpm shaking and harvested 72 hours after transfection by centrifugation. HyQ SFX insect serum medium containing 10 µg/mL gentamicin was used as the culture medium.

For pFBOH-MHL constructs, cells were lysed in 25mM Tris pH 8.0, 300 mM NaCl, 5% glycerol, 1 X protease inhibitor cocktail (1 µg/mL Aprotinin, 1 µg/mL Leupeptin, 2 µg/mL Pepstatin A and 1 µg/mL E-64), 2 mM imidazole, 0.6% NP-40 (v/v) and 5% glycerol (v/v) before lysates were clarified by centrifugation. Clarified lysates were bound with Ni-NTA for 30 mins with rocking and then washed twice with 25mM Tris pH 8.0, 300 mM NaCl, 5% (v/v) glycerol and 15 mM imidazole. Proteins were eluted with 30 µL 25mM Tris pH 8.0, 300 mM NaCl, 5% (v/v) glycerol and 500 mM imidazole. 10 µL 4x SDS-PAGE loading dye was added to eluted protein samples. Samples were analysed by 4-20 % Tris-Glycine SDS-PAGE.

For pBacMam2-DiEx-LIC constructs, cells were lysed in 25mM Tris pH 8.0, 500 mM NaCl, 5% glycerol, 1 X protease inhibitor cocktail (1 µg/mL Aprotinin, 1 µg/mL Leupeptin, 2 µg/mL Pepstatin A and 1 µg/mL E-64), 0.6% NP-40 (v/v) and 5% glycerol (v/v) before lysates were clarified by centrifugation. Clarified lysates were bound with anti-FLAG resin (Sigma M2) for 60 mins with rocking and then washed twice with 25mM Tris pH 8.0, 500 mM NaCl and 5% (v/v) glycerol supple. Proteins were eluted with 30 µL 0.1 M Glycine pH 3.0. 10 µL 4x SDS-PAGE loading dye was added to eluted protein samples. Samples were analysed by 4-20 % Tris-Glycine SDS-PAGE.

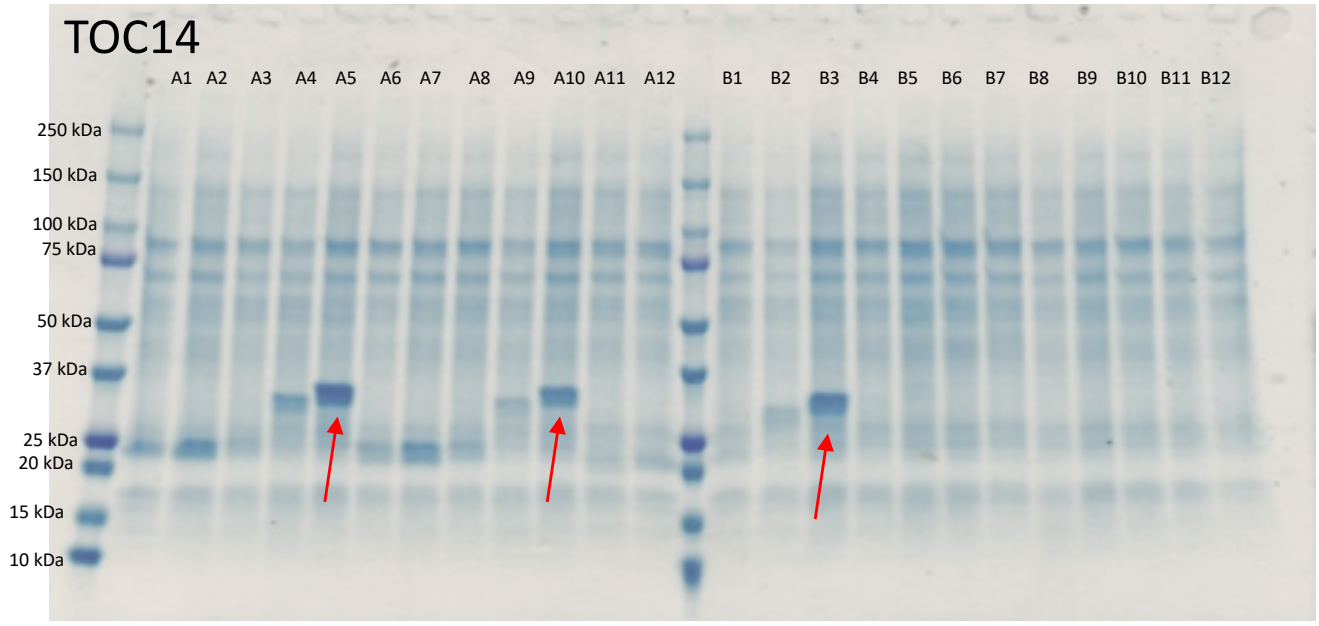
3. Large-scale expression and purification in Sf9 cells:

4 L of Sf9 cell culture at a density of ~4.5 million cells per mL were infected with 8 mL of P3 recombinant baculovirus and grown at 130 rpm and 27 °C. HyQ SFX insect serum medium containing 10 µg/mL gentamicin was used as the culture medium. Infected cells were harvested when viability dropped to 80%–85%, normally after ~72 h post-infection. Samples were purified through FLAG-affinity chromatography in scaled version of the test expression protocol.

NB: all production, test expression and large scale expression and purification was completed by Ashley Hutchinson, Alma Seitova, Linda Lin and I.

Results:

SDS-PAGE analysis of the small-scale test expression samples of pFBOH-MHL constructs shows very few expressed reasonable levels of soluble HTT protein. The highest levels of expression were seen for constructs TOC014-A05, A10 and B03. All three of these constructs correspond to the N-HEAT 1 domain, spanning aa. 76-404.



4-20 % Tris-Glycine SDS-PAGE of 1st round of HTT fragments cloned into pFBOH-MHL expression vector. Bands of the expected size for each construct are indicated with red arrows.

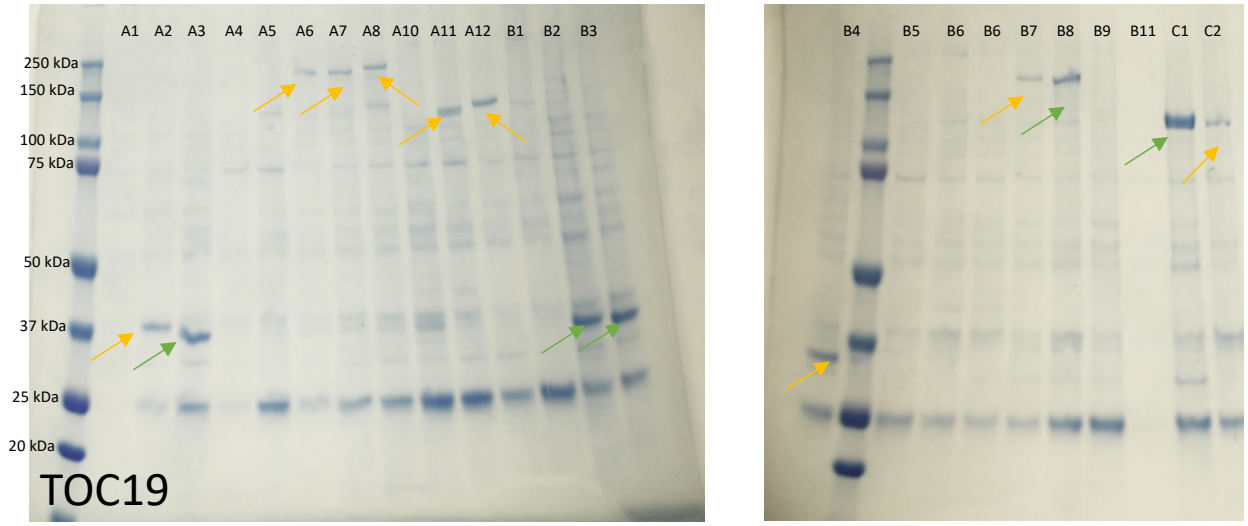
To follow up, Sf9 production of these three constructs was scaled to 4 L cultures but yielded no soluble HTT protein. This suggests that the protein is likely unstable or aggregated.

Construct ID	Construct AA start	Construct AA end	Batch and Plate Well	Corresponding Domain(s)	Tag	Follow up production
HTT_76_404-pFBOH-MHL	76	404	TOC014A05	N-HEAT 1	MHHHHHHSSGRENLYFQG…	4 L
HTT_81_404-pFBOH-MHL	81	404	TOC014A10	N-HEAT 1	MHHHHHHSSGRENLYFQG…	4 L
HTT_93_404-pFBOH-MHL	93	404	TOC014B03	N-HEAT 1	MHHHHHHSSGRENLYFQG…	4 L

Details of pFBOH-MHL HTT fragment constructs which showed some expression in small-scale Sf9 culture.

To overcome this issue, we cloned critical constructs comprising larger fragments or "complete" domains of HTT into the pBacMam2-DiEx-LIC expression vector. Previous experience in the lab, full-length HTT clones have yielded excellent expression levels of folded and soluble protein using this vector backbone. The C-terminal FLAG tag for purification permits more stringent purification from a large amount of cellular material compared to His-tag purification. Additionally, this construct allows for immediate transfer from insect cell to mammalian cell production systems, should HTT fragments prove extremely difficult to make *in vitro* in insect cell expression systems.

Good expression was seen for some of the pBacMam2-DiEx-LIC constructs. Encouragingly, constructs with the same insert sequence but different C-terminal tags show similar expression levels.



4-20 % Tris-Glycine SDS-PAGE of 2nd round of HTT fragments cloned into pBacMam2-DiEx-LIC expression vector. Bands of the expected size for each construct are indicated with green arrows for those showing good expression levels and yellow for those with lower levels of expression.

Construct ID	Construct AA start	Construct AA end	Pipeline Position	Batch and Plate Well	Corresponding Domain(s)	Tag	Follow up production
HTT_76_404-pBacMam2-DiEx-LIC	76	404	Cloned	TOC019A01	N-HEAT 1	…AENLYFQSHHHHHHHHHHDYKDDDDK	TestX
HTT_81_404-pBacMam2-DiEx-LIC	81	404	Cloned	TOC019A02	N-HEAT 1	…AENLYFQSHHHHHHHHHHDYKDDDDK	100 mL
HTT_93_404-pBacMam2-DiEx-LIC	93	404	Cloned	TOC019A03	N-HEAT 1	…AENLYFQSHHHHHHHHHHDYKDDDDK	4 L
HTT_663_1643-pBacMam2-DiEx-LIC	663	1643	Cloned	TOC019A04	N-HEAT 2	…AENLYFQSHHHHHHHHHHDYKDDDDK	TestX
HTT_663_1715-pBacMam2-DiEx-LIC	663	1715	Cloned	TOC019A05	N-HEAT 2	…AENLYFQSHHHHHHHHHHDYKDDDDK	TestX
HTT_76_1643-pBacMam2-DiEx-LIC	76	1643	Cloned	TOC019A06	N-HEAT 1 + 2	…AENLYFQSHHHHHHHHHHDYKDDDDK	100 mL
HTT_81_1643-pBacMam2-DiEx-LIC	81	1643	Cloned	TOC019A07	N-HEAT 1 + 2	…AENLYFQSHHHHHHHHHHDYKDDDDK	100 mL
HTT_76_1715-pBacMam2-DiEx-LIC	76	1715	Cloned	TOC019A08	N-HEAT 1 + 2	…AENLYFQSHHHHHHHHHHDYKDDDDK	100 mL
HTT_81_1715-pBacMam2-DiEx-LIC	81	1715	Cloning Failed	TOC019A09	N-HEAT 1 + 2	…AENLYFQSHHHHHHHHHHDYKDDDDK	N/A
HTT_1731_2064-pBacMam2-DiEx-LIC	1731	2064	Cloned	TOC019A10	BRIDGE	…AENLYFQSHHHHHHHHHHDYKDDDDK	TestX
HTT_2088_3100-pBacMam2-DiEx-LIC	2088	3100	Cloned	TOC019A11	C-HEAT	…AENLYFQSHHHHHHHHHHDYKDDDDK	100 mL
HTT_2088_3144-pBacMam2-DiEx-LIC	2088	3144	Cloned	TOC019A12	C-HEAT	…AENLYFQSHHHHHHHHHHDYKDDDDK	100 mL
HTT_2094_3100-pBacMam2-DiEx-LIC	2094	3100	Cloned	TOC019B01	C-HEAT	…AENLYFQSHHHHHHHHHHDYKDDDDK	TestX
HTT_76_404-pBacMam2-DiEx-LIC	76	404	Cloned	TOC019B02	N-HEAT 1	…DYKDDDDK	4 L
HTT_81_404-pBacMam2-DiEx-LIC	81	404	Cloned	TOC019B03	N-HEAT 1	…DYKDDDDK	4 L
HTT_93_404-pBacMam2-DiEx-LIC	93	404	Cloned	TOC019B04	N-HEAT 1	…DYKDDDDK	100 mL
HTT_663_1643-pBacMam2-DiEx-LIC	663	1643	Cloned	TOC019B05	N-HEAT 2	…DYKDDDDK	TestX
HTT_663_1715-pBacMam2-DiEx-LIC	663	1715	Cloned	TOC019B06	N-HEAT 2	…DYKDDDDK	TestX
HTT_76_1643-pBacMam2-DiEx-LIC	76	1643	Cloned	TOC019B07	N-HEAT 1 + 2	…DYKDDDDK	100 mL
HTT_81_1643-pBacMam2-DiEx-LIC	81	1643	Cloned	TOC019B08	N-HEAT 1 + 2	…DYKDDDDK	4 L
HTT_76_1715-pBacMam2-DiEx-LIC	76	1715	Cloned	TOC019B09	N-HEAT 1 + 2	…DYKDDDDK	TestX
HTT_81_1715-pBacMam2-DiEx-LIC	81	1715	Cloning Failed	TOC019B10	N-HEAT 1 + 2	…DYKDDDDK	N/A
HTT_1731_2064-pBacMam2-DiEx-LIC	1731	2064	Cloned	TOC019B11	BRIDGE	…DYKDDDDK	TestX
HTT_2088_3100-pBacMam2-DiEx-LIC	2088	3100	Cloned	TOC019B12	C-HEAT	…DYKDDDDK	TestX
HTT_2088_3144-pBacMam2-DiEx-LIC	2088	3144	Cloned	TOC019C01	C-HEAT	…DYKDDDDK	4 L
HTT_2094_3100-pBacMam2-DiEx-LIC	2094	3100	Cloned	TOC019C02	C-HEAT	…DYKDDDDK	100 mL

Details of pBacMam2-DiEx-LIC HTT fragment constructs expression profiles in small-scale Sf9 culture. Constructs with bands of the expected size for each construct on SDS-PAGE are highlighted in green for good levels of expression and yellow for those with lower levels of expression.

Next Steps:

To verify the findings of this second round of test expression, all pBacMam2-DiEx-LIC constructs will be followed up. Small-scale 4 mL cultures will be repeated for those which gave no band on the SDS-PAGE to prevent assumption of a false-negative result (common when using high throughput expression and purification protocols). Constructs with low levels of expression will be verified for expression in 100 mL culture volume to allow generation of sufficient HTT fragment material to verify protein identity by mass spectrometry and anti-FLAG Western blot analysis. Constructs with high levels of expression will be scaled to 4 L culture.