Impaired Coenzyme A metabolism affects histone and tubulin acetylation in Drosophila and human cell models of Pantothenate Kinase Associated Neurodegeneration

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Supplementary Figures

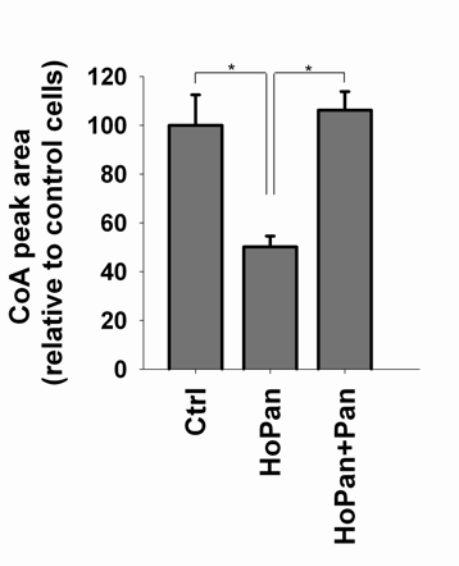


Figure S1. *CoA levels are decreased in HoPan treated S2 cells.* S2 cells were left untreated, treated with HoPan (0.5 mM) or treated with HoPan and pantethine (0.1 mM) and CoA levels were measured (see Supp Methods) after for 48 hours.

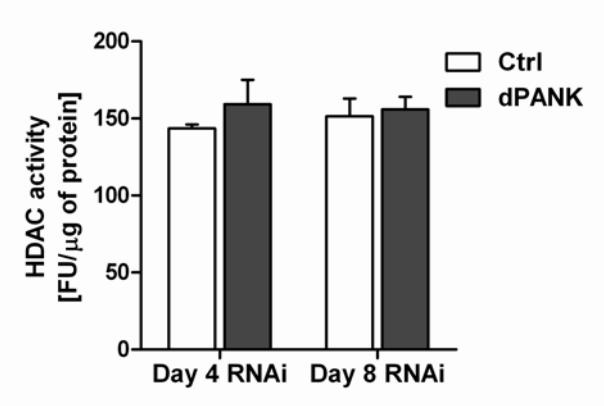


Figure S2. *dPANK/fbl RNAi treatment in Drosophila S2 cells has no effect on HDAC activity.*

HDAC activity was measured (see Supp. Methods) in control cells and in dPANK/Fbl depleted cells after 4 and 8 days of RNAi treatment.

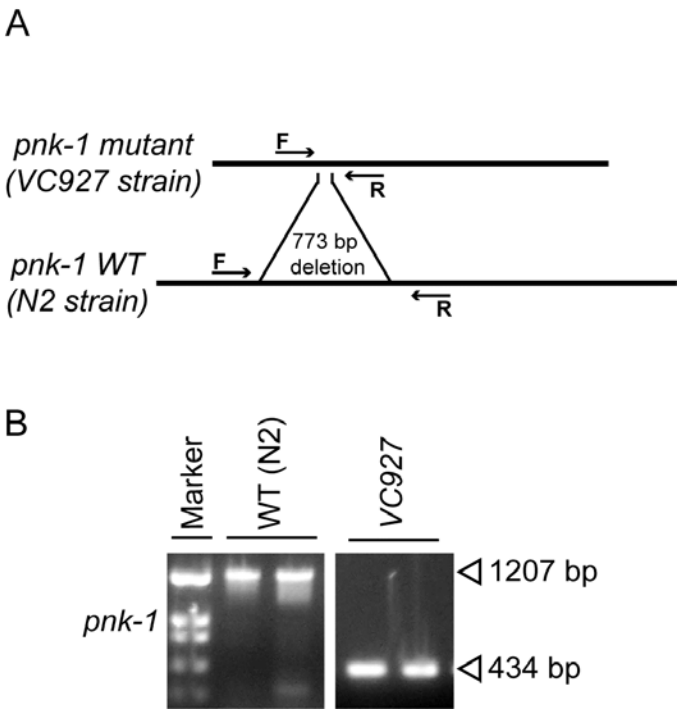


Figure S3. *C. elegans mutant strain VC927 carries a deletion in pantothenate kinase gene pnk-1*. **A,** Schematic representation of the 773 bp deletion region in *pnk-1* gene present in the *C. elegans* VC927 strain. Arrows indicate the position of primers (forward - F and reverse - R) used to confirm the presence of the deletion. **B,** Single worm PCR amplification of a fragment of *C. elegans pnk-1* gene was performed to confirm the 773 bp deletion in VC927 strain used in this study. N2 worms were used as a wild type control. See supplementary material and methods for the details on the PCR reaction and primer sequences

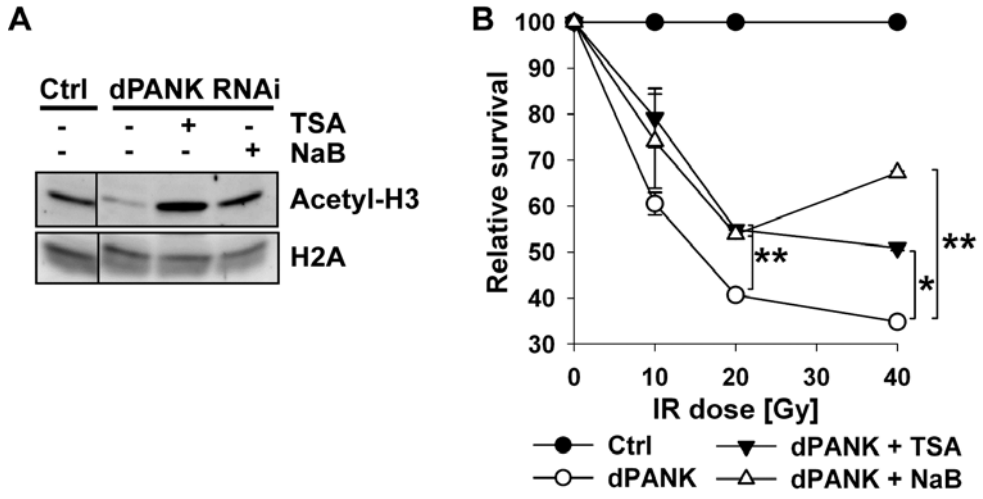


Figure S4. *Decreased levels of acetylated histones coincide with radiation sensitivity of dPANK/Fbl-depleted S2 cells.* **A,** Cells were treated with HDAC inhibitors (TSA or NaB) 5 hours before the exposure to ionizing radiation. Acetylation of histone 3 was assayed with western blotting to demonstrate the effect of inhibitors. H2A was used as a loading control. **B,** Cell survival was determined after treating the control cells and dPANK/Fbl-depleted cells with HDAC inhibitors (TSA or NaB) followed by the exposure to increasing doses of ionizing radiation.

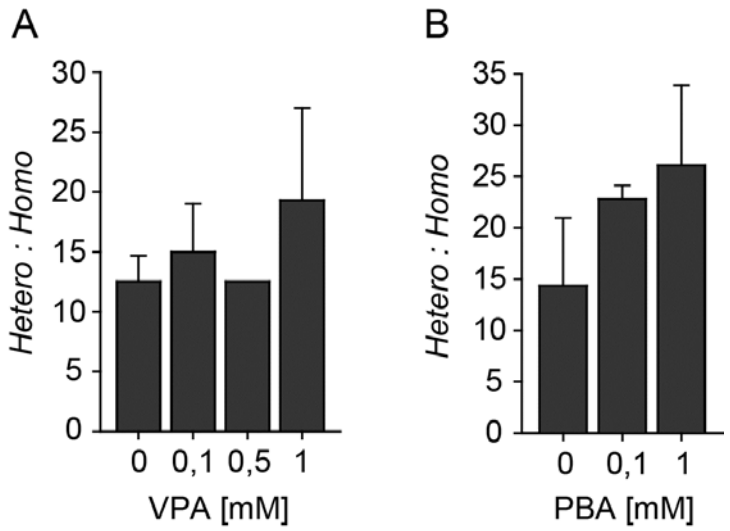
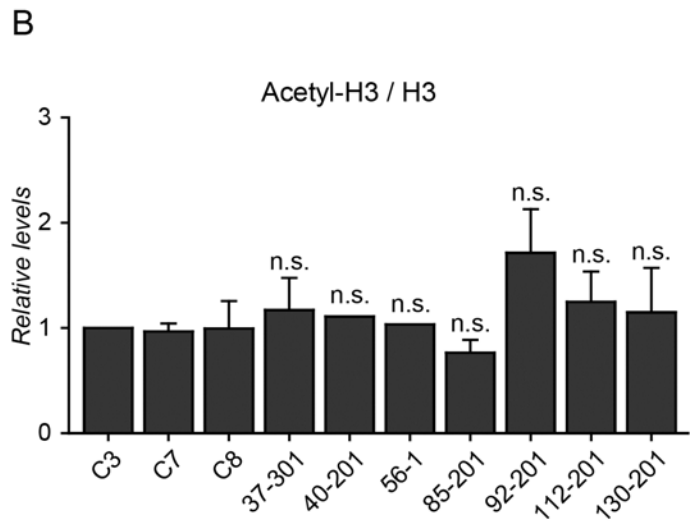


Figure S5. *VPA or PBA feeding fails to improve the eclosion rates of dPANK/fbl mutant flies. dPANK/fbl* mutant flies were raised on fly food supplemented with increasing doses of valproic acid (VPA) **(A)** or sodium phenyl-butyrate (PBA) **(B)**. The eclosion rates were determined as described in Figure 5D and material and methods section. For both compounds, concentrations above 1 mM were toxic for all genotypes, resulting in low numbers of eclosed flies.

A

Sample ID	*hPANK2* mutation
C3	None
C7	None
C8	None
37-301	206-228 del 243 del
40-201	502 C>T 1231 G>A
56-1	1231 G>A homozygous
85-201	526 C>T 1231 G>A
92-201	519 C>G 1231 G>A
112-201	1021 C>T 1231 G>A
130-201	993-996 del homozygous



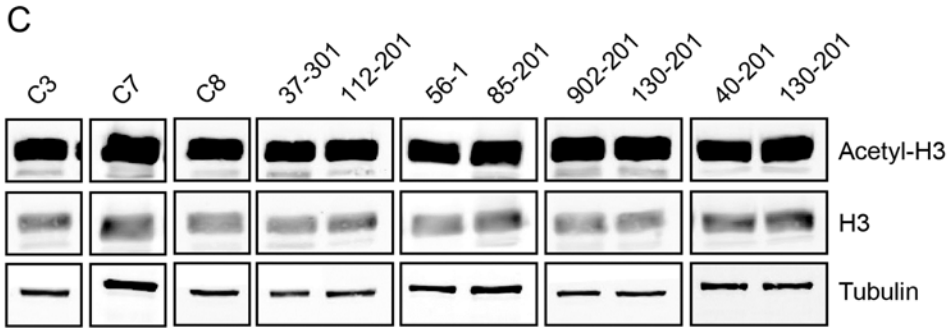


Figure S6. *No significant changes in the levels of H3 acetylation can be observed in PKAN patients-derived lymphoblasts.* Levels of acetylated histone 3 were determined by Western blot analysis using lymphoblasts derived from various PKAN patients and control healthy individuals. **A,** Three control lymphoblast lines (C3, C7 and C8) were analyzed in addition to seven patient-derived lines (37-301, 40-201, 56-1, 85-201, 92-201, 112-201, 130-201). A table indicates mutations present in the *hPANK2* gene in each cell line tested. **B,** Quantification of the relative levels of acetyl-histone 3 in the lymphoblast lines described in A. Total H3 was used as a loading control and the value obtained for C3 control sample was set us 1. Error bars represent st.dev.; n.s. – not significant (vs. C3 control) **C,** Representative blots used for the analysis of acetyl-H3 levels as shown in B. As an additional loading control tubulin was visualized.

Supplementary Methods

Measurement of total CoA levels by HPLC

The levels of total CoA in Drosophila Schneider's S2 cells were measured after pre-column derivatization with ammonium 7-flurobenzo-2-oxa-1,3-doazole-4-sulfonate (SBD-F) using reverse phase HPLC-fluorescence detection. The pre-column derivatization procedure for both S2 cell samples and CoA standard dilutions was performed as previously described [1]. Briefly 10 million Drosophila Schneider's S2 cells were pelletized and washed with ice cold phosphate buffered saline. 100 uL of borate buffer (0.1M containing 1 mM EDTA disodium, pH 9.5) was added to the cell pellets and sonicated three times to lyse the cells. To the lysate 20uL Tributylphosphine (10% v/v in DMF) was added and allowed to react at room temperature for 10 min. Proteins were removed and 5 µL of ammonia (12.5%) was added to an aliquot of the resulting solution (50 µL) and derivatized with 45 µL solution of SBD-F (1mg/ml in borate buffer). The derivatized sample was then analyzed using High-Performance Liquid Chromatography (HPLC) in combination with a fluorescence detector with excitation at 385 nm and emission at 515 nm, using optimized chromatographic conditions (manuscript of complete chromatographic conditions and CoA derivatization/stability is under preparation and will be published elsewhere).

HDAC activity assay

S2 cells (5 million cells/sample) were pelleted, washed once with PBS and resuspended in 200 µl of cold HDAC Cell Lysis Buffer (Enzo Life Sciences). Lysates were incubated on ice for 15 min, followed by a brief homogenization by passing 5 times through a 26G needle. Homogenates were centrifugated at 10000 rpm, 4 ^{0}C for 10 minutes and the supernatant was used for protein estimation and for the HDAC activity measurement. Protein content was determined with a DC Protein Assay (BioRad). HDAC activity was assayed with the fluorymetric HDAC Assay Kit (Sigma) according to manufacturer instructions. Fluorescence readings for each sample were averaged and corrected for the total protein levels.

C. elegans single worm PCR

Total DNA was obtained by a single worm lysis in 5 µl lysis buffer containing 10 µl/ml ProtK. Worms were lysed at 60 ^{0}C for one hour, followed by 95 ^{0}C for 15 minutes. 1 µL of the lysate was used for a standard PCR reaction. Primers were designed in front and behind the deletion part in the pnk-1 gene: TTTGTCGCGAGTTCTTGTAAAGGCT (forward) and GAGCAGGTGTGAGCAGGCTTCC (reversed).

Measurement of the histone acetylation levels in PKAN patient lymphoblasts

Lymphoblasts from healthy and PKAN diagnosed individuals were derived from blood samples and cultured as previously described [2]. The exception to this was Control 8, which was obtained from the National Institute on Aging through the Coriell Cell Repositories (New Jersey, USA) and then cultured similarly to the other samples. Cells were counted, and equal numbers were spun down and harvested in Laemmli buffer, sonicated on ice, then boiled. Western blots were run using equal sample volumes and developed with secondary fluorescent antibodies. Blots were visualized/analyzed with a Licor Odyssey Infrared Imaging System and associated Odyssey software. Primary antibodies included Active Motif Histone H3 acetyl antibody (rabbit, 1:1500), Active Motif Histone H3, C-terminal antibody (rabbit, 1:25000), and monoclonal anti-α-tubulin (mouse, 1:4000). Secondary antibodies

included Invitrogen Alexa Fluor 680 anti-rabbit IgG (goat, 1:10000) and Rockland anti-mouse IgG Antibody IRDye800CW Conjugated (goat, 1:10000).

References:

1. Imai, K., Toyo'oka, T., and Watanabe, Y. (1983). A novel fluorogenic reagent for thiols: ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. Anal Biochem *128*, 471-473.
2. Wall, F.E., Henkel, R.D., Stern, M.P., Jenson, H.B., and Moyer, M.P. (1995). An efficient method for routine Epstein-Barr virus immortalization of human B lymphocytes. In Vitro Cell Dev Biol Anim *31*, 156-159.