Supporting Information

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SI Materials and Methods

SNO Peptide Enrichment and Preparation for MS-Based Analysis. Approximately 400 μg of each iodoTMT^6 (or cysTMT^6) sample was combined and digested with trypsin (1:30 trypsin/protein; Promega). The digested peptides were incubated with anti-TMT antibody-affinity resin at room temperature for 2 h. Unlabeled peptides were washed with Tris-buffered saline, and the TMT-labeled peptides were eluted with 3 × 0.6 mL of 500 mM triethylammonium bicarbonate (TEAB) (pH 8.5) buffer containing 10 mM *N*,*N*-diisopropylethylamine, as previously described (1). Samples were then cleaned using solid-phase extraction with an Oasis HLB (Waters) according to the manufacturer's protocol.

Peptide Identification by Liquid Chromatography/Tandem MS. Liquid chromatography/tandem MS analysis was performed using an UltiMate 3000 RSLCnano System (Thermo Fisher Scientific) connected to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) equipped with an EasySpray ion source. Peptides were first loaded onto a trap column (PepMap100 C18, 5 μm, 100 Å, 300-μm i.d. × 5 mm; Thermo Fisher Scientific), followed by separation on a PepMap RSLC C18 column (2 μm, 100 Å, 75-μm i.d. × 25 mm; Thermo Fisher Scientific) using a flow rate of 300 nL·min^{-1} with a linear gradient of 2–20%B for 70 min, 20–35%B for 10 min, and 35–98%B for 2 min; holding at 98%B for 8 min; and reequilibrating at 2%B for 20 min at 400 nL·min^{-1} (mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile). The nanosource capillary temperature was set to 275 °C, and the spray voltage was set to 2 kV. MS1 scans were acquired in the Orbitrap Elite mass spectrometer at a resolution of 60,000 FWHM (380–2,000 *m/z*) with an automatic gain control (AGC) target of 1×10^6 ions over a maximum of 250 ms. MS2 spectra were acquired for the top 10 ions from each MS1 scan in higher-energy collisional dissociation (HCD) mode in the Orbitrap mass spectrometer with 30,000 FWHM and a target setting of 3×10^5 ions, an accumulation time of 50 ms, and an isolation width of 1.2 Da. The normalized collision energy was set to 33%, and one microscan was acquired for each spectrum. Monoisotopic precursor selection was enabled, and only MS1 signals exceeding 500 counts triggered the MS2 scans, with +1 and unassigned charge states not being selected for MS2 analysis. Dynamic exclusion was enabled with a repeat count of 1, repeat duration of 30 s, and exclusion duration of 80 s.

Each biological sample was analyzed twice, resulting in a total of 10 technical replicates per cohort.

MS Data Analysis for Determining SNO Identification and SNO Change. Raw MS data were searched using open mass spectrometry search algorithm (OMSSA) against the mouse UniProt database as previously described (1). Once TMT-modified peptides were extracted from the search, SNO "sites" were searched based on a FAST-All (FASTA) sequence using customized code. A site of S-nitrosylation was determined only when the TMT intensity in the ascorbate-treated sample was greater than negative control (not ascorbate-treated). The TMT value of the ascorbate-treated sample decreased by that of control was considered as the SNO magnitude for each specific site. To verify a true change and exclude a possible false change caused by process variation, we applied more stringent criteria to include only those found in a minimum of two biological replicates and showing a WT versus DMD difference greater than the average of the coefficient of variation. Of the sites that satisfied the stringent criteria, if this level of SNO was greater in the DMD than control, this residue was considered as "hypernitrosylated"; if the opposite, then it was marked as "hyponitrosylated." A histogram was used for hypernitrosylated residues in DMD, and it plotted the percentage of SNO in DMD (compared with WT) over the percent change of SNO in DMD/Trpc6$^{-/-}$ (compared with WT), showing that 70% of the changes observed between DMD and WT were reversed by 30% or more in DMD C6$^{-/-}$. A binominal distribution was used for the nine hyponitrosylated sites, which showed that seven sites had increased nitrosylation levels with Trpc6 deletion ($P = 0.07$). The MS data have been deposited in the PeptideAtlas repository (www.peptideatlas.org; identifier no. PASS01074).

1. Chung HS, et al. (2015) Dual labeling biotin switch assay to reduce bias derived from different cysteine subpopulations: A method to maximize S-nitrosylation detection. *Circ Res* 117:846–857.

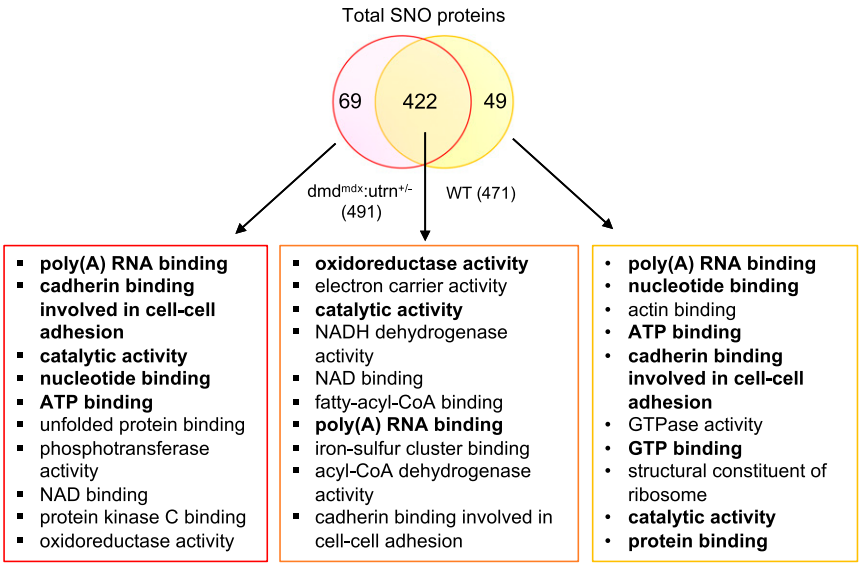

Fig. S1. Cluster analysis for unique proteins versus shared proteins. The number of SNO proteins in dmd^{mdx}:utrn$^{+/-}$ and WT was shown as a Venn diagram. Cluster analysis was performed with a total of 69 unique proteins to dmd^{mdx}:utrn$^{+/-}$, 49 unique proteins to WT, and 422 shared proteins to both, based on Gene Ontology molecular function. Details are listed in Dataset S2. Lowest to highest (top to bottom) *P* values are listed in boxes. When the number (%) of the proteins for the terms is >10%, the term is marked in bold. Similar or duplicated terms were removed.

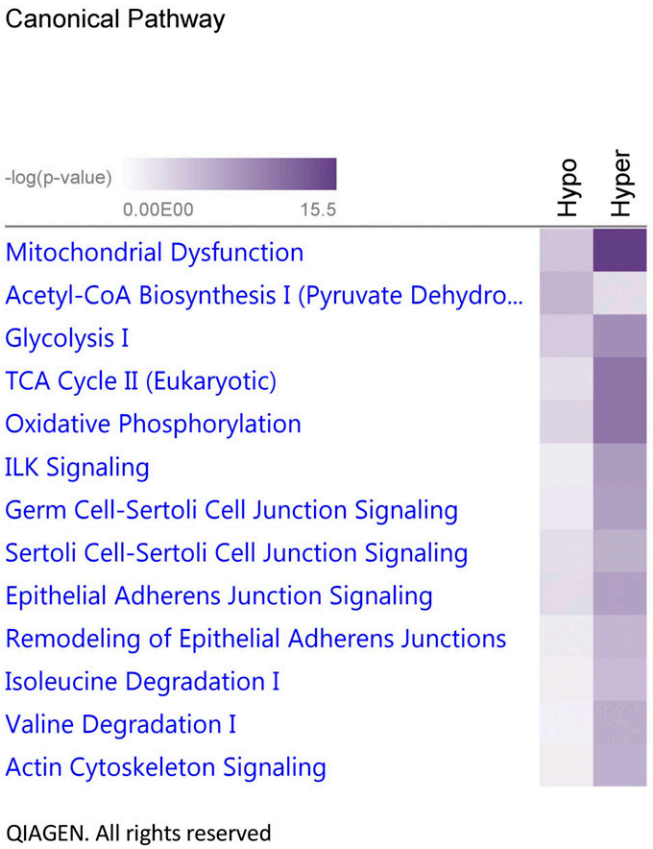

Fig. S2. Canonical pathway analysis for hyper-SNO protein versus hypo-SNO. Bioinformatics analysis revealed both hyper- and hypo-SNO proteins function similarly in mitochondrial pathways and metabolisms. The heat map was created by pathway enrichment analysis using the Ingenuity Pathway Analysis (QIAGEN) tool.

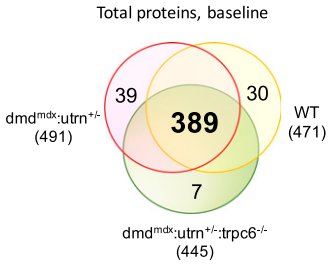

Fig. S3. Three-way comparison of SNO sites at a protein level. A majority of the modified proteins were detected in all three mouse models.

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)
Dataset S3 (XLSX)
Dataset S4 (XLSX)
Dataset S5 (XLSX)
Dataset S6 (XLSX)
Dataset S7 (XLSX)