***Instructions:*** *Fill in your name, institution, project title, and date of submission in the header. Use this form to report on all of your work during the entire funding period. For any data and figures, we are much more interested in the information they provide than in their artistic value, so please feel free to share all graphs and observations even if preliminary. Use as much space as necessary.*

**I. Progress Report Summary:** provide an overall summary of your progress, and describe the significance of your work to HD patients.

The major finding of the first year of the Berman-Topper fellowship was that many proteins that interact with the huntingtin protein bear a molecule involved in the DNA repair process: poly ADP ribose (PAR). This led us to investigate a connection between huntingtin and PAR signaling in the second year of the fellowship. Methods for detecting PAR were optimized, and a hyper-PAR phenotype was identified in HD patient fibroblasts and in HD mouse striatal precursor cells. The huntingtin-PAR interaction was confirmed in cells and *in vitro*.

Elevated PAR levels may arise from unrepaired DNA damage and lead to cellular energy deficits, both of which have been observed in many HD models and tissues. Our results introduce the exciting possibility that inhibitors of poly ADP ribose polymerase (PARP), drugs that have already been through clinical trials for a number of cancers, can be explored as a therapeutic option for HD. This is an avenue that has not been considered in the past, and shows promise for other neurodegenerative diseases including Parkinson’s, Alzheimer’s, and ALS.

What is needed at this point is rigorous pre-clinical testing of

* Whether PARP inhibitors affect HD model phenotypes
* Safety profiles of PARP inhibitor drugs that would make promising therapeutic leads

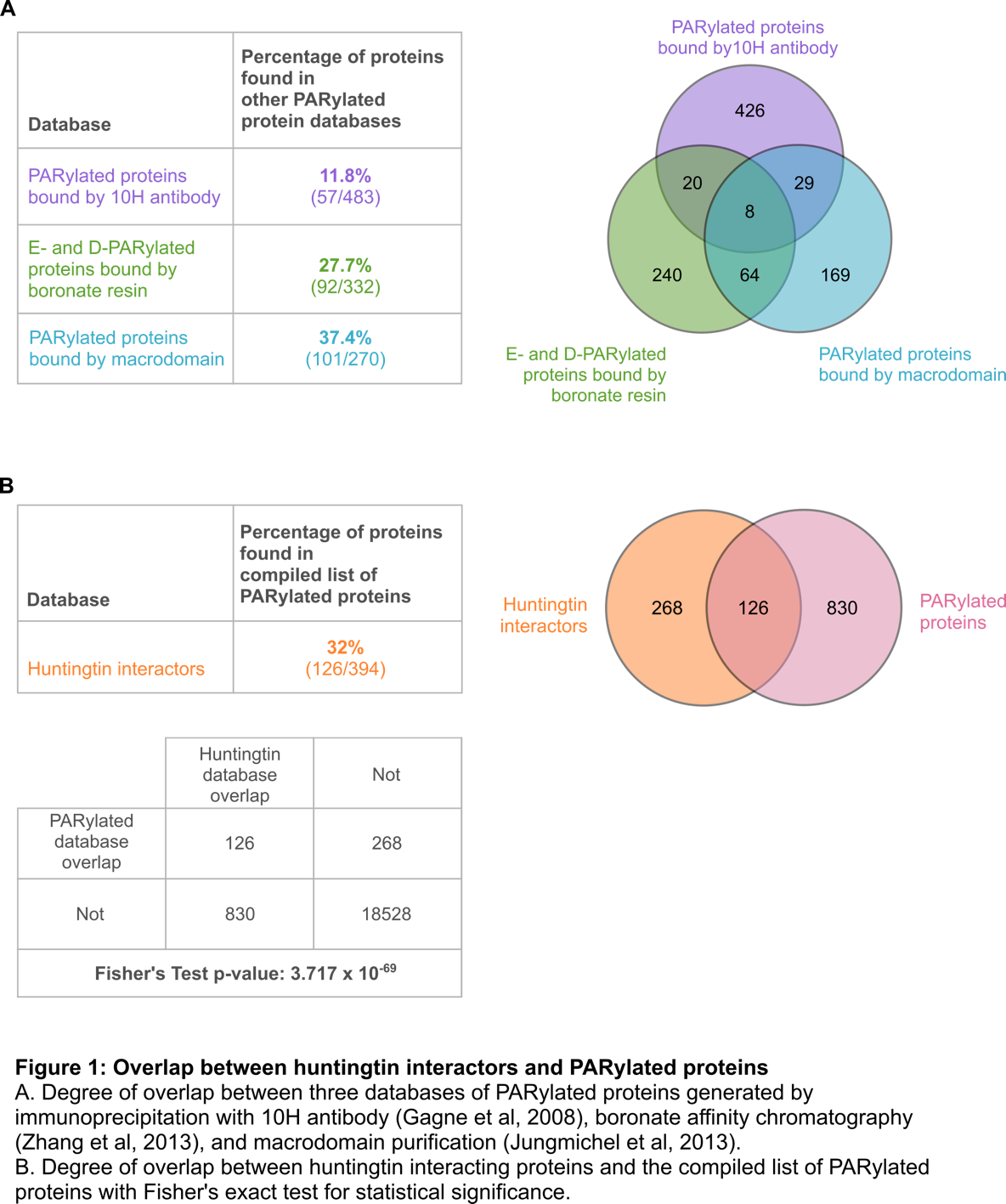
Preliminary experiments suggest that PARP inhibitors, which trap PARP at DNA, are co-trapping huntingtin at DNA. This is not likely to be beneficial as it may interfere with huntingtin function. As such, PARP lowering strategies will also be considered in the final year of the fellowship. Complete characterization of the mechanism and function of huntingtin PAR binding is also underway.

**II. Project Milestones:** these can be taken from the proposal.

|  |  |
| --- | --- |
| Milestone 1: Optimization of detection methods for proteins of interest | Complete |
| Milestone 2: Phenotypic characterization of immunofluorescence patterns | Complete |
| Milestone 3: Characterization of mechanism/function of huntingtin PAR binding | On-going |
| Milestone 4: Testing PAR phenotypes in additional HD tissues and models | On-going |

**III. Research Progress Toward Milestones:** use as much space as necessary to describe your progress towards the milestones in your proposal. Please include data and figures that are representative of your progress, even if preliminary.

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| **Background** |

 In the first year of the fellowship, we used mass spectrometry to identify huntingtin-interacting proteins under conditions of oxidative stress, and found that a significant proportion were common to three independently produced databases of PARylated proteins. The first database, generated by immunoprecipitation with the PAR-specific 10H antibody following alkylation-induced DNA damage of SK-N-SH (human neuroblastoma) cells, lists 483 PARylated proteins [1]. The second database was generated by alkylation-induced DNA damage of HCT116 (human colon carcinoma) cells followed by affinity chromatography with boronic acid-agarose, which forms an ester bond with a 1,2-*cis*-diol moiety of ADP-ribose. Elution by NH2OH leaves a hydroxamic acid derivative on aspartic acid and glutamic acid residues that can be distinguished by mass spectrometry. The resulting database lists 332 proteins representing the aspartic acid- and glutamic acid-ADP-ribosylated proteome [2]. The third database lists 270 proteins purified by the PAR-binding macrodomain, GST-Af1521, from U2OS (human bone osteosarcoma) cells after a panel of genotoxic stresses [3].

As would be expected, the different purification strategies yielded different sets of proteins, with some degree of overlap (Fig 1A). We then compiled the three databases and removed replicates to generate a final list of 956 PARylated proteins and compared it to the list of huntingtin-interacting proteins. As shown in Figure 1B, 126 of the 394 (32%) huntingtin-interacting proteins were also found in the list of PARylated proteins. Fisher’s test for significance returned a p-value of 3.7 x 10-69. Thus, a significant proportion of huntingtin-interacting proteins are reported to be modified by PAR.

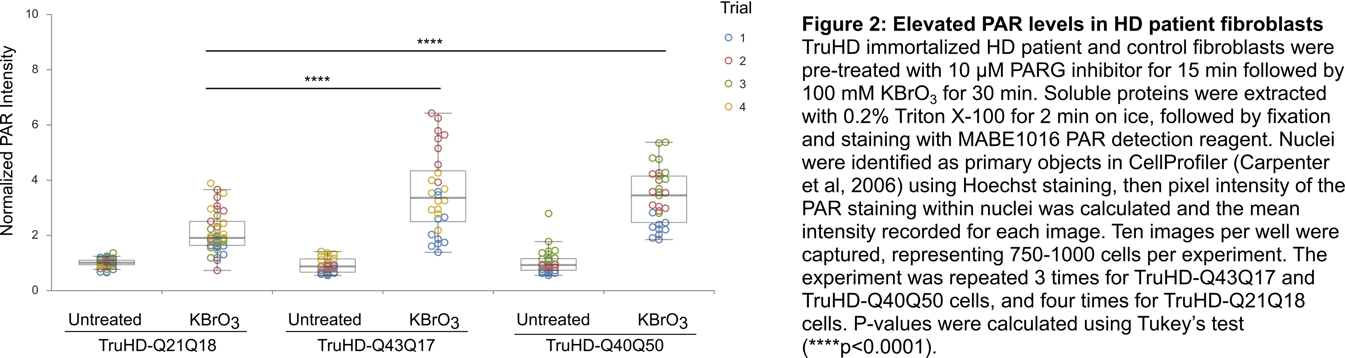
This led us to investigate a connection between huntingtin and PAR signaling.

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| **Milestone 1: Optimization of detection methods for proteins of interest** |

Optimization of the method for PAR detection was described in previous reports and a detailed analysis has been deposited to Zenodo ([link](https://zenodo.org/record/3251044#.XQvNmNNKh-U)). Briefly, pre-treatment with PARG inhibitor was necessary to prevent the rapid depolymerization of PAR chains. The pan-ADP ribose binding reagent MABE1016, a commercially available recombinant PAR-binding macro domain fused to rabbit Fc tag (Millipore), gave the best results. Extraction of soluble proteins before fixation was not necessary.

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| **Milestone 2: Phenotypic characterization of immunofluorescence patterns** |

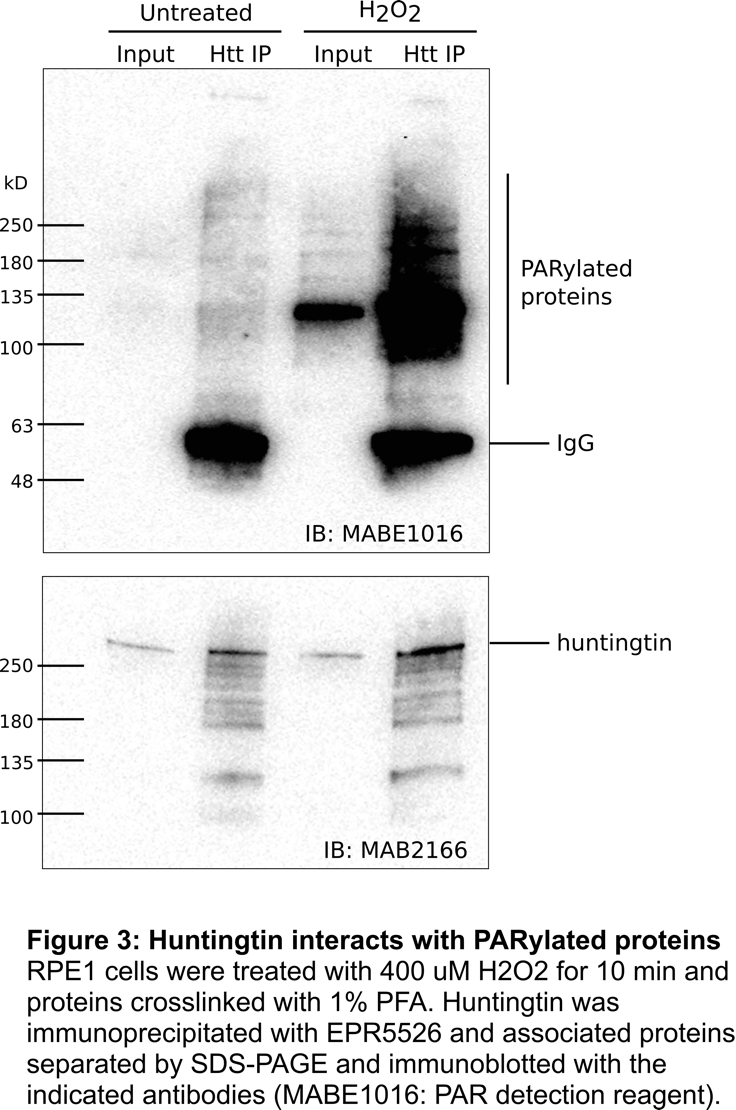
Immunofluorescence analysis of immortalized fibroblasts from HD patients and controls (TruHD cells, [4]) revealed a hyper-PARylation phenotype (Fig 2). Details of the experiments have been deposited to Zenodo ([link](https://zenodo.org/record/1464979#.XQvOcS0ZN-U)). This readout can now be used to investigate potential beneficial effects of small molecules.

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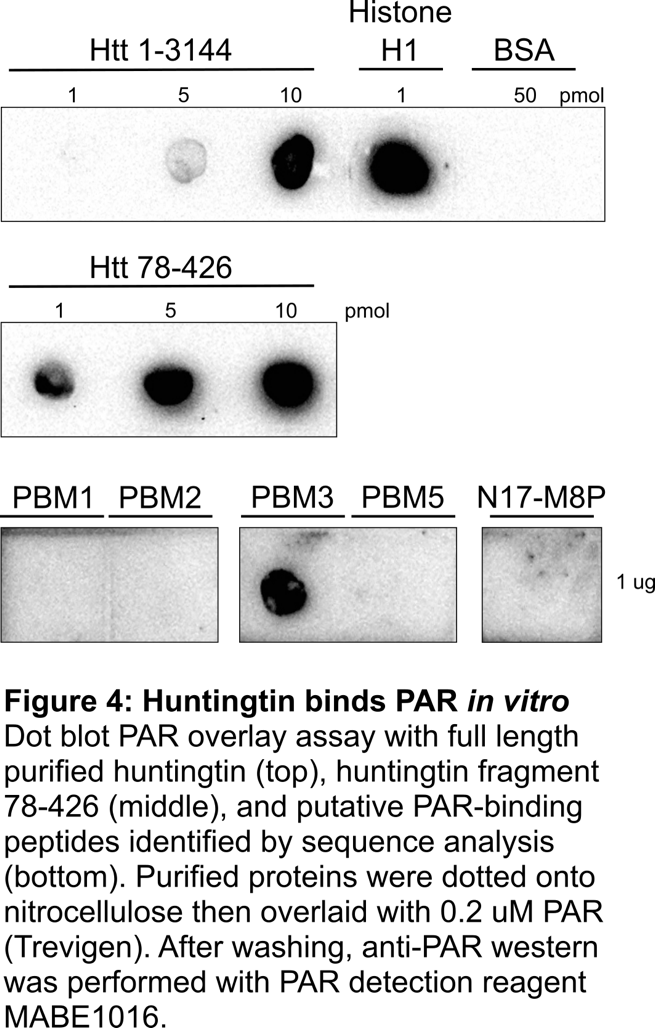
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| **Milestone 3: Characterization of mechanism/function of huntingtin PAR binding** |

***3. 1 Confirmation of mass spec results by co-immunoprecipitation***

Four trials of the huntingtin co-immunoprecipitation experiment measuring PARylated proteins have now been completed (Fig 3). The experimental details and results have been deposited to Zenodo (<https://zenodo.org/record/2586924>). Huntingtin reproducibly interacts with PARylated proteins in response to oxidative stress.



***3.2 Identification of PAR-binding motifs***

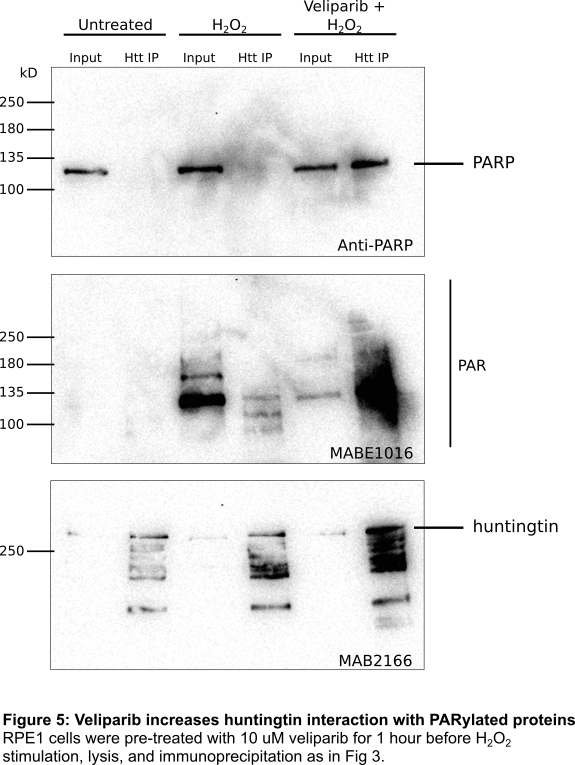
** Purified huntingtin (full length or a fragment spanning amino acids 78-426) binds PAR *in vitro* (Fig 4; experimental details deposited to Zenodo: [link](https://zenodo.org/record/2539483#.XQvOqS0ZN-U)). Huntingtin sequence analysis identified potential PAR-binding motifs, peptides of which were tested in the PAR overlay assay. The peptide residing within amino acids 78-426, PBM1, did not bind PAR *in vitro*. In addition, a PBM1 deletion mutant of huntingtin fragment 1-586 retained its ability to bind chromatin in response to oxidative stress (which may or may not represent a readout for the function of huntingtin PAR binding; see section 3.5). It therefore remains unknown which sequence within the 78-426 fragment mediates PAR binding, and which sequence within 1-586 mediates chromatin retention.

PBM3, a peptide corresponding to amino acids acids 1782-1804 of the huntingtin sequence (with the PBM at 1790-1798) bound PAR *in vitro*. Two YFP-tagged fragments of huntingtin containing this sequence were previously generated in the lab: 1208-1810 and 1775-2413 (Walpole et al, manuscript in preparation). These fragments were tested for their ability to bind chromatin in response to oxidative stress, however their low expression levels made it difficult to assess chromatin retention (data not shown). Further, both fragments appeared to reduce the expression of H2B-mCherry, which is included as a transfection control. Future experiments will compare transfection efficiency of the fragments with mCherry versus H2B-mCherry to see if the effect is specific to histone H2B, or whether these fragments affect global expression levels. Upon optimization of expression, PBM3 mutants will be made within the fragments for functional analysis.

Dr. Rachel Harding has now purified additional fragments of huntingtin, including the N- and C-terminal HEAT domains. These will be tested in the PAR overlay assay to further elucidate the mechanism of huntingtin PAR binding.

***3.3 Effect of PARP enzymatic inhibition on huntingtin-PAR interaction***

Despite reducing PAR levels, veliparib increased the interaction between huntingtin and PARylated proteins, including PARP (Fig 5). PARP inhibitors are known to trap PARP at the chromatin [5]. It is therefore possible that the huntingtin-PARP interaction results in the co-trapping of huntingtin, which would in turn cause PARylated proteins to be enriched in the IP.



***3.4 Huntingtin-PAR co-localization***

We have previously reported the localization of S13/S16-phosphorylated huntingtin at SC35-postive nuclear speckles [4], as well as the localization of PAR to speckles [6]. The October 2018 quarterly report also included super-resolution (SIM) images showing co-localization of huntingtin with PAR at SC35-positive nuclear speckles, as well as their co-ordinated redistribution to diffuse nuclear staining upon oxidative stress. Unfortunately, the SIM data could not be reproduced. The optimal antibodies for detection of PAR and chromatin-bound huntingtin are both recognized by anti-rabbit secondary antibodies, precluding their use in concert. The SIM data was acquired using mouse anti-huntingtin (MAB2166), however this antibody has proven highly variable in several different immunofluorescence experiments.

As an alternative, we have turned to the YFP-tagged huntingtin-specific intrabody (nucHCB2). Unlike S13/S16-phosphorylated huntingtin, which resides at speckles under basal conditions, the pool of huntingtin bound by the intrabody is diffuse throughout the nucleus. This makes it impossible to test whether huntingtin and PAR co-redistribute out of speckles upon oxidative stress, as was seen with the MAB2166 antibody. However, it is still possible to test whether PAR and the intrabody co-localize upon oxidative stress, which causes the intrabody to bind chromatin in a non-uniform pattern (as seen in the FRAP assay, Fig 7). Coordinated redistribution of huntingtin and PAR upon oxidative stress would support their functional interaction. These experiments are underway.

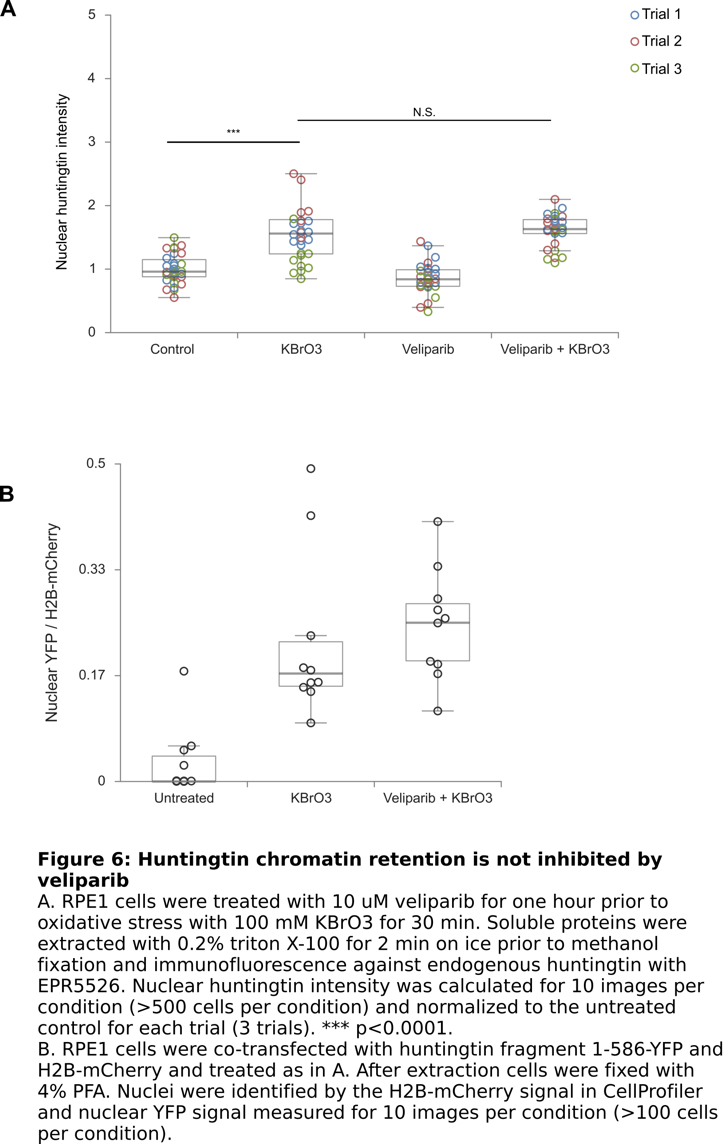
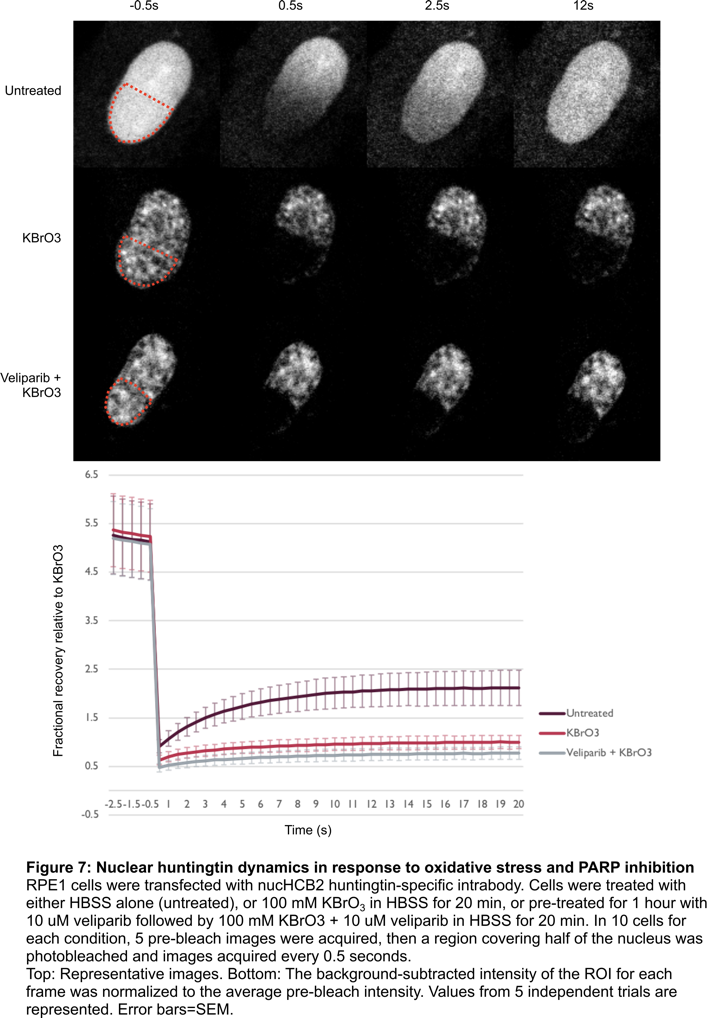
***3.5 Analysis of huntingtin chromatin retention as a possible function of PAR-binding***

*3.5.1 PARP enzymatic inhibition strategies*

We hypothesized that huntingtin uses PAR binding to recruit to chromatin in response to oxidative stress, and that inhibiting the production of PAR would diminish huntingtin chromatin retention. As measured by immunofluorescence of endogenous huntingtin, veliparib did not affect the final amount of huntingtin retained at chromatin in response to oxidative stress (Fig 6A). Similarly, there was no effect of veliparib on chromatin retention of the huntingtin 1-586-YFP fragment (Fig 6B).

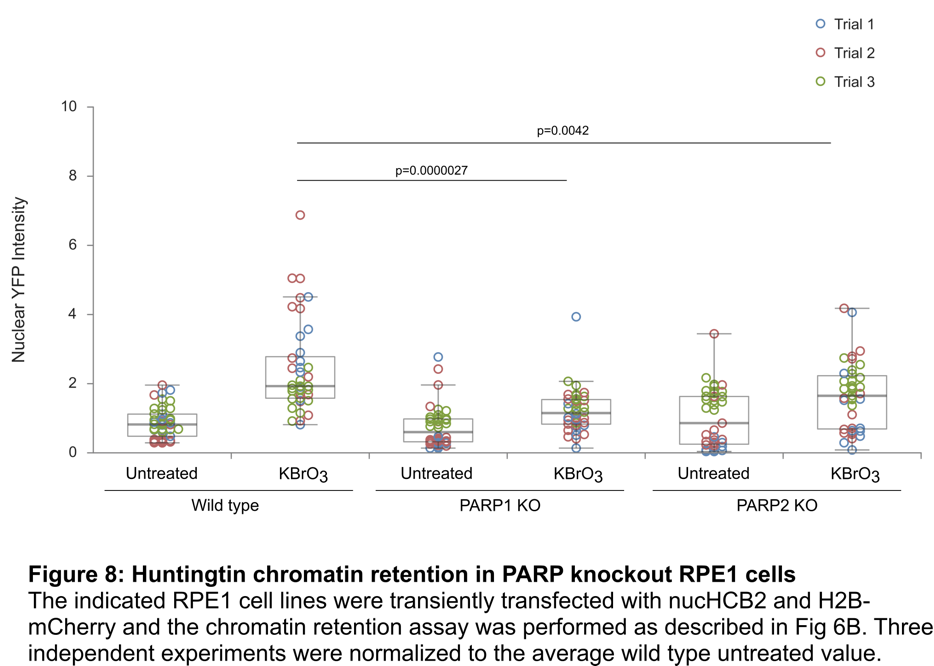
While the majority of PAR is produced by PARP1 upon DNA damage, PARP2 and PARP3 can also contribute [7,8]. Since veliparib inhibits PARPs 1 and 2, we reasoned that huntingtin chromatin retention could be mediated by PARP3 under these conditions. As reported previously, inhibition of all three PARPs did not block huntingtin chromatin retention and instead caused a striking increase in the *absence* of oxidative stress. This could be due to the trapping of huntingtin at chromatin, or a non-specific cell death response to the multiple inhibitors.

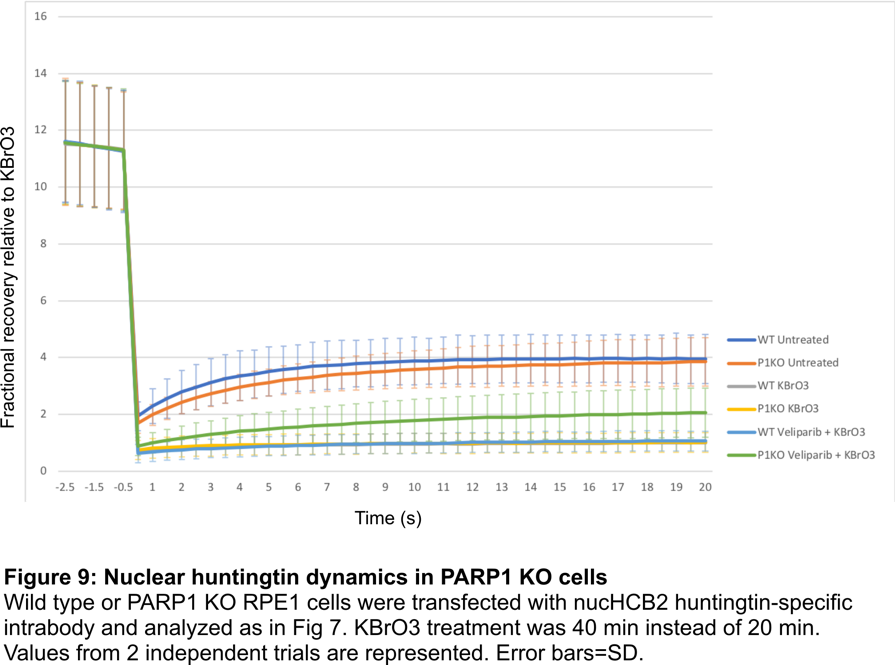
In an effort to capture the dynamics of huntingtin chromatin recruitment in response to oxidative stress, we used Fluorescence Recovery After Photobleaching (FRAP) of the YFP-tagged intrabody that recognizes endogenous huntingtin. As previously reported, KBrO3 slows huntingtin mobility, as would be expected upon huntingtin chromatin binding. If huntingtin uses PAR binding as a mechanism of chromatin retention, then inhibition of PAR formation would be expected to restore huntingtin mobility. In contrast, veliparib significantly reduced huntingtin mobility beyond the degree caused by KBrO3 alone. Since the last report, these experiments have been repeated with consistent results (Fig 7).

These results do not directly support a role for PAR binding in the chromatin recruitment of huntingtin. However, together with the effect of veliparib on huntingtin interaction with PARylated proteins (Fig 5), they suggest that huntingtin may exist in a complex with PARP, and that enzymatic inhibition of PARP causes co-trapping of PARP and huntingtin at chromatin. Enzymatic inhibition strategies are therefore not ideal for testing the role of PAR binding in huntingtin chromatin recruitment.

*3.5.2 PARP knock out strategies*

 To avoid the confounding effects of trapping by enzymatic inhibition, we used RPE1 cells deleted for either PARP1, PARP2, or both [9]. Chromatin retention of the huntingtin-specific intrabody nucHCB2 was significantly reduced in PARP1 KO and PARP2 KO cells (Fig 8). As reported previously, nucHCB2 formed inclusions when expressed in PARP1/PARP2 double knock out cells. The intense inclusion fluorescence over nuclei makes it impossible to accurately measure chromatin retention by this method. However, dysregulated huntingtin localization in PARP1/PARP2 double knock out cells supports a role for PAR in huntingtin subcellular localization. These results and experimental details have been deposited to Zenodo ([link](https://zenodo.org/record/3251038#.XQvGmdNKh-U)).



We next investigated the dynamics of huntingtin chromatin recruitment in PARP1 KO cells using the FRAP assay. To date, two experiments have been completed. In this case, KBrO3 treatment was longer than the experiments described in Fig 7 (40 min instead of 20 min). Under these conditions, huntingtin mobility was slowed to the point that veliparib treatment did not cause a further reduction (Fig 9). We did not detect an increase in huntingtin mobility in PARP1 KO cells, as would be expected if huntingtin uses PAR binding to recruit to chromatin. However, it is possible that PARP2 activity generates enough PAR to compensate for the lack of PARP1. Consistent with this, veliparib treatment of the PARP1 KO cells resulted in an increase in huntingtin mobility (Fig 9). This suggests that PARP2 enzymatic inhibition reduces PAR levels enough to detect a difference in huntingtin mobility, and that PARP2 is *not* trapped by veliparib, which is consistent with previous reports [10].

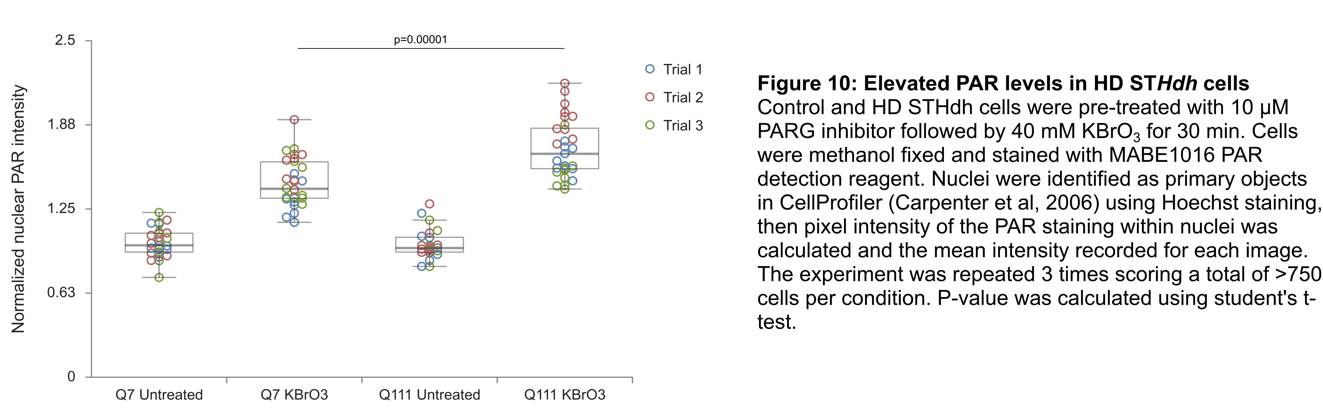
These results suggest that huntingtin may use PAR binding for chromatin recruitment, but that PARP2 can compensate for PARP1 and vice versa. Unfortunately, the intrabody forms inclusions in the PARP1/PARP2 double knock out cell line, making it impossible to test the dynamics of huntingtin chromatin recruitment by FRAP. This experiment will be repeated in PARP1 KO cells for statistical power. Given the compensatory effects of permanent PARP1 or PARP2 deletion, we will move to transient knock down strategies going forward.

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| **Milestone 4: Testing PAR phenotypes in additional HD tissues and models** |

***4.1 PAR levels***

Consistent with elevated DNA damage in HD tissues [6, 11, 12], we observed increased PAR levels in fibroblasts from HD patients (Fig 2). This led us to collaborate with the Dawson labs at John Hopkins, who found PAR levels to be elevated in the CSF from Parkinson’s disease patients [13]. They have received blinded CSF samples from HD patients and controls from the HD-CSF study run by Ed Wild and we await the results of their analysis. We also asked the Sipione lab to test PAR levels in wild type versus Q140 mice. They did not observe significantly increased PAR levels in the cortex from four Q140 mice compared to two WT mice (data not shown).

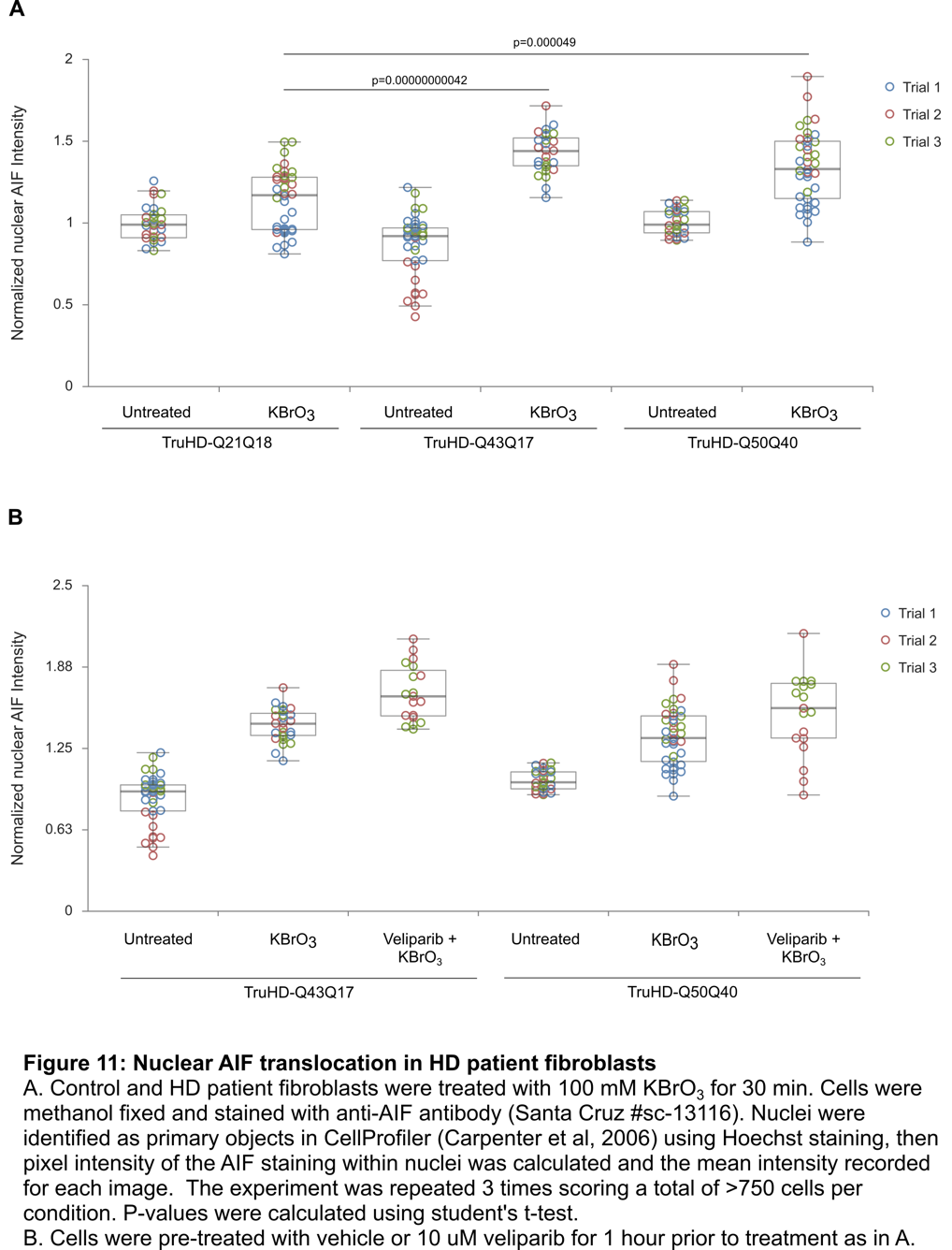
Similar to patient fibroblasts, we measured PAR levels in Q7/Q7 and Q111/Q111 ST*Hdh* cells and found them to be elevated in the HD model (Fig 10). This supports the hypothesis that PAR levels are elevated in HD. An additional collaboration with a student from the Tabrizi lab was initiated at the 2019 GRC on CAG Triplet Repeat Diseases. Joseph Hamilton has expressed interest in measuring PAR levels in HD iPSC cells and an open line of communication is being maintained with this group.



***4.2 Huntingtin chromatin retention***

Whether increased PAR levels would translate to increased huntingtin chromatin retention in HD patient fibroblasts was originally tested using the MAB2166 antibody. As with several experiments using this reagent, preliminary results showing elevated levels of huntingtin chromatin retention in TruHD-Q50Q40 cells compared to controls were not reproduced. These experiments will be repeated with the EPR5526 antibody. Current efforts are focused on identifying optimal conditions to measure the disassociation of huntingtin from chromatin and the recovery of cells after removal of oxidative stress.

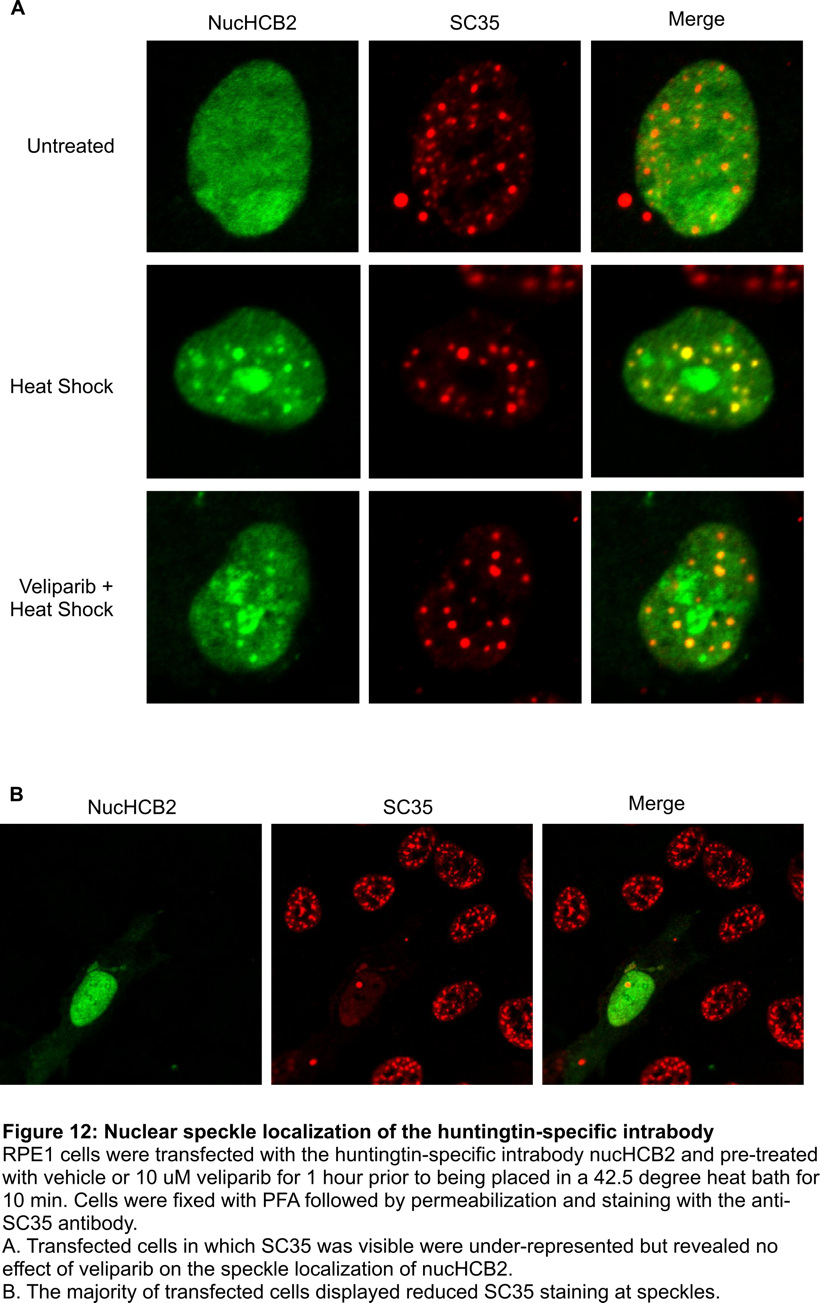
***4.3 Parthanatos***

 Parthanatos is a form of programmed cell death, distinct from apoptosis, that is mediated by PAR. We therefore wished to test whether HD patient fibroblasts undergo this process. One measurement of parthanatos is the nuclear translocation of the mitochondrial‐associated apoptosis‐inducing factor (AIF) [14,15]. In response to KBrO3, nuclear AIF was elevated in HD patient fibroblasts compared to controls (Fig 11A). However, nuclear AIF translocation can occur during other forms of programmed cell death, and to confirm that cells are undergoing parthanatos this effect must be rescued by PARP inhibition. As shown in Fig 11B, veliparib treatment did not block nuclear AIF translocation, indicating that increased parthanatos is not responsible for the observed phenotype. Data in Fig 11B is shown for HD patient fibroblasts, however a similar effect was seen in control fibroblasts. Review of the literature revealed that N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), not KBrO3, is the ideal reagent for inducing parthanatos in patient fibroblasts [14, 16]. These experiments will therefore be repeated with MNNG.

**IV. Future Experiments/Troubleshooting:** describe any further steps you plan to take to reach your milestones and any future experiments planned. Please make us aware of any challenges that require troubleshooting so that we can help you to meet your goals.

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| Milestone 1: Optimization of detection methods for proteins of interest | Complete |
| Milestone 2: Phenotypic characterization of immunofluorescence patterns | Complete |

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| Milestone 3: Characterization of mechanism/function of huntingtin PAR binding | On-going |

**** Experiments testing PAR binding, huntingtin-PAR co-localization and huntingtin chromatin retention are on-going.

Another possible readout for the function of huntingtin PAR binding is the effect of PARP inhibition/knock down on huntingtin localization to SC35-positive nuclear speckles. PAR localizes to speckles and is thought to nucleate liquid-liquid phase transition at this subnuclear domain [17,18]. As mentioned, the pool of huntingtin bound by the YFP-tagged intrabody is diffuse throughout the nucleus, however it localizes to speckles upon heat shock stress (Fig 12A). Preliminary experiments showed that veliparib treatment had no effect on huntingtin speckle localization. Unfortunately, quantitative data is impossible to acquire because expression of the intrabody disrupts anti-SC35 staining (Fig 12B), making it impossible to score huntingtin speckle localization in more than a few cells. It remains unknown whether binding of the intrabody to huntingtin simply excludes the anti-SC35 antibody from speckles, or whether it has some effect on speckle formation. As an alternative, huntingtin speckle localization can be tested using the anti-pS13pS16 antibody, which robustly stains speckles.

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| Milestone 4: Testing PAR phenotypes in additional HD tissues and models | On-going |

***PARylation of huntingtin***

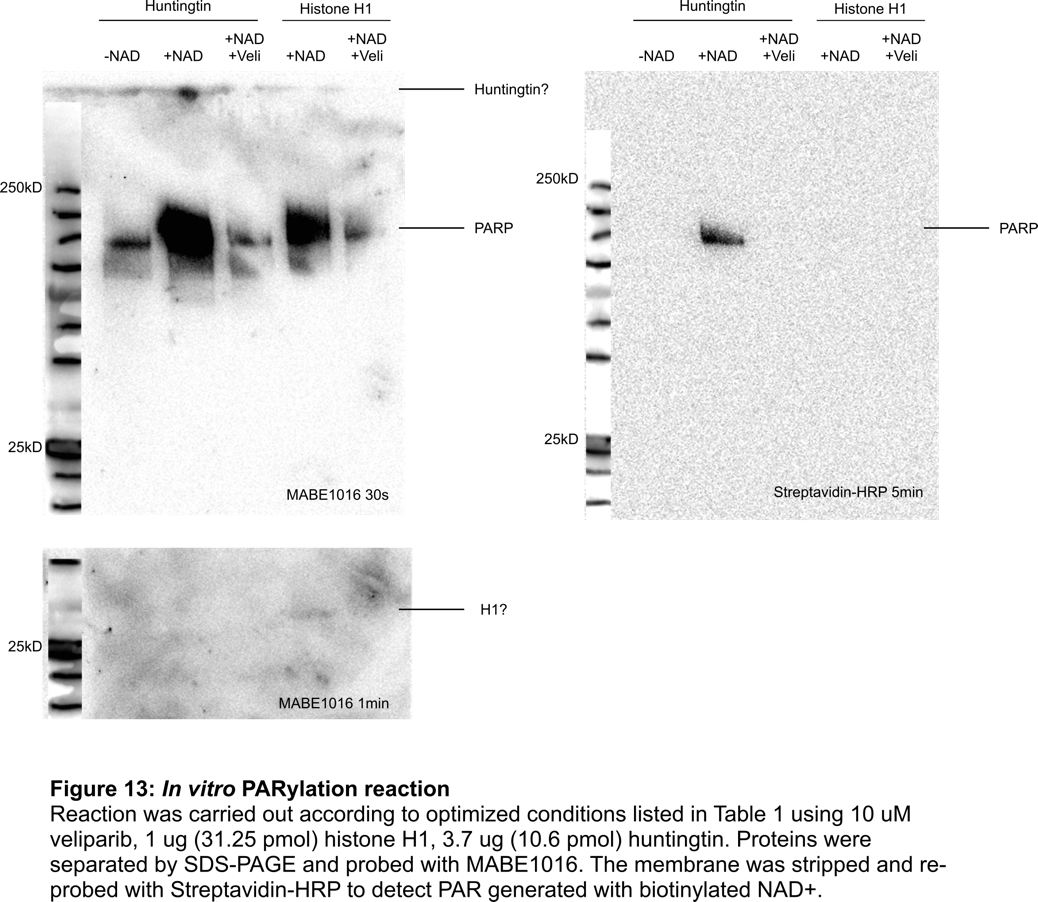
Given that huntingtin interacts with PARP, it is possible that huntingtin itself is modified by PARylation. Huntingtin immunoprecipitated from oxidatively stressed cells did not produce a signal by western with the PAR detection reagent (data not shown). However, PAR chains cause a significant shift in molecular weight, therefore PARylated huntingtin may not migrate into the gel or transfer to membrane efficiently. An *in vitro* PARylation reaction is currently under optimization. Different conditions were taken from the literature and tested in house, with the following conditions showing the most promise to date:

Table 1

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| --- | --- | --- | --- | --- |
| Kam et al [13] | Rahkimova et al [19] | Ame et al [20] | Wacker et al [21] | **In house** |
| 1 ug recombinant PARP1 (Trevigen) | 100 ng His-tagged Adprt1a | 800 ng PARP-2 | 2.5 pmol purified PARP-1 | **10 U PARP1 (Trevigen)** |
| 2 ug alpha-synuclein or 10 ug histone 2B (590 pmol) | 100 ng His-tagged histone | -- | -- | **1 uL of 3.7 ug/uL stock Q23 (10.6 pmol)** |
| Activated DNA (Trevigen) | 100 μg/ml of sheared salmon sperm DNA | 200 ng calf thymus DNA previously treated with DNase I | Sheared salmon sperm DNA | **Commercial 5X activated DNA**  **(BPS Bioscience cat #80605)** |
| Biotinylated NAD+ | 1.5 mM NAD+, 250 μM Biotinylated NAD+ (the stock is 250 uM…?) | 800 nM NAD+ | 670 nM NAD+ | **750 nM NAD+** |
| PARP assay buffer (Trevigen) | 50 mM Tris pH 8, 2 mM MgCl2, | 100 mM Tris pH 8, 10 mM MgCl2, 10 mM DTT | 30 mM HEPES pH 7.4, 12.5 mM MgCl2 | **30 mM HEPES pH 7.4, 12.5 mM MgCl2** |
| Room temp 30 min | Room temp 30 min | 100 uL reaction  25℃ 1-32 min | 15 uL reaction  Room temp 20 min | **15 uL reaction**  **Room temp 30 min** |

A high degree of background PARP auto-ribosylation is obtained with the MABE1016 PAR detection reagent. This could be due to basal PARylation of the commercial PARP preparation, in which case detection of only the PAR generated in the reaction with biotinylated NAD+ would have less background. Detection with streptavidin-HRP was cleaner but far less sensitive on a stripped blot (Fig 13).

There was no indication of huntingtin PARylation, however the reaction conditions may not be optimal since PARylation of the histone H1 positive control was also not detectable (Fig 13). It is also possible that PARylated huntingtin does not efficiently enter the gel during SDS-PAGE.



***Protein aggregation***

PAR is involved in the aggregation of alpha-synuclein [13] and the liquid-liquid phase transition of TDP-43, FUS, and hnRNPA1 [22]. We are therefore testing whether PARP inhibition/knock down affects the aggregation properties of huntingtin Exon1.

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| **Milestones for months 24-36: PARP pathway modulation** |

* Determine the effect of PARP enzymatic inhibition (panel of available inhibitors already in trials/tested) versus knockdown (siRNA, shRNA, knockout cells) on HD phenotypes of
  + Genomic integrity: comet assays [6], ɣH2AX [12], qPCR measurement of DNA damage [11, 23]
  + Mitochondrial integrity: ATP/ADP ratio [4], markers of mitochondrial activity [11]
* Test whether N6FFA and derivatives work through the PARP pathway or have synergistic effects

**V. Provide a list of references (optional)**

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