HTT interaction studies with HMGB1 and nanobody iVHH4 by pull-down analysis (2018/01/15)

Background and rationale:

HTT has been reported to interact with HMGB1 by coimmunofluorescence microscopy and coimmunoprecipitation (https://www.ebi.ac.uk/intact/query/id:EBI-466029%20AND%20id:EBI-389432 Min et al (2013) J Immunol - Chaperone-like activity of high-mobility group box 1 protein and its role in reducing the formation of polyglutamine aggregates, PMID 23303669). From the current literature, it is unclear if this is a direct physical interaction and confirmation of the interaction with purified proteins has not been published to date.

Full-length and C-terminally truncated HMGB1 protein samples were recently purified from *E. coli* https://zenodo.org/record/1116432. HTT nanobody iVHH4 was purified from *E. coli* previously https://zenodo.org/record/1066182 which binds HTT proline rich domain at aa. This should act as a positive control for the pull-down assay with HTT. N-terminal FLAG-tagged HTT https://zenodo.org/record/803343 and C-terminal FLAG-tagged HTT https://zenodo.org/record/1116434 samples were purified previously. Both tagged versions of the HTT protein were assayed to circumvent possibility that the (exon1) binding site might be occluded through HTT-FLAG interaction.

Construct ID	Protein Expressed	Molecular Weight (kDa)	Protein Concentration (mg/mL)
TOC009-C01	HTT aa. 1-3144 N-terminal FLAG tag https://zenodo.org/record/803343	349	8.4
TOC009-D01	HTT aa. 1-3144 C-terminal FLAG tag https://zenodo.org/record/1116434	349	8.2
TOC011-A02	HMGB1 aa. 11-160 N-terminal His tag https://zenodo.org/record/1116432	19.6	10.3
TOC011-A05	HMGB1 aa. 1-215 N-terminal His tag https://zenodo.org/record/1116432	27.1	12.2
TOC011-A07	Nanobody iVHH4 aa. 1-126 cleaved C-terminal His tag https://zenodo.org/record/1066182	14.9	1.7

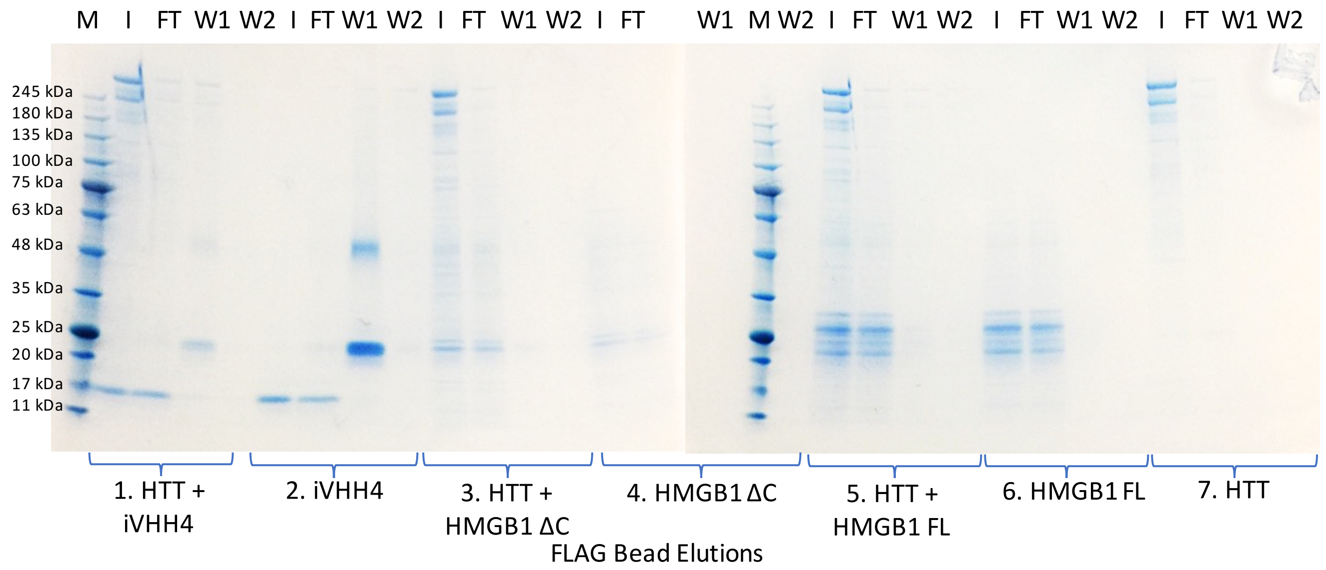
Pull down buffer:

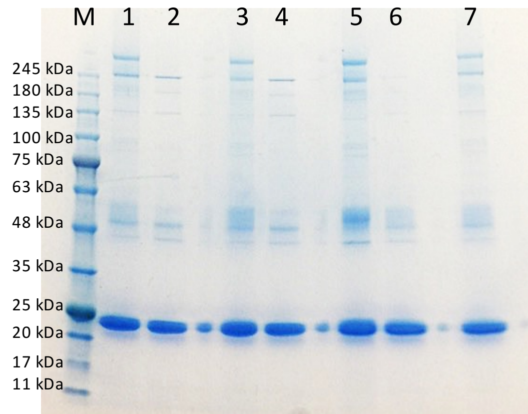
50 mM Tris pH 8, 150 mM NaCl, 0.005 % Tween-20.

Protocol:

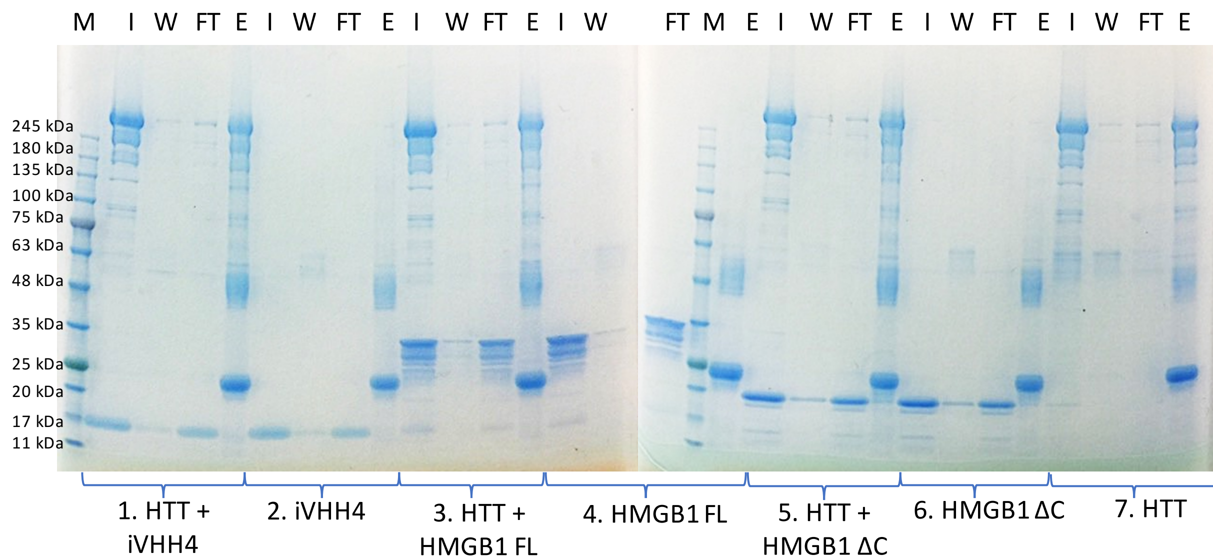
1. 20 µg HTT protein used with 5:1 molar excess of HTT binding partner.
2. Samples were incubated at 4 °C for 30 mins before binding to 20 µl FLAG beads per sample rocking at 4 °C for 30 mins.
3. Beads were washed with 3 x 1 mL buffer.
4. Samples eluted in 200 µL 1 x SDS loading buffer at 95 °C for 2 mins.
5. 10 µL (5 %) of input (I), flow through (FT) wash (W) and elution (E) loaded onto 4-20 % Tris-Glycine SDS-PAGE for each sample.

SDS-PAGE of Pull-Downs with N-terminal FLAG-tagged HTT:





SDS-PAGE of Pull-Downs with C-terminal FLAG-tagged HTT:



Analysis:

- Non-specific binding of HMGB1 and iVHH4 to the FLAG beads was not observed in the negative control pull-down experiments run without HTT.
- HMGB1 full-length (FL) and HTT constructs show signs of degradation due to the protein ladder seen under the dominant band on the SDS-PAGE.
- For both constructs, HTT does bind and can be eluted from the FLAG beads.
- For both the N-terminal and C-terminal FLAG-tagged HTT pull downs, neither HMGB1 nor iVHH4 protein samples bind HTT. In each case, HMGB1 and iVHH4 is seen in the input but nearly all of the sample comes out in the flow through (FT) fraction, with a small amount in the wash (W) and none in the HTT elution (E) fraction.
- iVHH4 should bind HTT protein samples according to published literature https://zenodo.org/record/1144553 so should act as a positive control for the pull-down assay.
- Possible reasons for not observing binding:
    - iVHH4 and HTT interact weakly
        - This is eluded to in the publication https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4341019/. iVHH4-HTT binding has not been characterised to determine affinity or specificity but co-IP was successful in PBS with post-mortem tissue samples and inverse i.e. iVHH protein immobilised on protein A beads.
    - The pull-down wash was too stringent
        - Try using less stringent conditions – will need to balance with not causing non-specific binding of the nanobody to the beads or HTT which would give a false positive readout.
    - iVHH4 is not folded properly
        - Mass spec confirms that the protein is the expected mass less 4 Da which are assumed to be due to the loss of 4 hydrogen atoms from the formation of 2 disulphide bonds which would indicate correct binding.
    - The motif for iVHH4 binding on HTT is not accessible to iVHH4 or has been cleaved off
        - N-tagged HTT must have the exon 1 sequence as otherwise the HTT would not bind the FLAG beads but perhaps it is not accessible once the HTT is bound. However this should be circumvented as the HTT and iVHH4 were incubated together on ice for 30 mins prior to FLAG bead binding. C-tagged HTT should have exposed and available exon 1 sequence but it may have been cleaved through partial degradation – this would be difficult to assess as the protein is too large for mass spectrometry ID.

Next steps:

- Repeat assay in PBS
- Repeat assay with protein A beads to immobilise the iVHH4 and both HTT constructs in solution.