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Multilevel Analysis in Rural Cancer Control: A Conceptual Framework and Methodological Implications

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Syntaphilin-mediated docking of mitochondria at the growth cone is dispensable for axon elongation *in vivo*

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Syntaphilin-mediated docking of mitochondria at the growth cone is dispensable for axon elongation *in vivo*

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HH and MH contributed unpublished reagents and tools.

FEP designed and performed research, analyzed data and wrote the paper.

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Abstract

Mitochondria are abundantly detected at the growth cone, the dynamic distal tip of developing axons that directs growth and guidance. It is however poorly understood how mitochondrial dynamics relate to growth cone behavior *in vivo*, and which mechanisms are responsible for anchoring mitochondria at the growth cone during axon pathfinding. Here, we show that in retinal axons elongating along the optic tract in zebrafish, mitochondria accumulate in the central area of the growth cone and are occasionally observed in filopodia extending from the growth cone periphery. Mitochondrial behavior at the growth cone *in vivo* is dynamic, with mitochondrial positioning and anterograde transport strongly correlating with growth cone behavior and axon outgrowth. Using novel zebrafish mutant lines that lack the mitochondrial anchoring proteins Syntaphilin a and b, we further show that Syntaphilins contribute to mitochondrial immobilization at the growth cone. Syntaphilins are however not required for proper growth cone morphology and axon growth *in vivo*, indicating that Syntaphilin-mediated anchoring of mitochondria at the growth cone only plays a minor role in elongating axons.

Significance statement

Proper axon elongation and pathfinding are essential for nervous system wiring. The growth cone, a dynamic structure at the distal end of axons, mediates axonal growth and guidance. Here, we describe for the first time *in vivo* the behavior of mitochondria at the growth cone of elongating axons. We show that mitochondria accumulate in the growth cone central area and are also present in its periphery. We further provide evidence that Syntaphilin, which immobilizes mitochondria along mature axons, also docks mitochondria at the growth cone. However, loss of Syntaphilin did not cause a complete depletion of mitochondria from the growth cone and did not affect axon elongation, indicating that other mitochondria-docking factors regulate axon growth during development.

Introduction

Nervous system formation and function critically rely on mitochondria. The ability of mitochondria to produce ATP via oxidative phosphorylation and to buffer cytosolic calcium is especially important in neurons that have a high energy demand and require proper ion homeostasis. Mitochondrial dynamics, including transport, fission, and fusion, contribute to the correct distribution of mitochondria in axons and are therefore essential regulators of mitochondrial functions (Trevisan et al., 2018). Mitochondria are targeted to regions distant from the cell body, such as the axonal growth cone and synaptic terminals, through their active transport along microtubules (Melkov and Abdu, 2018). Overall, the importance of mitochondrial dynamics for proper neuronal development and function is emphasized by the large number of neurological disorders caused by mutations affecting mitochondrial proteins (Misko et al., 2010, Bertholet et al., 2016).

During development, distal growing axons and growth cones contain higher densities of mitochondria compared to proximal axonal regions (Morris and Hollenbeck, 1993). This asymmetrical distribution is thought to be necessary for axon growth (Smith and Gallo, 2018). For instance, *in vitro*, increasing mitochondrial density in the distal axon and growth cone by overexpressing the mitochondrial biogenesis regulator PGC-1α was shown to increase axonal length (Vaarmann et al., 2016). Other studies have reported that factors promoting or inhibiting axon growth regulate mitochondrial localization at the growth cone (Beck et al., 2012, Sainath et al., 2017). For example, adding the growth-promoting factor BDNF to cultured neurons increases distal mitochondrial density, whereas adding the repulsive guidance cue ephrin-A5 or using chondroitin sulfate proteoglycans as a non-permissive substrate both cause mitochondria to leave the growth cone (Beck et al., 2012, Sainath et al., 2017). While these *in vitro* studies have highlighted a significant role for mitochondria in axon extension and growth cone

morphology, mitochondrial dynamics at the growth cone during axon pathfinding have never been assessed *in vivo*.

The mechanisms responsible for maintaining mitochondria in distal growing axons are not yet fully understood. Mitochondria are able to attach to both microtubule and actin cytoskeletons (Boldogh and Pon, 2006), and could therefore be immobilized on microtubules in the growth cone central area after being transported along developing axons. Such stalling might be mediated by Syntaphilin (Snph), a mitochondrial membrane protein able to directly tether mitochondria to microtubules, thereby stalling mitochondrial transport (Kang et al., 2008). By immobilizing mitochondria, Snph was shown to reduce synaptic plasticity (Kang et al., 2008) and modulate axon branching (Courchet et al., 2013). Snph was further found to prevent mitochondrial transport towards the distal axon and inhibit axon regeneration after injury (Zhou et al., 2016). However, whether Snph also docks mitochondria at the growth cone during axon elongation has never been tested.

The zebrafish embryo offers a unique accessibility and transparency to monitor mitochondrial distribution and transport along axons *in vivo* (Plucinska et al., 2012, Campbell et al., 2014, Drerup et al., 2017, Wehnekamp et al., 2019). Here, we used time-lapse imaging of mitochondria in single retinal axons as they elongate along the optic tract in zebrafish to characterize for the first the time *in vivo* mitochondrial dynamics at the growth cone during axon pathfinding. We show that mitochondrial distribution at the growth cone correlates with axon growth status, and that mitochondrial transport towards the growth cone correlates significantly with axon elongation. We further demonstrate that Snph contributes to mitochondrial docking at the growth cone during axon pathfinding. However, growth cone morphology and axon elongation are unaffected in *snph* mutants, indicating that the direct anchoring of mitochondria to growth cone microtubules by Snph only plays a minor role in axon elongation.

Material and Methods

Zebrafish husbandry

All animal procedures were performed in accordance with the author's University Institutional Animal Care and Use Committee (IACUC). Zebrafish (*Danio rerio*) wild-type (WT, AB strain) and *lakritz* (*lak*) mutant (Kay et al., 2001) embryos were obtained from natural matings, raised at 28.5 ˚C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) in the presence of 150 mM 1-phenyl-2-thiourea (PTU) (Sigma) to prevent pigment formation, and staged by age and morphology (Kimmel et al., 1995). Zebrafish possess a polygenic sex determination system, and sex-associated chromosomal regions are not fixed for the species (Liew et al., 2012). Our experiments were conducted on embryos before the onset of sexual differentiation, which only occurs at about 2.5 months after metamorphosis is complete (Maack and Segner, 2003).

TALEN-mediated mutagenesis and genotyping of snph *mutants*

The TALENs to target zebrafish *snpha* and *snphb* were designed using TAL Effector Nucleotide Targeter 2.0 (https://tale-nt.cac.cornell.edu/node/add/talen) (Cermak et al., 2011). The TALENs for *snpha* contained the following repeat variable di-residues (RVDs): HD NI HD NN HD HD NI HD NG NN HD NI NN HD NI NN and NI NN HD HD NN NG NN HD NI HD NN NG NI NN NI NG NN HD, which targeted the sequences CACGCCACTGCAGCAG and AGCCGTGCACGTAGATGC, respectively. The TALENs for *snphb* contained the following RVDs: NN NI NN HD NI NI NG NI HD HD NG HD NI HD HD HD and NN HD HD NG NN NI NG NN HD NI HD NI HD HD NG HD, which targeted GAGCAATACCTCACCC and GCCTGATGCACACCTC, respectively. The TALEN cDNAs were assembled as described previously (Sakuma et al., 2013) and subcloned into pCS2pTAL3DD and pCS2pTALRR (Dahlem et al., 2012). Capped RNAs were synthesized from NotI-digested TALEN expression plasmids using SP6 RNA polymerase (Promega) in the presence of m'G(5')ppp(5')G RNA Cap Structure Analog (NEB). One nanoliter of solution

containing a pair of TALEN RNAs (0.4 µg/µL each) was injected into zebrafish embryos at one-cell stage. Deletion mutations in the target region were detected using heteroduplex mobility analysis (HMA) (Ota et al., 2013). The following primers were used: AGAATCATGGCATTCGTCCTC and TGAAGCCTCTCCACATTTTCTT to detect the 14-bp deletion in *snpha*; and AATGATAACCATGGCATTCGAC and CTTTAAGCCGTGCTCTCAGGT to detect the 4-bp deletion in *snphb*. PCR products were separated on 12% or 20% TBE (Tris-borate-EDTA) acrylamide gels or on a 4% Metaphor gel (Lonza). The *snphb* mutation was additionally detected using high-resolution melting analysis (HRMA) (Parant et al., 2009) using the following primers: AGCAATACCTCACTCCACTG and GCCTGATGCACACCTCTTTC.

Cloning of* snpha and snphb *coding sequences

mRNA from embryos at 24 hours-post-fertilization (hpf) was isolated using Trizol (ThermoFisher) and the RNeasy mini kit (Qiagen). cDNA was prepared from RNA using SuperScript III First-Strand Synthesis System (ThermoFisher). The following primers were used to amplify zebrafish *snpha* and *snphb* cDNA: *snpha* fw: TGTCCTTCTGCATCCATGTC; *snpha* rv: TCAGATAGGTGTCGCTCTTTC; *snphb* fw: ATGTCTTCGCCTTCAAATAAAAG; *snphb* rv: TCATATATTCATTCCCCCTGG. Amplicons were subcloned into PCRII-TOPO (Invitrogen) and sequenced to verify gene identity and confirm sequence orientation for the generation of sense and antisense RNA probes.

DNA plasmid constructs

All expression vectors were constructed using the Tol2kit Gateway cloning system (Kwan et al., 2007). We generated a pME-mitoEGFP entry clone by adding the mitochondrial targeting signal of the zebrafish *cox8a* gene to the 5' end of EGFP sequence using a BP-compatible forward primer. We generated a pME-Lifeact-TagRFP entry clone by fusing the Lifeact sequence (Riedl et al., 2008) upstream of TagRFP using a BP-compatible forward primer. Lifeact-TagRFP was used to label F-actin in retinal axons and improve the visualization of growth cone filopodia. We generated a pME-TagBFP-*snphb* by adding the

coding sequences of TagBFP (Evrogen) and a linker peptide (SGLRSRV) to the 5' end of *snphb*. The p3E-2A-TagRFPCAAX-pA entry clone that encodes a 2A peptide (Provost et al., 2007) and TagRFP targeted to the plasma membrane by the prenylation motif of Ras (Moriyoshi et al., 1996) was a generous gift from Dr. Kristen Kwan (University of Utah). The p5E-*isl2b* plasmid that drives specific expression in retinal ganglion cells (RGCs) was described previously (Pittman et al., 2008). Final isl2b:mitoEGFP-2A-TagRFPCAAX, isl2b:Lifeact-TagRFP, and isl2b:TagBFP-*snphb* plasmids were generated using LR reactions with the pDestTol2pA2 backbone (Kwan et al., 2007).

RNA isolation and reverse transcriptase (RT)-coupled droplet digital PCR (ddPCR)

For RNA isolation from whole embryos, 15 dechorionated WT embryos were lysed and homogenized in 500 µL Trizol at cleavage, pharyngula prim-5 (24 hpf), long-pec (48 hpf), protruding mouth (72 hpf), larval day 4 (96 hpf), and larval day 5 (120 hpf). For RNA isolation from eyes at 72 hpf, 50 eyes were manually dissected from WT or *lak* mutant embryos. Total RNA was isolated using the Direct-zol RNA Miniprep kit (Zymo Research) and eluted into 20 µL nuclease-free water. cDNA was synthesized with 5 µg of purified RNA as an input using the SuperScript III System. Digital droplet PCR (ddPCR) was then performed on a QX200 AutoDG instrument (Bio-Rad) using predesigned Taqman primers and probes for zebrafish *snpha*, *sphnb*, *gapdh*, and *18s* (Table 1). Briefly, ddPCR reactions were assembled using Bio-Rad 2x ddPCR Supermix for Probes (no dUTP) and contained 1 µg of cDNA and 250 nM primers/probe. PCR amplification was performed with the following parameters: 39 cycles of 94°C for 30 seconds and 60°C for 1 minute.

In situ *hybridization*

In vitro transcription of digoxigenin-labeled probes was performed using the RNA Labeling Kit (Roche Diagnostics Corporation) according to manufacturer's instructions. Embryos were dechorionated at the appropriate developmental stages and fixed in 4% paraformaldehyde in phosphate buffered saline (pH

7.4) for 2 hours at room temperature and overnight at 4˚C. Whole-mount *in situ* hybridization was performed as previously described (Thisse and Thisse, 2008). Sense probes were used as controls. After staining, embryos were cleared in 80% glycerol. Images were acquired using an Olympus SZX16 stereomicroscope equipped with an Olympus DP80 dual color camera and Cellsens standard software.

Imaging of mitochondria in retinal axons

Both isl2b:mitoEGFP-2A-TagRFPCAAX (30 pg) and isl2b:LifeAct-TagRFP (10 pg) plasmids were injected together with transposase mRNA into one-cell stage WT or *snph db* embryos. At 30-32 hpf, embryos were sorted for fluorescence, anesthetized in 0.015% tricaine, and embedded in a lateral view in 1% low-melt agarose in E3 medium + PTU. Their right eye was removed using a pulled glass pipette with a short taper as described in previous studies (Poulain et al., 2010, Poulain and Chien, 2013, Gaynes et al., 2015). Of note, removal of the contralateral eye preserves the underlying neuroepithelium, which ensures that the optic tract environment is not changed by the surgery and that retinal axon elongation and guidance are not affected. Embryos were allowed to recover until 46 hpf, when they were re-anesthetized and about one-third of the yolk was removed by squeezing it out through a small hole torn with sharpened tungsten needles. Embryos were allowed to recover, re-anesthetized at 50-52 hpf, and remounted in a lateral view in 1% low-melt agarose in E3 medium + PTU + tricaine in a membrane-bottomed petri dish for time-lapse imaging on a Leica TCS SP8X laser-scanning confocal microscope equipped with LAS X software, HyD detectors, and a 40x objective (digital zoom 3, pinhole 1.25). Z-series were acquired for up to 3 hours with 512x512 pixel resolution at 1-min intervals to minimize photobleaching. Z-intervals were 1 µm with a z-range of around 35-40 µm to account for potential movement of the embryo. Chamber temperature was maintained at 28.5°C. Maximal intensity projections for each time point were compiled and aligned using ImageJ software (Schindelin et al., 2012, Schneider et al., 2012)(RRID: SCR_002285) and the StackReg plugin (Thevenaz et al., 1998). Kymograph analyses of mitochondrial movement were performed using the ImageJ plugins

KymoToolBox (Zala et al., 2013)(RRID:SCR_016098) and KymoAnalyzer (Neumann et al., 2017). The number of mitochondria arriving at and leaving the growth cone were counted manually from the time-lapse sequences.

For the visualization of Snphb at the growth cone, isl2b:mitoEGFP-2A-TagRFPCAAX (15 pg), isl2b:LifeAct-TagRFP (10 pg), and isl2b:TagBFP-*snphb* (25 pg) plasmids were co-injected with transposase mRNA at one-cell stage. Embryos were prepared in the same manner as for time-lapse imaging (described above). Single z-stacks (z-interval of 0.42 µm) were acquired around 50-52 hpf using a 40x objective with digital zoom 4, pinhole 1.0, and 1024x1024 pixel resolution.

For the visualization of mitochondria in single mature retinal axons at 120 hpf, embryos were injected with isl2b:mitoEGFP-2A-TagRFPCAAX (15 pg) and transposase mRNA at one-cell stage and selected for fluorescence in the optic tract at 96 hpf, after which the right eye was removed as described above. Larvae were re-anesthetized at 120 hpf and mounted in a lateral view in 1.5% low-melt agarose in E3 medium + PTU + tricaine in a membrane-bottomed petri dish. Imaging was performed using a 40x objective with digital zoom 2.5 and pinhole 1.0. Time-lapse recordings were done in 35 z-planes (0.80 µm z-interval), allowing acquisition at 1 frame every 15 seconds over 10 min.

Time-lapse image analysis

Image analysis was carried out using ImageJ (Schindelin et al., 2012, Schneider et al., 2012). For 2D analyses, the TagRFP/TagRFPCAAX signals from stack maximal projections were used to manually segment the growth cone total and central areas. The growth cone central area was determined by tracing the perimeter of the growth cone body, not including filopodia. As such, the central area is an arbitrary outline based on growth cone morphology and fluorescent intensity and presumably corresponds to growth cone central domain and transition zone (it does not necessarily correspond to what is commonly defined as the growth cone central domain based on cytoskeletal components).

Regions of interest (ROIs) defined as the total and central areas were used to segment the mitoEGFP signals on binary images using the 'Particle analysis' tool. Two different thresholds were used to analyze mitochondrial particles: a threshold of 55 was applied to the growth cone central area to segment the main mitochondrial cluster, and a threshold of 20 was applied to the growth cone total area to segment smaller individual mitochondria present in the peripheral area (see Fig. 1B). A threshold of 20 was also applied to analyze pioneering mitochondrial clusters using the `particle analysis' tool. The number, area, and presence (% time of advance) of pioneering mitochondrial clusters were analyzed in every frame of an advancing growth cone central area. The peripheral area, comprising filopodia, was calculated by subtracting the growth cone central area from the growth cone total area. The number of filopodia was counted manually. We drew a straight line from the mitochondrial cluster boundary to the most distal outline of the growth cone central area where two filopodia join together to measure the distance between the main mitochondrial cluster and the growth cone leading edge. We also defined the proximal growth cone as the most proximal boundary of the growth cone central area, where the growth cone transitions into the axon shaft. A growth cone was classified as advancing when the proximal growth cone advanced > 1 µm after an elongation of the growth cone central area (i.e. major > 3 x minor axis length of the best fitting ellipse of the central area). Elongation rates were quantified by measuring the displacement between the proximal growth cone boundaries before and after advance and expressed in µm per min.

Visualizations of 3D growth cones and mitochondria were prepared using FluoRender (RRID:SCR_014303) (Wan et al., 2017). For volumetric analysis of mitochondrial occupancy at the growth cone, z-stacks were processed and analyzed with ImageJ. Images were thresholded to the lowest level that excludes the majority of noise pixels to obtain binary z-projections similar to raw z-projections, after which volumes were calculated using the Voxel Counter plugin. Volume data of mitochondria and

247 the growth cone, expressed in μm^3, were divided to obtain percentages of occupancy. Figures were
248 prepared using Adobe Photoshop and Illustrator, time-lapse videos were assembled using ImageJ.

249 ***Statistics***

250 Data were analyzed and graphs were produced using Prism (Graphpad Software Inc.). Data are
251 presented as means + s.e.m. Statistical tests were applied as indicated in the Results and Figure legends.
252 Additional statistical details are provided in Table 2. Normal distribution was determined by column test.

253 **Results**

254 ***Mitochondrial localization at the growth cone correlates with axon outgrowth***

255 To monitor mitochondrial behavior in developing axons *in vivo*, we performed high-resolution confocal
256 time-lapse imaging of mitochondria in retinal axons elongating along the optic tract of zebrafish
257 embryos between 50 and 55 hours-post-fertilization (hpf) (Fig. 1A). We co-expressed EGFP targeted to
258 mitochondria (mitoEGFP) and TagRFP targeted to the plasma membrane (TagRFPCAAX) and to actin
259 filaments (Lifeact-TagRFP) in single retinal axons, and monitored labeled axons elongating along the
260 contralateral optic tract towards the optic tectum (Fig. 1, Movie 1). As previously described (Bovolenta
261 and Mason, 1987, Holt and Harris, 1993), growth cones of retinal axons in the tract were slender with
262 numerous filopodia protruding and retracting dynamically. We could distinguish two classes of axons
263 depending on their outgrowth status (Fig. 2A). Advancing axons had very elongated growth cones,
264 whereas pausing, not growing, axons had rounder growth cones with filopodia oriented in all directions,
265 supporting the notion that growth cone shape correlates with growth cone behavior (Bovolenta and
266 Mason, 1987, Mason and Wang, 1997). For the duration of our time-lapse videos (ranging from 30 to
267 160 min), several growth cones were found to alternate between pausing and advancing behaviors and
268 could therefore be classified into both categories (Movies 2-4).

As previously observed *in vitro* (Morris and Hollenbeck, 1993), mitochondria were abundantly detected at the growth cone (Fig. 2A, Movies 1-4). The majority of mitochondria clustered in the microtubule-rich growth cone central area, whereas smaller mitochondria regularly appeared in the peripheral area along protrusions or actin filopodia (e.g. Movie 3). We analyzed mitochondrial localization and distribution at the growth cone by quantifying mitochondrial occupancy in the growth cone total and central volumes (Fig. 2B,C), peripheral mitochondrial volume (Fig. 2D), mitochondrial occupancy in the growth cone total and central areas (Fig. 2B',C'), the number of mitochondria in the peripheral area (Fig. 2D'), and the distance between mitochondria and the growth cone leading edge (Fig. 2E, see also Fig. 1B,C). Mitochondrial occupancy was chosen as measure to evaluate mitochondrial density in the growth cone since spatial overlap of mitochondria within the growth cone central zone did not allow the visualization and quantification of single mitochondria. Mitochondrial occupancy in the total and central volumes and areas were similar in advancing versus pausing growth cones (Fig. 2B-C'). We did note that when we exclusively compared growth cones that frequently alternated between pausing and advancing behaviors, a significant decrease in mitochondrial occupancy in the central area of advancing growth cones was observed (paired *t*-test, $P = 0.006$, $t_{(6)} = 4.2$). Furthermore, the distance of the main mitochondrial cluster to the leading edge was significantly different between pausing and advancing growth cones (Fig. 2E). When a growth cone advanced, its central area elongated substantially, but this forward extension was not accompanied with a forward advance of mitochondria (See Fig. 3A, Movie 5). Hence, the distance between them and the leading edge increased two-fold (Fig. 2E). Mitochondria repositioned near the leading edge when the growth cone regained a rounder shape (Fig. 3A), which often indicated a transition to a pausing state. Interestingly, although the majority of mitochondria lagged behind during growth cone extension, a smaller subset of mitochondria was always detected adjacent to the leading edge (Fig. 3, Movie 5). We analyzed the number and area of these 'pioneering mitochondrial clusters', as well as the amount of time they were present when a growth cone advanced.

We found that pioneering mitochondrial clusters had on average a total area of 1.1 ± 0.4 μm^2, and that 2.6 ± 0.3 mitochondrial clusters positioned near the leading edge in 43 ± 9.5% of the time of advance. Importantly, pioneering mitochondria were consistently observed in every growth cone that elongated.

Altogether, our data show that mitochondria localize in the growth cone central area *in vivo* and are also present along filopodia extending from the growth cone periphery. The position of mitochondria in the growth cone central area further changes with the growth status of the axon.

Mitochondrial transport is coordinated with growth cone behavior and axon growth in vivo

Next, we asked whether transport of mitochondria in the axon shaft proximal to the growth cone also relates to growth status. We performed kymograph analysis to measure net mitochondrial transport as well as the percentage of time mitochondria spent in motion (Fig. 4A-C). In agreement with a higher mobility of mitochondria observed in developing versus mature axons (Lewis et al., 2016), less than five percent of all mitochondria were stationary (Fig. 4C). The majority of mobile mitochondria moved anterogradely, with a significantly higher percentage of anterograde versus retrograde trafficking in both pausing and advancing growth cones (Fig. 4B). An analysis of mitochondrial transport over shorter time scales showed a similar proportion of mitochondria moving anterogradely (71 +/- 5.4% in long movies, 78 +/- 7.3% over shorter time scales), retrogradely (26 +/- 4.5% in long movies, 20 +/- 6.7% over shorter time scales) or remaining stationary (2.3 +/- 1.6% in long movies, 2.3 +/- 2.3% over shorter time scales), indicating that the length of time-lapse movies had no effect on mitochondrial transport parameters (Two way ANOVA, effect of time, $P = 0.9994$, $F_{(1.66)} < 0.0001$). Mitochondria spent more time moving when a growth cone advanced (Fig. 4C), and we observed a trend towards more mitochondria arriving versus leaving in both pausing and advancing growth cones (Fig. 4D). We finally found a strong correlation between the number of mitochondria arriving at the growth cone and the distance the axon elongated (Fig. 4E). Altogether, these results illustrate that mitochondrial transport towards the growth

316 cone correlates with growth cone advance, suggesting that mitochondrial motility and axon elongation
317 may be functionally linked or co-regulated in vivo.

318 ***Zebrafish Syntaphilins are expressed in RGCs and localize to the growth cone***

319 How mitochondria are maintained in the growth cone remains poorly understood. One possible
320 mechanism involves the attachment, or docking, of mitochondria to microtubules by Snph. Snph was
321 identified as an outer mitochondrial membrane protein able to dock mitochondria directly to
322 microtubules via its microtubule-binding domain (MTB) (Kang et al., 2008) (Fig. 6A). Snph was shown to
323 be important in axons for the regulation of branching (Courchet et al., 2013) and synaptic plasticity
324 (Kang et al., 2008). We decided to test whether Snph also plays a role in docking mitochondria at the
325 growth cone during axon elongation *in vivo*. Due to the whole-genome duplication that occurred in the
326 teleost lineage (Glasauer and Neuhauss, 2014), two *snph* genes, *snpha* and *snphb,* are present in
327 zebrafish. Both Snpha and Snphb share a high sequence conservation with human SNPH in their MTB
328 and mitochondrial transmembrane domains (TMs), strongly suggesting a conserved mitochondrial
329 anchoring function (Fig. 6A, Extended Data Fig. 6-1). We first analyzed the expression of *snpha* and
330 *snphb* during zebrafish development. Quantification of *snpha* and *snphb* mRNA levels using reverse
331 transcriptase (RT)-coupled droplet digital PCR (ddPCR) showed that both *snpha* and *snphb* are
332 maternally expressed and that mRNA levels for both *snphs* increase over later developmental periods,
333 albeit at much lower levels for *snpha* (Fig. 5A). *In situ* hybridization (ISH) further revealed a high
334 expression of *snphb* in the nervous system at 48, 72, and 120 hpf (Fig. 5B), whereas *snpha* expression
335 was not detectable using this approach (data not shown). Importantly, *snphb* was detected in the RGC
336 layer at the time of retinal axon elongation (Fig. 5B), suggesting a possible role in anchoring
337 mitochondria in retinal growth cones. To further test whether *snphb* is expressed by RGCs, we
338 performed ISH on WT and RGC-deficient *lakritz* (*lak*) mutant embryos (Kay et al., 2001) at 72 hpf (Fig.
339 5C). *Snphb* expression was strongly reduced in the retina of *lak* compared to WT, while it appeared

unchanged in the brain (Fig. 5C). This decreased retinal expression was further confirmed by RT-ddPCR performed on dissected eyes (Fig. 5D). *Snphb* mRNA levels were highly detected in WT but significantly reduced in *lak*, indicating that *snphb* is strongly expressed by RGCs. Interestingly, *snpha* transcripts were also detected in WT, albeit at much lower levels. Like *snphb*, *snpha* eye expression was significantly decreased in *lak* (Fig. 5D), indicating that both Snphs are present in RGCs at the time of axon elongation.

As *snphb* is highly expressed in RGCs, we finally tested whether Snphb could localize to the growth cones of retinal axons elongating along the tract. As we could not identify an antibody directed against mammalian Snph that would specifically recognize zebrafish Snphs (data not shown), we decided to co-express TagBFP-tagged Snphb, mitoEGFP, TagRFPCAAX, and Lifeact-TagRFP in single retinal axons, and monitor labeled growth cones advancing along the contralateral optic tract (Fig. 5E). We detected TagBFP-Snphb in the central area of retinal growth cones, where it appeared to co-localize with the main mitochondrial cluster. Altogether, our results show that Snphs are expressed by RGCs and can localize to the growth cone of developing axons, suggesting they might participate to mitochondria docking at the growth cone during axon elongation *in vivo.*

Syntaphilins contribute to mitochondrial immobilization at the growth cone

To test the function of zebrafish Snphs in retinal axons, we generated *snpha* and *snphb* mutants using TALEN-mediated genome editing (Fig. 6A,B). We targeted a region upstream the MTB to ensure full loss-of-function and obtained two alleles consisting of a 14 and 4 bp deletion for *snpha* and *snphb*, respectively, predicted to cause premature termination of translation (Fig. 6B). As both *snpha* and *sphnb* are expressed in RGCs (Fig. 5D) and to avoid potential compensation mechanisms, we generated maternal zygotic double mutants (*db*) and confirmed by RT-PCR and cDNA sequencing that both mutant mRNAs were expressed and contained the corresponding mutations (Fig. 6B). No alternative transcripts were detected in *db* (data not shown). *Db* were viable and fertile, and *db* embryos did not show any

363 obvious morphological abnormalities, as was reported for *Snph* knockout (KO) mice (Kang et al., 2008).
364 To test whether zebrafish Snphs also contribute to mitochondrial anchoring in axons, we quantified
365 mitochondrial transport in mature retinal axons of WT and *db* embryos at 120 hpf (Fig. 6C), when most
366 mitochondria are known to become immobile (Lewis et al., 2016, Smit-Rigter et al., 2016). As expected,
367 mitochondrial mobility was strongly increased in axons of *db* (Fig. 6D,E), demonstrating that the
368 mitochondrial docking function of Snph is conserved in zebrafish.

369 We next performed time-lapse imaging of mitochondria in retinal axons elongating along the optic tract
370 of *db* embryos to test whether Snphs also play a role in mitochondrial tethering at the growth cone of
371 growing axons (Movies 6-8). We compared *db* with WT embryos that have a similar genetic background
372 (datasets analyzed in Fig. 2-4). Interestingly, mitochondrial distribution at the growth cone was modified
373 in *db* compared to WT (Fig. 7A-D'). While a same mitochondrial volume and number were observed in
374 the growth cone peripheral volume and area in WT and *db* (Fig. 7C,C'), mitochondria occupied a smaller
375 proportion of the growth cone total and central volumes and areas in *db* (Fig. 7A-B', Movies 6-8).
376 Moreover, mitochondria were located further from the growth cone leading edge in *db*, which was most
377 pronounced in pausing growth cones (Fig. 7D,D'). Interestingly, the decreased mitochondrial occupancy
378 of the growth cone in *db* was accompanied with changes in mitochondrial flux. While a similar number
379 of mitochondria arrived at the growth cone, more mitochondria left the growth cone in *db* versus WT
380 (Fig. 7E), suggesting that Snphs dock mitochondria once they have reached the growth cone. The
381 increased departure of mitochondria from the growth cone of *db* was associated with changes in net
382 mitochondrial transport proximally to the growth cone. More mitochondria moved retrogradely in *snph*
383 *db*, which was accompanied by a decreased percentage of mitochondria moving in the anterograde
384 direction (Fig. 7F). No differences were found between *WT* and *db* in the amount of time that
385 mitochondria moved in both directions (Fig. 7G).

Finally, in addition to the decreased mitochondrial occupancy of the growth cone in *db*, we observed some changes in pioneering mitochondrial clusters. As in WT, we detected pioneering clusters in every *db* growth cone that advanced (Fig. 8A-C). The number of clusters (Fig. 8E) and the amount of time they were present at the growth cone (Fig. 8F) did not vary, however the total area occupied by pioneering mitochondrial clusters was significantly decreased in *db* compared to WT (Fig. 8D). Altogether, these observations demonstrate for the first time that Snphs contribute to mitochondrial clustering at the growth cone *in vivo*. Snphs play a role in anchoring mitochondria in the growth cone central domain, including both the main cluster and the pioneering mitochondria that dynamically appear along the leading edge of advancing growth cones.

Mitochondrial docking at the growth cone by Syntaphilins is not required for axon outgrowth

As Snphs contribute to mitochondrial docking at the growth cone (Figs. 7A-G, 8), we then tested whether the loss of Snphs would affect axon elongation. Analysis of the total and central growth cone areas (Fig. 7H) and the number of filopodia (Fig. 7I) did not reveal any differences in growth cone morphology between WT and *snph db*. Moreover, retinal axon growth rate was similar between WT and *db* mutants (Fig. 7J), indicating that Snph-mediated mitochondrial docking at the growth cone is dispensable for axon elongation. Thus, we identified Snph as a mediator of mitochondrial docking at the growth cone in elongating axons *in vivo*, but this function plays only a minor role, if any, in axon growth.

Discussion

By performing confocal live imaging in the zebrafish embryo, we provide the first detailed characterization of mitochondrial dynamics during growth cone behavior in elongating axons *in vivo*. We show that mitochondrial distribution at and trafficking towards the growth cone are coordinated with axon outgrowth, which is in agreement with previous *in vitro* observations (Morris and Hollenbeck, 1993, Sainath et al., 2017), and highlights that mitochondria might play a role in axon outgrowth and

409 pathfinding. We further provide evidence that Snph contributes to mitochondrial docking at the growth
410 cone. However, growth cone morphology and axon elongation are unaffected in *snph* db mutants,
411 indicating that the direct anchoring of mitochondria to growth cone microtubules by Snph only plays a
412 minor role in axon elongation.

413 An intriguing finding of our study is the localization of mitochondria along growth cone filopodia that is
414 independent of Snph. Since filopodia dynamics are highly dependent on actin filament polymerization
415 and turnover, this observation suggests that mitochondria might associate with the growth cone actin
416 cytoskeleton. Mitochondria have indeed been shown to be able to attach to actin via the myosin 19
417 molecular motor (Shneyer et al., 2016). Interaction with myosin 19 positioned mitochondria into actin-
418 rich filopodia of U-2 OS osteosarcoma cells following stress (Shneyer et al., 2017). A localization of
419 mitochondria to leading edge lamellipodia, filopodia, and invadopodia of cancer cells has further been
420 shown to play an important role in cancer cell migration and metastatic potential (Cunniff et al., 2016,
421 Smith and Gallo, 2018). Whether the interaction between mitochondria, myosin 19, and actin is relevant
422 in the growth cone remains unknown. Interestingly, mitochondria appeared along growth cone filopodia
423 in a transient manner, suggesting that they might also be transported along the dynamic microtubules
424 that explore the growth cone periphery and play an essential role in growth cone adhesion and turning
425 (Buck and Zheng, 2002, Suter et al., 2004). Our observation of pioneering mitochondrial clusters at the
426 leading edge of the growth cone during elongation further supports the hypothesis that mitochondria
427 might frequently attach to the plus-end of microtubules. An intriguing consequence of mitochondrial
428 targeting to microtubule plus ends in filopodia would be the possibility to predict the net direction of
429 axon growth based on mitochondrial appearance. Unfortunately, our imaging conditions did not have
430 the resolution required for correlating mitochondrial peripheral distribution with filopodia protrusion,
431 retraction, or stabilization. It will be important in the future to optimize imaging approaches in order to
432 quantify fine aspects of mitochondrial behavior *in vivo* and test the possible interaction between

mitochondria and the cytoskeleton in the growth cone. Interestingly, the presence of pioneering mitochondrial clusters close to the leading edge is at least partially Snph-dependent, suggesting that the targeting of mitochondria to peripheral filopodia and to the leading edge rely on different mechanisms. While mitochondria might be transported on actin filaments or dynamic microtubules in the periphery, they may accumulate on the stable microtubules that "end" in the central area of the growth cone and be captured there by Snph and other factors.

The interdependence between mitochondrial transport and axon outgrowth we observed is consistent with *in vitro* work demonstrating a net anterograde mitochondrial trafficking in growing axons that is abolished when axons encounter a physical barrier (Morris and Hollenbeck, 1993). We now establish such a correlation for the first time *in vivo* by comparing various parameters of mitochondrial transport in pausing versus advancing retinal axons. Together with the observation of a growth status-dependent mitochondrial distribution at the growth cone, our data indicate that mitochondrial dynamics in the distal axon and growth cone are coordinated with growth cone behavior and axon outgrowth *in vivo*. Vaarmann et al. (2016) previously showed that axon length could be increased by overexpressing PGC-1α, a central inducer of mitochondrial biogenesis, supporting the notion that mitochondria provide energy for axon growth. In line with this, pharmacological and genetic disturbance of the mitochondrial fission-fusion balance in cultured retinal neurons affected neurite length and caused neurite guidance errors (Steketee et al., 2012). On the other hand, other studies hinted at the ability of axons to grow with dysfunctional mitochondria (Yoon et al., 2012, Campbell et al., 2014). While depletion of the intermediate filament protein Lamin B2 caused defects in mitochondrial membrane potential, morphology, and transport, it did not affect retinal axon initial growth and guidance along the optic tract *in vivo* (Yoon et al., 2012). In a similar manner, lack of Kif5Aa, which transports mitochondria anterogradely into axons, causes a lack of mitochondria in distal peripheral axons that leads to degeneration. Yet, axons grow normally in *kif5Aa* zebrafish mutant embryos (Campbell et al., 2014). As it

457 remains unclear which cellular processes are specifically dependent on mitochondrial respiration (Smith
458 and Gallo, 2018), it might be conceivable that ATP production through glycolysis can, at least partially,
459 sustain axon growth. Previous studies have even suggested that embryonic neurons predominantly rely
460 on glycolytic ATP (Surin et al., 2012). As growth cone turning is regulated by local calcium levels
461 (Gasperini et al., 2017), the ability of growth cone mitochondria to buffer calcium might be more
462 important than its capacity to generate energy. Future work analyzing mitochondrial calcium dynamics
463 at the growth cone *in vivo* might thus be highly interesting.

464 We identified Snph as a mediator of mitochondrial docking at the growth cone in elongating axons *in*
465 *vivo*. This function is consistent with its previously established role in axonal mitochondrial stalling (Kang
466 et al., 2008, Chen and Sheng, 2013). Yet, our data suggest that Snph does not play an important role in
467 stalling mitochondria along the axon at this early developmental stage. The lack of difference in the
468 number of mitochondria arriving at the growth cone in WT versus *snph db* axons suggests that Snph
469 does not inhibit mitochondrial anterograde transport towards the growth cone as it does in the context
470 of axon regeneration (Zhou et al., 2016). An increased halting of mitochondria along the axon over time
471 is instead consistent with an increased expression of Snph in the brain during development, both in
472 zebrafish in our study and in the mouse (Das et al., 2003, Kang et al., 2008). Alternatively, Snph
473 localization and functions might be differently regulated in the axon shaft versus growth cone during
474 development.

475 While the loss of Snphs reduced the number of mitochondria at the growth cone, it did not cause a
476 complete depletion of mitochondria and did not affect axon growth. The remaining mitochondria at the
477 growth cone might thus compensate for a lower density by increasing their activity. In this regard, it is
478 noteworthy that *Snph* KO mice sciatic nerves show enhanced regrowth capacity after injury (Zhou et al.,
479 2016). Cultured cortical neurons from these *Snph* KO mice displayed increased ATP content upon
480 axotomy (Zhou et al., 2016), but this difference in energy production was not observed in baseline

481 conditions. Alternatively, the number of mitochondria remaining at the growth cone in absence of Snph
482 might be enough for normal axon development, especially considering that glycolytic ATP might have an
483 important role in sustaining axon growth (Surin et al., 2012). These remaining mitochondria are likely
484 stabilized at the growth cone central domain by other anchoring factors, of which the outer
485 mitochondrial membrane GTPases Miro1 and 2 are especially good candidates. Miro proteins are indeed
486 involved in mitochondrial trafficking along microtubules (Devine et al., 2016) and can arrest
487 mitochondrial movement in a calcium-dependent manner (Saotome et al., 2008), which is particularly
488 relevant at the growth cone where calcium signaling regulates pausing, extension, and turning
489 (Sutherland et al., 2014, Gasperini et al., 2017). In that context, an elegant study recently demonstrated
490 that growth inhibiting substrates such as MAG or CSPGs decrease mitochondrial axonal transport by
491 promoting the deacetylation of Miro1 by HDAC6 in a calcium-dependent manner (Kalinski et al., 2019).
492 Finally, Miros have also been discovered to play a role in mitochondrial trafficking and positioning along
493 actin filaments (Lopez-Domenech et al., 2018), which might regulate mitochondrial positioning along
494 filopodia in the growth cone peripheral area. Future studies examining the role of Miros in
495 mitochondrial docking at the growth cone *in vivo* might thus be a great interest to decipher how
496 mitochondrial positioning is regulated during axonal development.
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Figure legends

Figure 1. Live imaging approach and analysis. (A) Individual retinal axons and mitochondria were mosaically labeled by co-injecting isl2b:mitoEGFP-2A-TagRFPCAAX and isl2b:Lifeact-TagRFP plasmids at one-cell stage. isl2b:Lifeact-TagRFP was used to improve the visualization of single axons and growth cone (GC) filopodia (see Main text and Materials and Methods for more details). After removal of the contralateral eye, axons growing along the optic tract towards the optic tectum (OT) and their mitochondria were imaged in a lateral view between 50 and 54 hpf (Δt = 1 min). A: anterior, D: dorsal, hpf: hours post fertilization, OT: optic tectum, P: posterior, V: ventral. (B) Growth cone total and central areas visualized with TagRFP/TagRFPCAAX were manually segmented and used as ROIs for segmenting mitochondrial signals using the particle analysis tool in ImageJ. Two different thresholds were used to analyze mitochondrial particles: a threshold of 55 was applied to the growth cone central area to segment the main mitochondrial cluster, and a threshold of 20 was applied to the growth cone total area to segment smaller individual mitochondria present in the peripheral area (red arrowheads). Time-lapse recordings were classified according to growth cone behavior. Growth cone leading edge and proximal growth cone are indicated in the merged image, see Materials and Methods for definitions. Lateral view, confocal maximal projections. Scale bar: 5 µm. (C) Volumes of mitochondria and GC total and central volumes were calculated using 3D analysis. GC total and central volumes visualized with TagRFP/TagRFPCAAX were calculated on binary z-projections and used as VOIs for segmenting mitochondrial volumes using the voxel counter plugin in ImageJ (see also Movie 1).

Figure 2. Mitochondrial distribution changes with growth cone behavior. (A) Representative time-lapse images of mitochondria (mitoEGFP, white) in distal retinal axons and growth cones (labeled with TagRFP, delineated in orange and shown in left panels) classified as pausing or advancing (see Movies 2-4). Most growth cones alternate periods of pausing and advancing/elongating, resulting in net axon growth, as

shown in the lower panels. The red asterisk indicates mitochondria in neighboring axons. Scale bar: 5 µm. (B-C') Quantification of the mitochondrial occupancy (in %) of the growth cone total volume (B), total area (B'), central volume (C) and central area (C'). (D-D') Quantification of the mitochondrial volume in the peripheral growth cone (D), and of the number of mitochondria in the growth cone peripheral area (D'). (E) Quantification of the distance between the largest mitochondrial cluster and the growth cone leading edge. When growth cones elongate, the distance to the growth cone leading edge increases significantly. Data from 12 independent experiments (pausing: n = 11, advancing: n = 8) are shown as mean + s.e.m. Statistical analysis (B-E): unpaired *t*-test, ***$P < 0.001$.

Figure 3. Pioneering mitochondrial clusters localize near the leading edge in advancing growth cones. (A) Representative time-lapse images of mitochondria (mitoEGFP, white) in an advancing growth cone (see Movie 5). While the main mitochondrial cluster (green arrow) lags behind during growth cone advance, some small pioneering mitochondrial clusters (blue arrows) appear in close proximity to the leading edge. Lateral view, confocal maximal projections. Scale bar: 3 µm. (B,C) EGFP intensity profiles calculated along a line between the initial proximal growth cone and final leading edge to analyze the distribution of mitochondria along the advancing growth cone at successive time points shown in panel A. (C) The orange and green lines correspond to the fluorescent intensities of mitochondria in the growth cone and at the proximal growth cone, respectively (see panel B: Analysis).

Figure 4. Anterograde mitochondrial transport correlates with axonal outgrowth. (A) Representative kymograph of mitochondria (mitoEGFP, white) in a distal axon whose growth cone alternates between advancing and pausing. The first and last frames of the time-lapse recording (Movie 4) are shown with axon and growth cone delineated in orange. Confocal maximal projections. Scale bar: 5 µm. The growth cone leading edge and proximal growth cone (green line) are indicated on the kymograph and kymograph analysis panels. (B) Quantification of net transport, analyzed by counting the number of mitochondria moving anterogradely or retrogradely. Data from 12 independent experiments (pausing: n

= 11, advancing: n = 8) are shown as mean + s.e.m. Statistical analysis: Two-way ANOVA with *post-hoc* Bonferroni test, ** $P < 0.01$, ***$P < 0.001$. (C), Quantification of the percentage of time mitochondria spent in a mobile or stationary state. A significant increase in time spent in motion (C) is observed proximally to growth cones that are advancing ($P = 0.013$). Data from 12 independent experiments (pausing: n = 11, advancing: n = 8) are shown as mean + s.e.m. Statistical analysis: unpaired *t*-test. (D) Quantification of mitochondrial flux, showing a trend towards more mitochondria arriving versus leaving the growth cone in both pausing and advancing growth cones. Data from 12 independent experiments (pausing: n = 11, advancing: n = 8) are shown as mean + s.e.m. Statistical analysis: unpaired *t*-test. (E) Linear regression analysis between the number of mitochondria arriving at a growth cone and growth cone advance (n = 12).

Figure 5. Zebrafish Syntaphilins are expressed in RGCs during development. (A) Quantification of *snpha* and *snphb* mRNA levels during embryonic development by RT-ddPCR. mRNA levels were normalized to that of *gapdh* used as a control. Data from 3 independent experiments are shown as mean + s.e.m. (B) Lateral views of whole embryos stained for *snphb* by ISH show predominant expression in the brain at 48, 72, and 120 hpf. *Snphb* is also increasingly expressed in the RGC layer over time. Scale bar: 400 µm (whole embryos) and 200 µm (eyes). (C) Dorsal views of WT and RGC-deficient *lak* mutant embryos stained for *snphb* by ISH at 72 hpf. Expression of *snphb* is decreased in the retina of *lak* embryos (arrows). Scale bar: 200 µm. (D) Quantification of *snpha* and *snphb* mRNA levels in the eyes of WT and *lak* embryos at 72 hpf analyzed by RT-ddPCR. Transcripts levels were normalized to that of *18s* used as a control. Data from 3 experiments are shown as mean + s.e.m. Statistical analysis: unpaired *t*-test, ***$P < 0.001$. (E) TagBFP-Snphb localizes to the growth cone of elongating axons *in vivo*. Isl2b:TagBFP-*snphb,* isl2b:mitoEGFP-2A-TagRFPCAAX, and isl2b:Lifeact-TagRFP were co-expressed in individual RGCs. TagBFP-Snphb and mitochondria are both present in the growth cone (arrow in merged image). Lateral view, confocal maximal projections. Scale bar: 5 µm.

728 **Figure 6.** Zebrafish Syntaphilins anchor mitochondria in mature retinal axons. (A) Domain structure of
729 human SNPH. Both the microtubule-binding domain (MTB) and transmembrane domains (TMs) are
730 highly conserved in zebrafish Snpha and Snphb (see also extended data Figure 6-1). The red asterisk
731 indicates the position of the TALEN target region. (D) Mutations in *snpha* and *snphb* were introduced by
732 TALEN mutagenesis. Red lines indicate the deleted sequences. Changes in amino acids are shown in red.
733 RT-PCR analysis of *snpha* and *snphb* in WT and *db* embryos demonstrate the presence of shorter
734 transcripts in the mutants. (C) Individual retinal axons and mitochondria were mosaically labeled by
735 injecting isl2b:mitoEGFP-2A-TagRFPCAAX at one-cell stage. After removal of the contralateral eye,
736 mature axons of the optic tract and their mitochondria were imaged in a lateral view at 120 hpf (Δt = 15
737 sec). (D,E) Quantification of the percentage of stationary mitochondria using kymograph analysis shows
738 a reduction in stalled mitochondria in axons from *snph db* mutants. Data from 2 independent
739 experiments (WT: n = 7, *db*: n = 8) are shown as mean + s.e.m. Statistical analysis: unpaired *t*-test, $**P <$
740 0.01.

741 **Extended Data Figure 6-1.** Zebrafish Syntaphilins have a highly conserved microtubule-binding domain
742 and mitochondrial attachment sites. (A) Depiction of the domain structure of human SNPH and zebrafish
743 Snpha and Snphb. The microtubule-binding domain (MTB) and transmembrane domains (TMs) are
744 indicated in orange and green, respectively. (B) Sequence alignment of human SNPH and zebrafish
745 Snpha and Snphb. The MTB (orange) and TMs (green) are highly conserved, suggesting a conserved
746 function in anchoring mitochondria to microtubules.

747 **Figure 7.** Syntaphilins participate in mitochondrial docking at the growth cone but do not regulate axon
748 elongation. (A-B') Quantification of the mitochondrial occupancy (in %) of the growth cone total volume
749 (A), total area (A'), central volume (B) and central area (B') in WT and *snph db*. Mitochondrial occupancy
750 is decreased in *snph db.* (C-C') Quantification of the mitochondrial volume in the peripheral growth cone
751 (C), and of the number of mitochondria in the growth cone peripheral area (C') in WT and *snph db*.

(D,D') Quantification of the distance between the largest mitochondrial cluster and the growth cone leading edge in WT and *snph db*. We quantified the distance from the leading edge separately in pausing and advancing growth cones as it varies depending on growth cone status (see Fig. 2E). Mitochondria are located further from the leading edge in retinal growth cones of *db* compared to WT. (E-G) Reduced mitochondrial occupancy in the growth cone of *snph db* is accompanied by an increased removal of mitochondria from the growth cone. More mitochondria leave the growth cone per minute in *db*, while no difference is detected for arriving mitochondria (E). Quantification of net mitochondrial transport proximally to the growth cone (F) shows more mitochondria moving retrogradely in *snph db*, which is accompanied by a decreased percentage of mitochondria moving in the anterograde direction. No differences in percentage time in anterograde or retrograde motion were found (G). (H,I) Quantification of growth cone morphology shows no differences in growth cone total and central areas (H) and number of filopodia (I) between *db* and WT embryos. (J) Axon elongation is not statistically different between *db* and WT embryos. Data from 12 independent experiments per genotype are shown as mean + s.e.m. Statistical analysis: unpaired *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 8. Syntaphilin contributes to the localization of pioneering mitochondrial clusters near the leading edge in advancing growth cones. (A) Representative time-lapse images of mitochondria (mitoEGFP, white) in an advancing growth cone (delineated in orange) in a *db* embryo. Like in WT (Fig. 3), the main mitochondrial cluster lags behind during growth cone advance while some pioneering mitochondria appear in close proximity to the leading edge. Lateral view, confocal maximal projections. Scale bar: 3 µm. (B,C) Fluorescent intensity profiles show the distribution of mitoEGFP fluorescence at various time points along the advancing growth cone shown in panel A. Orange and green lines correspond to fluorescent intensities of mitochondria in the growth cone and the proximal growth cone, respectively. Arrows show peaks of fluorescence corresponding to localizations of mitochondria in the growth cone (see panel A). (D-F) Quantification of pioneering mitochondrial cluster total area (D), number (E), and

776 dynamics (% of time present during advance) (F). Pioneering mitochondrial clusters occupy a reduced
777 area in *snph db* embryos compared to WT. Data from 12 independent experiments per genotype are
778 shown as mean + s.e.m. Statistical analysis: unpaired *t*-test.

779 **Multimedia legends**

780 **Movie 1.** Representative 3D visualization of mitochondria in a pausing growth cone. Video
781 corresponding to Figure 1C showing mitochondria (green) in a distal retinal axon and growth cone (red)
782 pausing along the optic tract. Scale bar: 5 µm.

783 **Movie 2.** Representative time-lapse recording of mitochondrial dynamics in a pausing growth cone.
784 Time-lapse video corresponding to Figure 2A showing mitochondria (green, white) in a distal retinal
785 axon and growth cone (red) pausing along the optic tract. Images were acquired at 1 min intervals for 48
786 min. Each frame is a confocal image stack maximal projection, lateral view, and anterior is on the left.
787 Time stamp format: hr:min. Scale bar: 5 µm.

788 **Movie 3.** Representative time-lapse recording of mitochondrial dynamics in an advancing growth cone.
789 Time-lapse video corresponding to Figure 2A showing mitochondria (green, white) in a distal retinal
790 axon and growth cone (red) elongating along the optic tract. Images were acquired at 1 min intervals for
791 48 min. Each frame is a confocal image stack maximal projection, lateral view, and anterior is on the left.
792 Time stamp format: hr:min. Scale bar: 5 µm.

793 **Movie 4.** Representative time-lapse recording of mitochondrial dynamics in a growth cone that
794 combines pausing and advancing. Time-lapse video corresponding to Figure 2A showing mitochondria
795 (green, white) in a distal retinal axon and growth cone (red) that alternates between pausing and
796 elongating behaviors. The growth cone is pausing during the majority of the recording, but advances
797 intermittently (e.g. from 00:00 to 00:18, 01:39 to 01:55, and 02:24 to 02:39). Images were acquired at 1

798 min intervals for 160 min. Each frame is a confocal image stack maximal projection, lateral view, and
799 anterior is on the left. Time stamp format: hr:min. Scale bar: 5 µm.

800 **Movie 5.** A subset of mitochondria localizes to the leading edge of the growth cone during elongation.
801 Representative time-lapse video corresponding to Figure 3A showing mitochondria (green, white) in a
802 distal retinal axon and growth cone (red) elongating along the optic tract. Images were acquired at 1 min
803 intervals for 36 min. Note that the growth cone is elongating from 00:10 to 00:25. During this
804 elongation, most mitochondria lag behind, but a subset of smaller mitochondria localizes adjacent to the
805 leading edge. Each frame is confocal image stack maximal projection, lateral view, and anterior is on the
806 left. Time stamp format: hr:min. Scale bar: 3 µm.

807 **Movie 6.** Representative 3D visualization of mitochondria in a pausing growth cone of a *snph db mutant*
808 embryo. Video showing mitochondria (green) in a distal retinal axon and growth cone (red) pausing
809 along the optic tract. Scale bar: 5 µm.

810 **Movie 7.** Representative time-lapse recording of mitochondrial dynamics in a growth cone of a *snph db*
811 mutant embryo. Time-lapse video showing mitochondria (green, white) in a distal retinal axon and
812 growth cone (red) elongating along the optic tract of a *snph db* embryo. Images were acquired at 1 min
813 intervals for 120 min. Note that the growth cone is pausing during the first minutes (00:00 to 00:47),
814 after which it advances. Each frame is a confocal image stack maximal projection, lateral view, and
815 anterior is on the left. Time stamp format: hr:min. Scale bar: 5 µm.

816 **Movie 8.** Representative time-lapse recording of mitochondrial dynamics in a growth cone of a *snph db*
817 mutant embryo. Time-lapse video showing mitochondria (green, white) in a distal retinal axon and
818 growth cone (red) elongating along the optic tract of a *snph db* embryo. Images were acquired at 1 min
819 intervals for 96 min. Note that the growth cone is combining periods of pausing with advancing (from

820 00:00 to 00:10 and 00:53 till end). Each frame is a confocal image stack maximal projection, lateral view,
821 and anterior is on the left. Time stamp format: hr:min. Scale bar: 5 µm.

822 **Tables**

823 **Table 1.** Primers and probes used for ddPCR.

Gene	Forward primer	Reverse primer	Taqman probe	Amplicon size
snpha	GCAGCAGTTACTCAGCATCA	TGCCATGATTCTCACCACAG	TCCTGCAAGTGCACAGAGAGCATT	117
snphb	CACCTGTCAGTAACCGTGAT	TATGTGACGCCTATGGGTTG	AGCAGCAGTAGCAATTCAGGGTCA	107
gapdh	CCAAGGCTGTAGGCAAAGTA	GACTGTCAGATCCACAACAGAG	ACACGGAAGGCCATACCAGTAAGC	101
18s	GCCGCTAGAGGTGAAATTCT	TCGGAACTACGACGGTATCT	CAAGACGGACGAAAGCGAAAGCAT	129

824

825 **Table 2.** Summary of statistical analyses.

Figure	Measurement	Data structure	Type of test	Comparison	Statistical value
Fig. 2B	Mitochondrial occupancy (GC total volume)	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P = 0.4065$ $t_{(17)} = 0.8511$
Fig. 2B'	Mitochondrial occupancy (GC total area)	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P = 0.4310$ $t_{(17)} = 0.8067$
Fig. 2C	Mitochondrial occupancy (GC central volume)	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P = 0.2829$ $t_{(17)} = 1.109$

Fig. 2C′	Mitochondrial occupancy (GC central area)	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P = 0.2325$ $t_{(17)} = 1.238$
Fig. 2D	Mitochondrial volume (peripheral volume)	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P = 0.4444$ $t_{(17)} = 0.7831$
Fig. 2D′	Number of mitochondria in GC peripheral area	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P = 0.7028$ $t_{(15)} = 0.3889$
Fig. 2E	Distance from leading edge	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P < 0.0001$ $t_{(17)} = 6.740$
Fig. 4B	% Net mitochondrial transport	Normal	Two-way ANOVA *(post-hoc* Bonferroni)	Pausing vs. advancing GC	$P = 0.9955$ $F_{(1,34)} = 0.00003$
Fig. 4C	% Time mitochondria spent mobile	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P = 0.0131$ $t_{(17)} = 2.772$
Fig. 4C	% Time mitochondria spent stationary	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P = 0.0075$ $t_{(17)} = 3.034$
Fig. 4D	Mitochondrial flux (arriving mitochondria)	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P = 0.4666$ $t_{(17)} = 0.7448$
Fig. 4D	Mitochondrial flux (leaving mitochondria)	Normal	Unpaired *t*-test	Pausing vs. advancing GC	$P = 0.3617$ $t_{(17)} = 0.9374$
Fig. 4E	Number of arriving mitochondria vs. axon growth	Normal	Linear regression		$P = 0.0071$ $r^2 = 0.5324$

Fig. 5D	*snpha* expression	Normal	Unpaired *t*-test	WT vs. *lak*	$P < 0.0001$ $t_{(4)} = 18.33$
Fig. 5D	*snphb* expression	Normal	Unpaired *t*-test	WT vs. *lak*	$P < 0.0001$ $t_{(4)} = 17.84$
Fig. 6E	% Stationary mitochondria	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.0074$ $t_{(13)} = 3.168$
Fig. 7A	GC total occupancy (% volume)	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.0023$ $t_{(22)} = 3.446$
Fig. 7A’	GC total occupancy (% area)	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.0178$ $t_{(22)} = 2.562$
Fig. 7B	GC central occupancy (% volume)	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.0184$ $t_{(22)} = 2.543$
Fig. 7B’	GC central occupancy (% area)	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.0071$ $t_{(22)} = 2.967$
Fig. 7C	Peripheral mitochondrial volume	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.2765$ $t_{(22)} = 1.116$
Fig. 7C’	Number of mitochondria in peripheral area	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.6030$ $t_{(21)} = 0.5278$
Fig. 7D	Distance from leading edge (pausing GC)	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.0003$ $t_{(20)} = 4.403$

Fig. 7D′	Distance from leading edge (advancing GC)	Normal	Unpaired *t*-test	WT vs. *snph db*	*P* = 0.1080 $t_{(16)}$ = 1.703
Fig. 7E	Mitochondrial flux (arriving mitochondria)	Normal	Unpaired *t*-test	WT vs. *snph db*	*P* = 0.4976 $t_{(22)}$ = 0.6904
Fig. 7E	Mitochondrial flux (leaving mitochondria)	Normal	Unpaired *t*-test	WT vs. *snph db*	*P* = 0.0412 $t_{(22)}$ = 2.168
Fig. 7F	% Net mitochondrial transport (anterograde)	Normal	Unpaired *t*-test	WT vs. *snph db*	*P* = 0.0178 $t_{(22)}$ = 2.561
Fig. 7F	% Net mitochondrial transport (retrograde)	Normal	Unpaired *t*-test	WT vs. *snph db*	*P* = 0.0529 $t_{(22)}$ = 2.046
Fig. 7G	% Time mitochondria spent in motion (anterograde)	Normal	Unpaired *t*-test	WT vs. *snph db*	*P* = 0.3330 $t_{(22)}$ = 0.9900
Fig. 7G	% Time mitochondria spent in motion (retrograde)	Normal	Unpaired *t*-test	WT vs. *snph db*	*P* = 0.5596 $t_{(22)}$ = 0.5924
Fig. 7H	GC total area	Normal	Unpaired *t*-test	WT vs. *snph db*	*P* = 0.2904 $t_{(22)}$ = 1.083
Fig. 7H	GC central area	Normal	Unpaired *t*-test	WT vs. *snph db*	*P* = 0.3869 $t_{(22)}$ = 0.8829
Fig. 7I	Number of filopodia	Normal	Unpaired *t*-test	WT vs. *snph db*	*P* = 0.6775 $t_{(22)}$ = 0.42154

Fig. 7J	Growth rate	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.8256$ $t_{(15)} = 0.2243$
Fig. 8D	Pioneering cluster area	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.0166$ $t_{(15)} = 2.697$
Fig. 8E	Number of pioneering clusters	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.1865$ $t_{(15)} = 1.385$
Fig. 8F	Mitochondria presence (% time)	Normal	Unpaired *t*-test	WT vs. *snph db*	$P = 0.1994$ $t_{(15)} = 1.343$

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