TSG101 negatively regulates mitochondrial biogenesis in axons

Tzu-Huai Lin, Dana M. Bis-Brewer, Amy E. Sheehan, Louise N. Townsend, Daniel C. Maddison, Stephan Züchner, Gaynor A. Smith, Dr. John T. Macdonald Foundation Department of Human Genetics, University of Miami, Miami, FL 33136; Dr. John T. Macdonald Foundation Department of Human Genetics, University of Miami, Miami, FL 33136; UK Dementia Research Institute, School of Biosciences, Cardiff University, Cardiff CF24 4HQ, United Kingdom; and UK Dementia Research Institute, School of Medicine, Cardiff University, Cardiff CF24 4HQ, United Kingdom

There is a tight association between mitochondrial dysfunction and neurodegenerative diseases and axons that are particularly vulnerable to degeneration, but how mitochondria are maintained in axons to support their physiology remains poorly defined. In an in vivo forward genetic screen for mutants altering axonal mitochondria, we identified tsg101. Neurons mutant for tsg101 exhibited an increase in mitochondrial number and decrease in mitochondrial size. TSG101 is best known as a component of the endosomal sorting complexes required for transport (ESCRT) complexes; however, loss of most other ESCRT components did not affect mitochondrial numbers or size, suggesting TSG101 regulates mitochondrial biology in a noncanonical, ESCRT-independent manner. The TSG101-mutant phenotype was not caused by lack of mitophagy, and we found that autophagy blockade was detrimental only to the mitochondria in the cell bodies, arguing mitophagy and autophagy are dispensable for the regulation of mitochondrion number in axons. Interestingly, TSG101 mitochondrial phenotypes were instead caused by activation of PGC-1α/Nrf2-dependent mitochondrial biogenesis, which was mTOR independent and TFEB dependent and required mitochondrial fission–fusion machinery. Our work identifies a role for TSG101 in inhibiting mitochondrial biogenesis, which is essential for the maintenance of mitochondrial numbers and sizes, in the axonal compartment.

Mitochondria are vital organelles for all cells, but the highly polarized architecture of neurons presents a unique challenge in distributing these organelles to support cellular physiology. Axons in particular, which can be extremely long, must be supported at great distances from the cell bodies. How mitochondrial populations in axons are established and maintained at the correct density and distribution is not understood, but it is clear that axons lacking mitochondria can exhibit blocked growth or frank degeneration (1, 2). Alterations in mitochondrial function are tightly associated with nearly all neurodegenerative diseases, and axons, perhaps due to their enormous size, seem to be particularly vulnerable to degeneration (3–6). Unraveling the mechanisms by which axons receive and maintain appropriate numbers of healthy mitochondria is of paramount importance to defining the basic cellular principles of axonal maintenance, and it is likely that such insights will shed light on mechanisms that drive neurodegeneration. Mitochondria are intricately linked to many cellular functions. It is important to keep a constant functional pool of mitochondria through the generation of new mitochondria and eliminating malfunctioning ones. Mitochondria undergo fission to produce new mitochondria from existing ones, and the master transcriptional coactivator PPAR gamma coactivator, PGC-1α, is required to express nuclear-encoded mitochondrial genes to supply newly generated mitochondria with the molecules essential for function (7). When mitochondria are dysfunctional and need to be turned over, Pink1 can accumulate on mitochondria in response to a loss of mitochondrial membrane potential, where it recruits Parkin to initiate mitophagy or mitochondrial autophagy, whereby Parkin ubiquitinates proteins on the mitochondrial surface in order for autophagy machinery to recognize the doomed mitochondrion and target it for degradation (8–13).

While Pink1/Parkin-mediated mitophagy has been considered a major mechanism for disposing of mitochondria, recent studies have revealed a link between the endosomal sorting complexes required for transport (ESCRT) machinery and mitochondrial clearance. CHMP2A/Vps2 in the ESCRT-III complex is required during mitophagy for phagophore closure to form functional autophagosomes (14, 15). Snf8/Lsn in the ESCRT-II and TSG101 in the ESCRT-I complexes are involved in autophagy-independent but Parkin-dependent mitochondrial clearance (16). How and whether ESCRT complexes might regulate mitochondrial clearance in vivo has not been explored nor have roles for ESCRT complexes in mitochondrial biogenesis.

Despite our growing understanding of molecules regulating mitochondrial biology, the mechanisms by which cells maintain a properly sized, functional pool of mitochondria in vivo remain enigmatic. Given that many molecules known to regulate mitochondria...
were discovered in nonneuronal cells in an in vitro setting, determining how these mechanisms translate into in vivo mitochondrial regulation is also an important and often outstanding question. Hence, we performed an in vivo forward genetic screen in *Drosophila* (17) for mutations that exhibited changes in axonal mitochondrial size, number, or distribution. After screening over 6,000 genomes, we identified TSG101 as a regulator of axonal mitochondria. **tsg101** mutants exhibited elevated mitochondrial

---

![Image of diagram and graphs](https://doi.org/10.1073/pnas.2018770118)
numbers and reduced mitochondrial length. TSG101 is a component of the ESCRT-I complex; however, other key components of the ESCRT complexes were not required for normal axonal mitochondrial number or size, suggesting TSG101 functions in this context in a noncanonical way. Blocking either Pink1/Parkin-mediated mitophagy or macroautophagy exerted no significant effect on axonal mitochondrial morphology, suggesting mitophagy/autophagy are also dispensable in the axons. Instead, TSG101 loss activated mitochondrial biogenesis in an mTOR-independent and TFEB-dependent manner, to increase number and reduce the size of mitochondria. Our work argues that TSG101 is a critical regulator of axonal mitochondrial populations and that canonical

Fig. 2. Blocking mitophagy/autophagy in neurons did not recapitulate the axonal mitochondrial phenotype of tsg101 mutants. (A and B) Mito-QC was utilized to visualize mitochondrial turnover event: normal mitochondria in yellow (green + red) and lysosome-associated mitochondria in red (mitolysosomes, indicated by white arrowheads). Flies were grown in 29 °C and checked at 7 dpe. Number of mitolysosomes per axon in A was quantified and shown in B, analyzed by unpaired t test. n = 10. (C–E) Homozygous mutant neurons were sparsely generated and labeled red and mitochondria in green. Null alleles of Pink1 (Pink15) and parkin (parkinΔ21) were used to suppress mitophagy. Flies were grown in 25 °C and checked at 14 dpe. Mitochondria were subsequently quantified and analyzed in D and E. n ≥ 20. Data were analyzed via two-way ANOVA. *P < 0.05. **P < 0.01. (F–H) Blocking autophagy by knocking out atg6 (Beclin-1) and/or overexpressing dominant-negative atg1 (ULK1). Flies were grown in 25 °C and checked at 14 dpe. Quantification of axonal mitochondria is shown in G and H; analyzed via one-way ANOVA. n ≥ 20. Error bar: SEM. (Scale bar, 10 μm.)

Lin et al. PNAS | 3 of 11
TSG101 negatively regulates mitochondrial biogenesis in axons
https://doi.org/10.1073/pnas.2018770118
TSG101 and autophagy-dependent mechanisms for mitochondrial turnover do not predominate in the axonal compartment.

Results

tsg101 Mutants Exhibited Increased Mitochondrial Number and Decreased Mitochondrial Size in Axons. To identify genes involved in the maintenance of axonal mitochondria in vivo, we performed a forward genetic screen for mutants with altered mitochondria in the axons of adult Drosophila L1 wing-vein glutamatic sensory neurons (17). We mutagenized animals with ethyl methanesulfonate (EMS) and used an F1 mosaic analysis with a repressible cell marker (MARCM)-based genetic approach (18) to generate sparse, homozygous mutant clones, where the plasma membrane was labeled with td-Tomato (mmy::td-Tomato) and mitochondria with GFP (mito::EGFP). Potential mitochondrial regulating molecules were indicted based on changes in mitochondrial morphology and number or distribution in either the axon and/or cell body, with the preliminary screen identifying five candidates, including one previously mapped and characterized regulator gsf2 (17). We quantified mitochondrial populations 1, 7, and 14 d posteclosion (dpe) in individual axons and bred F1 animals with altered mitochondrial profiles. We identified one mutant, l(3)387, that bred true and was subsequently named tsg101 after rigorous tests, including complementation test (SI Appendix, Fig. S1A) and next-generation whole genome sequencing (Fig. S1B) and identified a gain-of-stop in the gene tsg101 as the causal lesion (Fig. S1A). Mutations in the gene caused early larval lethality and hence phenotypes were studied in neuronal clones. tsg101 mutants exhibited elevated mitochondrial number and shortened mitochondrial length. At 14 dpe, while control animals averaged below 10 mitochondria (per 90 μM of axon scored), tsg101 mutant axons had more than 21 mitochondria in the same area of axon, although they were reduced in size by over 30% (Fig. 1 B–D), resulting in an increase in overall axonal occupation by mitochondria (SI Appendix, Fig. S1B). This change in mitochondrial morphology manifested as early as 1 dpe, and the phenotypical differences widened as the time elapsed (SI Appendix, Fig. S1 D–F). In addition to the mitochondrial phenotype, we also observed a neuronal phenotype, in which the cell bodies of the mutant neurons were consistently larger than the control (SI Appendix, Fig. S1F), and at 30 dpe neurons underwent spontaneous neurodegeneration (SI Appendix, Fig. S1C). Next, we determined the localization of TSG101 using antibody detection in primary mouse neurons. TSG101 was ubiquitously expressed, partly colocalizing with both mitochondria and endosomes in projections (SI Appendix, Fig. S1 G and H). Lastly, we found a dramatic increase in endolysosomes in both the axons and the cell bodies of the mutant neurons, and the mutant axons exhibited more stationary endolysosomes in close proximity to the mitochondria than the control did (SI Appendix, Fig. S2), indicating the importance of TSG101 for mitochondrial maintenance, neuronal integrity, and endolysosomal homeostasis.

TSG101 and Shrb, but Not Other ESCRT Complex Components, Regulate Axonal Mitochondria and Axon Integrity. TSG101 is best known as a component of the ESCRT-I complex, one of several ESCRT complexes (ESCRT-I, ESCRT-II, ESCRT-III, and the accessory ESCRT complex) that drive the functional maturation of endosomes into multivesicular bodies. To determine whether other ESCRT-associated molecules also regulate axonal mitochondria, we knocked down most components of all ESCRT complexes by RNA interference (RNAi). To our surprise, knockdown of core ESCRT pathway components did not phenocopy the increased mitochondrial number and decreased mitochondrial length, as observed in the tsg101 mutant, with the exception of shrb-RNAi (SI Appendix, Table S1). Shrb is a homolog of vps32/snf7 in the ESCRT-III complex. Knockdown of shrb fully phenocopied mitochondrial phenotypes in tsg101 loss-of-function mutants (Fig. 1 H–J). However, we were unable to generate clones for analysis of adult axons as shrb mutant glutamaticergic neurons underwent cell death by 1 dpe. To further rule out a role for the major ESCRT complexes in regulation of axonal mitochondria, we used more rigorous loss-of-function genetic methods. We generated MARCM clones using tested and published null alleles of at least one component of each major ESCRT complex and found that knockout (KO) of lsn (ESCRT-II), vps2 (ESCRT-III), or ALIX and map (accessory complex) did not recapitulate the tsg101 mutant phenotype (Fig. 1 E–G). Together, these data support the notion that TSG101 does not regulate axonal mitochondria through canonical ESCRT signaling.

Turnover of Mitochondria in Axons Is Attenuated in tsg101 Mutants, and the Turnover Does Not Require Parkin/Pink1-Mediated Mitophagy or Autophagy. The increase in mitochondrial number in axons that we observed in tsg101 mutants might be the result of failed mitochondrial clearance or activated mitochondrial biogenesis. Given that some ESCRT complex components have recently been found to be required for mitochondrial clearance (14–16), we first assayed mitochondrial clearance. We used the well-established genetic tool mito-QC, which allows for monitoring of mitochondrial turnover induction using a pH-sensitive fluorophore, whose fluorescence is altered upon mitochondrial entry into acidic vesicles during degradation (Fig. 2 A) (19, 20). In control animals, we observed normal (yellow) mitochondria, along with (red) mitolysosomes, which represent mitochondria undergoing lysosomal degradation, in both axons and the cell body. However, in tsg101 mutants, mitolysosomes were nearly undetectable (Fig. 2 A and B), indicating that mitochondrial turnover was largely attenuated. Live cell imaging revealed that lysosomes accumulated in axons, were more stationary, and were found in close proximity to mitochondria (SI Appendix, Fig. S2), indicating that loss of mitophagy may occur through defective mitolysosome trafficking.

To determine if loss of mitophagy could phenocopy the alterations in axonal mitochondria observed in tsg101 mutants, we blocked mitophagy by knocking out Parkin or Pink1. We found that neither parkin nor Pink1 loss-of-function mutants phenocopied tsg101 mutant phenotypes. We also found that neither mutant exerted significant effects on axonal mitochondrial numbers or size nor long-term axon maintenance (Fig. 2 C–E). We conclude that inhibition of Pink1/Parkin-mediated mitophagy does not explain the abnormal mitochondrial properties observed in the tsg101 mutants. These data argue strongly that Pink1/Parkin-mediated mitophagy is not the primary mechanism for turnover of mitochondria in axons, and emerging evidence supports the notion that basal mitophagy can be Parkin and Pink1 independent (12, 19, 21).

We next blocked mitophagy via a more extreme means—impeding the entire autophagy process by knocking out atg6 (Beclin-1 homolog) and/or overexpressing dominant-negative atg1 (ULK1 homolog). atg6 loss did not affect axonal mitochondrial number or size nor did combining atg6 loss with simultaneous expression of atg1DN (Fig. 2 F–H). However, we did observe that the cell bodies drastically increased in size (Fig. 2 F and SI Appendix, Fig. S3A). Moreover, somal mitochondria changed from linear shapes to more globular ones (reflected in lower aspect ratio in tsg101 mutants; SI Appendix, Fig. S3B), and the mitochondrial mass significantly increased (SI Appendix, Fig. S3C), although the increase is proportionate to that in cell body size, leading to no change in overall mitochondrial coverage (SI Appendix, Fig. S3D). This phenotype persisted up to 30 dpe, where the cell body phenotype progressively worsened, while axonal mitochondria remained morphologically similar to wild type, although occasional axon blebs were apparent (SI Appendix, Fig. S3E). As we confirmed that mitochondria were no longer able to undergo mitophagy when autophagy is genetically blocked (SI Appendix, Fig. S3 F and G), we conclude that axonal mitochondrial populations can be maintained at normal levels even in the absence of Parkin, Pink1, or autophagy. This suggests that the
TSG101-mediated, autophagy-independent but Parkin-dependent mitochondrial clearance reported previously (16) is mechanistically distinct from TSG101-mediated mitochondrial clearance in axons. Furthermore, our data support the premise that mitochondrial clearance mechanisms are compartmentalized in neurons, since we observe changes in mitochondria in cell bodies with manipulation of autophagy but not in axons.

Mitochondria in tsg101-Mutant Neurons Have Lower Reactive Oxygen Species and Generate Increased Levels of ATP. TSG101 could be involved in mitochondrial clearance through a mechanism that does not require Pink1/Parkin or autophagy. If that is the case, we would predict that tsg101-mutant neurons would accumulate dysfunctional mitochondria and potentially have decreased ATP levels. To explore this possibility, we utilized two different roGFP genetic tools (GSSG-sensing and H2O2-sensing) to measure redox potential in mitochondria, which is an indicator of overall mitochondrial health—an increased oxidation state, which increases the 405/488-nm ratio, correlates well with increased mitochondrial damage and dysfunction (22). Interestingly, mitochondria in tsg101-mutant axons were less oxidized than in controls. As a further control for the sensors, we overexpressed human mitochondrial uncoupling protein (hUCP2), which artificially renders mitochondria more oxidized, and observed a >50% increase in the 405/488-nm ratio (Fig. 3 A–D). These data suggest that mitochondria in tsg101 mutants were less oxidized and healthier than in controls. Moreover, when we utilized the ATP sensor ATPsnFR (23), we found that neurons lacking TSG101 had significantly increased levels of cytoplasmic ATP, and this increase was abolished when oxidative phosphorylation (OXPHOS) was disrupted with hUCP2 overexpression (24), indicating that a lack of TSG101 results in more mitochondria synthesizing increased levels of axonal ATP (Fig. 3 E and F). These results suggest that the lack of mitochondrial turnover in tsg101 mutants may be due to the fact that the mitochondria were hyperfunctional and thereby resistant to mitochondrial clearance.

Loss of TSG101 Activates Mitochondrial Biogenesis through PGC-1α, Nrf2, and TFEB. The majority of mitochondrial genes are encoded by the nuclear genome, and the master transcription coactivator PGC-1α and Nrf2 are responsible for activating nuclear-encoded mitochondrial gene expression. To investigate whether the hyperfunctional mitochondria in tsg101 mutants were the result of activated mitochondrial biogenesis, we knocked down srl (the Drosophila PGC-1α homolog) and cnc (the Drosophila Nrf2 homolog) in both controls and tsg101-mutant neurons. Knockdown of srl or cnc in controls resulted in smaller mitochondria but normal numbers of mitochondria in the axon (Fig. 4 A–C). This is consistent with a decrease in overall mitochondrial content, which is expected after blocking mitochondrial biogenesis. When we knocked down srl or cnc in tsg101mutant mutants, the tsg101mutant mitochondrial phenotypes were rescued, and this was true when we tested two different RNAi lines for srl and cnc (Fig. 4 A–C).

TFEB is a positive regulator of PGC-1α-mediated mitochondrial biogenesis during conditions such as nutrient deprivation and exercise (25, 26). We therefore investigated the role of TFEB as a potential activator of PGC-1α-mediated mitochondrial biogenesis in tsg101-mutant axons. Knockdown of mitf (the Drosophila TFEB homolog), with two independent RNAi lines, rescued tsg101-mutant mitochondrial phenotypes (Fig. 5 A–C), indicating that TFEB also acts downstream of TSG101 in controlling mitochondrial biogenesis. The mTOR pathway is also a modulator of mitochondrial biogenesis (7, 27) and can control TFEB activation (28, 29). Interestingly, a mutation in the gene encoding the mTOR inhibitor, Tsc1, was also uncovered in our forward genetic screen, with a phenotype of increased mitochondria numbers in axons; loss of tsc1 has also been found to activate mitochondrial biogenesis (30). We therefore knocked down tsc1 in control and tsg101-mutant clones and assayed mitochondrial phenotypes. We found that mitochondrial number was significantly increased after depletion of Tsc1, at levels even higher than in TSG101 mutants alone, although we detected no difference in mitochondrial size. In addition, we found that knockdown of Tsc1 in TSG101 null clones had no additive effect on the number of mitochondria in axons (SI Appendix, Fig. S4 A–C). This finding suggests that TSG101 controls mitochondrial biogenesis in an mTOR-independent manner or at least not through Tsc1. Therefore, our model suggests that TSG101 functions in a parallel pathway to mTOR, which converges on TFEB to regulate mitochondrial biogenesis.

Mitochondrial Biogenesis in tsg101 Mutants Requires Drp1-Dependent Mitochondrial Fission. Mitochondria are generated by fission of existing mitochondria, which can be achieved by activating fission or inhibiting fusion. We first sought to determine whether the mitochondrial biogenesis activated in tsg101 mutants required mitochondrial fission. We blocked fission by knocking out the critical fission gene Dnp1, which significantly rescued both the mitochondrial number and size in axons caused by tsg101 knockdown (SI Appendix, Fig. S5). This indicates that Drp1-mediated mitochondrial fission is downstream of TSG101 loss and is required for activation of mitochondrial biogenesis.

We next explored the effects of blocking mitochondrial fusion by knocking down the essential fusion gene Marf in attempt to recapitulate tsg101-mutant phenotype. Knockdown of Marf in control clones successfully shortened mitochondria to levels indistinguishable from tsg101mutant mutants at 7 dpe yet had no effect on number (SI Appendix, Fig. S6 A–C). To our surprise, Marf knockdown rescued mitochondrial number in tsg101-mutant axons to near control levels (SI Appendix, Fig. S6 A and B). We further investigated the effect of Marf knockdown at the earliest time point, 1 dpe, where the mitochondrial phenotype was not fully manifested and Marf knockdown had little effect (SI Appendix, Fig. S6 D–F). This suggests that the age-dependent increase of mitochondrial number from 1 to 7 d requires Marf, and fusion is a necessary initial step for biogenesis.

TSG101 Regulates Mitochondria and Neurodegeneration through Independent Pathways. Loss of TSG101 leads to spontaneous neurodegeneration in mutant clones as animals age (SI Appendix, Fig. S1C). We wished to determine whether this phenotype could also be rescued by manipulating mitochondrial biogenesis and to explore the molecular nature of the signaling pathways driving this axon degeneration. Given that knockdown of TFEB or Nrf2 rescued the defects in mitochondrial numbers and size in tsg101 mutants, we explore whether these manipulations also prevented neurodegeneration. We found that knocking down TFEB (Mitf) or Nrf2 (Cnc) did not affect neurodegeneration nor axon survival rate, despite complete rescue of mitochondrial phenotypes (SI Appendix, Fig. S7 A and B). This argues strongly that TSG101 has two roles in axons, one to inhibit mitochondrial biogenesis and another to maintain long-term axon integrity. What is the nature of the neurodegeneration we observe? To explore this, we used a variety of manipulations to block Wallerian degeneration and apoptotic pathways using well-established genetic tools: Wldε, p53DN, or p35 (31–33). Interestingly, neither blockade of Wallerian degeneration signaling (via Wldε expression) or apoptosis signaling (via p53DN or p35 expression) were able to suppress the neurodegeneration in tsg101-mutant clones (SI Appendix, Fig. S7C). These data argue that the neurodegeneration caused by lack of TSG101 occurs through mechanisms independent of canonical Wallerian degeneration and apoptotic signaling pathways.

Discussion

The remarkable size and morphological complexity of axons present an enormous challenge for neurons—how does the neuron establish and maintain a healthy population of mitochondria at

Lin et al.
sufficient density throughout their axonal arbors? This problem, termed neuronal mitostasis (34), is ongoing throughout the animal’s lifespan and must be dealt with successfully in order to sustain the energetically expensive process of neurotransmission and to maintain neuronal integrity. Mitochondrial populations in axons are believed to be regulated primarily by mitochondrial

Fig. 3. Mitochondria in tsg101 mutants were less oxidized and generated more ATP. (A–D) Redox-sensing GFPs (roGFps) were utilized and expressed in mitochondria to reactive oxygen species. Fluorescence intensities were measured with 405-nm (pseudocolored red) and 488-nm (pseudocolored green) laser excitations. roGFP ratios were determined by the measured fluorescence intensity from 405 nm divided by that from 488 nm. 405/488-nm ratios were normalized to the control. A higher ratio indicates a more oxidized mitochondrion. Overexpression of human uncoupling protein (hUCP2) was used to make mitochondria more oxidized by disrupting mitochondrial membrane potential in order to reassure the reliability of the sensors. Flies were grown in 25 °C and checked at 14 dpe. Data were analyzed via unpaired t test. n ≥ 10. Error bar: SEM. *P < 0.05. **P < 0.01. (E and F) ATP-sensing GFP (ATPsnFR, green) was expressed in the cytosol to measure cytosolic ATP level. ATPsnFR intensities were normalized to the fluorescence intensity of mitochondria (mito::td-Tomato, red), and the normalized ATPsnFR intensities were then normalized again to that of control. Flies were raised in 29 °C and examined at 7 dpe. n ≥ 14. Data were analyzed via two-way ANOVA. Error bar: SEM. (Scale bar, 10 μm.) **P < 0.01.
transport into the axon (i.e., motility), anchoring, and fission and fusion to increase or decrease numbers, respectively. Turnover of damaged mitochondria or their components is mediated by the shedding of mitochondria-derived vesicles (MDVs), which are used to degrade packages of lipids and proteins (35, 36), mitophagy, or the general cellular degradative macroautophagy pathway. The critical importance of neuronal mitostasis is borne out by the fact that mutations in many genes that regulate mitochondrial function (37) or dynamic fission/fusion events (38, 39) lead to devastating neurological disorders and neurodegeneration.

To identify molecules required for neuronal mitostasis in axons, we performed an unbiased forward genetic screen for mutants that altered the number or size of mitochondrial in sensory axons in the Drosophila adult L1 wing vein in vivo. We identified TSG101, a well-known component of the ESCRT-I complex, as required for maintaining normal mitochondrial numbers and sizes. Although we found Shrb, a component of the ESCRT-III complex, was also required for axonal mitostasis like TSG101, we found no evidence for other components or the ESCRT-I, -II, or -III complexes being involved in regulating mitochondria in axons, even when we used null alleles of key components for each complex. These observations support the notion that TSG101 and Shrb regulate mitochondrial numbers and size in a noncanonical ESCRT pathway.

Other components of the ESCRT complexes have recently been linked to mitophagy or autophagy in cultured cells: CHMP2A/Vps2 of ESCRT-III was found to promote mitophagy by mediating phagophore sealing during autophagy, and Snf8/Lsn of ESCRT-II was discovered important in autophagy-independent but endosome-dependent mitochondrial clearance (14–16). However, we found that KO of either of these genes, or full blockade of Parkin/Pink1 mitophagy or autophagy, failed to recapitulate tsg101-mutant phenotype or alter mitochondrial numbers or distribution in axons. Live cell imaging of lysosomes revealed their accumulation within the axon, which may occur through the disinhibition of TFEB resulting in their biogenesis. Loss of TSG101 caused an increased proportion of stationary lysosomes in close proximity to mitochondria without subsequent sequestration, since overall mitophagy

Fig. 4. Loss of TSG101 activates PGC-1α (srl)/Nrf2 (crc)-dependent mitochondrial biogenesis. (A–C) Homozygous mutant neurons were generated and labeled using the same approach as in Fig. 1. Two independent RNAi were used to knock down PGC-1α (srl) or Nrf2 (crc). All four independent RNAi were able to successfully rescue the mitochondrial phenotype resulted from TSG101 ablation. Quantification of (B) number and (C) length. n ≥ 30. Data were analyzed via two-way ANOVA. Error bar: SEM. **P < 0.01. (Scale bar, 10 μm.)
levels were reduced, indicating deficits in trafficking of mitochondria to lysosomes. It is also possible that accumulation of late endosomes/lysosomes in close proximity may contribute to mitochondrial biogenesis, by serving as translation hubs for mitochondrial encoded proteins, as previously reported (40). Although mitophagy is critical for mitochondrial turnover in many settings, these mechanisms have been studied primarily in the context of cellular stress (e.g., perturbation of mitochondrial membrane potential), while we are examining turnover in wild-type axons in vivo, which might account for some differences in the genetic pathways deployed. Indeed, others have also found that loss of Pink1 or Parkin does not alter axonal mitochondria significantly in vivo (12, 19, 21). We interpret our findings to indicate that at least one component of the ESCRT complex, TSG101, may function in mitophagy regulation under chronic stress yet under normal physiological conditions functions instead as a negative regulator of mitochondrial biogenesis. Turnover of mitochondria appears to happen in Drosophila sensory neuron axons, given that we find small vesicles in axons.

Fig. 5. TFEB (mitf) is downstream of TSG101 in mitochondrial regulation. (A–C) Homozygous mutant neurons were generated and labeled using the same approach as in the previous figure. Two independent RNAi were used to knock down TFEB (mitf), and both of them successfully rescued TSG101-mutant phenotype. The result is quantified in B and C and analyzed via two-way ANOVA, n ≥ 30. Error bar: SEM. **P < 0.01. (Scale bar, 10 μm.) (D) A schematic model shows the genetic pathway of TSG101-controlled mitochondrial biogenesis and its relation with the mTOR pathway. In axons mutant for TSG101, TFEB-dependent mitochondrial biogenesis is activated, which requires PGC-1α and Nrf2. As studies have shown that mTOR pathway suppresses TFEB activity and activates mitochondrial biogenesis through transcription factor other than Nrf2, TSG101-controlled mitochondrial biogenesis is parallel to the mTOR pathway.
consistent with the mito-QC shifting into an acidified compartment. These may be MDVs that are normally shed in control animals and turned over through lysosomal-dependent mechanisms to maintain a functional mitochondria pool in axons. The mechanisms by which turnover occurs in axons is unclear, since loss of Pink1, Parkin, or full blockade of autophagy did not alter mitochondrial numbers in axons. Our data likely indicates that another lysosome-mediated pathway selectively targets MDVs for turnover. Whatever the pathway, it appears sensitive to loss of TSG101: in tsg101 mutants we found a near complete blockade of turnover of mitochondrial components based on mito-QC dynamics.

In the context of mature axons, how TSG101 and Shrb regulate mitochondrial numbers through regulation of biogenesis is an interesting question. Loss of TSG101 resulted in increased numbers of smaller mitochondria, and multiple measurements of mitochondrial physiology, including sensors of redox status and ATP levels in axons, suggested these mitochondria had less oxidative species and heightened OXPHOS than in controls. These features suggest that TSG101-mutant axons have shifted toward increased mitochondrial biogenesis and therefore argue that TSG101 normally functions in axons to constitutively suppress mitochondrial biogenesis. It would be interesting to see if loss of TSG101 also enhances mitochondrial membrane potential in future studies. TSG101 may regulate signaling back to the nucleus to inhibit activation of the nuclear transcriptional program mediated by PGC-1α, Nrf2, and TFEB, which are key regulators of nuclear mitochondrial genes (41). Interestingly, previous work has shown that TFEB is held on lysosomes when it is not active, and it is released and translocates to the nucleus for transcription upon deactivation of the mTOR pathway (42). It is therefore possible that TSG101 might control TFEB translocation in a similar fashion. TFEB activation is also tightly linked with lysosomal biogenesis and may therefore explain enhanced lysosomal numbers and TSG101-mutant neurons.

Changes in mitochondria occur in Drosophila axons with aging. In wild-type animals, mitochondrial numbers remain fairly constant, but average mitochondrial length slowly increases with age. We found that tsg101-mutant phenotype manifested as early as 1 dpe and that mitochondrial numbers continued to increase at later time points. Interestingly, mitochondrial length in TSG101 mutants increased even at late time points, in contrast to wild type where an age-dependent increase was observed. We found that mitochondrial number and size in TSG101 knockdown conditions could be rescued by KO of Drp1, which is consistent with the notion that the final step of mitochondrial biogenesis requires fission (43). Strangely, we also found that elevated mitochondrial numbers in TSG101-null clones could be rescued by knockdown of Marf, which should enhance fusion. That manipulating fusion can rescue mutant phenotypes seems counterintuitive and requires further explanation. We note that PGC-1α can activate Mfn2 (Marf homolog) in mammalian cells (44, 45), and to expand to a sizable number of mitochondria (i.e., mitochondrial biogenesis), mitochondrial fusion needs to happen in order to grow mitochondria in size before fission initiates, severs and generating mitochondria from the existing ones. Certainly, overexpression of PGC-1α has also been found to increase the phosphorylation of Drp1 to enhance fusion (46). In the TSG101-KO and Marf knockdown condition, mitochondrial fusion is blocked, and fission is no longer required, hence the inhibition of TSG101-dependent growth in mitochondrial number. How high levels of fission and fusion coordinate with each other to expand a mitochondrial pool in a developing neuron is an interesting and important question for future studies. There is also a possibility that manipulating fusion/fission dynamics can feed back and change how PGC-1α signals downstream of TSG101. Understanding how this feedback mechanism might work is potentially important and requires further investigation.

Our data show that loss of TSG101 causes age-dependent neurodegeneration that occurs independently in mitochondrial dynamics. Prior to cell death, axons are swollen and dysmorphic, recapitulating spongiform phenotypes observed in MGRN1 KO mice (47, 48), associated with loss of TSG101. Genetic blockade of either apoptosis or Wallerian degeneration did not prevent such neurodegeneration, indicating a unique form of cell death, warranting further investigation. Spongiform-like degeneration could be the direct consequence of the accumulation of endosomes or loss of the receptor cargos they contain. Blocking many key components of the mitophagy pathway did not cause detrimental effects to the axons or their mitochondria, regardless of animal age. This complements the emerging findings that Pink1/Parkin-dependent mitophagy is dispensable in mature neurons under normal physiological conditions (12, 19) and may be protein selective (49). Pink1/Parkin may only be required for mitophagy under scenarios of acute oxidative stress, such as that caused by toxins and rapid depolarization, a context in which Pink1/Parkin–Optineurin signaling is often studied (50–52). It is also interesting to note that blockade of autophagy, and hence all known forms of mitophagy, also had no effect on mitochondria number in axons; however, its blockade did dramatically alter mitochondria in the cell bodies, arguing that mitochondrial regulatory mechanisms in the axon versus cell body are genetically distinct. Genetic evidence has recently linked complex I deficiency and trafficking dysfunction to long axon maintenance and disease, and the compartmentalization of such processes requires further investigation (53).

To the extent of our knowledge, where biogenesis happens in neurons (i.e., in the axon versus the cell body) is an open question. BrdU labeling studies in isolated chick neurons support the notion that at least in vitro mitochondrial biogenesis can occur in axons (43) and could be triggered by oxidative stress (54), and it seems likely that biogenesis happens in axons in vivo, especially in very long axons that cannot as easily derive such support from the soma. Mitochondrial numbers or position may also meaningfully regulate neurophysiology. Intriguingly, loss of Syntaphilin, a molecule that anchors mitochondria to the cytoskeleton, leads to widespread mitochondrial motility in neurites. Enhanced mitochondrial motility correlated with increased pulse-to-pulse variability in synaptic strength properties, while anchoring mitochondria reduced variability (55). The primary effect of these synaptic changes were found to occur through local modulation of ATP levels (55), which we also observed in tsg101-mutant axons. An exciting next question is whether axon-specific regulation of mitochondrial biogenesis or clearance can also provide an additional mechanism to contribute to cell-specific plasticity in circuits.

Materials and Methods

Drosophila Strains. Drosophila strains for mutagenesis and MARCM clone generation were as follows: OK371-Gal4, 10x UAS-IVS-myc::tdTomato, 5x UAS-mito::GFP, asense-FLP2α; tub-Gal80, FRT2A (males) and OK371-Gal4, 10x UAS-IVS-myc::tdTomato; FRT2A, FRT2B (males). Fly lines used for MARCM clone generation in epistatic analyses or general experimental procedures are the following: OK371-Gal4, n54-Gal4, FRT19A, FRT40A, FRT2A, FRT2B, FRT82B, asense-FLP2α; asense-FLPα; asense-FLPα; hs-FLP22; tub-Gal80, 10x UAS-IVS-myc::tdTomato, 5x UAS-mito::GFP, 5x UAS-mito::tdTomato, and 5x UAS-lamp1::GFP. MARCM tools and resources were generated by ref. (56). The following null mutant alleles, overexpression constructs, and commercially available RNAi lines were used in epistasis or general experimental procedures: tsg101 58649 (BDSC: 19659), tsg101 5209 (Bloomington Drosophila Stock Center [BDSC: 63105], tsg101 58649 (Vienna Drosophila Resource Center [VDRC]: 330271), 5x UAS-Tsg101RNAi (VDR: 103355), 5x UAS-cncRNAi (VDR: 40854), 5x UAS-cncRNAi (VDR: 37673), 5x UAS-mitfRNAi (VDR: 44561), 5x UAS-mitfRNAi (VDR: 109184), 5x UAS-StampRNAi (VDR: 55189), 5x UAS-srmRNAi (Vienna Drosophila Resource Center [VDRC]: 330271), 5x UAS-srmRNAi (VDR: 103355), 5x UAS-srmRNAi (VDR: 40854), 5x UAS-srmRNAi (VDR: 37673), 5x UAS-mitfRNAi (VDR: 44561), 5x UAS-mitfRNAi (VDR: 109184), 5x UAS-StampRNAi (VDR: 55189), 5x UAS-srmRNAi (Vienna Drosophila Resource Center [VDRC]: 330271), 5x UAS-srmRNAi (VDR: 103355), 5x UAS-cncRNAi (VDR: 40854), 5x UAS-cncRNAi (VDR: 37673), 5x UAS-mitfRNAi (VDR: 44561), 5x UAS-mitfRNAi (VDR: 109184), 5x UAS-StampRNAi (VDR: 55189), 5x UAS-srmRNAi (Vienna Drosophila Resource Center [VDRC]: 330271), 5x UAS-srmRNAi (VDR: 103355), 5x UAS-cncRNAi (VDR: 40854), 5x UAS-cncRNAi (VDR: 37673), 5x UAS-mitfRNAi (VDR: 44561), 5x UAS-mitfRNAi (VDR: 109184), 5x UAS-StampRNAi (VDR: 55189), 5x UAS-srmRNAi (Vienna Drosophila Resource Center [VDRC]: 330271), 5x UAS-srmRNAi (VDR: 103355), 5x UAS-cncRNAi (VDR: 40854), 5x UAS-cncRNAi (VDR: 37673), 5x UAS-mitfRNAi (VDR: 44561), 5x UAS-mitfRNAi (VDR: 109184), 5x UAS-StampRNAi (VDR: 55189), 5x UAS-srmRNAi (Vienna Drosophila Resource Center [VDRC]: 330271), 5x UAS-srmRNAi (VDR: 103355), 5x UAS-cncRNAi (VDR: 40854), 5x UAS-cncRNAi (VDR: 37673), 5x UAS-mitfRNAi (VDR: 44561), 5x UAS-mitfRNAi (VDR: 109184), 5x UAS-StampRNAi (VDR: 55189), 5x UAS-srmRNAi (Vienna Drosophila Resource Center [VDRC]: 330271), 5x UAS-srmRNAi (VDR: 103355), 5x UAS-cncRNAi (VDR: 40854), 5x UAS-cncRNAi (VDR: 37673), 5x UAS-mitfRNAi (VDR: 44561), 5x UAS-mitfRNAi (VDR: 109184), 5x UAS-StampRNAi (VDR: 55189), 5x UAS-srmRNAi

Lin et al.
MARCM Clone Induction and In Vivo Mitogenesis Screen for Changes in Mitochondrial Morphology. Induction of MARCM clones in Drosophila wings was described in our previous work (17), which was adapted from an odon-detection screen (56). The screen started off by starving male flies for 8 h following by feeding mutagen EMS (Sigma-Aldrich catalog no. M80880) for 12 h (25 mM EMS in 1% sucrose). The males were kept in fresh vials to recover for another 12 h before mating with females. The F1 progeny, aged 7 dpe at 25°C were checked by clipping off one wing per fly, mounting the wings directly in Halocarbon Oil 27 (Sigma-Aldrich catalog no. 87773) on a microscope slide with cover slip on top, and immediately examining under microscope. Axonal mitochondria (labeled with mito-GFP residing in the L1 vein of each wing were checked for any phenotypic changes. Flies that exhibited phenotypes were saved, bred, outcrossed, and made into stocks for further analysis including checking for any phenotypic changes. Flies that exhibited phenotypes were washed three times with D-PBS, counterstained with Hoechst for 30 min and cells were fed every 3 d. At 12 days in vitro, mouse cortical neurons were processed in Zen Blue and Fiji (version 2.0). Fiji was used to quantify mitochondrial populations in axons of parkin-mediated mitochondrial quality control in the Drosophila nervous system. Data Availability. All study data are included in the article and/or SI Appendix.

ACKNOWLEDGMENTS. We thank the Looger laboratory in the Janelia Research Campus for providing the backbone plasmids for making the AttoPhNR transgenic fly, the Whittow laboratory at University of Cambridge for the mito-qtc transgenic fly, Lukas Neukenm for MARCM flies, the Baehrke laboratory at the University of Massachusetts Medical School for the atg5 KO fly, and Bloomington Drosophila Stock Center, Kyoto Stock Center, and Vienna Drosophila Resource Center for the rest of the reagents. We also thank Dr. Kelly Monk, Dr. Philip Copenhaver, Dr. Alex Nechiporuk, and Dr. Gail Mandel at the fly, and Bloomington Drosophila Stock Center, Kyoto Stock Center, and Vienna Drosophila Resource Center for the rest of the reagents. We also thank Dr. Kelly Monk, Dr. Philip Copenhaver, Dr. Alex Nechiporuk, and Dr. Gail Mandel at the

Marine androgen receptor (AR) is a ligand-activated transmembrane hormone receptor that mediates the physiological effects of androgens. It is predominantly expressed in the male reproductive tract, including the prostate, seminal vesicles, and epididymis. In females, AR expression is low and primarily restricted to the brain and reproductive organs. The activation of AR by its ligands, such as dihydrotestosterone (DHT) and testosterone, leads to the transcription of target genes involved in prostate growth, development, and function. Understanding the molecular mechanisms underlying AR signaling in prostate cancer is crucial for developing effective treatments. In this study, we investigated the role of AR in the progression of prostate cancer by characterizing the AR expression pattern in various prostate cell lines and primary human prostate epithelial cells. We used immunofluorescence staining and qRT-PCR analyses to determine the expression level of AR in different prostate cell lines and primary prostate epithelial cells. Our results showed that AR expression is significantly higher in prostate cancer cell lines compared to normal prostate epithelial cells, indicating a potential role of AR in prostate cancer progression. These findings suggest that targeting AR could be a promising strategy for the treatment of prostate cancer. However, further studies are needed to elucidate the specific mechanisms by which AR contributes to prostate cancer development and progression.
26. C. Settembre et al., TFEB controls cellular lipid metabolism through a starvation-induced autoregulatory loop. Nat. Cell Biol. 15, 647–658 (2013).
27. J. T. Cunningham et al., mTOR controls mitochondrial oxidative function through a YY1-PGC-1α transcriptional complex. Nature 450, 736–740 (2007).
28. J. A. Martina, Y. Chen, M. Gueck, R. Puertollano, MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. Autophagy 8, 903–912 (2012).
29. G. Napolitano et al., mTOR-dependent phosphorylation controls TFEB nuclear export. Nat. Commun. 9, 3312 (2018).
30. C. Chen et al., TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species. J. Exp. Med. 205, 2397–2408 (2008).
31. M. A. Avery et al., WlsD prevents axon degeneration through increased mitochondrial flux and enhanced mitochondrial Ca2+ buffering. Curr. Biol. 22, 596–600 (2012).
32. B. A. Hay, T. Wolff, G. M. Rubin, Expression of baculovirus P35 prevents cell death in Drosophila. Development 120, 2121–2129 (1994).
33. M. Ollmann et al., Drosophila p53 is a structural and functional homolog of the tumor suppressor p53. Cell 101, 91–101 (2000).
34. T. Misgeld, T. L. Schwarz, Mitostasis in neurons: Maintaining mitochondria in an extended cellular architecture. Neuron 96, 651–666 (2017).
35. R. F. Roberts, M. Y. Yang, E. A. Fon, T. M. Durcan, Defending the mitochondria: The pathways of mitophagy and mitochondrial-derived vesicles. Int. J. Biochem. Cell Biol. 79, 427–436 (2016).
36. E. Shlevkov, T. L. Schwarz, Have you seen? For parkin, it’s not all or nothing. EMBO J. 33, 277–279 (2014).
37. M. T. Lin, M. F. Beal, Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443, 787–795 (2006).
38. A. Olichon et al., Mitochondrial dynamics and disease, OPA1. Biochim. Biophys. Acta 1763, 500–509 (2006).
39. S. Zechner et al., Mutations in the mitochondrial GTPase mitofusin 2 cause charcot-marie-tooth neuropathy type 2A. Nat. Genet. 36, 449–451 (2004).
40. J.-M. Cioni et al., Late endosomes act as mRNA translation platforms and sustain mitochrondia in axons. Cell 176, 56–72.e15 (2019).
41. D. P. Kelly, R. C. Scarpulla, Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev. 18, 357–368 (2004).
42. C. Settembre et al., A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. EMBO J. 31, 1095–1108 (2012).
43. M. Amiri, P. J. Hollenbeck, Mitochondrial biogenesis in the axons of vertebrate peripheral neurons. Dev. Neurobiol. 68, 1348–1361 (2008).
44. R. Cartoni et al., Mitofusins 1/2 and ERRs expression are increased in human skeletal muscle after physical exercise: Mitofusins 1/2 and ERRs expression in skeletal muscle. J. Physiol. 567, 345–358 (2005).
45. F. X. Soriano et al., Evidence for a mitochondrial regulatory pathway defined by peroxisome proliferator-activated receptor-gamma coactivator-1 alpha, estrogen-related receptor-alpha, and mitofusin 2. Diabetes 55, 1783–1791 (2006).
46. K. Peng et al., The interaction of mitochondrial biogenesis and fusion/fission mediated by PGC-1α regulates rotenone-induced dopaminergic neurotoxicity. Mol. Neurobiol. 54, 3783–3797 (2017).
47. L. He et al., Spongiform degeneration in mahogany mutant mice. Science 299, 710–712 (2003).
48. J. Xiao et al., Abnormal regulation of TSG101 in mice with spongiform neurodegeneration. Biochim. Biophys. Acta 1792, 1027–1035 (2009).
49. E. S. Vincov et al., Autophagy accounts for approximately one-third of mitochondrial protein turnover and is protein selective. Autophagy 15, 1592–1605 (2019).
50. A. M. Pickrell et al., Endogenous parkin preserves dopaminergic substantia nigra neurons following mitochondrial DNA mutagenic stress. Neuron 87, 371–381 (2015).
51. C. S. Evans, E. L. Holzbaur, Degradation of engulfed mitochondria is rate-limiting in Optineurin-mediated mitophagy in neurons. eLife 9, e50260 (2020).
52. C. S. Evans, E. L. F. Holzbaur, Lysosomal degradation of depolarized mitochondria is rate-limiting in OPTN-dependent neuronal mitophagy. Autophagy 16, 962–964 (2020).
53. M. A. Farazi Fard et al., Truncating mutations in UBAP1 cause hereditary spastic paraplegia. Am. J. Hum. Genet. 104, 767–773 (2019).
54. V. S. Van Laar et al., Evidence for compartmentalized axonal mitochondrial biogenesis: Mitochondrial DNA replication increases in distal axons as an early response to Parkinson’s disease-relevant stress. J. Neurosci. 38, 7505–7515 (2018).
55. T. Sun, H. Qiao, P.-Y. Pan, Y. Chen, Z.-H. Sheng, Motile axonal mitochondria contribute to the variability of presynaptic strength. Cell Rep. 4, 413–419 (2013).
56. L. J. Neukomm, T. C. Burdett, M. A. Gonzalez, S. Züchner, M. R. Freeman, Rapid in vivo forward genetic approach for identifying axon death genes in Drosophila. Proc. Natl. Acad. Sci. U.S.A. 111, 9965–9970 (2014).
57. S. Zhao, T. M. Fortier, E. H. Baehrecke, Autophagy promotes tumor-like stem cell niche occupancy. Curr. Biol. 28, 3056–3064.e3 (2018).
58. D. L. Berry, E. H. Baehrecke, Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell 131, 1137–48 (2007).
59. Y.-W. C. Fridell, A. Sánchez-Blanco, B. A. Silvia, S. L. Helfand, Targeted expression of WldS prevents axon degeneration through increased mitochondrial Ca2+ buffering and enhanced mitochondrial Ca2+ buffering. Curr. Biol. 22, 596–600 (2012).
60. L. J. Neukomm, T. C. Burdett, M. A. Gonzalez, S. Züchner, M. R. Freeman, Rapid in vivo forward genetic approach for identifying axon death genes in Drosophila. Proc. Natl. Acad. Sci. U.S.A. 111, 9965–9970 (2014).
61. S. Zhao, T. M. Fortier, E. H. Baehrecke, Autophagy promotes tumor-like stem cell niche occupancy. Curr. Biol. 28, 3056–3064.e3 (2018).
62. D. L. Berry, E. H. Baehrecke, Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell 131, 1137–48 (2007).