**Project:** ROS-specific Huntingtin Interactions

**Experiment:** ROS source optimization in mouse striatal cells

**Purpose:** To identify the ROS source resulting in the most efficient co-immunoprecipitation of DNA repair factors with huntingtin from mouse striatal cells

**Date:** 2017-11-07

Treatment and crosslinking

Treated 4 x 75% confluent 15-cm plates of Q7s each with HBSS containing either 50 mM 3NP (15 min), 40 mM KBrO3 (30 min), 40 mM KBrO3 (60 min), 100 uM H2O2 (10 min), or 100 uM H2O2 (60 min). Have 20 plates total, and based on previous experiments, 3NP treatment will not induce DDR protein interactions and will act as a negative control, therefore did not include untreated control to maximize protein concentration of treated samples. Used trypsin to dissociate cells. Washed once with 10 mL cold PBS. Cell pellets were each 100 uL, except H2O2 10 min sample, which was 75 uL.

Resuspended cell pellets in 3.5 mL of 1% PFA in PBS. 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, snap froze, stored at -80℃.

Lysis

Thawed cell pellets. Resuspended in 3 volumes CSK buffer containing inhibitors. Incubated 5 min on ice.

Spun at 5000 x *g* for 3 min. Kept supernatant and measured protein concentration:

* 3NP 15 min: 3.7 ug/uL
  + Input: moved 10 uL to new tube, added 5 uL lysis buffer and 7.5 uL 3X SDS loading buffer = 37 ug
  + IP: 300 uL = 1.11 mg @ 3.7 ug/uL
* H2O2 10 min: 5.1 ug/uL
  + Input: moved 7.3 uL to new tube, added 7.7 uL lysis buffer and 7.5 uL 3X SDS loading buffer = 37 ug
  + IP: 250 uL = 1.275 mg @ 5.1 ug/uL
* H2O2 1 h: 4.5 ug/uL
  + Input: moved 8.2 uL to new tube, added 6.7 uL lysis buffer and 7.5 uL 3X SDS loading buffer = 37 ug
  + IP: 300 uL = 1.35 mg @ 4.5 ug/uL
* KBrO3 30 min: 3.7 ug/uL
  + Input: moved 10 uL to new tube, added 5 uL lysis buffer and 7.5 uL 3X SDS loading buffer = 37 ug
  + IP: 300 uL = 1.11 mg @ 3.7 ug/uL
* KBrO3 1 h: 4.1 ug/uL
  + Input: moved 9 uL to new tube, added 6 uL lysis buffer and 7.5 uL 3X SDS loading buffer = 37 ug
  + IP: 300 uL = 1.23 mg @ 4.1 ug/uL

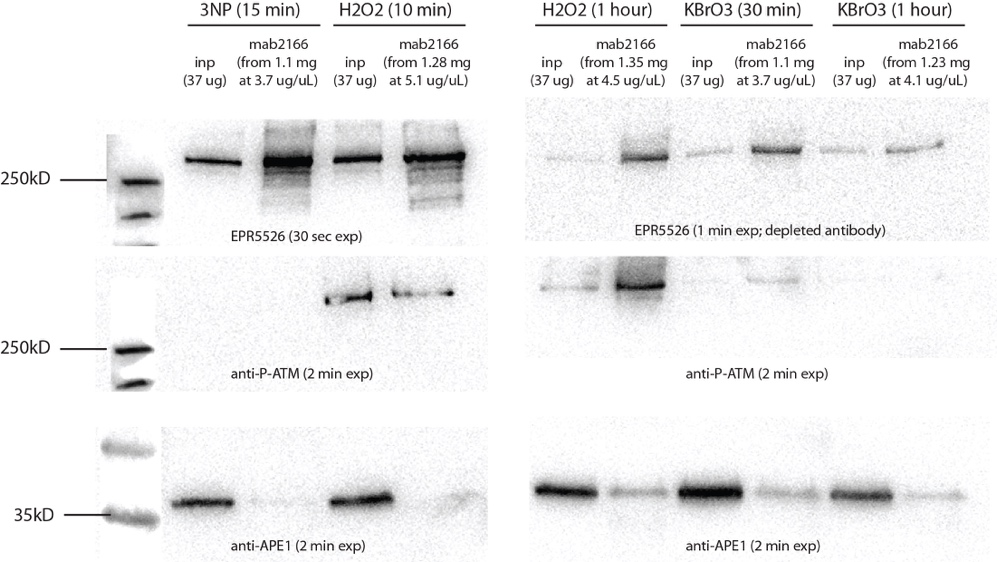
Boiled input in SDS loading buffer 10 min then stored at -80℃.

CO-IP

Added 2 uL mab2166 and 30 uL of 50:50 slurry protein G-agarose beads to each IP, rotated at 4deg for 2h. Washed IPs 3X 500 uL CSK, eluted in 40 uL 3X SDS loading buffer with boiling 10 min.

Ran samples on two 4-20% gradient gels. Transferred at 90V for 1 hour. Cut blots and performed westerns using anti-ATM-phospho-S1981 and anti-APE1, then stripped the top blot and performed EPR5526 western. NB: tested stripping efficiency and signal remained on the second blot, therefore repeated stripping and EPR5526 western. EPR5526 antibody preparation may have been depleted as signal was weaker on second round (see below).

Results



Conclusion

One hour H2O2 treatment gave the most robust P-ATM and APE1 pull downs, therefore use these conditions for mass spec sample preparation.