SUPPLEMENTAL DATA

Quantitative profiling of protein palmitoylation in mutant neuronal stem cells

Experimental Procedures

Materials. DMEM:F12 1:1, minus lysine and arginine) from Pierce Biotechnology (catalog number 88215), L-lysine (L5501), L-arginine (A5006), L-lysine-$^{13}C_6$,$^{15}N_2$-HCl (608041) and L-arginine-$^{13}C_6$,$^{15}N_4$ -HCl (608033) and 17-octadecynoic acid (17-ODYA) were purchased from Sigma-Aldrich. Papain and DNase I were purchased from Worthington Biochemical Corporation. Dispase II was purchased from Stemcell Technologies. Mouse brain primary cortex neurons were purchased from Lonza. PBS buffer was purchased from Invitrogen. Gelatin was purchased from Chemicon. Paraformaldehyde was purchased from USB Corporation.

Preparation and characterization of NSCs. NSCs (neural stem cells) were prepared according to previously published methods (1-2). DHHC5-WT and DHHC5-GT cells were derived from heterozygotes littermates at generation F2. (The original gene-trap insertion was obtained from mouse embryonic stem cell line (RRD553, strain 129/Ola, BayGenomics) and bred into C57BL/6, and therefore the DHHC5-GT allele is present in these animals on a mixed background). The NSCs were further characterized by semi-quantitative reverse transcription-PCR for Sox2, nestin and actin (3), immunoblotting (4-5) and immunofluorescent staining using antibodies to Sox2.

Stable isotope labeling with amino acids (SILAC) of NSCs. NSCs were grown in SILAC light medium (lysine and arginine-free DMEM:F12 (1:1) containing supplemental L-lysine (100 μg/ml), L-arginine (100 μg/ml), and N2 supplement (1X), 20 ng/ml EGF, 20 ng/ml FGF, 5 μg/ml heparin, and antibiotic-antimycotic (1X) or SILAC heavy medium (in which heavy lysine and arginine (each at 100 μg/ml) were substituted for the supplemental lysine and arginine) for over 6 passages (~24 days) (6). The cells were then grown in the SILAC heavy or light media plus 25 μM 17-ODYA (17-octadecynoic acid) overnight (7), then washed 3 times with PBS and frozen at -80°C.

Click chemistry and enrichment. Cell pellets were sonicated and separated into soluble and insoluble (membrane) fractions by ultracentrifugation. For fluorescent gel-based analysis, sonicated membranes corresponding to 50 μg of membrane protein were mixed with 20 μM rhodamine-azide, 1 mM Tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich), 100 μM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (Sigma-Aldrich), and 1 mM CuSO4 in PBS in a 50 μL at room temperature. After 1 hour, samples were mixed with SDS sample loading buffer and loaded without boiling on a 10% SDS-PAGE gel and analyzed using a Hitachi FMBIO-II flatbed fluorescence scanner. For proteomic analysis, 1.5 mg of both light and heavy membrane proteomes were mixed equally in a 1:1 ratio, then extracted once with chloroform/methanol (1:1, v/v) to remove lipids and unincorporated probe. Samples treated with 1M hydroxylamine were precipitated by chloroform/methanol extraction, and then mixed with the isotopic paired sample. The precipitated proteome was sonicated in PBS and combined with 500 μM biotin-azide, 100 μM TBTA, 1 mM TCEP, and 1 mM CuSO4 in 2 mL PBS for 1 hour. Samples were centrifuged to pellet the precipitated protein, washed 1x with cold methanol, and extracted 1x with chloroform/methanol. The protein interphase was then washed with methanol and solubilized in 6 M urea in PBS, then reduced with 10 mM TCEP and alkylated with 20 mM iodoacetamide. SDS was added to a final concentration of 2%, and the sample was diluted 10x in PBS. Streptavidin beads (Thermo) (100 μL slurry) were added and rotated at room temperature for 2 hours. Beads were washed 10x with 1mL 1% SDS in PBS, then 20x with 1mL PBS. Beads were resuspended in 2 M urea/PBS supplemented with 1 mM calcium chloride and sequence grade porcine trypsin (Promega) for overnight digestion at 37°C. The eluant was collected the following day and acidified with 5% formic acid.

Mass spectrometry. Mass spectrometry was performed using a Thermo Orbitrap Velos mass spectrometer. Peptides were eluted using a 6-step MudPIT protocol (using 0%, 10%, 25%, 50%, 80%, and 100% salt bumps of 500 mM aqueous ammonium acetate, each step followed by an increasing gradient of aqueous acetonitrile/0.1% formic acid) and data were collected in data-dependent acquisition mode (2 MS1 microscans (400-1800 m/z) and 30 data-dependent MS2 scans) with dynamic exclusion enabled (repeat count of 1, exclusion duration of 20 s) with monoisotopic precursor selection enabled. All other parameters were left at default values. Unenriched samples were eluted in a 12-step MudPIT using 0%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% salt bumps of 500 mM ammonium acetate. SEQUEST searches allowed for variable oxidation of methionine (+15.9949), static modification of cysteine residues (+57.0215 due to alkylation), and no enzyme specificity. For the heavy SEQUEST search, static modifications on lysine (8.0142) and arginine (10.0082) were specified. The precursor ion mass tolerance was set to 50 ppm and the fragment ion mass tolerance was left at the default assignment of 0. The data was searched using a mouse reverse-concatenated non-redundant (gene-centric) FASTA database that combines IPI and Ensembl identifiers. The resulting MS2 spectra matches were assembled into protein identifications and filtered using DTASelect (version 2.0.47) with the --trypstat option. Peptides were also restricted to fully tryptic using the -y 2 option with a defined peptide false positive rate of 1% (--fp 0.01) and single peptides per locus were allowed (-p 1). Redundant peptide identifications common between multiple proteins were allowed, but the database was restricted to a single consensus splice variant. SILAC ratios were quantified using in-house software (8). The program was updated to identify cases where complete inhibition could not be quantified based on light/heavy peak pairs due the absence of a MS1 signal from either the heavy or light sample. In order to identify these cases, all single MS1 chromatographic peaks (from either the light or the heavy sample) were identified within a retention time window. Next, these peaks were aligned with the corresponding sequence SEQUEST/DTASelect identification and the charge state and monoisotopic mass were validated using the "envelope correlation score" filter (8). Finally, the candidate peak was cross-checked to ensure there was no corresponding (heavy or light) peak co-eluting around the same retention time window. Only after all these conditions were met, the peptide was assigned as the case of complete inhibition with an artificial threshold ratio of 20.

Supplemental Figure Legends

Fig. S1. Characterization of neuronal stem cells (NSCs) derived from DHHC5-gene targeted (DHHC5-GT). Total RNA was extracted from primary mouse cortical neurons (Con, control), or mouse NSCs as indicated and subjected to RT-PCR as described under Supplemental Materials and Methods. For immunoblotting (lower panels) to detect DHHC5, Sox2 and actin, 30 μg of whole cell lysate prepared in RIPA buffer were loaded. Right panels, immunofluorescence for Sox2 and DAPI (nuclear stain) are shown. Virtually every nucleus is stained for Sox2. Bar, 40 μm.

Legends to Tables in Dataset S1

Dataset S1, Table 1. Peptides derived from candidate palmitoylated proteins identified in WT NSCs using 17-ODYA in comparison with a palmitic acid (PA) control. Runs 1 and 2: Light = 17-ODYA, Heavy = PA. Runs 3 and 4: Light = PA, Heavy = 17-ODYA. Ratios are displayed as 17-ODYA divided by PA. A protein is identified in one line that includes the IPI number, description, Ensembl identifier, median ratio across all quantified peptides, and notation to signify runs containing quantified peptides assigned to the specific protein. Below this line is a list of peptides identified by SEQUEST that includes the peptide sequence, monoisotopic mass, and run where the peptide was quantified. Peptides displayed with a ratio of 0 were identified by SEQUEST, but failed to pass the criteria assigned for MS1 quantification, including peak shape, co-elution, and isotopic envelop score. Peptides with a ratio of 20 were classified as "singletons" and arbitrarily assigned a ratio of 20, based on the criteria listed in the Supplemental Methods. Peptides with M* signify oxidized methionine. Data is filtered based on proteins

passing the criteria described in the Supplemental Methods (Median ratio ≥ 5, at least 1 quantified peptide from Runs 1 - 2 AND at least 1 quantified peptide from Runs 3 - 4) for the 17-ODYA / PA experiments.

Dataset S1, Table 2. Peptides derived from candidate palmitoylated proteins identified in WT NSCs using 17-ODYA in with or without NH_2OH as a control. Runs 1 and 2: Light = 17-ODYA + NH_2OH, Heavy = 17-ODYA. Runs 3 and 4: Light = 17-ODYA, Heavy = 17-ODYA + NH_2OH. A protein is identified in one line that includes the IPI number, description, Ensembl identifier, median ratio across all quantified peptides, and notation to signify runs containing quantified peptides assigned to the specific protein. Below this line is a list of peptides identified by SEQUEST that includes the peptide sequence, monoisotopic mass, and which replicate run where the peptide was quantified. Peptides displayed with a ratio of 0 were identified by SEQUEST, but failed to pass the criteria assigned for MS1 quantification, including peak shape, co-elution, and isotopic envelop score. Peptides with a ratio of 20 were classified as "singletons" and arbitrarily assigned a ratio of 20, based on the criteria listed in the Supplemental Methods. Peptides with M* signify oxidized methionine. Data is filtered based on proteins passing the criteria described in the Supplemental Methods (Median ratio ≥ 5, at least 1 quantified peptide from Runs 1 - 2 AND at least 1 quantified peptide from Runs 3 - 4) for the 17-ODYA / NH_2OH experiments.

Dataset S1, Table 3. Candidate palmitoylated protein substrates of DHHC5 identified by SILAC-based proteomic profiling of 17-ODYA enriched proteins in WT and DHHC5-GT NSCs. Runs 1, 2, and 3: Light = wild type, Heavy = DHHC5-GT. Runs 4, 5, and 6: Light = DHHC5-GT, Heavy = wild type. Data is filtered based on the list of specifically enriched proteins identified in the two control groups (Dataset S1, Tables 1 and 2). A protein is identified in one line that includes the IPI number, description, Ensembl identifier, median ratio across all quantified peptides, and notation to signify runs containing quantified peptides assigned to the specific protein. Below this line is a list of peptides identified by SEQUEST that includes the peptide sequence, monoisotopic mass, and which replicate run where the peptide was quantified. Peptides displayed with a ratio of 0 were identified by SEQUEST, but failed to pass the criteria assigned for MS1 quantification, including peak shape, co-elution, and isotopic envelop score. Peptides with a ratio of 20 were classified as "singletons" and arbitrarily assigned a ratio of 20, are displayed but not included in the median calculation. Peptides with M* signify oxidized methionine.

Dataset S1, Table 4. SILAC proteomic analysis of unenriched membrane proteomes from WT and DHHC5-GT NSCs with relative ratios are shown. Run 1: Light = wild type, Heavy = DHHC5-GT. Run 2: Light = DHHC5-GT, Heavy = wild type. Data is filtered based on the list of specifically enriched proteins identified in the two control groups of experiment (Dataset S1, Tables 1 and 2). A protein is identified in one line that includes the IPI number, description, Ensembl identifier, median ratio across all quantified peptides, and notation to signify runs containing quantified peptides assigned to the specific protein. Below this line is a list of peptides identified by SEQUEST that includes the peptide sequence, monoisotopic mass, and which replicate run where the peptide was quantified. Peptides displayed with a ratio of 0 were identified by SEQUEST, but failed to pass the criteria assigned for MS1 quantification, including peak shape, co-elution, and isotopic envelop score. Peptides with a ratio of 20 were classified as "singletons" and arbitrarily assigned a ratio of 20, are displayed but not included in the median calculation. Peptides with M* signify oxidized methionine.

Dataset S1, Table 5. Data from Dataset S1, Tables 1, 2, 3, and 4 are summarized. The “Validation” column lists the control experiment(s) where the protein was identified as specifically enriched (Experiment 1 = ODYA, Experiment 2 = NH_2OH, or both experiments 1 and 2 = BOTH). The “N” column lists the number of peptides for a specific protein that reported quantifiable peptides. Singleton peptides are not counted in the calculation of the median, mean, and number of quantified peptide events per protein. In the case where only singletons (and no quantified peptides) are identified for a protein, these proteins are reported as “S”. Proteins with a reported ratio of “0” did not report quantifiable peptides. Standard errors (“St.err.”) are reported.

Supplemental Data References:

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Figure S1