**Project:** ROS-specific Huntingtin Interactions

**Experiment:** ROS source optimization and immunoprecipitation from soluble fraction

**Purpose:** To identify the most effective/consistent treatment for ROS stress and test huntingtin immunoprecipitation from the more concentrated fraction

**Date:** 2017-07-17

In the series of optimization experiments using CSK buffer, the protein yield of the soluble fraction has been much higher than that of the chromatin fraction, and appears to have histone H3 and ample huntingtin. Therefore try immunoprecipitating from SOL fraction. Since KBrO3 effectiveness is questionable, try H2O2 until new order comes in. Once optimal conditions reached, repeat in TruHD-Q43 fibroblasts to move forward with what will be sent for mass spec. Also try LDS loading buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 1% LDS, 0.005% Bromophenol Blue, 166.25 mM DTT) instead of SDS based on recommendations from this paper: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4752115/>

Treatment and crosslinking

Treated 4 x 50% confluent 10-cm plates of HEK 293 cells with HBSS or HBSS containing 200 uM H2O2 (old stock) for 10 min. Dissociated cells with PBS/EDTA, washed with PBS. Pellets were ~50 uL for +H2O2, slightly more for -H2O2.

Resuspended cell pellets in 3.5 mL of 1% PFA in PBS (diluted from 10% stock in freezer). Incubated 10 min at room temperature with gentle agitation. Added 500 uL of 1 M glycine (final concentration 125 mM), incubated 5 min at room temperature with gentle agitation. Washed 2 times with 10 mL cold PBS.

Fractionation

Resuspended -H2O2 cell pellet in 200 uL, +H2O2 pellet in 150 uL (3 volumes) CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 0.5% (v/v) Triton X-100) containing 1X protein and phosSTOP inhibitor cocktails (Roche). Incubated 3 min on ice.

Spun at 5000 x *g* for 3 min

* Supernatant: soluble fraction. Measured protein concentration:
  + -H2O2 SOL: 4.0 ug/uL
    - Input: moved 10 uL to new tube, added 5 uL lysis buffer and 5 uL 4X LDS loading buffer = 40 ug
    - IP: moved 140 uL to new tube = 560 ug
  + +H2O2 SOL: 3.9 ug/uL
    - Input: moved 10 uL to new tube, added 5 uL lysis buffer and 5 uL 4X LDS loading buffer = 39 ug
    - IP: moved 140 uL to new tube = 546 ug
* Pellet: cytoskeletal frameworks, membranes, and chromatin

Resuspended -H2O2 pellet in 200 uL, +H2O2 pellet in 150 uL (3 volumes) CSK buffer containing 10 ug/mL DNase I. Incubated 15 min at 37deg. At 5 min point, tested DNA denaturation by combining 5 uL 1 M ammonium sulfate and 15 uL nuclear DNase suspension. No viscous pellet.

Moved 75 uL nuclei suspension to a new tube and added 25 uL of 1 M ammonium sulfate. Incubated on ice 5 min.

Spun full speed for 10 min.

* Supernatant: chromatin fraction containing solubilized chromatin-bound proteins and DNA. Measured protein concentration:
  + -H2O2 CHROM: 3.2 ug/uL
    - Input: moved 12 uL to new tube, added 3 uL lysis buffer and 5 uL 4X LDS loading buffer = 38.4 ug
    - IP: moved 60 uL to new tube = 192 ug
  + +H2O2 CHROM: 3.4 ug/uL
    - Input: moved 12 uL to new tube, added 3 uL lysis buffer and 5 uL 4X LDS loading buffer = 40.8 ug
    - IP: moved 60 uL to new tube = 204 ug
* Pellet: nuclear matrix

Incubated inputs in LDS loading buffer 1h at 37℃ then stored at -80℃.

CO-IP

Equilibrated protein G-Agarose in CSK buffer. Pre-coupled 160 uL of 50:50 slurry protein G-Agarose with 8 uL mab2166 2 h at 4℃.

To the IP samples described above, added 20 uL of 50:50 slurry protein G beads to each for pre-clearing. Incubated 1h at 4℃.

Spun out beads, moved pre-cleared lysates to a new tube. Added 20 uL of 50:50 slurry mab2166-coupled protein G beads. Incubated overnight at 4℃.

Collected beads and washed 3 x 500 uL CSK buffer. Resuspended beads in 30 uL 4X LDS loading buffer and incubated at 37℃ for 1 hour.

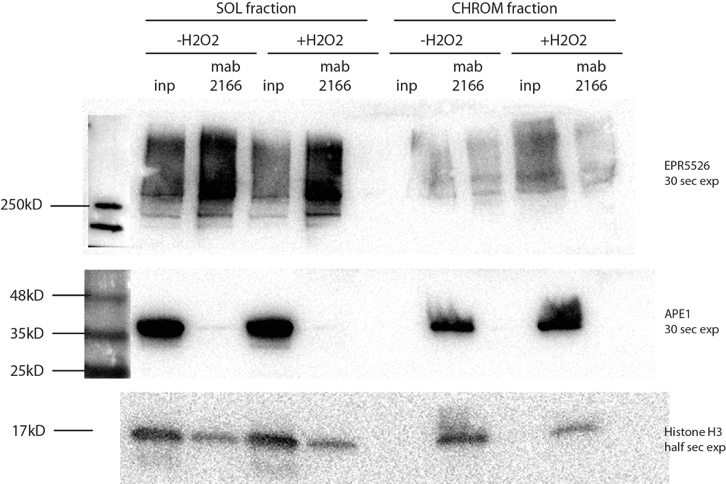
Ran as follows on gradient gel:

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Ladder | SOL fraction | | | | Ladder | CHROM fraction | | | |
| -H2O2 | | +H2O2 | | -H2O2 | | +H2O2 | |
| Input (40 ug) | IP  (from 560 ug) | Input (39 ug) | IP  (from 546 ug) | Input (38.4 ug) | IP  (from 192 ug) | Input (40.8 ug) | IP  (from 204 ug) |

Transferred at 90V for 1 hour, blocked for 30 min, incubated in primary antibodies 1h:

* EPR5526
* APE1
* Histone H3

Results



Conclusions

* Ample huntingtin pulled out of SOL fraction by mab2166, not so for CHROM fraction. Likely because SOL fraction had 2.5X as much protein in the IP.
* Smearing of bands in huntingtin western--LDS loading buffer problem?
* APE1 did not come down in the huntingtin IP from H2O2-treated cells. This old stock of H2O2 is not effective. Try 3NP.
* Histone H3 came down in both untreated and treated for SOL fraction
* SOL fraction contains histone H3 and other chromatin proteins (APE1) in excess of CHROM fraction. Not really a cytoplasmic fraction, obviously solubilizes nuclear proteins.