Pull-Down with Purified Huntingtin and Putative interaction partners:

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Objective

Huntington's disease (HD) is a progressive neurological disorder caused by a mutation in the huntingtin gene, which encodes the huntingtin protein. Last year, the first structure of this protein was published. With a global resolution of approximately 4 Angstroms, this was a fantastic leap forward in our understanding of this protein. This structure helped our knowledge of Huntingtin, but there is still much more to find out. The structure published by this group is the only representative of about 75% of the protein, the other 25% being too mobile to capture using many high-resolution mapping techniques. One such mobile area is Exon 1, the location of the triplet repeat expansion responsible for HD. This critical region of the protein requires more structural information to understand. To obtain higher resolution images of this region, we are looking for huntingtin interaction partners that bind this region. These interaction partners will hopefully stabilize the structure enough to image the area. Interaction partners will be verified by a Pull-Down assay. Utilizing the FLAG-Tag and His-Tag present on HuntingtinPolyQ54+HAP40(HTT) complex used in this assay, we will look to identify reliable interaction partners.

Materials and Methods

All proteins used for this interaction assay are described in the table below. For specific volumes, concentrations, and experimental documentation, look to the supplementary information document.

Protein	Tag(s)	Mol. Weight (kDa)	Prot. Conc. (µg/µL)	Methods
HTTQ54HAP40	FLAG / His	390	4	10.5281/zenodo.3383264
RAB1A	His	24.7	120	10.5281/zenodo.3252165
RAB1B	His	20.1	48	10.5281/zenodo.3252174
SH3G3		39	16	10.5281/zenodo.3256579
PACN1		51	27	10.5281/zenodo.3256589
UBE2K		22.4	20	10.5281/zenodo.3267176
OPTN		65	1.2	10.5281/zenodo.3367278
PIAS1		41.1	5.5	10.5281/zenodo.3367282
HIP1		116	1.9	10.5281/zenodo.3367298
VCP		89	3.1	10.5281/zenodo.3367290
PCNA		28.8	0.45	Provided by collaborator
MutS α	FLAG	256	0.9	Provided by collaborator
MutS β	FLAG	231	1.5	Provided by collaborator
PFN1	His	19.9	5	Provided by collaborator

Initially, all proteins were tested using the His-Tag present on HAP40 for Nickel based Pull-Downs. Protein mixtures were made to accommodate a 5:1 molar ratio for the interaction partner and HTT respectively, then were diluted to a final volume of 75 µL in binding buffer [20mM HEPES pH 7.5, 150 mM NaCl]. The proteins were left to incubate together for 1 hour and 15 minutes (Initial – I). After the incubation period, 70 µL of ~1:1 Qiagen Ni NTA slurry was added and the protein mixture which then was rocked for 30 minutes. Subsequently, 3 x 1 mL washes of the Ni NTA beads were completed, the first with binding buffer and the next two with wash buffer [binding buffer + spiked in 10 mM Imidazole]. Elution of the beads took place in 80 µL of elution buffer [binding buffer + spiked in 300 mM Imidazole] (Elution – E). The resulting elution was concentrated to a volume of ~20 µL using a spin filter (MWCO 10 000) (Concentrated Elution – Conc. E).

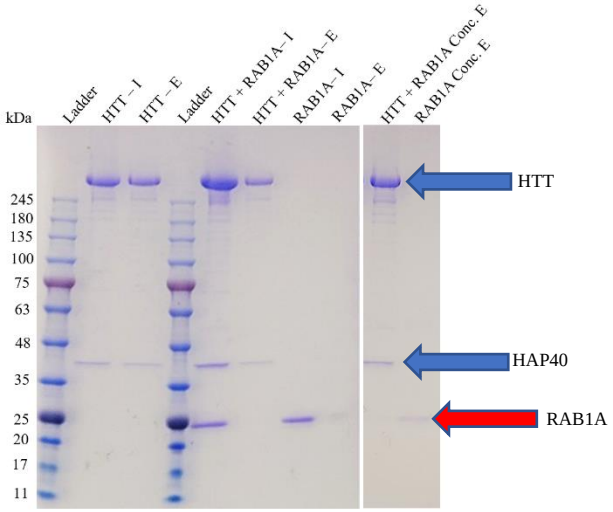
All of the proteins with the exception of those with their own FLAG-Tags, were also ran through a FLAG based Pull-Down. Much like the Nickel based Pull-Down, a 5:1 molar ratio was conserved, and the proteins were incubated together for 1 hour 15 minutes in 75 µL of binding buffer (Initial – I). The sample was then inoculated with 200 µL of FLAG resin 1:7 slurry and rocked for two hours. The resin was then washed 3 x 1 mL of binding buffer. 100 µL of Elution buffer [Binding Buffer + 10mM FLAG antibodies] was then rocked with the resin for 30 minutes (Elution – E). After this, 80 µL of the elution was concentrated to a volume of ~20 µL using a spin filter (MWCO 10 000) (Concentrated Elution – Conc. E).

Samples I, E and Conc. E for each protein were then run on a 4-20% Tris-Glycine SDS Page and stained with Imperial Stain. 17 µL of Sample I (15 µL sample: 5 µL loading dye) was loaded onto the gel for each protein. For E and Conc. E, 28 µL samples were loaded (21 µL sample: 7 µL loading dye) for each protein as well. This amount of loading sample was chosen to keep the overall amount of protein somewhat proportional, although the use of concentrators for the Conc. E samples did not maintain consistent protein levels.

Results

RAB1A

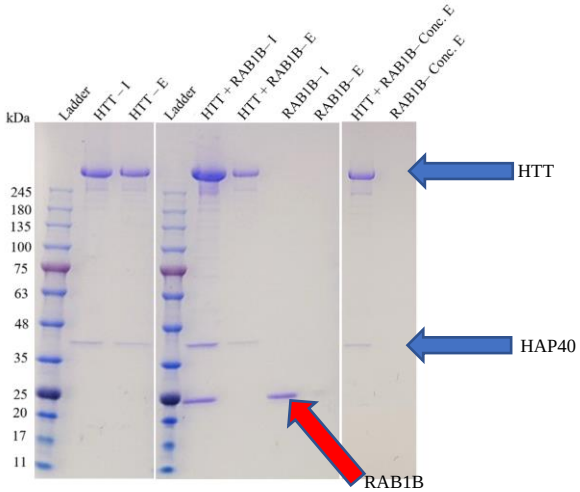
FLAG Pull-Down



There is no RAB1A observed from the HTT pull-down.

RAB1B

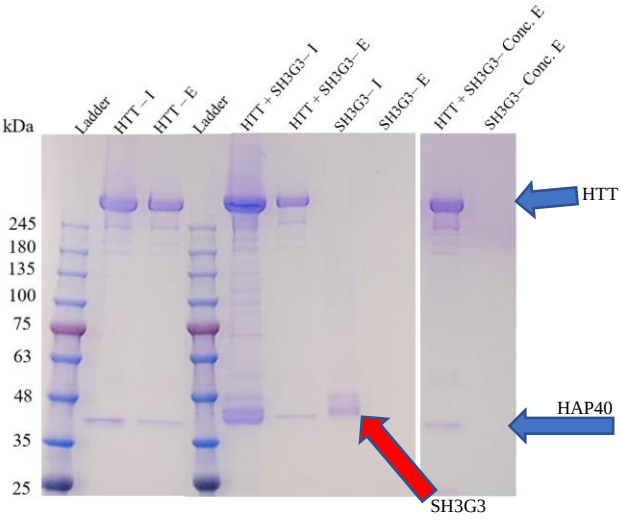
FLAG Pull-Down



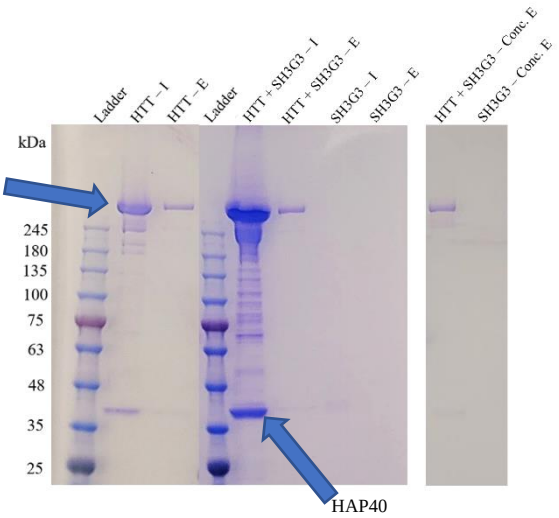
There is no RAB1B present in HTT + RAB1B – E, meaning that no interaction was detected between HTT-HAP40 Q54 and RAB1B by this assay.

SH3G3

FLAG Pull-Down



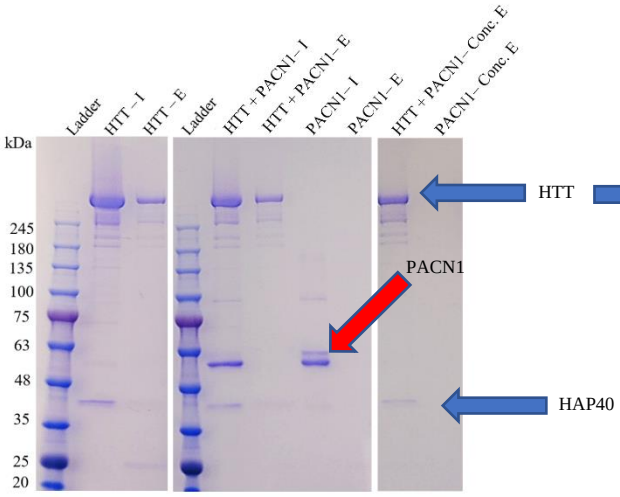
Nickel Pull-down



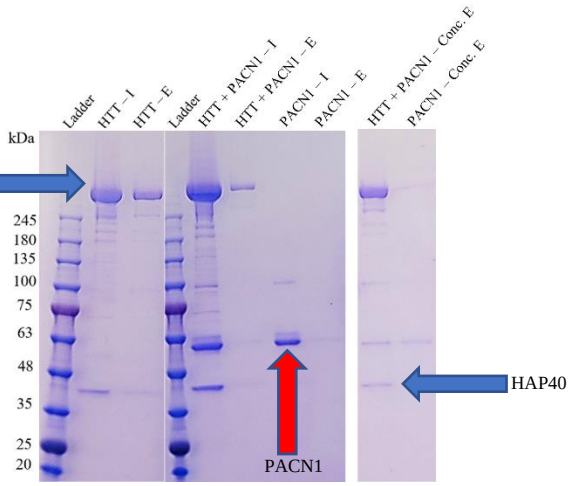
There is no SH3G3 present in HTT + SH3G3 – E, meaning meaning that no interaction was detected between HTT-HAP40 Q54 and SH3G3 by this assay. SH3G3 is not present at high enough quantities to observe in Nickel Pull-Down, this may have been due to degradation of sample over time. NB: that also the SH3G3 and HAP40 bands overlap so this is in no way conclusive.

PACN1

FLAG Pull-Down



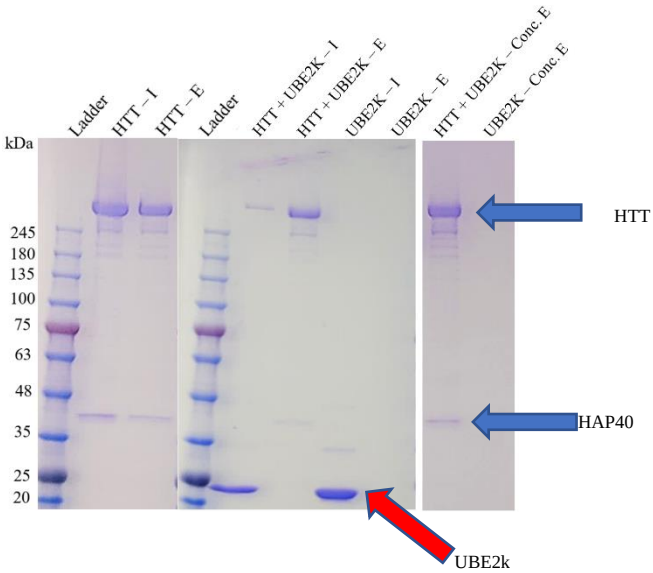
Nickel Pull-Down



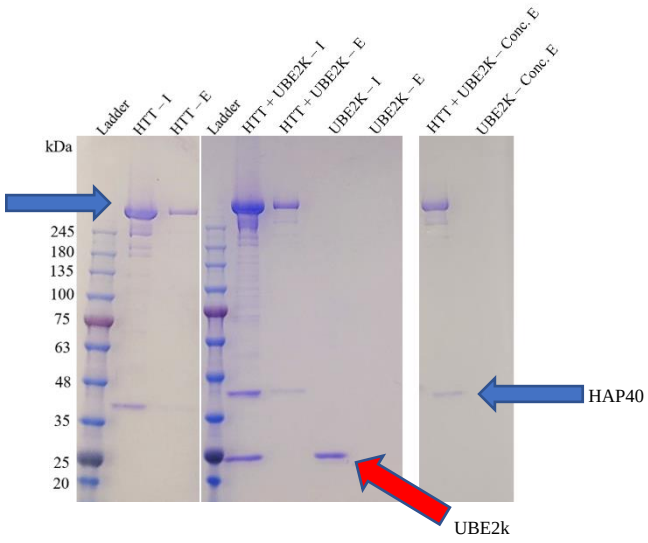
In the FLAG Pull-Down there is no PACN1 present in HTT + PACN1 – E, meaning meaning that no interaction was detected between HTT-HAP40 Q54 and PACN1 by this assay. For the Nickel Pull-Down, the protein is present by itself and with HTT indicating non-specific binding to Nickel Resin

UBE2K

FLAG Pull-Down



Nickel Pull-Down

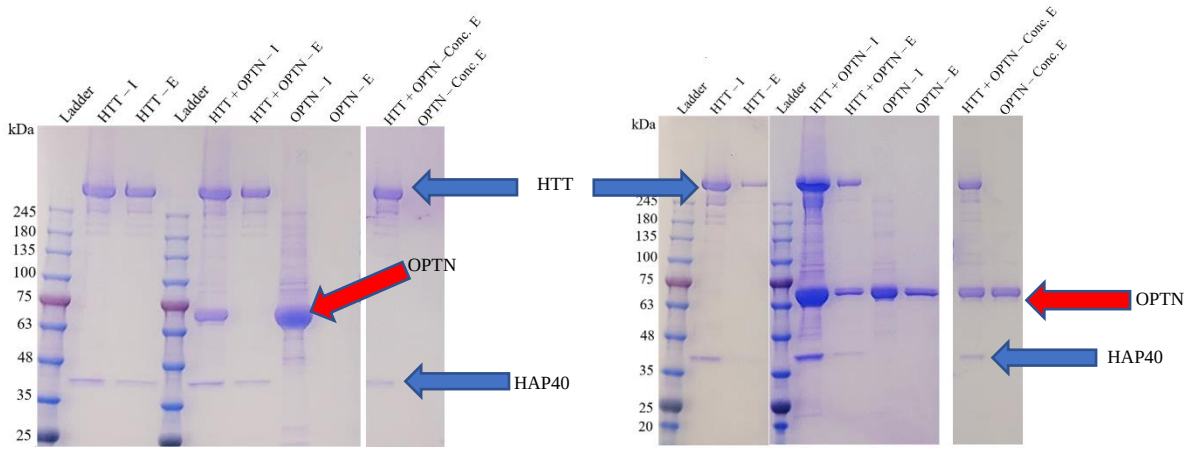


There is no UBE2K present in HTT + UBE2K – E, meaning meaning that no interaction was detected between HTT-HAP40 Q54 and UBE2K by this assay.

OPTN

FLAG Pull-Down

Nickel Pull-Down

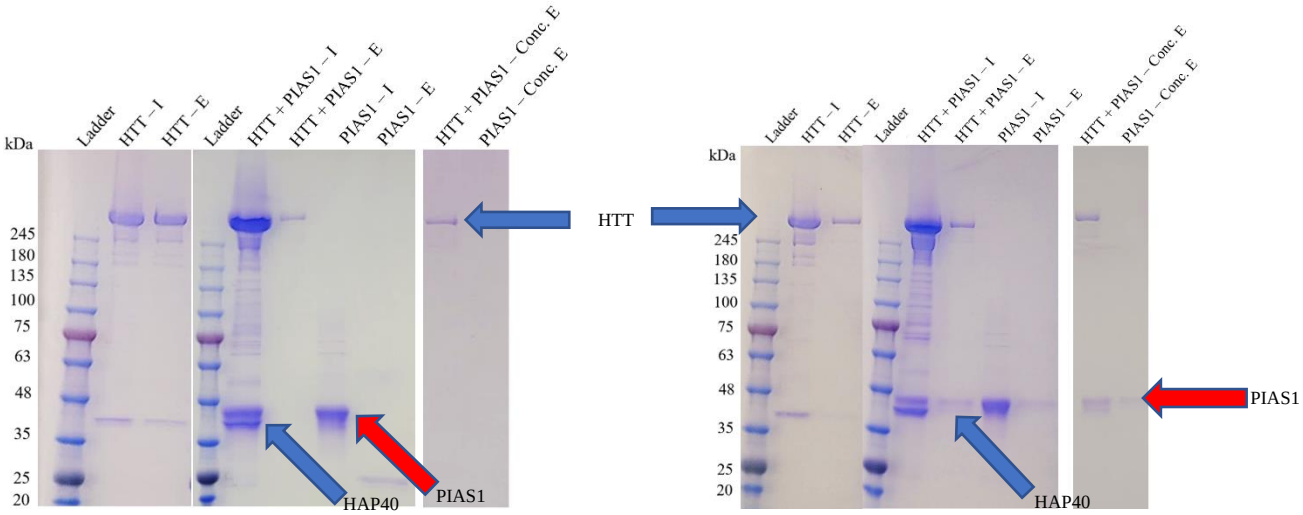


In the FLAG Pull-Down, no OPTN present in HTT + OPTN – E, meaning meaning that no interaction was detected between HTT-HAP40 Q54 and RAB1B by this assay. OPTN is in the OPTN – E for the Nickel Pull-Down sample which indicates non-specific binding.

PIAS1

FLAG Pull-Down

Ni Pull-Down

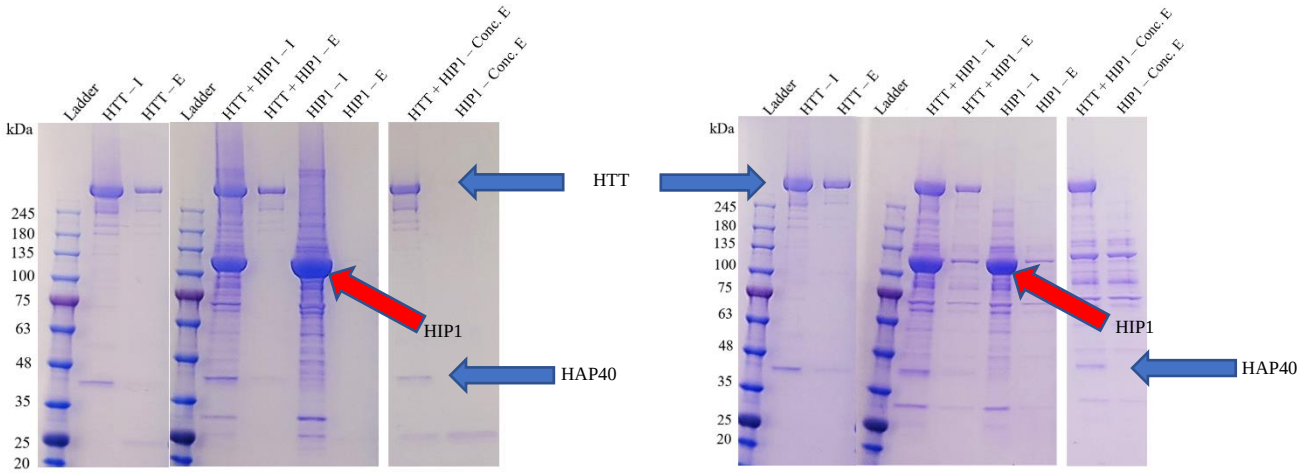


In the FLAG Pull-Down, no PIAS1 present in HTT + PIAS1 – E, meaning meaning that no interaction was detected between HTT-HAP40 Q54 and PIAS1 by this assay. PIAS1 is in the PIAS1 – E for the Nickel Pull-Down sample which indicates non-specific binding. PIAS1 is very close to HAP40 in molecular weight so it hard to distinguish the two. Further analysis must be done to ensure the Nickel Pull-Down was not a successful pull-down.

HIP1

FLAG Pull-Down

Nickel Pull-Down

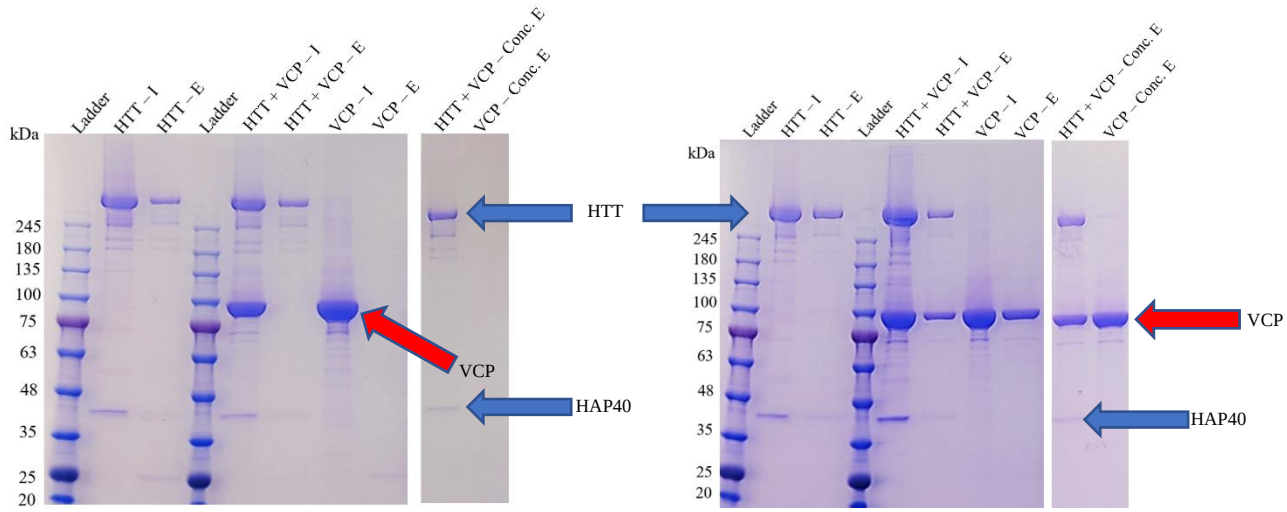


Especially visible in the Nickel Pull-Down, there is a high proportion of contaminants in this HIP1 sample. A few of these seemed to be carried by the pull-down but none of the HIP1 was pulled down. This was another lack of interaction detected between HTT-HAP40 Q54 and HIP1 by this assay.

VCP

FLAG Pull-Down

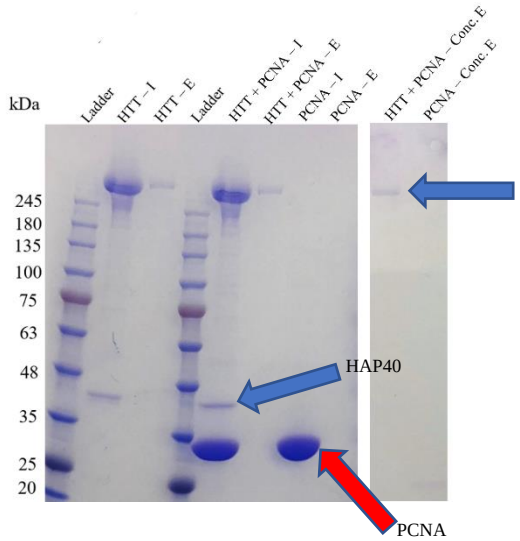
Nickel Pull-Down



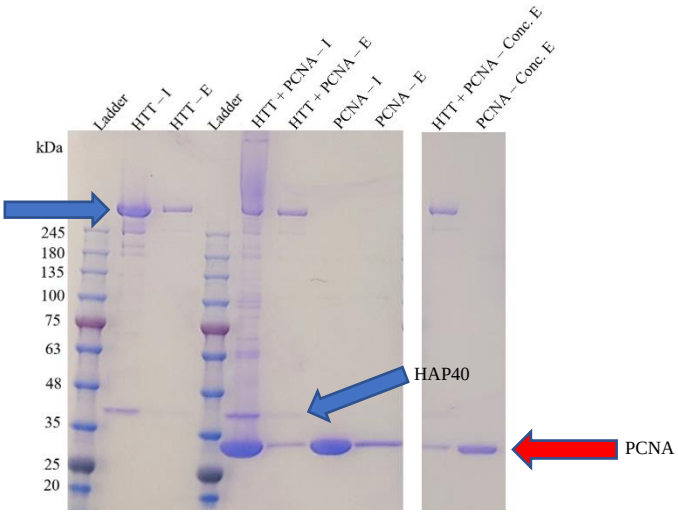
In the FLAG Pull-Down, no VCP present in HTT + VCP – E, meaning that no interaction was detected between HTT-HAP40 Q54 and VCP by this assay. VCP is in the VCP – E for the Nickel Pull-Down sample which indicates non-specific binding.

PCNA

FLAG Pull-Down



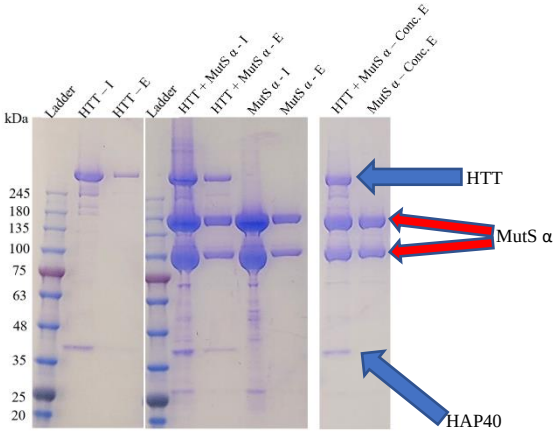
Nickel Pull-Down



In the FLAG Pull-Down, no PCNA present in HTT + PCNA – E, meaning that no interaction was detected between HTT-HAP40 Q54 and PCNA by this assay. PCNA is in the PCNA – E for the Nickel Pull-Down sample which indicates non-specific binding.

MutS α

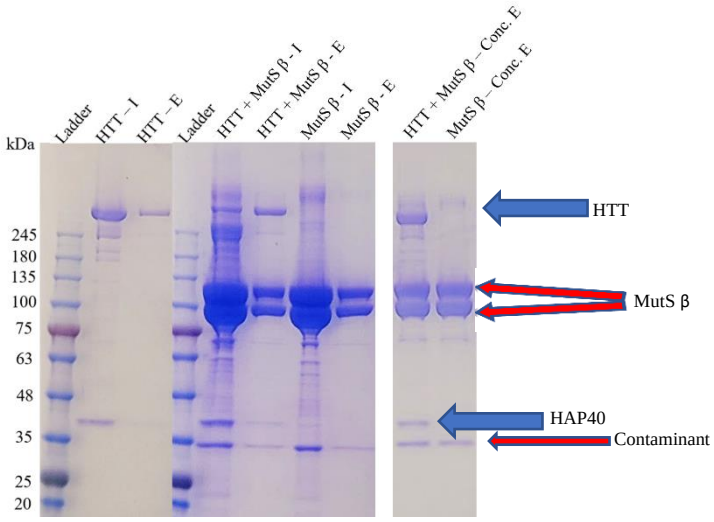
Nickel Pull-Down



MutS α is a heterodimeric complex, which is why separate bands are shown as MutS α. MutS α is in the MutS α – E sample for the Nickel Pull-Down sample which indicates non-specific binding.

MutS β

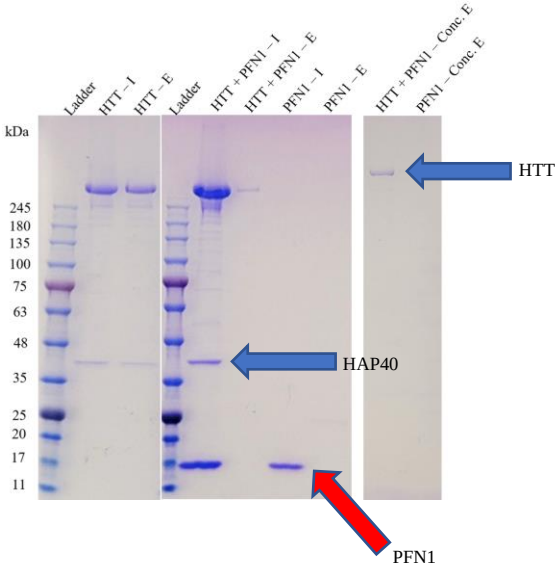
Nickel Pull-Down



MutS β is a heterodimeric complex, which is why separate bands are shown as MutS β. MutS β is in the MutS β – E sample for the Nickel Pull-Down sample which indicates non-specific binding.

PFN1

FLAG



Discussion

The goal of this set of experiments was to verify previously flagged HTT interaction partners through the method of HTT linked tag pull-downs. Through His and Flag tag pull-downs, no interactions were seen. The negative results are not necessarily because none of these proteins interact with HTT. This preliminary test was an assay chosen for its aptitude for medium throughput method, although some issues may have impaired our ability to observe interactions. The initial problem that was faced was false

positives. These false positives were particularly an issue with the Nickel-based pull-down. Non-specific binding was found to occur with many of the proteins. The non-specific binding led to several false positives, but by running the experiment alongside the interaction partner by itself, we were able to rule out these interactions as not with HTT but specifically with the NiNTA resin. Due to the increased stringency of FLAG resin, we did not see any false positives using this resin. Although in a few samples, there were low molecular weight bands present in Initial and Elution samples, it seems likely that these were just degradation products.

A complication arose concerning the relative strengths of the bands. The HTT molecule is roughly 390 kDa, while many of the interaction partners we are testing are a fraction of that size. Due to their disproportional size, if we expect to see a 1:1 reaction, the amount of protein loaded onto the final gel becomes an issue. For the interaction partner to be visible, the HTT in the sample would have to be overloaded, distorting the gel. If HTT is prioritized for proper loading amount, the amount of protein of the interaction partner would be too small to see. Two strategies resolved this issue. First, the staining of the gel was done by Imperial Stain, allowing for the colouring of bands containing $\geq$0.3 μg of protein. The use of a concentrated sample also improved our chances of seeing any interaction partners that were successfully pulled down. The experiment itself also presents a few issues; because we don't know the nature of these interactions, we were unable to ensure the tags used for the pull-down are not inhibiting the interactions that we could have otherwise seen; this is also true of HAP40 protein. Although it is essential for biochemical work with huntingtin, increasing its stability, we are unsure of how this protein works in vivo, as such, we don't know if the introduction of HAP40 is forcing conformational stability in HTT that would inhibit its interaction with any of the flagged putative interaction partners. Some proteins that have been tested are also known to bind to HTT inversely proportionally to PolyQ length, considering all testing here has been done with a PolyQ length of 54, this may also be decreasing the likelihood of some interactions, going forward using this method with a shorter PolyQ length will possibly provide better results. Using another technique, such as DLS may allow for more sensitive detection of interactions as well. There is still a lot to learn, but this has been a stride forward in the hunt for a better understanding of interactions of HTT protein.