A redox-mediated conformational change in NQO1 controls binding to microtubules and α-tubulin acetylation

Supplementary Material

Knockout of NQO1 in 16HBE cells using CRISPR/cas9 gene editing

Knockout of NQO1 in 16HBE cells was performed using human NQO1 CRISPR/cas9 plasmids (sc400326) in combination with CAS HDR plasmid (sc417159-HDR) as described by the manufacturer (Santa Cruz Biotechnology). Briefly, 16HBE cells (2.5 x105 cells) were seeded into each well of a 6-well plate in 3ml of Dulbecco's modified Eagles medium containing 10% fetal bovine serum (no antibiotics). The next day plasmids (3.3µg of NQO1 CRISPR/CAS9 and 3.3 µg CAS HDR) were combined, and 33 µl was diluted in 117 µl Plasmid Transfection Medium (Santa Cruz Biotechnology, sc108062). UltraCruz Transfection Reagent (Santa Cruz Biotechnology, sc395739) was diluted in Plasmid Transfection Medium (5 µl transfection reagent and 150 µl Plasmid Transfection Medium). Mediums containing plasmids and transfection reagent were gently mixed and 300 µl was then added to each well for 72 h at 37°C. After 72 h the plasmid containing medium was aspirated and replace with 3 ml of Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 units/ml penicillin and 5 µg/ml puromycin (selection medium) for the selection of successful transfections. After one week surviving cells were trypsinized and seeded onto a T75-mm flask in 10 ml of selection medium (above). After another week of growth the cells were trypinized, resuspended in selection medium containing 10 µg/ml puromycin for limited dilutional cloning (one cell/100 µl/well, http://www.sciencegateway.org/protocols/cellbio/cell/cbld.htm). After approximately 25 days in culture at 37°C wells which had turned slightly yellowish were selected and expanded in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum and 100 µg/ml streptomycin and 100 units/ml penicillin (no puromycin). Clones were assayed for NQO1

catalytic activity using the reduction of DCPIP at 600 nm in the absence and presence of dicumarol (10 μM). NQO1 protein expression was measured using immunoblot analysis on whole cell lysates using two separate anti-NQO1 antibodies (A180, C-terminal).

Pyridine nucleotide LC-MS methods [1,2]

Materials. NAAD, NAD^+, $NADP^+$, NADH, NADPH, NaM, NmN, and adenosine triphosphate, cytidine monophosphate-13C9-15N3, 200 proof ethanol were obtained from Sigma Aldrich (St. Louis, MO). Sodium hydroxide and HEPES were obtained from Fisher Scientific (Fairlawn, NJ). Adenosine triphosphate (ribose-d4) and metabolite yeast extract (U13C, 98%, product #ISO1) were obtained from Cambridge Isotope Laboratories (Tewksbury, MA). NaM (13C6) was obtained from Cerilliant (Round Rock, TX). Nicotinamide riboside and doubly labeled nicotinamide riboside (13C1, H2-1) were obtained from Chromadex Inc. (Irvine, CA). All HPLC solvents and extraction solvents were HPLC grade or better.

Preparation of calibration standards. Individual stock standards for NAAD, NAD^+, $NADP^+$, NaM, NmN, and adenosine triphosphate were prepared by dissolving 10 mg/ml in deoxygenated buffered ethanol solution (3:1 ethanol:1mM HEPES pH 7.1) and then combining to obtain a stock mixture at various concentrations depending on the amount expected for each compound (mix A). The NADH and NADPH combined stock standard (mix B) was prepared separate from the other compounds. both were frozen at −70°C until use.

The internal standard solution was prepared at 250 μg/ml adenosine triphosphate-d4, doubly labeled nicotinamide riboside and 2.5 μg/ml of NaM-13C6 in deoxygenated buffered ethanol solution. The metabolite yeast extract containing the U13C labeled NAD^+ and $NADP^+$ was prepared by adding 2 ml of deoxygenated buffered ethanol solution to 15 mg of lyophilized yeast extract and vortexed until the extract was completely reconstituted. The reconstituted yeast extract was then centrifuged at 14,000 RPM for 5 min at 4°C, and the clear supernatant was then stored in 50 μl aliquots in 1.5 ml microfuge tubes at -70°C until use. Immediately prior to use, 250 μl of

deoxygenated buffered ethanol solution was added to the 50 µl aliquot of yeast extract and vortexed for 10 s.

A calibration stock standard was prepared by adding 20 µl of internal standard, 10 µl of yeast extract, 15 µl of mix A, 15 µl of mix B and 90 µl of deoxygenated buffered ethanol solution (total volume=150 µl). The calibration stock solution was then diluted into 9 additional calibration standards in deoxygenated buffered ethanol solution, with the internal standard and yeast extract concentration kept constant in the 10 calibration levels. The concentration of NAD^+ in the 10 calibration standards was 30.2, 15.1, 7.55, 3.77, 1.88, 0.944, 0.472, 0.236, 0.0472 and 0.0236 µM respectively. HPLC separation of NAD_+ metabolites was performed using a method described by Hsiao et al with minor modifications[(Hsiao et al., 2018)]. Separation of NAD^+ metabolites was performed on a 1200 series HPLC with a 150 × 2.1 mm Poroshell 120 HILIC-Z column from Agilent Technologies (Santa Clara, CA). Buffer A consisted of 10mM ammonium acetate adjusted to pH 9.0 with ammonium hydroxide, and buffer B consisted of 90:10 acetonitrile:water with 10 mM ammonium acetate adjusted to pH 9.0 with ammonium hydroxide. 5 uM of methylenediphosphonic acid was added to both buffers. Two microliters of the extracted sample was analyzed using the following gradient at a flow rate of 0.5 ml/min: Hold at 90% B for 2 min, then 90% B to 60% B from 2 to 12 min, hold at 60% B from 12 to 15 min, then 60% B to 90% B from 15 to 6 minutes followed by re-equilibration at 90% B for 5 minutes. The column temperature was held at 25°C for the entire gradient. Mass spectrometric analysis was performed on an Agilent 6490 triple quadrupole mass spectrometer with an electrospray source in positive ionization mode. The drying gas was 130°C at a flow rate of 15 L/min. The nebulizer pressure was 35 psi. The sheath gas temperature was 350°C at a flow rate of 12 L/min. The capillary voltage was 3000 V. Data for NAD^+ metabolites was acquired in MRM mode using experimentally optimized conditions obtained by flow injection analysis of authentic standards.

Data Analysis. Calibration curves for each NAD^+ metabolite were constructed using Agilent Masshunter Quantitative Analysis software. The peak areas for U13C-NAD^+, U13C-$NADP^+$ and

U13C-NADH from the yeast extract were used as internal standards for their corresponding unlabeled metabolites. The peak area for U13C-$NADP^+$ in the yeast extract was used as the internal standard for unlabeled NADPH.

1 Demarest, T. G. *et al.* Assessment of NAD^+ metabolism in human cell cultures, erythrocytes, cerebrospinal fluid and primate skeletal muscle. *Anal Biochem* **572**, 1-8, (2019).

2 Hsiao, J. J., Potter, O. G., Chu, T. W. & Yin, H. Improved LC/MS Methods for the Analysis of Metal-Sensitive Analytes Using Medronic Acid as a Mobile Phase Additive. *Anal Chem* **90**, 9457-9464, (2018).

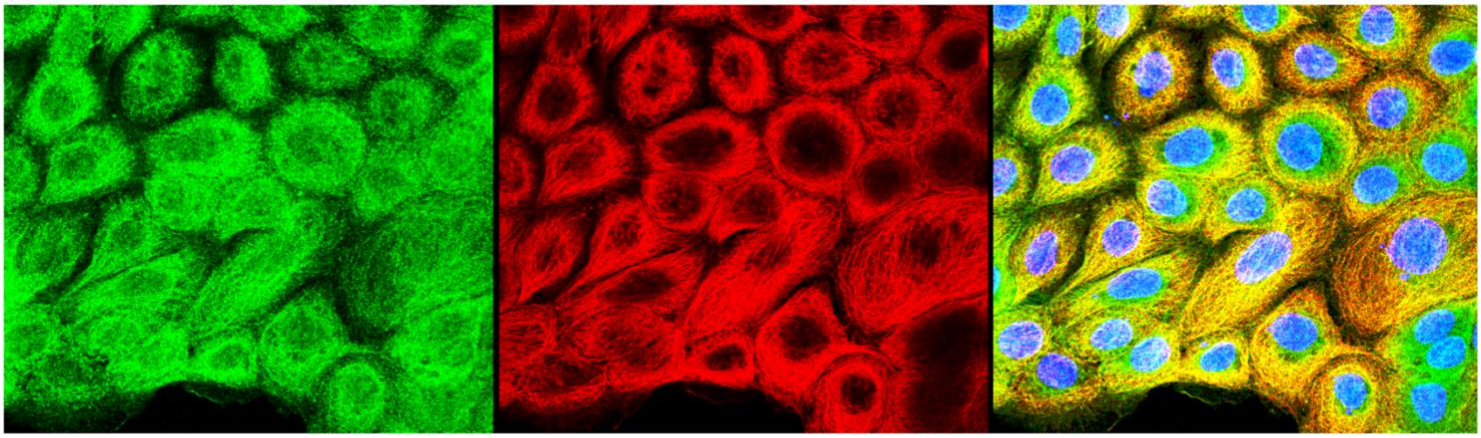

Fig. S1. Immunostaining for NQO1 and α-tubulin in 16HBE cells. Untreated 16HBE cells were prepared for immunocytochemistry as described in Materials and Methods and immunostained with mouse anti-NQO1 (A180, Novus) and rabbit anti-α-tubulin (Abcam #15246) for 1h at room temperature. FITC-labeled anti-mouse and Texas red-labeled anti-rabbit was added with DAPI (1µg/ml) for 30min at room temperature.

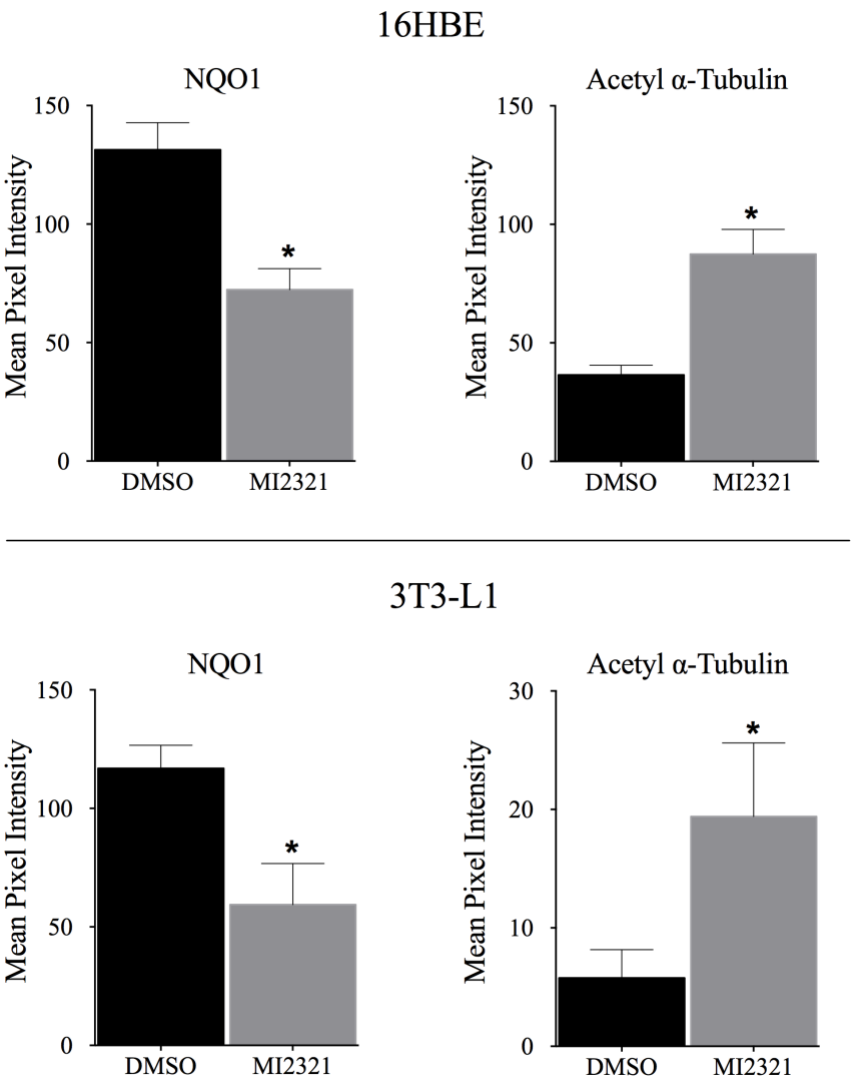

Fig. S2. Quantification of fluorescent immunostaining for NQO1 and acetyl α-alpha tubulin. Mean pixel intensity was determine in ImageJ for NQO1 and acetyl α-tubulin in 16HBE cells (n= 7) and 3T3-L1 fibroblasts (n= 5) treated with DMSO or MI2321. Treatment with MI2321 resulted in a decrease in immunostaining for NQO1 and an increase in immunostaining for acetyl α-tubulin in both cell lines (*$p < 0.05$, unpaired *t*-test).

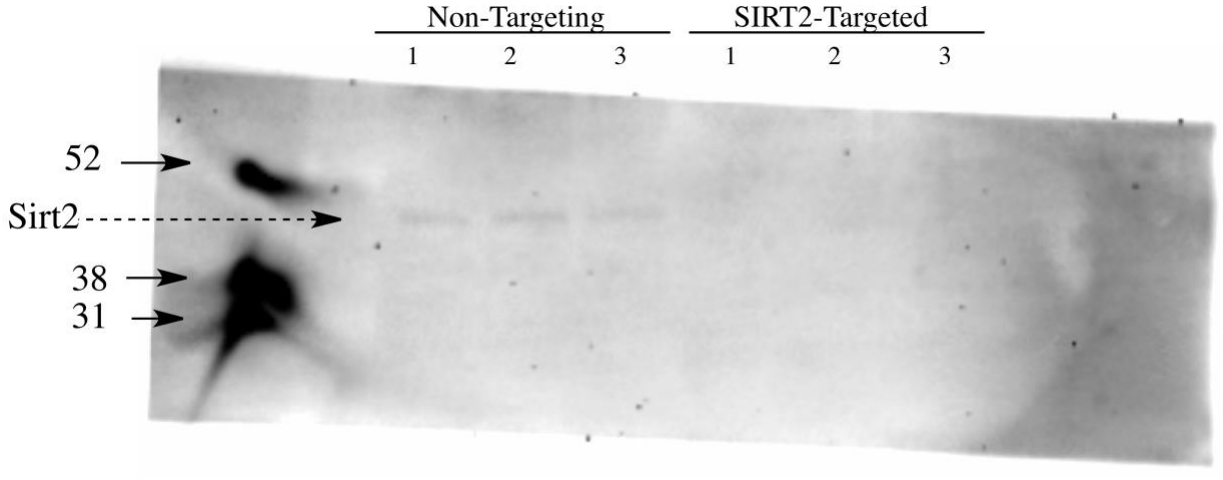

Fig. S3. Immunoblot analysis for Sirt2 following transient knockdown in 16HBE cells. 16HBE were treated with non-targeted siRNA (left) or siRNA targeted to Sirt2 (right) for 48h. Immunoblot analysis was performed on 20µg of cell lysate. Rabbit anti-Sirt2 antibody was obtained from Cell Signaling Technologies (#12672) and was allowed to react for 16h at 4ºC.