Supplemental Information

Coupled Control of Distal Axon Integrity
and Somal Responses to Axonal Damage
by the Palmitoyl Acyltransferase ZDHHC17

Jingwen Niu, Shaun S. Sanders, Hey-Kyeong Jeong, Sabrina M. Holland, Yue Sun, Kaitlin M. Collura, Luiselys M. Hernandez, Haoliang Huang, Michael R. Hayden, George M. Smith, Yang Hu, Yishi Jin, and Gareth M. Thomas
A

B

C

D

Niu, Sanders, Jeong et al., Figure S1, related to Fig 1
Figure S1, related to Figure 1: Characterization of Optic Nerve Crush (ONC) response and confirmation of AAV-mediated DLK knockdown and AAV co-infectivity in vivo.

A: Retinal flat-mounts were isolated from uninfected mice three days after sham injury (Sham) or optic nerve crush (ONC) and immunostained with the indicated antibodies. ONC markedly downregulates Brn3a, a marker of healthy RGCs, and increases phosphorylation of the injury response transcription factor c-Jun (P-c-Jun), consistent with prior reports (Watkins et al. 2013; Welsbie et al. 2013).

B: Retinas of mice that were injected with control AAV or AAV-DLKsh and subjected to sham injury or ONC 10 days later. Three days after ONC or sham injury, retinas were isolated and immunostained with the indicated antibodies. DLK is upregulated after ONC in retinas infected with control AAV, consistent with prior reports (Watkins et al. 2013; Welsbie et al. 2013). In contrast, anti-DLK antibody detects only background signals and Brn3a signal is not reduced following ONC in DLK ‘knockdown’ retinas, consistent with (Watkins et al. 2013; Welsbie et al. 2013).

C: Quantified data from B reveal that ONC upregulates DLK fluorescent signal in retinas infected with control, but not DLKsh-expressing, AAV. ****; p<0.0001, **; p<0.01. 2-way ANOVA: virus p=0.0004 [F (1,12) = 23.50], treatment p=0.0021 [F (1,12) =15.31], interaction p=0.0043 [F(1,12) = 12.36. N=3-5 per condition.

D: Flat-mounted retinas from Fig 1B were assessed to determine the fraction of GFP-positive cells that were also positive for either HA-tagged DLK-WT* or –CS*. Quantified data (4 images per retina, n=3-5 retinas per condition) confirm that >80% of GFP-positive RGCs are also HA-positive i.e. coinfected with both AAVs. All data are mean ± SEM.
Figure S2, related to Fig 2
Figure S2, related to Figure 2: Genomic details of *C. elegans* PAT mutant strains and evidence that ZDHHHC17 regulates DLK distribution and palmitoylation via an AnkR-zDABM interaction.

**A:** Schematics of *dhhc*-13 and *dhhc*-14 genomic loci, with red lines below indicating the locations of mutations. *dhhc*-13(*gk36*) deletion primarily removes sequences 5’ to ATG. To obtain a definitive null of *dhhc*-13, we generated *dhhc*-13(*ju1673*) by CRISPR/Cas9, which deletes 3.7kb sequence from exon 1 to exon 10. *dhhc*-14(*gk330*) deletion removes exons 7 and 8 and is predicted to induce a frameshift, likely leading to genetic null.

**B:** HA immunoprecipitates (IPs, top and second panels) and parent lysates (third and fourth panels) from HEK293T cells transfected to express GFP-tagged wtDLK (DLK-GFP) plus the indicated HA-tagged ZDHHHC-PATs, blotted with the indicated antibodies. Similar results were obtained in two other experiments.

**C:** Schematic of ZDHHHC17 and DLK domain structure, showing locations of mutations disrupting AnkR domain (N100A) and predicted zDABM (PVAA) in ZDHHHC17 and DLK, respectively.

**D:** ZDHHHC17 palmitoylation of DLK involves an AnkR-zDABM interaction. HEK293T cells transfected to express the indicated cDNAs were immunostained with the indicated antibodies. Right column shows merged images of columns 1-3 for each condition. In HEK293T cells, targeting of DLK-GFP to the Golgi (detected by GM130 marker) is palmitoylation-dependent (Martin et al. 2019).

**E:** Quantified data from D confirm that Golgi targeting of DLK-GFP is enhanced by HA-ZDHHHC17WT but not by HA-ZDHHHC17-N100A mutant, and is also disrupted by DLK zDABM mutation (PV-AA). **; p<0.01, ****; p<0.0001, 1-way ANOVA, Bonferroni post hoc test. N=17-27 cells per condition.

**F:** Acyl- Biotinyl Exchange (ABE) samples (left) and parent lysates (right) from HEK293T cells transfected to express the indicated forms of DLK-GFP and HA-ZDHHHC17, blotted with the indicated antibodies. Center: signals from parallel ABE assays lacking the key reagent hydroxylamine (NH$_2$OH). ZDHHHC17-N100A mutation and DLK-GFP PV--AA mutation both reduce DLK-GFP palmitoylation by ZDHHHC17.

**G:** Quantified DLK-GFP palmitoylation from multiple determinations from F. (N=4); *; p<0.05, ***; p<0.001 relative to wild type, 1-way ANOVA, Bonferroni post hoc test.

**H:** Quantified intensities of ZDHHHC17 protein levels from Fig 2E, normalized to tubulin, confirm efficacy of Zdhhc17 shRNAs. N=9-10 individual cultures per condition; ***; p<0.001, **; p<0.01 versus control virus condition, Kruskal Wallis non-parametric test with Dunn’s multiple comparison post hoc analysis.

**I:** Quantification of DLK total protein levels from Zdhhc17 knockdown experiments (Fig 2D-E) confirm slight, but not statistically significant, reduction of DLK levels by Zdhhc17 shRNAs. n=5 determinations per condition. **; p<0.01, 1-way ANOVA, Bonferroni post hoc test.

**J:** Combined knockdown of Zdhhc5 and Zdhhc8 does not reduce DLK palmitoylation in DRG neurons. Cultured DRG neurons were infected with control lentivirus (expressing GFP alone) or with lentiviruses expressing GFP plus shRNAs against Zdhhc5 and Zdhhc8. Cultures were lysed 7 days later and processed for ABE. Western blots of ABE fractions (top) and total lysates (2$^{nd}$-4$^{th}$ panels) reveal that combined Zdhhc5/8 knockdown does not reduce DLK palmitoylation. Efficacy of Zdhhc5/8 knockdown in these samples was confirmed in (Collura et al., 2020). Right-hand histogram: quantified data, n=3 determinations per condition. n.s.; not significant, t-test with Welch’s correction.

**K:** Zdhhc17 knockdown also reduces JNK3 palmitoylation. ABE fractions and total lysates from Fig 2D were immunoblotted to detect JNK3. Quantified data (right hand histogram) confirms that Zdhhc17 knockdown reduces JNK3 palmitoylation, consistent with a prior report that ZDHHHC17 is a PAT for JNK3 (Yang et al. 2013), although JNK3 palmitoylation has not been reported to be functionally important for DLK-JNK pro-degenerative axonal signaling. n=5 determinations per condition. **; p<0.01, 1-way ANOVA, Bonferroni post hoc test.
Niu, Sanders, Jeong et al., Figure S3, related to Fig 3
Figure S3, related to Figure 3: Importance of DLK palmitoylation for pro-degenerative signaling in DRG neurons and additional links between ZDHHC17 and DLK.

A: Images of GFP signal (left) and merged p-cJun and NeuN signals (right) from cultured DRG neurons infected with the indicated lentiviruses, subjected to TD for 4h and immunostained. DLK knockdown greatly reduces TD-induced c-Jun phosphorylation. ShRNA-resistant WT-DLK (WT-DLK*), but not shRNA-resistant DLK-CS (DLK-CS*), rescues this effect.

B: Quantified data from A. **P<0.01 versus control virus TD condition. 1-way ANOVA, Bonferroni post hoc test, n= 3-6 determinations per condition.

C: Images of GFP (left), activated caspase-3 (Casp3, middle) and merged GFP/Casp3 fluorescent signals (right) from cultured DRG neurons infected with the indicated lentiviruses, subjected to TD for 24h and immunostained. DLK knockdown greatly reduces TD-induced cleaved caspase-3 levels. ShRNA-resistant WT-DLK (WT-DLK*), but not shRNA-resistant DLK-CS (DLK-CS*), rescues this effect.

D: Quantified data from C. ****P<0.0001 versus control virus TD condition. 1-way ANOVA, Bonferroni post hoc test, n= 3-4 determinations per condition.

E: Correlation of Zddhc17 and DLK (Map3k12) expression in the nervous system. DLK expression in 265 mouse nervous system cell types was plotted individually against expression of each of the 23 mouse ZDHHC PATs (from quantitative single cell RT-PCR data; www.mousebrain.org). Linear regression analysis was performed and the r² value was determined for each of the 23 pair-wise comparisons.

F: Histogram of R-squared value for each of the 23 pairwise comparisons in E confirms that Map3k12 expression correlates better with Zdhhc17 expression than with any other PAT.
Figure S4, related to Figure 4. Additional evidence that ZDHHC17 is a major NMNAT2 PAT.

A: NMNAT2 binds ZDHHC17. Western blots of HA immunoprecipitates (top, second panels) and parental cell lysates (third, bottom panels) from HEK293T cells transfected with the indicated cDNAs and blotted with the indicated antibodies.

B: ZDHHC17 palmitoylates NMNAT2. ABE samples (left panels) and total lysates (right panels) from HEK293T cells transfected with the indicated cDNAs. Palmitoylation of NMNAT2 is greatly increased by HA-ZDHHC17.

C: Histogram of quantified NMNAT2 total levels, from samples in Fig 4C. Zdhhc17 knockdown reduces NMNAT2 total expression. ***; p<0.001 versus control virus condition, Kruskal Wallis non-parametric test with Dunn’s multiple comparison post hoc analysis.

D: NMNAT2 palmitoylation by ZDHHC17 involves an AnkR-zDABM interaction. Western blots of ABE samples (left) and parent lysates (right) from HEK293T lysates expressing the indicated forms of ZDHHC17-HA and NMNAT2-myc. NMNAT2 palmitoylation is reduced by ZDHHC17-N100A mutation, or by NMNAT2-4A and 3BR mutations individually, and is reduced further in the NMNAT2-4A-3BR combination mutant.

E: Quantified data from n=7 determinations per condition from D. *<0.05, **<0.01, ***<0.001; 1-way ANOVA, Bonferroni post hoc test.

F: Lysates from DRG cultures infected as in Fig 4E were blotted with the indicated antibodies.

G: Quantified NMNAT2-myc expression from n=4 infections per condition from F confirms that WT and 4A-3Br forms of NMNAT2-myc express similarly: n.s.: not significant, t-test.

H: Correlation of Zddhc17 and Nmnat2 expression in the nervous system. Nmnat2 expression in 265 mouse nervous system cell types was plotted individually against expression of each of the 23 mouse ZDHHC PATs (from quantitative single cell RT-PCR data, www.mousebrain.org). Linear regression analysis was performed and the r^2 value was determined for each of the 23 pair-wise comparisons.

I: Histogram of R-squared value for each of the 23 pairwise comparisons in H confirms that Nmnat2 expression correlates better with Zdhhc17 expression than with any other PAT.
Niu, Sanders, Jeong et al., Figure S5, related to Fig 5
Figure S5, related to Figure 5. Additional evidence that loss of ZDHHC17 causes NMNAT-dependent axon degeneration *in vivo.*

*A:* Flat-mounted retinas from the same mice assessed for optic nerve integrity in Fig 5E (13 days post-AAV injection), immunostained with the indicated antibodies.

*B:* Quantified data from *A* confirm that RGC somal viability in Zdhhc17<sup>f/f</sup> mice is unaffected 13 days post AAV-Cre delivery. n.s., not significant, 1-way ANOVA, Bonferroni *post hoc* correction, n=4-8.

*C:* Immunostained distal optic nerves from Zdhhc17<sup>f/f</sup> mice intravitreally injected with the indicated AAVs and fixed 17 days later.

*D:* Quantified data from *C* confirm that distal optic nerve axonal fragmentation and microgliosis at 17 days post-AAV injection are greatly increased in Zdhhc17 CKO and rescued by cytoNMNAT1-HA. n=5-8. **,** p<0.01, 1-way ANOVA, *post hoc* Bonferroni test.

*E:* As *C*, except that images show proximal optic nerves close to the retina. Images are representative of 5-8 mice per condition.

*F:* Retinal flat mount images immunostained with the indicated antibodies at 17 days post AAV injection.

*G:* Quantified data from *F* reveal slightly lower RGC somal viability (16% reduction) with prolonged Zdhhc17 loss. n=4. **,** p<0.01, t-test.
Table S1, related to STAR Methods: Oligonucleotides used in this study

| OLIGONUCLEOTIDE | SOURCE | IDENTIFIER |
|-----------------|--------|------------|
| shRNA#1 against rat Zdhhc17: 5'-ATGAATGCCAGGAGATACAGCACTTTAA-3' | This paper | N/A |
| shRNA#2 against rat Zdhhc17: 5'-CATTAAGCTACAGAAGAA-3' | This paper | N/A |
| shRNA against DLK (Map3k12): 5'-GCACCTGGACACACCTTT-3' | Ghosh et al., 2011 | N/A |
| Genotyping primer1 for Zdhhc17/mice: 5'-GGAGAATGTTAGAAGAGCTCGTACC-3' | Sanders et al., 2016 | N/A |
| Genotyping primer2 for Zdhhc17/mice: 5'-GAGGAAAGCATGCAAGAGCAGCTCTTCTC-3' | Sanders et al., 2016 | N/A |
| Primer: dhhc-13(gk36) genotyping  Forward: 5' GAGGAAGTAAACTGCAGCGCG  Reverse: 5' CGAATAACGAGGAACGCGCAG | This paper | YJ9595 YJ9596 |
| Primer: dhhc-14(gk330) genotyping  Forward: 5' GAGGAAGTAAACTGCAGCGCG  Reverse: 5' CGAATAACGAGGAACGCGCAG | This paper | YJ9597 YJ9598 |
| Alt-R CRISPR-Cas9 crRNA for dhhc-13  5' GAGAAACUGAAACUCCACUGG  5' AUUAUGUUCUGGCGAUGUUGG | This paper | YJ12383 YJ12384 |
| Alt-R CRISPR-Cas9 crRNA for dpy-10 as co-CRISPR marker  GCUACCAUAGGCACCAC  GAGGUUUGAGAGCUAUGCU | Arribere et al., 2014 | N/A |
| Alt-R CRISPR-Cas9 tracrRNA | IDT | Cat#1072532 |
| Primer: dhhc-13(ju1673) genotyping  Forward: 5' TTGATTACCAATGCCCGGACG  Reverse: 5' ACTGAGTGAGGAGACGATC  Internal: 5' TCGAGTGAGTGAAGCAG | This paper | YJ12385 YJ12386 YJ12387 |
| shRNA#1 against rat Zdhhc17: 5'-ATGAATGCCAGGAGATACAGCACTTTAA-3' | This paper | N/A |
| shRNA#2 against rat Zdhhc17: 5'-CATTAAGCTACAGAAGAA-3' | This paper | N/A |
| shRNA against Zdhhc5: 5'-CCTCATGTATTCCAAGAGAT-3' | Thomas et al., 2012 | N/A |
| shRNA against Zdhhc8: 5'-CAGGATGCCACTCTCAGTGAGCCTCCAGC-3' | Collura et al., 2020 | N/A |