Appendix Information

Stress-induced vesicular assemblies of dual leucine zipper kinase are signaling hubs involved in kinase activation and neurodegeneration

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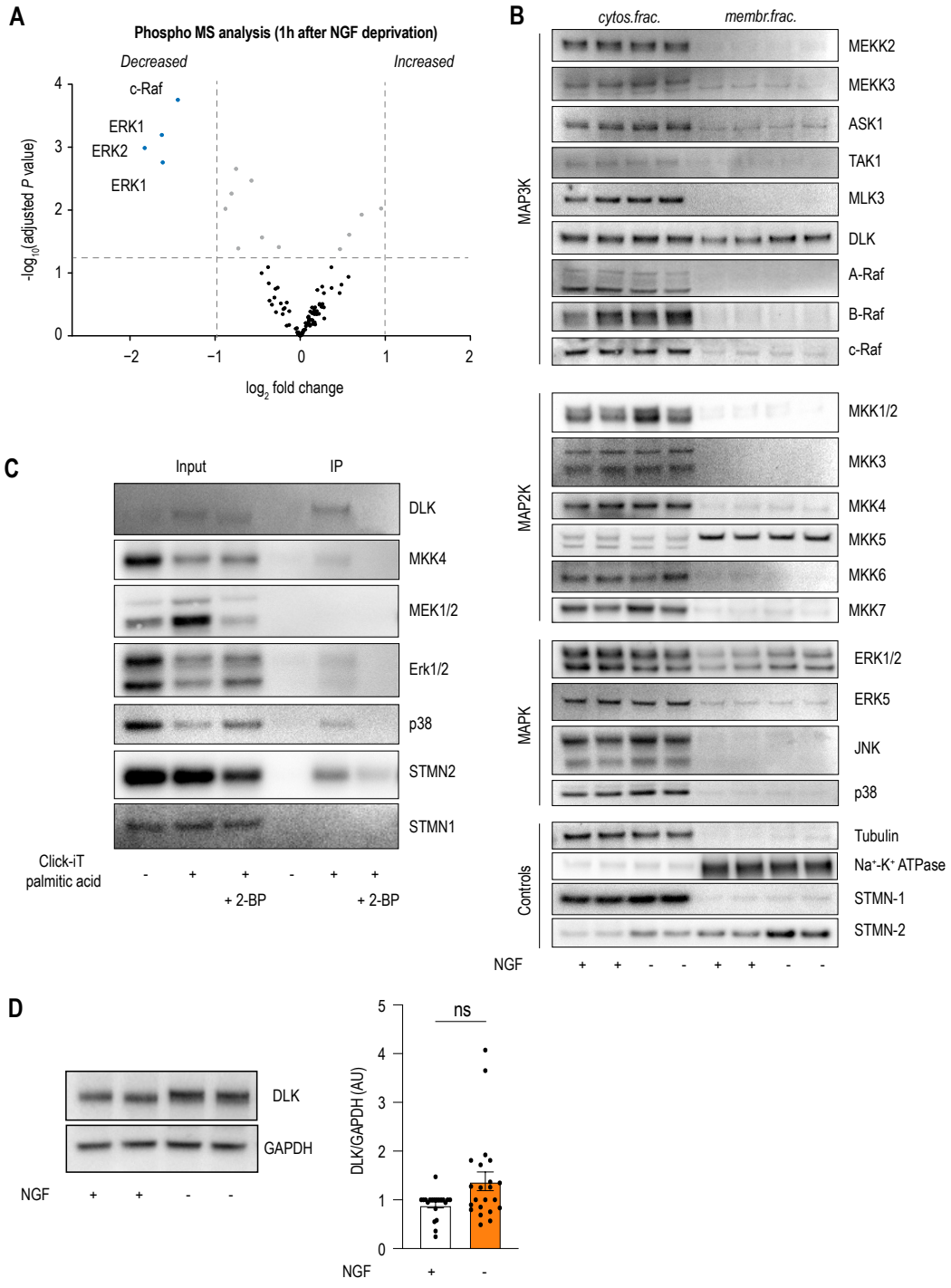
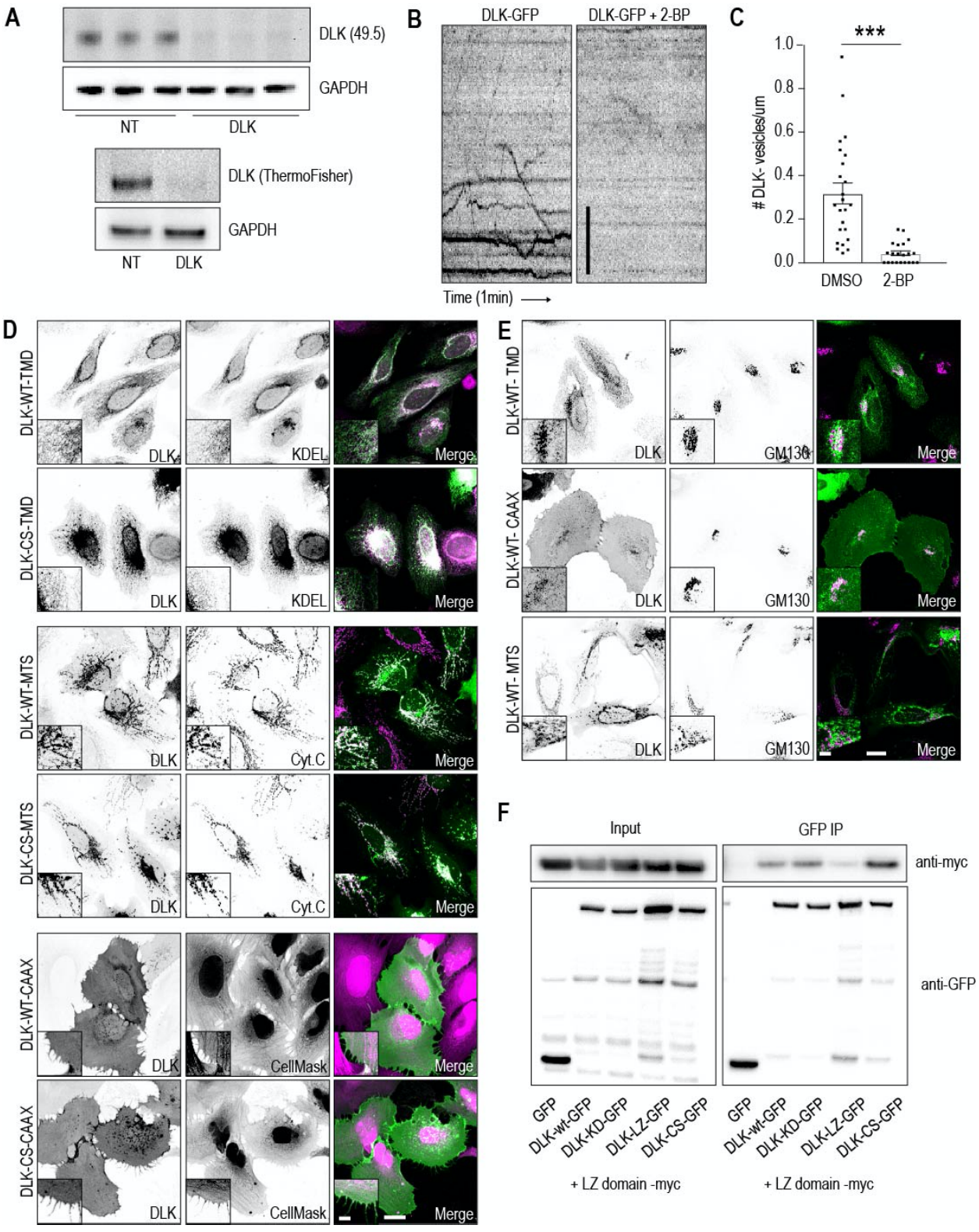
APPENDIX INFORMATION INVENTORY

Appendix Figures S1-S4



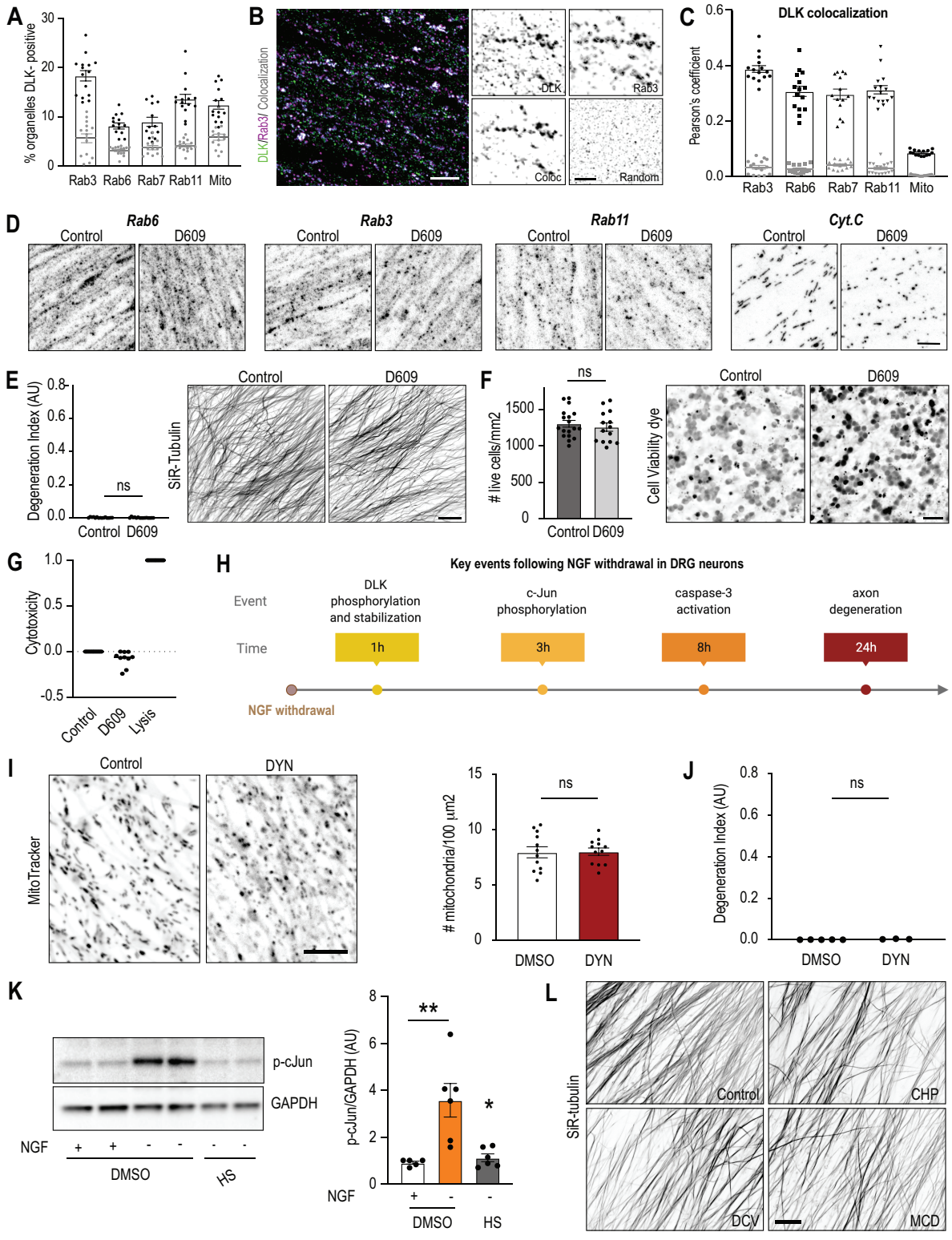
Appendix Figure S1 (related to Figure 1). Characterization of MAPK cascades components during NGF deprivation. **A**. Volcano plot of differently-regulated phosphosites of MAPK cascade components in 3DIV cultured embryonic DRG neurons upon 1h of NGF deprivation, with protein-level cutoffs set at log2 fold change >1.0 and –log10 adjusted P value >1.3 (P< 0.05), marked by dashed lines. Downregulated sites represented as blue points (n = 2 independent

experiments). **B.** Representative Western blots of the different MAPKs expressed in DRG neurons (DIV3) cultured in the presence or absence of NGF for 3h, and subjected to a membrane/cytosolic fractionation. α-tubulin and STMN-1 are used as controls of cytosolic proteins and Na^+/K^+-ATPase and STMN-2 as controls of membrane proteins. **C**. Representative Western blots of total and palmitoylated MAPKs detected by click-chemistry-based assays in the presence and absence of click-it palmitic acid and 2-bromopalmitate (2-BP) in DRG neurons. **D**. Representative Western blots of DLK and GAPDH, and quantifications of the relative levels of DLK protein in 3DIV cultured embryonic DRG neurons maintained for 3h in the presence and absence of NGF (n = 20-22 biological replicates, Mann-Whitney U test). All graphs represent mean ± SEM.



Appendix Figure S2 (related to Figure 2 and 3). Validation of different DLK antibodies and constructs. A. Representative Western blots of DIV3 DRG neurons nucleofected with a non-

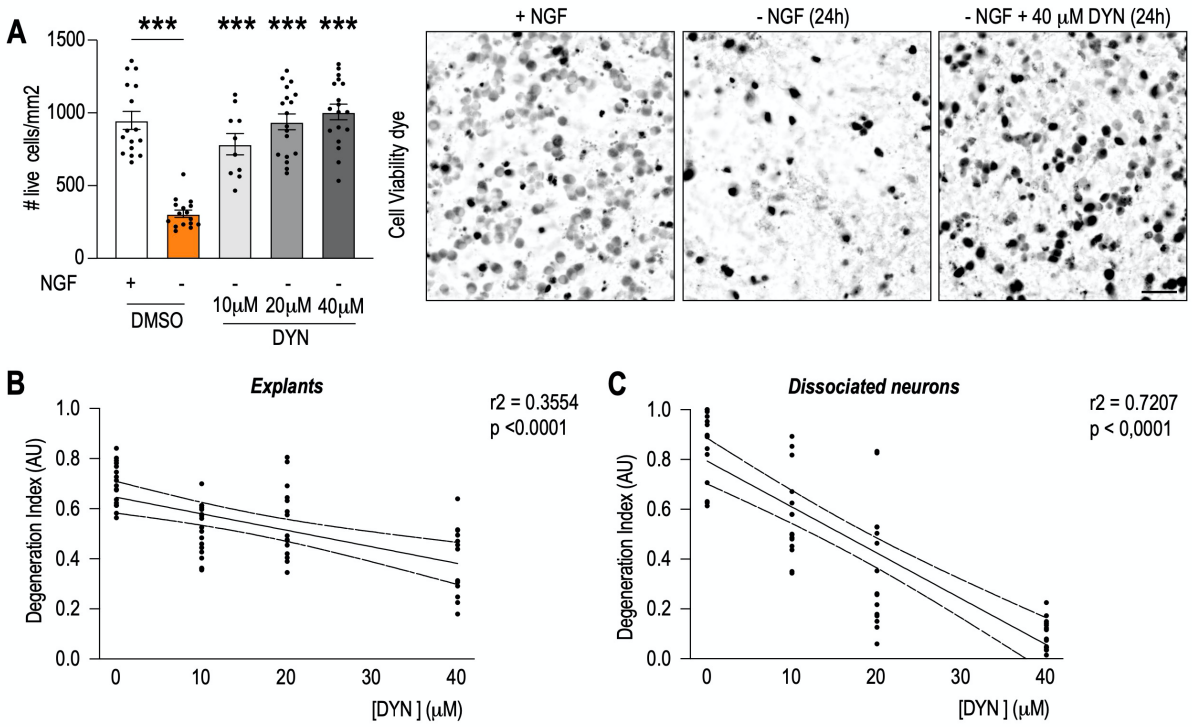
targeting siRNA (NT) or a siRNA against DLK (DLK), and blotted for endogenous DLK with two different antibodies (clone 49.5 and a commercial one from ThermoFisher) and GAPDH. **B-C.** Representative kymographs (B) and quantification of GFP-positive particle number (C) from DLK-GFP time-lapse recordings in DIV6 DRG neurons, in control situation DMSO or after 3h of 2-bromopalmitate (2-BP) treatment (n = 21-45 neurons/ condition; Mann-Whitney U test). **D**. Representative images of HeLa cells expressing the different HA-tagged DLK constructs (DLK-WT-TMD, DLK-CS-TMD, DLK-WT-CAAX, DLK-CS-CAAX, DLK-WT-MTS, DLK-CS-MTS), and stained with markers of endoplasmic reticulum (KDEL), mitochondria (cytochrome C) and membrane (CellMask). **E.** Representative images of HeLa cells expressing the different HA-tagged DLK constructs (DLK-WT-TMD, DLK-WT-CAAX, DLK-WT-MTS), and stained with a marker for Golgi apparatus (GM130) and an antibody against DLK. **F**. Western blots of whole lysate (Input) and GFP immunoprecipitated (GFP IP) from HeLa cells co-transfected with a myc-tagged DLK leucine zipper domain mutant together with GFP alone (GFP), GFP-tagged DLK wild-type (DLK-WT-GFP), kinase dead mutant (DLK-KD-GFP), leucine zipper domain mutant (DLK-LZ-GFP) or a palmitoyl-site mutant (DLK-CS-GFP), and incubated with anti-myc and anti-GFP antibodies. All graphs represent mean ± SEM. Scale bar represents 20 μm in (D) and (E), 10 μm in (B) and 5 μm in zooms in (D) and (E).



Appendix Figure S3 (related to Figure 4 and 5). Characterization of DLK localization and neuron integrity upon sphingomyelin synthesis and endocytosis inhibition. A. Object-based

colocalization analysis between markers for different intracellular compartments (Rab3, Rab6, Rab7, Rab11 and mitochondria) and DLK (black values) or same image after 90° rotation (grey values) in DIV3 cultured embryonic DRG neurons (n = 13-17 images/condition). **B**. Representative confocal deconvolved images and zooms of DIV3 cultured embryonic DRG neurons co-stained for endogenous DLK (green) and the marker for synaptic vesicles Rab3 (magenta). Colocalization between both channels is shown in white. A representative example of a pixel randomization generated by Costes test is included. **C**. Colocalization analysis between DLK (black values) or randomized DLK image (grey values), and markers for different intracellular compartments (Rab3, Rab6, Rab7, Rab11 and mitochondria) in DIV3 cultured embryonic DRG neurons (n = 13-17 images/condition). **D**. Representative confocal images of DIV3 cultured embryonic DRG neurons treated with D609 for 3h and stained for markers of post-Golgi secretory vesicles (Rab6), synaptic precursor vesicles (Rab3), recycling endosomes (Rab11) and mitochondria (cytochrome C). **E.** Quantification of fragmented axons using the degeneration index in cultured embryonic DRG neurons cultured for 3 days and treated with D609 for 3h (n = 5 confocal images/condition from 3 independent cultures; Mann-Whitney U test). **F.** Quantification of live cells in cultured embryonic DRG neurons cultured for 3 days and treated with D609 for 3h (n = 14-18 confocal images from 3 independent cultures; Unpaired t-test). **G.** Quantification of lactate dehydrogenase leakage from cultured embryonic DRG neurons cultured for 3 days and treated with D609 for 3h (n = 12 technical replicates from 3 independent cultures)**.** **H**. Scheme of key events and timeline following NGF deprivation. **I**. Representative images and quantifications of number of mitochondria (labeled with MitoTracker) from cultured embryonic DRG neurons treated with dynasore (DYN) for 3h (n = 12 confocal images/condition from 3 independent cultures, Unpaired t test). **J.** Quantification of fragmented axons using the degeneration index in cultured embryonic DRG neurons cultured for 3 days and treated with dynasore (DYN) for 3h (n = 3 confocal images/condition from 3 independent cultures; Mann-Whitney U test). **K**. Representative Western blots of p-cJun and GAPDH, and quantification of relative c-Jun phosphorylation levels in 3DIV cultured embryonic DRG neurons treated with a hypertonic sucrose solution (HS) for 3h in the absence of NGF (n = 5-6 biological replicates, Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test). **L**. Representative images of axons from 3DIV DRG neurons treated with chlorpromazine (CHP), dansylcadaverine (DCV) or methyl-β-cyclodextrin (MCD) and visualized by imaging the SiR-Tubulin probe. All graphs

represent mean ± SEM. Scale bar represents 50 μm in (E) and (F), 20 μm in (L), 10 μm in (I) and 5 μm in (B) and (D) and 2 μm in zoom on (B). Chart in H was created with BioRender.com.



Appendix Figure S4 (related to Figure 6). Characterization of axon degeneration in neurons upon dynasore treatments. A. Quantification of live cells in embryonic DRG neurons cultured for 2 days, and subsequently deprived of NGF and treated with different concentrations of dynasore (DYN) for 24 h (n = 10-18 images from 3 independent cultures, series of unpaired t-test/Mann-Whitney U tests with NGF withdrawal condition followed by a Holm-Sidak correction). **B**. Regression analysis of the axonal degeneration index in E12.5 DRG explants DRG explants cultured for 2 days, and subsequently deprived of NGF and treated with different concentrations of dynasore (DYN) for 24 h (n = 13-15 explants). **C**. Regression analysis of the axonal degeneration index in cultured embryonic DRG neurons cultured for 2 days, and subsequently deprived of NGF and treated with different concentrations of dynasore (DYN) for 24 h (n = 15 images from 3 independent cultures). Scale bar represents 50 μm in (A).