Tracking changes in mitochondrial acetylation by immunofluorescence provides new insight into HDACs class I/II and class III crosstalk

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P2394

Abstract

Lysine acetylation (Ac-K) is a critical post-translational modification that has been shown to play a fundamental role in epigenetic histone regulation[1]. Recent acetylome studies indicate that acetyl-lysine regulation expands well beyond histones, and can target thousands of proteins and many different cellular processes. Many of these acetylome studies often link changes in global acetylation to disease progression. Of interest, changes in the acetylated mitochondrial protein profiles are linked to neurologic, oncogenic, cardiac, and diabetic pathologies[2]. A recent study by Horton et al. specifically identified hyperacetylation of mitochondrial proteins in patients with heart failure[3]. As hyperacetylation of mitochondrial proteins appear to be a critical marker of metabolic disease, having tools to quickly access the acetylated mitochondrial state may be beneficial for diagnosis or treatment. A new, pan-acetyl-lysine antibody (AAC02) was developed that can identify acetylated mitochondrial proteins by immunofluorescence. Importantly, this antibody was tested in combination with mitotracker, and co-localized with the mitochondrial marker; conversely, the acetyl-lysine antibody did not co-localize with the LAMP 1 lysosomal marker. This acetyl-lysine antibody identified acetylated mitochondrial proteins in fibroblast, epithelial, cardiomyocytes, and fibroblast-like cell lines highlighting its range of utility. Hydrogen peroxide, an important regulator of glucose metabolism, as well as class I/II and class III HDAC inhibitors were used to examine the antibodies ability to detect changes in mitochondrial acetylation profile. Level of acetylated mitochondrial protein changes in response to these various treatments were detected with the acetyl-lysine antibody. Surprisingly, class I/II HDAC inhibitor, TSA, promoted significant cell specific down regulation of mitochondrial acetylation, which was reversed with class III HDAC inhibitor treatment. This result supports previous publications showing crosstalk between class I/II and class III HDACs[4,5]; however, this is the first report implicating acetylation of mitochondrial proteins as a target of this crosstalk. In summary, these data highlight the ability of this novel antibody to detect and track mitochondrial acetylation, and may be a critical to gain a better mechanistic understanding of the role of HDACs in disease, and ultimately, may be an important diagnostic tool for dysfunctional mitochondrial related diseases.

Results

1. AAC02 detects mitochondrial acetylation by IF

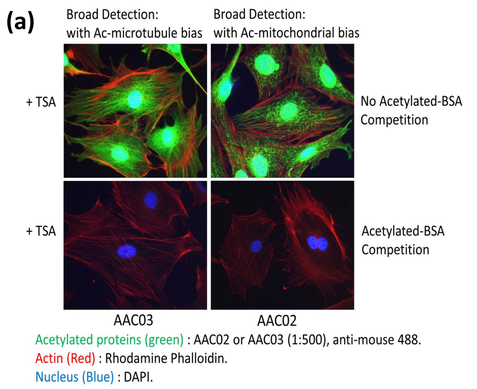


Figure 1a: Swiss 3T3 cells seeded on glass coverslip were treated with TSA (1 uM for 6 h). Cells were than fixed and permeabilized with 4% formaldehyde and 0.5% Triton-X 100 respectively. Acetylated proteins were detected by AAC02 or AAC03 and were visualized using a green fluorescent secondary. Actin fibers were visualized using a red Rhodamine Phalloidin and the nucleus was stained with DAPI. Acetylated BSA (10mg/ml) was used to compete for AAC02 and AAC03 binding as an indicator of their specificity for acetyl-lysine modifications.

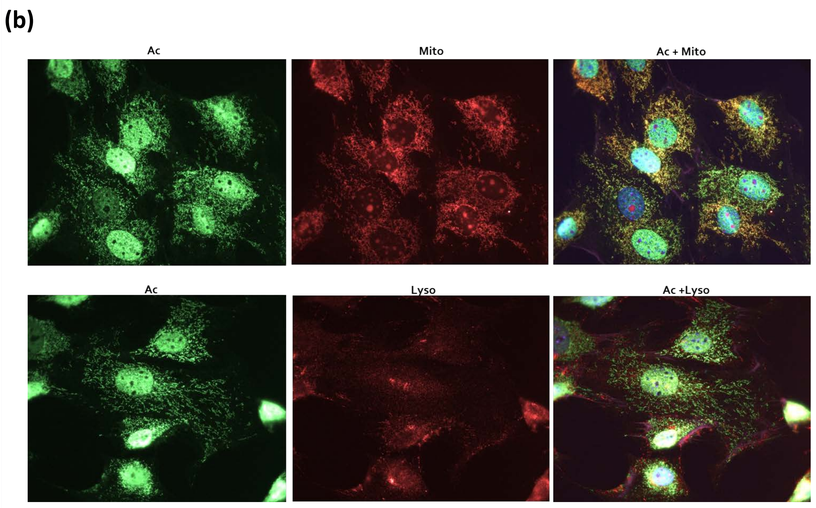


Figure 1b: Untreated Swiss 3T3 cells were stained as described previously. Acetylated proteins were detected by AAC02 and visualized in green fluorescence. Mitochondria were stained with MitoTracker orange (Thermo Fisher), while the lysosomes were detected with anti-Lamp1 antibody (Abcam) and visualized in red fluorescence. Merged images of mitochondrial and acetylation signals, or lysosomal and acetylation signals were performed to define vesicular staining by AAC02. Our results show that the vesicular staining by AAC02 colocalizes with mitochondrial marker MitoTracker orange but not lysosomal marker Lamp-1.

2. AAC02 detects dynamics changes in mitochondrial acetylation in A431 treated with H_2O_2

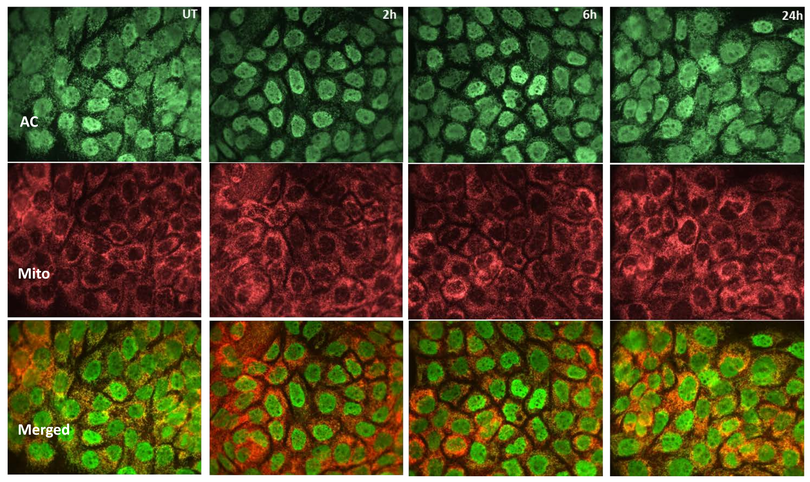


Figure 2a: A431 treated with 100uM H_2O_2 at different time intervals(0, 2, 6, and 24 hours) were stained as described previously. Acetylated proteins were visualized in green fluorescence. Mitochondria were visualized in red fluorescence using mitochondrial marker (anti-hexokinase1, Abcam). Merged images identified co-localization of mitochondrial and acetylation signals. The results show that AAC02 detects dynamics changes of mitochondrial acetylation in A431 cells with H_2O_2 treatment.

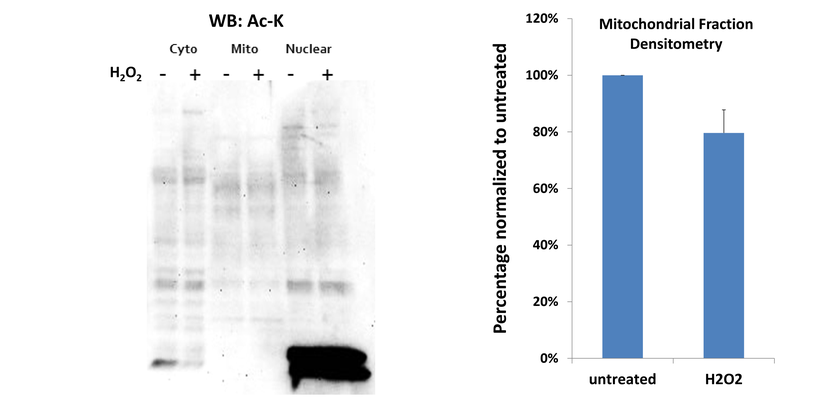


Figure 2b: A431 cells were either untreated (-) or treated (+) with 100uM H_2O_2 for 2 hours. Sample extracts (30 ug) from cytosol, mitochondrial and nuclear fractions were resolved in SDS-PAGE gel and then transferred to a PVDF membrane. Acetylated proteins were detected with AAC02 antibody. AAC02 detects distinctive acetylated protein profile in sample extracts from cytosol, mitochondrial and nuclear fractions.

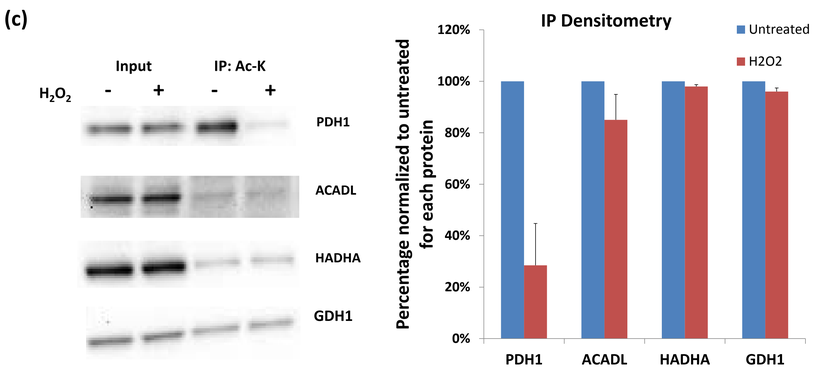


Figure 2c: Whole cell lysates from A431 cells either treated (+) or untreated (-) with H_2O_2 for 2 hours were obtained using BlastR buffer and filter system. Immunoprecipitation of acetylated proteins from 1 mg of whole cell lysates with pan acetyl-lysine AAC04 beads (Cytoskeleton) were performed per manufacturer's instruction. Eluted proteins were resolved in a SDS-PAGE gel and then transferred to a PVDF membrane. Change in acetylation level of mitochondrial proteins were examined with antibodies against specific mitochondrial proteins(PDH1: pyruvate dehydrogenase, ACADL: acyl-CoA dehydrogenase long chain, HADHA: trifunctional protein alpha subunit, GDH1: glutamate dehydrogenase). 10ug of total lysates were used as input.

3. Crosstalk between Class I/II and III HDACs may regulate mitochondrial acetylation

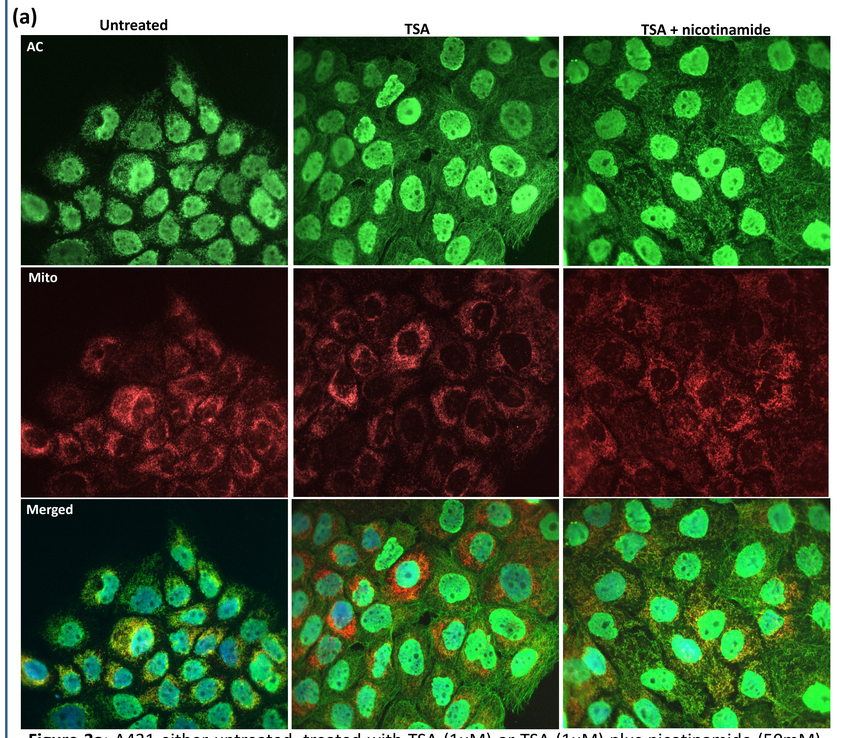


Figure 3a: A431 either untreated, treated with TSA (1uM) or TSA (1uM) plus nicotinamide (50mM) for 6 hours were stained as described. The results indicate that TSA (Class I/II HDAC inhibitor) promotes down regulation of mitochondrial acetylation which is attenuated by nicotinamide (Class III HDAC inhibitor). TSA induced loss of mitochondrial acetylation is cell specific since TSA treated Swiss 3T3 cells did not affect mitochondrial acetylation level (Fig 1a).

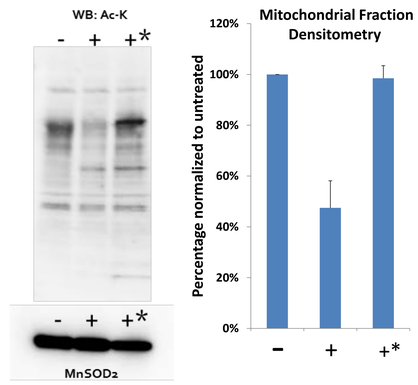
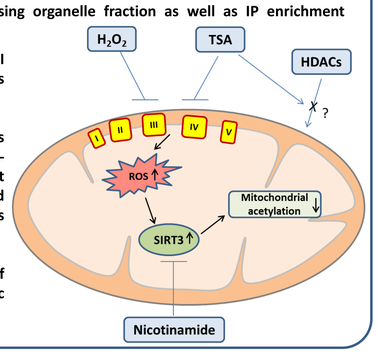


Figure 3b: Mitochondria were isolated from A431 either untreated (-), treated with 1uM TSA (+), or 1uM TSA plus 50mM nicotinamide (+*). 30ug of mitochondrial extracts were resolved in SDS-PAGE gel and transferred to a PVDF membrane. Acetylated proteins were detected with AAC02 antibody. Protein loading was determined by mitochondrial superoxide dismutase 2 (MnSOD2).

Conclusions

- **Investigation of mitochondrial acetylation using immunofluorescence is feasible with this newly developed acetyl-lysine antibody (AAC02).**
- **Importantly, this antibody is capable of tracking dynamic changes in the acetylated state of the mitochondria, as depicted using multiple cell types and stimulus[6,7].**
- **Specifically, this tool effectively visualized mitochondrial acetylation over a time-course of hydrogen peroxide treatment, and correlated with similar results obtained using organelle fraction as well as IP enrichment methods.**
- **Newly identified potential crosstalk was between class I/II and class III HDACs to regulate mitochondrial acetylation was discovered with this tool.**
- **Mounting evidence has shown that class I, II and III HDACs regulate mitochondrial dynamics such as biogensis, fission-fusion, movement, and mitophagy[8]. Here we speculate that the crosstalk between non mitochondrial class I/II HDACs and mitochondrial class III HDACs, mainly SIRT3, regulates mitochondrial acetylation.**
- **Further studies are required to decipher the mechanism of such crosstalk by investigating the effects of class specific HDACs on mitochondrial acetylation.**



References
1. Verdin E et al. 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. *Nat Rev Mol Cell Biol.* **2015**;16(4):258-64.
2. Iyer A et al. Lysine acetylation in obesity, diabetes and metabolic disease. *Immunol Cell Biol.* **2012**; 90(1):39-46.
3. Horton JL et al. Mitochondrial protein hyperacetylation in the failing heart. *JCI insight*. **2016**; 2(1):1-14.
4. Marin Poljak et al. Class I to III histone deacetylases differentially regulate inflammation-induced matrix metalloproteinase 9 expression in primary amnion cells. *Reproductive Science*. **2014**; 21(6):804-813.
5. Francesco Spallotta et al. A nitric oxide-dependent crosstalk between class I and III histone deacetylases accelerates skin repair. *JBC.* **2013**; 288(16):11004-11012.
6. Shu-Hui Dai et al. Sirt3 attenuates hydrogen peroxide-induced oxidative stress through the preservation of mitochondrial function in HT22 cells. *IJMM*. **2014**; 34:1159-1168.
7. Shujuan Sun et al. Trichostatin A targets the mitochondrial respiratory chain, increasing mitochondrial reactive oxygen species production to trigger apoptosis in human breast cancer cells. *PLOS one*. **2014**; 9(3): 1-9
8. Pedro Guedes-Dias et al. Lysine deacetylases and mitochondrial dynamics in neurodegeneration. *Biochimica et Biophysica Acta*. **2013**: 1345-1359