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Impacts of mitochondrial dysfunction on axonal microtubule bundles as a potential mechanism of neurodegeneration

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Mitochondrial dysfunction is an important cause for neurodegeneration, often associated with dyshomeostasis of reactive oxygen species, i.e., oxidative stress. However, apart from ATP production, mitochondria have many other functions the aberration of which may impact neurons in very different ways. Oxidative stress can cause the deterioration of axonal microtubule bundles, thus critically affecting the highways for life-sustaining transport and providing a potential path to neurodegeneration. We recently found that aberrant transport of mitochondria can have this effect by causing oxidative stress. We therefore asked which aberrations of mitochondrial physiology might impact microtubules, which of these might explain the observed consequences of aberrant mitochondrial transport, and whether mitochondria-induced microtubule phenotypes are always mediated by oxidative stress. Using one consistent *Drosophila* primary neuron system, we studied functional loss of 13 different mitochondrial factors known to be detrimental to neurons *in vivo*. Losses of five factors caused MT damage, namely pyruvate dehydrogenase A, succinate dehydrogenase A, adenine nucleotide translocase, frataxin and superoxide dismutase 2. All involved oxidative stress, hence supported the path from mitochondria via oxidative stress to microtubule deterioration; of these, we discuss superoxide dismutase 2 as potential candidate explaining effects of mitochondrial transport aberration. Six of the remaining factors not causing microtubule damage were important mitochondrial morphogenesis regulators, suggesting efficient protection mechanisms preventing oxidative stress upon mitochondrial contortion.

KEYWORDS

***Drosophila*, microtubules, reactive oxygen species, mitochondria, neurodegeneration**

Significance statement

We used one consistent *Drosophila* primary neuron system to study the deficiencies of 13 mitochondrial factors known to be detrimental to neurons *in vivo*. Five factors triggered axonal microtubule bundle decay mediated by oxidative stress, thus establishing a potential mechanism linking dysfunctional mitochondria to neurodegeneration. Six factors were important morphogenesis regulators of mitochondria and their cristae, and none of them affected microtubule bundles, suggesting efficient protection mechanisms preventing oxidative stress upon mitochondrial contortion.

Introduction

Neurodegenerative disorders are an important socioeconomic challenge to modern ageing societies (GBD 2016 Neurology Collaborators, 2019). One major cellular cause often highlighted in this context is the dysregulation of mitochondria (Wang et al., 2021), especially the excessive generation or inappropriate release of harmful reactive oxygen species (ROS) causing oxidative stress as a side product of the electron transfer chain (ETC) which drives oxidative phosphorylation (Figure 1; OXPHOS; Zorov et al., 2014).

However, mitochondria are far more than the cell's powerhouse. For example, astrocytes, activated immune cells, tumour cells or cells during migration often circumvent the resource-efficient but slow process of oxidative phosphorylation; they instead switch to aerobic glycolysis as resource-hungry but fast and locally available means of ATP generation, which also churns out pyruvate molecules as metabolic building blocks or turning them into lactate as secondary energy source (Bhattacharya et al., 2020; Magistretti and Allaman, 2018). It was even reported that mitochondria in neuronal axons often act as ATP sink rather than source (Hirabayashi et al., 2024). Ever more roles of mitochondria are coming to light (Pfanner et al., 2019). For example, they are essential to produce iron-sulphur clusters as obligatory components of many enzymes in mitochondria, the cytoplasm or nucleus (Marelja et al., 2018; Shi et al., 2021). Mitochondria are discussed as calcium-buffering stores at synapses (Walters and Usachev, 2023), play key roles in programmed cell death involving the release of signals such as cytochrome c or mitochondrial DNA (Bonora et al., 2022; Glover et al., 2024; Khatun et al., 2024) and display direct contacts with other organelles or with each other as means of cross-regulation (Picard et al., 2015; Voeltz et al., 2024). Furthermore, mitochondria appear to respond to oxidative and metabolic states or requirements of cells by adapting their activities, changing shape, sending signals to the cytoplasm, positioning themselves into subcellular locations as extreme as the tips of filopodia or even transiting between cells; in this way they seem to help maintain cellular homeostasis or change the activity states of cells (Borcherding and Brestoff, 2023; D'Angelo et al., 2023; Marlar-Pavey et al., 2025; Palma et al., 2020; Sturm et al., 2024).

It is conceivable that harmful ROS production can be the outcome when aspects of this complex mitochondrial physiology become derailed (see Discussion). To add to this spectrum of possibilities, we recently observed in *Drosophila* primary neurons that even axonal transport deficits of mitochondria, including their complete absence from axons, lead to axonal ROS dyshomeostasis (Liew et al., 2025). That study further showed that axonal ROS dyshomeostasis caused severe disintegration of axonal microtubule (MT) bundles which tend to display as areas of chaotic MT-curling (Figure 2; see also Shields et al., 2025). ROS may therefore provide a potential link between the dysfunction or mislocalization of mitochondria and MT bundles. This may provide a potential mechanism explaining mitochondria-induced neurodegeneration, because MT bundles provide the essential lifeline of axons: they run uninterrupted from the neuronal cell bodies to the axonal tips serving as the highways for cargo transport required to sustain axonal cell biology; interrupting these bundles turns them into an axon's Achilles heel on path to degeneration (Okenve-Ramos et al., 2024; Prokop, 2021; Smith et al., 2023).

Based on these observations, we therefore asked (1) whether different forms of mitochondrial dysfunction can lead to MT-curling, (2) whether this is always mediated by harmful ROS, and (3) how the absence of mitochondria from axons might lead to MT-curling. To address these questions, we carried out a pilot study systematically assessing the functional loss of 13 well-conserved mitochondrial proteins in one consistent *Drosophila* primary neuron system. Importantly, all results were compared to reports in the wider literature that will be discussed in detail.

Abbreviations: Acon, mitochondrial aconitase; ABCB7, ATP binding cassette subfamily B member 7; Aralar, mitochondrial aspartate/glutamate carrier; ATP6, mitochondrial ATPase subunit 6 (*Drosophila* name: mt:ATPase6); ATPsynC, ATP synthase, subunit C; DIC, dicarboxylate carrier 1; DIV, days *in vitro*; $F_{0/1}$, F_0 and F_1 subunits of the ATP synthase; Fh, frataxin; Gdh, glutamate dehydrogenase; Gls, glutaminase; Got2, glutamate oxaloacetate transaminase 2; HRP, horseradish peroxidase; i-AAA, ATPase associated with various cellular activities exposed to the inter-membrane space; ISC, ion-sulfur cluster; Ldh, lactate dehydrogenase; Marf, mitochondrial assembly regulatory factor; MIA40, mitochondrial intermembrane space import and assembly 40; MICOS, mitochondrial contact site and cristae organizing system; Mpc1, mitochondrial pyruvate carrier; OGDC, oxoglutarate dehydrogenase complex; Ogdh1, oxoglutarate dehydrogenase 1; Opa1, optic atrophy 1; QIL1, subunit of MICOS, orthologue of MICOS13; Pcb, pyruvate carboxylase; PDC, pyruvate decarboxylase complex; PdhA, pyruvate dehydrogenase E1 alpha subunit 1; PerX, peroxisomes; SesB, stress-sensitive B, orthologue of ANT (SLC25A4-6); Sod2, superoxide dismutase 2 (Mn); TIM22/23, mitochondrial import inner membrane translocase subunits 22/23; TOM40, translocase of the outer mitochondrial membrane 40; YME1L, YME1 like ATPase, i-AAA protease of the inner mitochondrial membrane.

Results

Experimental strategy

To address our question, we selected 13 candidate genes primarily based on their reported links to neurodegeneration, published information about their debilitating effects on *Drosophila* neurons *in vivo*, and the availability of established genetic tools for their manipulation (detailed rationales provided at start of each section; Supplementary Table 1; Brischigliaro et al., 2023; Marelja et al., 2018). To validate results, we aimed to deplete the function of each gene using at least two independent approaches, which included different loss-of-function mutant alleles, combinations of mutant alleles with deficiencies uncovering these genes, or the

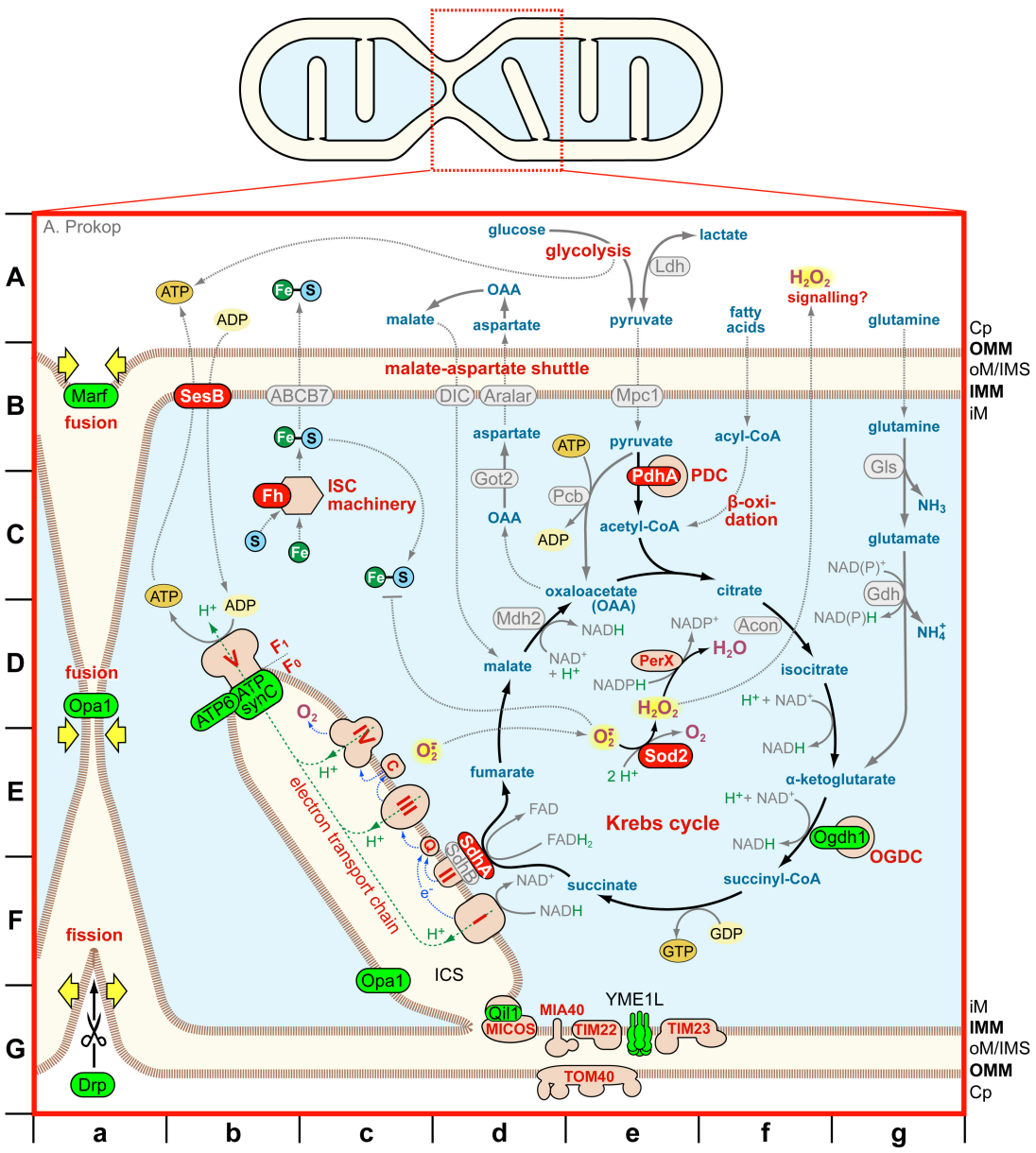

FIGURE 1
Processes and components referred to in the text that mediate mitochondrial physiology and dynamics. The sketch on top represents a mitochondrion during fusion/fission, with the red box indicating the position of the close-up shown below. Different compartments and membranes are colour-coded: cytoplasm (Cp) in white, outer mitochondrial matrix/inter-membrane space (oM/IMS) and inter-cisternal space (ICS) in light beige, inner mitochondrial matrix in light blue, inner and outer mitochondrial membranes (IMM, OMM) as stippled brown lines; for further acronyms see the dedicated abbreviation list. Protein complexes are shown in pink with red outlines; identified proteins are either in light green/dark red with black outline (proteins manipulated in this study without/with MT-curling), or in light-grey with grey outline (mentioned in the text but not investigated here). Organic and inorganic components are colour coded as follows: organic metabolites in blue, protons in green, oxygen derivatives in magenta (those being ROS highlighted in yellow), co-enzymes of redox reactions in grey, iron (Fe) in dark-green circles, sulphur (S) in blue circles, ATP/GTP in dark orange circles, ADP/GDP in light orange circles. Chemical reactions are shown as solid black or grey arrows, spatial translocations as stippled grey arrows. Letters on the left and at the bottom outside the box are grid coordinates referred to in the text (chevron followed by italics letters).

knock-down of genes using the targeted expression of interference RNA constructs with the pan-neuronal driver line *elav-Gal4* (see Methods). To achieve a level playing field for our analyses, all studies were carried out in one consistent *Drosophila* primary neuron system by harvesting neurons from either mutant embryos or embryos displaying gene knock-down. We used MT-curling as one consistent readout, for the underlying regulation of which we have longstanding experience (Hahn et al., 2021; Liew et al., 2025; Qu et al., 2017). Since most loss-of-function mutations used in this study are homozygous lethal, mothers have to be heterozygous and their eggs may contain deposited mRNA or protein from the healthy gene copy (referred to as maternal component) which can mask mutant phenotypes for a while. Based on our experience, a culture period of 5 days *in vitro* (DIV) is a reliable length to achieve full phenotypic penetrance (Liew et al., 2025) and was therefore chosen as standard for most analyses. However,

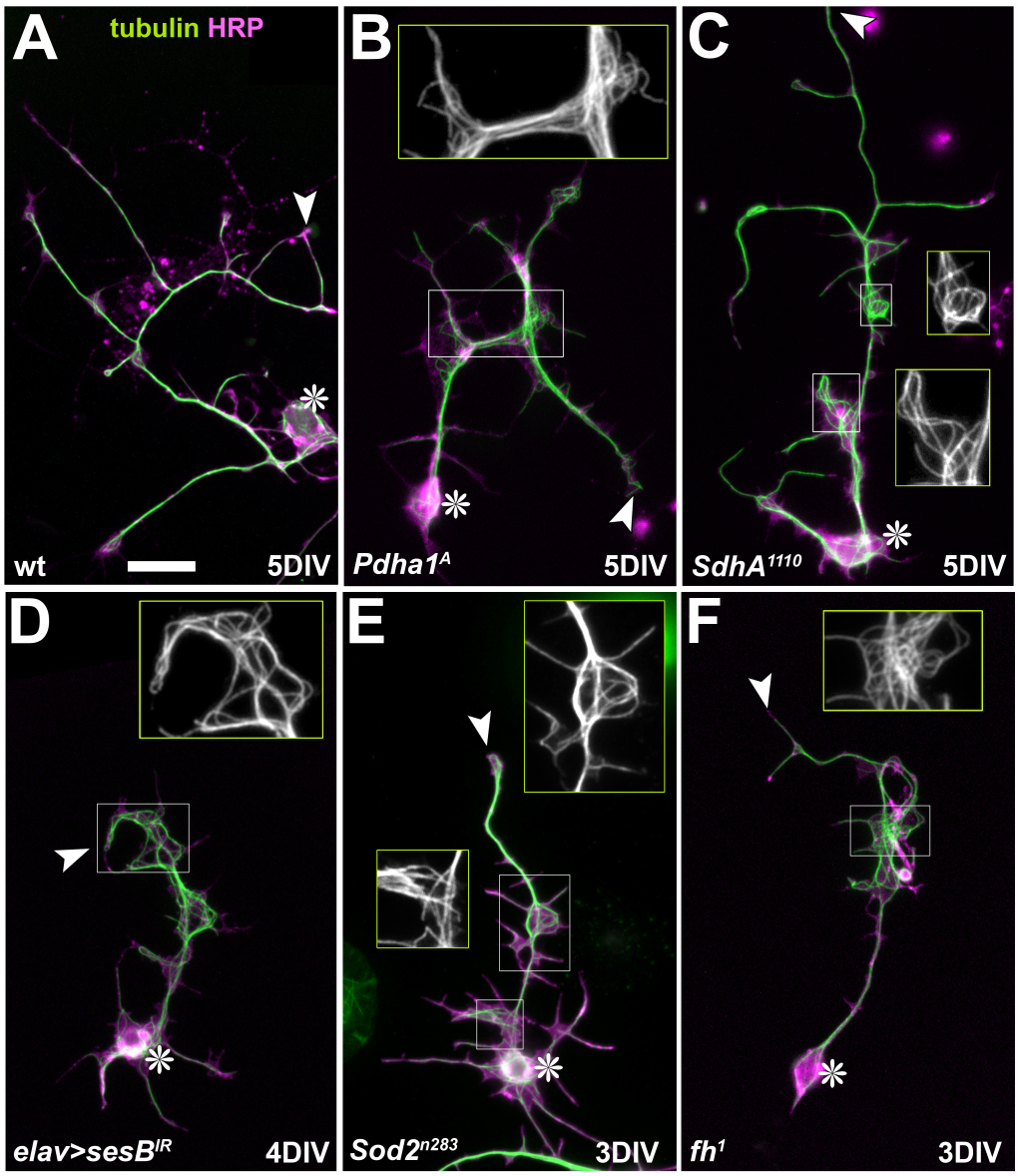

FIGURE 2
Representative images of MT-curling observed in mutant neurons. **(A–F)** *Drosophila* primary neurons of different genotypes (indicated bottom left; see text) cultured for 3, 4 or 5 days *in vitro* (DIV) stained for tubulin (green) and the neuronal surface marker HRP (magenta). Cell bodies are indicated by asterisks, axon tips by arrow heads, and emboxed areas are shown as two-fold magnified insets (tubulin channel only). Scale bar in A represents 20 μm in all images.

since phenotypes can often display as early as 6 hrs *in vitro*; Gonçalves-Pimentel et al., 2011; Qu et al., 2022), some follow-up experiments were done at shorter culture periods once phenotypes were established.

Loss of *PdhA* causes ROS-dependent MT-curling

We began our investigation with examples of factors that contribute to Krebs cycle function, starting with the pyruvate dehydrogenase complex (PDC) as the gate keeper which feeds the Krebs cycle with acetyl-CoA (Figure 1 > *A-C/e*; letters behind the chevron indicate the grid coordinates provided in the figure). PDC is composed of multiples of three enzymes (E1-3) converting pyruvate and CoA-SH into acetyl-CoA and CO_2 whilst generating NADH; PDHA (pyruvate dehydrogenase E1 subunit α1) is an obligatory subunit of the E1 enzyme which catalyses the first reaction step (Magistretti and Allaman, 2018; Naifeh et al., 2024; Patel et al., 2014).

In humans, PDHA1 mutations constitute ~80% of cases of Leigh disease displaying with severe early-onset neurodegenerative symptoms, PDC enzyme activity reduced to 30%, and lactic acidosis with ~4-fold increase in pyruvate and lactate plasma levels (Foucher and Tubben, 2024; Gopal et al., 2023). Rare patients with PDHB mutations show strong clinical overlap with PDHA mutant cases (Patel et al., 2012). In *Drosophila*, *in vivo* studies

showed that Pdha1-deficient photoreceptor cells degenerate when challenged with light-induced activation (Jaiswal et al., 2015). Also, strong knock-down of Pdhb (the β-subunit of E1) displayed clear neurodegenerative phenotypes (Dung et al., 2018).

To study loss of PDC function in primary neurons, we generated *Drosophila* primary neurons (see Methods) from embryos homozygous for the previously reported loss-of-function mutant allele $Pdha1^{A}$ (Liu et al., 2017; Yamamoto et al., 2014) or displaying nervous system-specific knock-down of Pdha1 (Huang et al., 2022). In both cases, primary neurons showed a robust increase in MT-curling when analysed at 5 DIV (Figures 2B, 3A), clearly confirming our hypothesis that axonal MT bundles can be affected by mitochondrial dysregulation.

To test whether the phenotype of $Pdha1^{A}$ mutant primary neurons was ROS-dependent, we supplied the culture medium for the entire culture period with 100 µM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Trolox is a vitamin E analogue that acts as a ROS-scavenger; it inhibits fatty acid peroxidation and quenches singlet oxygen and superoxide and was reported to have beneficial antioxidant effects in neuronal models of neurodegeneration (Chow et al., 1994; Janc and Müller, 2014). In Pdha1-deficient neurons, Trolox clearly suppressed the MT-curling phenotype (Figure 4C) suggesting that harmful ROS dyshomeostasis mediates the phenotype. PdhA loss provides therefore an example where mitochondrial dysfunction causes ROS-mediated MT-curling.

Loss of Ogdh1 seems not to cause excessive ROS

Another Krebs cycle component we selected is the oxoglutarate dehydrogenase complex (OGDC) which converts α-ketoglutarate (aka oxoglutarate) to succinyl-CoA (Figure 1 > *EF/fg*). Ogdh1/OGDH encodes the E1 component of the complex, which catalyses the initial step of the reaction (Nemeria et al., 2021).

In humans, OGDH deficiency causes movement disorders and hyperlactatemia (Yap et al., 2021b). In *Drosophila*, the $Ogdh1^{MI06026-TG4.1}$ loss-of-function allele is embryonic lethal, and likewise when loss of Ogdh1 is restricted to the nervous system; its knock-down in photoreceptors causes progressive loss of synaptic transmission (Yap et al., 2021a; Yap et al., 2021b; Yoon et al., 2017; Supplementary Table 1).

We therefore assessed primary neurons carrying the lethal $Ogdh1^{MI06026-TG4.1}$ mutant allele in homozygosis or over deficiency. These neurons displayed no obvious increase in MT-curling at 5 DIV, in one set of experiments even a potential bias to reduce MT-curling (Figure 3B).

Therefore, although loss of Ogdh1 is a lethal conditions, no MT phenotypes became apparent in neurons during the 5 day culture period.

SdhA deficiency causes MT-curling suppressed by Trolox

As a further Krebs cycle component we selected succinate dehydrogenase (SDH) which is an enzymatic complex formed by at least four distinct constituent subunits A to D. It participates in the Krebs cycle catalyzing the step from succinate to fumarate (Figure 1 > *EF/cd*). However, SDH is a special case in that it forms not only part of the Krebs cycle but also constitutes complex II of the ETC passing on electrons from its enzymatic reaction via ubiquinone to complex III (Figure 1 > *EF/cd*; Al-Rasheed and Tarjan, 2018; Rutter et al., 2010).

In humans, SDHA mutations cause neurodegeneration, muscle weakness and tumor formation (Al-Rasheed and Tarjan, 2018; Hadrava Vanova et al., 2020; Patel et al., 2012; Rutter et al., 2010). The *Drosophila* $SdhA^{1110}$ and $SdhA^{1404}$ mutant alleles reduce SDH activity substantially causing recessive larval lethality; upon mosaic analysis, photoreceptor cells homozygous for these alleles showed gradual synapse loss (Mast et al., 2008). Furthermore, heterozygous deficiency of *SdhA* strongly enhances premature death of flies lacking the SDH assembly factor Sirup/SDHAF4 (Van Vranken et al., 2014) and $SdhA^{1110}$ genetically interacts with the Parkinson gene *park* enhancing its motility deficits (O'Hanlon et al., 2022).

Our analyses of primary neurons homozygous for the above-mentioned $SdhA^{1404}$ or $SdhA^{1110}$ mutant alleles, revealed a robust MT-curling phenotype at 5 DIV (Figures 2C, 3C). To test whether the phenotype of *SdhA*-deficient primary neurons was ROS-dependent, we applied the ROS-scavenger Trolox (Chow et al., 1994) which resulted in robust suppression of the MT phenotype (Figure 4D). Functional loss of SdhA is therefore another condition demonstrating deteriorating impacts of mitochondrial dysfunction on axonal MT bundles mediated by oxidative stress.

Loss of ATP synthase does not induce MT-curling

We next focused on complex V of the ETC, also known as ATP synthase. ATP synthase is a multi-subunit complex clustered at the tips of cristae. Its channel-forming transmembrane sub-complex F_0 permits the flow of protons across the inner membrane which provides the energy that drives ATPase activity of the enzymatic matrix-facing sub-complex F_1 (Figure 1 > *D/b*). APT synthase also plays important roles in cristae morphogenesis by mediating the extreme membrane curvature of their tips (Guo et al., 2017; Hahn et al., 2016; Zhou et al., 2015), and it is proposed to form a component of the mitochondrial permeability transition pore (mPTP; Bonora et al., 2022).

Here, we chose two key components of the F_0 complex crucial for ATP synthase function: ATP5MC1-3 (ATPsynC in flies) constitutes the multimeric proton-conducting c-ring, whereas mitochondrially encoded MT-ATP6 (mt:ATPase6 in fly) closely associates with the c-ring and is required for its proton-conducting ability (Guo et al., 2017; Hahn et al., 2016; Zhou et al., 2015).

In humans, the dystonia-linked $ATP5MC3^{N106K}$ mutation causes a reduction in ATP production and oxygen consumption (Neilson et al., 2022), and MT-ATP6 mutations are linked to severe neurological conditions (Dautant et al., 2018; Galber et al., 2021). In *Drosophila*, loss of ATPsynC ($ATPsynC^{KG01914}$) causes a reduction in mitochondrial cristae and animals die as larvae after a prolonged developmental block (Lovero et al., 2018). Furthermore, ubiquitous expression of $ATPsynC^{N102K}$ (mimicking human $ATP5MC3^{N106K}$) caused strongly reduced ATP synthase

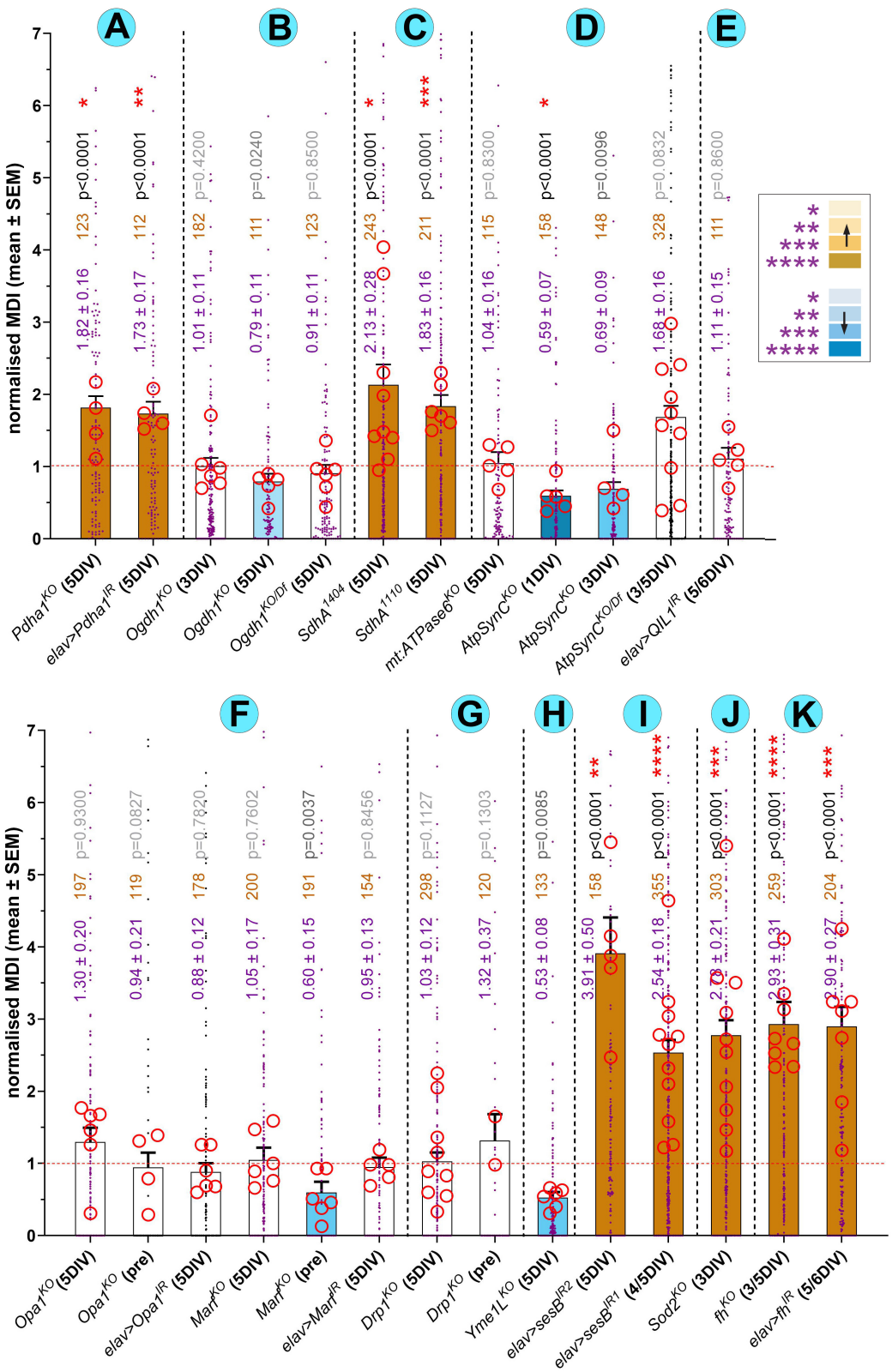

FIGURE 3
Quantification of MT-curling observed upon functional deficiency of factors involved in mitochondrial physiology. (A–K) Bars indicating the degree of MT-curling where genotypes are grouped and separated by stippled vertical lines as they are presented in dedicated sections in the main text; only in one case there is an additional subdivision separating fusion (F) from fission (G) factors. Bars indicate the degree of MT-curling indicated in blue font as the mean ± SEM MDI (MT disorganization index: the size of axonal areas displaying MT-curling relative to axon length); data are normalized to internal wild-type controls of each experiment (horizontal red dashed line); single data points are shown as blue dots and the means of repeats (independent coverslips from usually two to three independent replicates) are shown as red circles with their statistical significance (established using *t-tests*) indicated as red asterisks (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$); numbers of assessed neurons are shown in orange, their *p* values relative to controls (established by Mann-Whitney tests) in black or grey; the grey-to-black intensity of statistical values and the brown/blue bar colour (see inset) reflect the respective degrees of significance relative to wild-type.

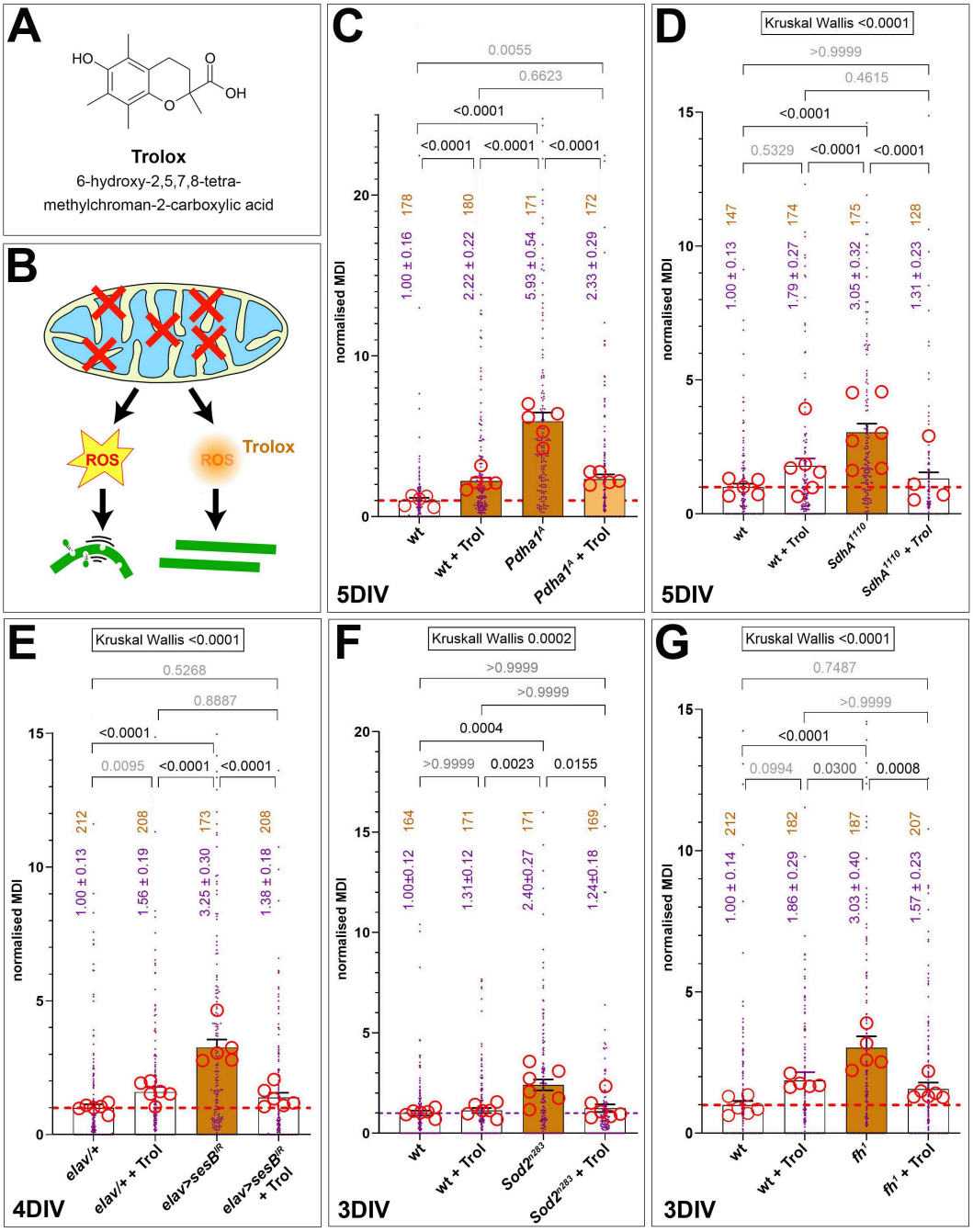

FIGURE 4
The phenotypes of all MT-curl-inducing conditions are rescued by Trolox. **(A)** The chemical structure of Trolox. **(B)** The design of Trolox experiments: mitochondrial phenotypes (red X) cause ROS (yellow star) which, in turn, causes curling and potential damage of MTs (left); upon application of Trolox (right), ROS is quenched and MT-curling abolished although the original mitochondrial phenotype is still present. **(C–G)** Each graph represents one set of experiments composed of wild-type without Trolox (wt), wt with Trolox (+ Trol) and the respective loss-of-function mutant condition (as indicated in **C–G**) without/with Trolox. Graphs are organized as explained in the legend of Figure 3. Kruskall-Wallis test results are indicated on top, Dunn's multiple comparison results are indicated above bars; the intensity of brown fill-colour of bars reflects the degree of statistical significance relative to wild-type control.

activity coupled to lethality (Neilson et al., 2022). Individuals carrying the strong *mt:ATPase6*^{1} loss-of-function allele (a G116E point mutation) were reported to be almost 100% mutant (homoplamic) leading to total loss of ATP synthase activity and aberrant mitochondria with severely rounded cristae; surprisingly, flies can eclose but display a vastly reduced adult lifespan (Celotto et al., 2011; Celotto et al., 2006; Demine et al., 2019). We similarly observed that *mt:ATPase6*^{1} mutant flies showed severely reduced mobility already at 2 weeks and a strong tendency to display seizures.

Our analyses of neurons homozygous for the above-mentioned lethal null mutant allele *ATPsynC*^{KG01914} (Lovero et al., 2018)

revealed no obvious increase in MT-curling compared to wild-type controls at 3 or 5 DIV; in some experiments it even caused a reduction in curling (Figure 3D). Likewise, *mt: ATP6*^{1} mutant neurons failed to reveal any increases in MT-curling at 5 DIV (Figure 3D). The combined outcome for both gene deficiencies strongly suggests that the loss of ATPase function, known to affect OXPHOS and cristae formation, seems not to cause harmful ROS, at least during the culture period assessed.

Depletion of QIL1 or fission and fusion factors does not cause MT-curling within 5 days

Surprised that the expected aberration of cristae upon loss of ATP synthase seems not to be a ROS-inducing condition, we challenged this finding further. For this, we studied other factors involved in cristae formation, namely the MICOS complex and Opa1. The MICOS complex is located at the neck of cristae (cristae junctions; Figure 1 > *G/d*) required for cristae formation, the stabilization of cristae junctions, and the assembly of various protein complexes at this site (Mukherjee et al., 2021). Upon loss of the QIL1/MICOS13 subunit, the entire MICOS complex fails to form, causing reduced ETC activity and severe cristae aberrations (Mukherjee et al., 2021).

In humans, QIL1 mutations are linked to diseases with neurodegenerative traits (for example COXPD37; omim.org #618329). In *Drosophila*, knock-down of QIL1 in the nervous system and muscles reduced expression to under 25% accompanied by severe aberration of mitochondria, an increase in mitophagy, but no obvious induction of cell death (Guarani et al., 2015; Wang et al., 2020). We used the same knock-down construct in primary *Drosophila* neurons, but no obvious MT-curling phenotype was detectable at 5 DIV (Figure 3E).

OPA1 is also positioned at the base of cristae and known to cause their disruption when dysfunctional (Figure 1 > *G/c*; Quintana-Cabrera and Scorrano, 2023). However, OPA1 has an additional function in that it also regulates mitochondrial fusion (Figure 1 > *D/a*). We therefore extended our study by including a second pro-fusion factor MFN (Figure 1 > *B/a*) and the pro-fission factor DNM1L (Figure 1 > *G/a*; Quintana-Cabrera and Scorrano, 2023).

In humans, all three pro-fission and -fusion factors have been linked to neurodegeneration (Supplementary Table 1; Chen W. et al., 2023), and in *Drosophila* their losses cause lethality. However, studies of the human, mammalian or fly genes draw an inconclusive picture as to whether the pathologies involve harmful ROS production (Supplementary Table 1; see section "Discussion"). We therefore assessed losses of Opa1 (lethal *Opa1*^{s3475} null allele and *elav* > *Opa1*^{IR}), the MFN orthologue Marf (lethal *Marf*^{B} null allele and *elav* > *Marf*^{IR}) and the DNM1L orthologue Drp1 (lethal *Drp1*^{T26} null allele) in *Drosophila* primary neurons. We observed no MT-curling at 5 DIV, even when using pre-culture to exclude potential maternal rescue (see section "Methods"; Figures 3F, G, 5B–D, F, G). Functional loss of the three factors in the assessed neurons was clearly indicated by fragmented mitochondria when depleting Opa1 or Marf (Figures 5A–C, E–G) and long stretches of continuous mitoTracker-labelled structures along primary axons of Drp-deficient neurons (Figure 5D) as similarly reported for mammalian neurons (Berthet et al., 2014; Uo et al., 2009; Yu et al., 2011).

Taken together, all six factors involved in mitochondrial morphogenesis or cristae formation assessed in our standardized neuron system do not induce MT-curling during the 5 day culture period, suggesting that contorted mitochondria are well protected from harmful ROS dyshomeostasis (see Discussion).

Loss of YME1L causes a robust reduction in MT-curling

The remaining factors addressed by our study cover a diverse range of further mitochondrial functions. For example, YME1L1 forms a homo-hexameric i-AAA protease which localizes in the vicinity of the translocase complexes of the outer and inner mitochondrial membranes (TOMM and TIMM; Figure 1 > *G/e*); YME1L1 plays important roles in proteolytic protein turn-over and the regulation of mitochondrial import and maturation (Kan et al., 2024).

Human patients homozygous for a hypomorphic YME1L1 mutation display onset of degeneration at childhood accompanied by an increase in lactate/pyruvate ratio indicative of glycolysis upregulation (Hartmann et al., 2016). In mice, ubiquitous knock-out is lethal due to heart dysfunction (Wai et al., 2015), and nervous system-specific loss causes late-onset neurodegeneration and aberration of mitochondrial morphology and transport (Sprenger et al., 2019). Similarly, also *YME1L*^{del} flies display neurodegeneration of photoreceptors, reduced locomotion and premature death at adult stage, correlating with severe mitochondrial pathology including reduced and malformed cristae and electron-dense inclusions likely due to unfolded protein stress (Qi et al., 2016).

Surprisingly, we found that primary neurons homozygous for the protein null allele *YME1L*^{del} displayed a consistent drastic reduction in MT-curling to about half of the values observed in parallel wild-type controls at 5 DIV (Figure 3H). These findings might suggest a reduction in ROS production below base levels present in control neurons. Baseline levels of ROS in *Drosophila* primary neurons might be slightly elevated because they are grown in an environment with higher oxygen levels than experienced *in vivo*. The primary neurons might therefore have some bias for default MT-curling which could be reduced when some natural sources of ROS production are removed. This same effect is not observed upon Trolox application (Figure 4), which may be because Trolox represents a rather crude and non-discriminatory intervention with potential negative side effects.

Loss of non-redundant ANT in neurons causes MT-curling

Adenine nucleotide translocase (ANT) is a highly abundant mitochondrial protein in the inner mitochondrial membrane that acts as an ADP/ATP antiporter ("SesB" in Figure 1 > *B/b*); it is also considered to be a component of the low-conductance mPTP helping to avoid mitochondrial calcium stress, and to mediate

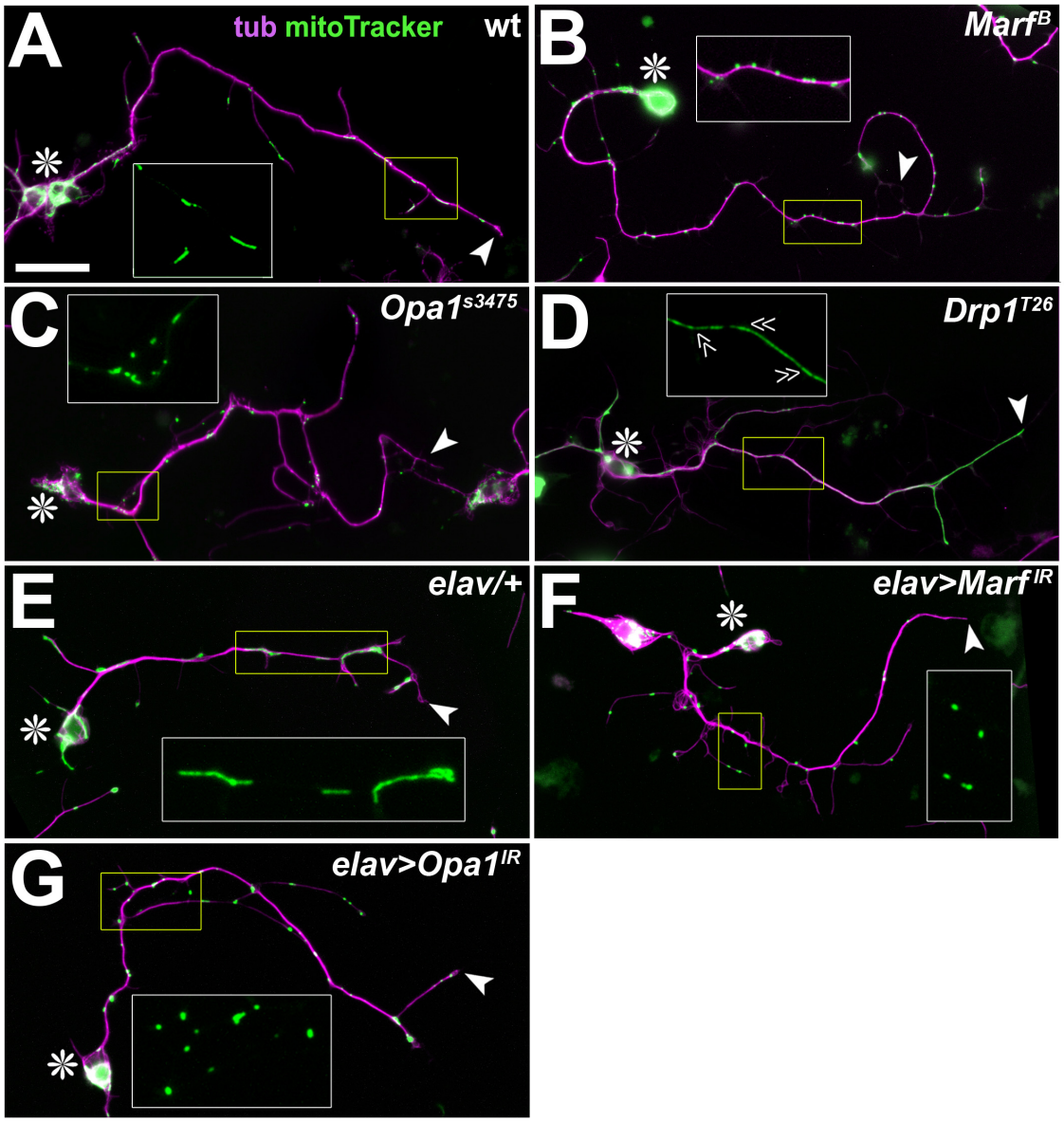

FIGURE 5
Genetic manipulations impairing mitochondrial fission/fusion processes. Neurons at 5 days *in vitro* (DIV) and stained with anti-tubulin (tub, magenta) and mitoTracker (green); they are wild-type **(wt; A)** or deficient for mitochondrial pre-fusion factors Marf **(B, F)** and Opa1 **(C, G)** and the mitochondrial pre-fission factor Drp1 **(D)**; asterisks indicate cell bodies, arrow heads axon tips, yellow emboxed areas are shown as 2-fold enlarged insets (green channel only); the scale bar in A represents 30 μm in A–D and 15 μm in E–G; note that mitochondria tend to appear as dashed lines in controls **(A)**, as sparse dots upon loss of fusion **(B, C)** and as a continuous structure excluded from many side branches upon loss of fission ("double-chevrons" in D). Quantification of MT-curling phenotypes under these mutant conditions are given in Figure 3.

proton leakage involved in mitochondrial uncoupling (Bround et al., 2020; Chen Y. et al., 2023; Demine et al., 2019).

In humans, ANTs are discussed in the context of neurodegenerative diseases including AD and PD (Chen Y. et al., 2023). However, there are 4 functionally redundant ANT genes, of which the well-studied ANT1/SLC25A4 gene is expressed in brain, heart, muscles, lung and testis (linked to cardiomyopathy; OMIM #103220) and the poorly researched ANT3/SLC25A6 gene is ubiquitously expressed; very little is known about ANT2/SLC25A5 and ANT4/SLC25A31 (Chen Y. et al., 2023). In *Drosophila*, ANTs are encoded by the *sesB* gene (highly expressed in the nervous system) and the paralogous *Ant2* gene (expressed at very low levels; Supplementary Material 1; Li et al., 2022). Therefore, loss of SesB can be considered a total loss of ANT function in the nervous system; *in vivo* studies in *Drosophila* larvae or flies revealed decreased ATP production, reduced calcium response of mitochondria, elevated peroxide levels, clear signs of age-related neurodegeneration and enhanced autophagy (Celotto et al., 2006; DeVorkin et al., 2014; Terhzaz et al., 2010; Vartiainen et al., 2014).

In agreement with these *in vivo* findings, knock-down of *sesB* with two independent constructs showed a strong increase in MT-curling in primary neurons at 4 or 5 DIV (Figures 2D, 3I). When treated with Trolox, the MT-curling was reduced to control levels, suggesting harmful ROS dyshomeostasis as the curl-inducing factor

(Figure 4E). This presents another example where mitochondrial dysfunction can affect neurons through impacting their axonal MT bundles in a ROS-dependent manner.

Sod2 deficiency causes ROS-dependent MT-curling

The mitochondrial manganese-containing superoxide dismutase SOD2 converts the highly toxic but short-lived superoxide (the main ETC-derived ROS; Andreyev et al., 2005; Murphy, 2008; Zorov et al., 2014) into the less aggressive H_2O_2 (Figure 1 > *DE/e*) that is longer-lived and believed to diffuse into the cytoplasm contributing to local signaling (Figure 1 > A/f; Palma et al., 2020).

Human SOD2 mutations are suggested to link to neurodegenerative diseases and conditions (Flynn and Melov, 2013; Houldsworth, 2024), although these associations are less clear than the ALS-links of SOD1 (Kim et al., 2020). SOD2 knock-out mice display increased apoptosis and urine acidity, cardiomyopathy and die after birth (Kokoszka et al., 2001; Li et al., 1995; Melov et al., 1998). If restricting SOD2 loss to the nervous system, severe neurodegeneration is observed (Melov et al., 1999; Oh et al., 2012; Qi et al., 2003). In the *Drosophila* brain, heterozygosity for $Sod2^{\Delta 2}$ and $Sod2^{n64}$ causes precocious axon decay and MT-curling (Shields et al., 2025), and the $Sod2^{n283}$ allele caused reduced life span (rescued by hypoxia), early onset neurodegeneration at tissue and behavioral levels, enhanced apoptosis of brain cells, and sensitivity to oxidative stress (Dias-Santagata et al., 2007; Duttaroy et al., 2003; Paul et al., 2007; Piazza et al., 2009; Vrailas-Mortimer et al., 2011; Wicks et al., 2009).

We therefore used the $Sod2^{n283}$ mutant allele in homozygosis in primary neuron culture at 3 DIV. These experiments revealed a strong increase in MT-curling, which was suppressed by Trolox, indicating involvement of harmful ROS (Figure 2E, 3J, 4F). Very similar results were recently reported by others using the $Sod2^{\Delta 2}$ and $Sod2^{n64}$ mutant alleles, clearly demonstrating validity of our findings (Shields et al., 2025) and identifying Sod2 deficiency as another example of mitochondrial dyshomeostasis that causes ROS-dependent MT-curling.

Loss of frataxin causes ROS-dependent MT-curling

FRATAXIN (FXN) is an iron-binding mitochondrial protein involved in the early steps of iron-sulphur cluster formation (ISCs; Figure 1 > C/b; Monfort et al., 2022). Ion-sulphur clusters are essential functional components of many proteins in mitochondria (including many ETC components), the cytoplasm and nucleus (Vallières et al., 2024). Although iron-sulphur cluster maturation and assembly into cytosolic and nuclear proteins takes place in the cytoplasm, the early steps always depend on mitochondria, making FXN a key factor in this functional context (Fan et al., 2022; Marelja et al., 2018; Marquez et al., 2023; Shi et al., 2021).

In humans, FXN mutations link to Friedreich's ataxia as the most common form of autosomal recessive ataxia displaying with severe neurodegenerative pathology (Delatycki et al., 2000; Williams and De Jesus, 2024). In mouse models, this pathology is reproduced and correlates with iron accumulations and oxidative stress (Al-Mahdawi et al., 2006; Simon et al., 2004).

In *Drosophila*, Frataxin (Fh) loss causes strong neurodegeneration with dying-back symptoms of peripheral axons, aberrant mitochondrial appearance, enhanced mitophagy, increased iron uptake in mitochondria of the nervous system, and higher sensitivity to iron intake (Chen et al., 2016; Edenharter et al., 2018; Llorens et al., 2007; Navarro et al., 2015; Shidara and Hollenbeck, 2010). A reduced lifespan of flies was also observed when knocking down *frataxin* specifically in neurons (Anderson et al., 2005). From all these studies in flies, there are contradicting opinions as to whether Frataxin loss induces ROS (Marelja et al., 2018).

Employing genetic tools used for the above-mentioned *in vivo* experiments (Supplementary Table 1; the lethal S136R point mutation fh^1 and *elav* > $fh^{RNAi.A2}$) in primary neurons, we found a strong increase in MT-curling at 3, 5 and 6 DIV (Figures 2F, 3K). When applying Trolox, we found a clear reduction of the phenotype down to control levels (Figures 4G), indicating harmful ROS as the mediating factor.

Taken together, 5 out of 13 factors clearly caused ROS-dependent MT-curling establishing MT bundle deterioration as a potential mechanisms leading from mitochondrial dysfunction to neurodegeneration.

Harmful ROS triggered by loss of Fh or SesB affects Eb1 amounts at MT plus ends

To test whether ROS produced upon loss of mitochondrial factors has an impact on other cell parameters, we used primary neurons mutant for fh^1 or with *elav-Gal4*-driven knock-down of *sesB* and assessed their morphological parameters. Neither axon length nor branch patterns (number of primary neurites) appeared affected (Figure 6). To assess whether other MT parameters were changed upon loss of these factors, we assessed the amount of Eb1 as an indicator of MT polymerization (Hahn et al., 2021) and found a robust reduction in both cases. This reduction aligns with previous publications reporting reduced MT polymerization upon ROS increase (Conze et al., 2025; Shields et al., 2025).

Discussion

Only certain forms of mitochondrial aberrations impact axonal MT bundles

Here we used one consistent *Drosophila* primary neuron system to apply over 30 different genetic and experimental manipulations to study 13 genes important for mitochondrial physiology, the loss of which has been reported to be detrimental to neurons *in vivo* (Supplementary Table 1). We asked (1) whether any of these assessed mitochondrial dysfunctions can affect axonal MT bundles, (2) whether such effects are mediated by harmful ROS, and (3) through which mechanisms even the absence of

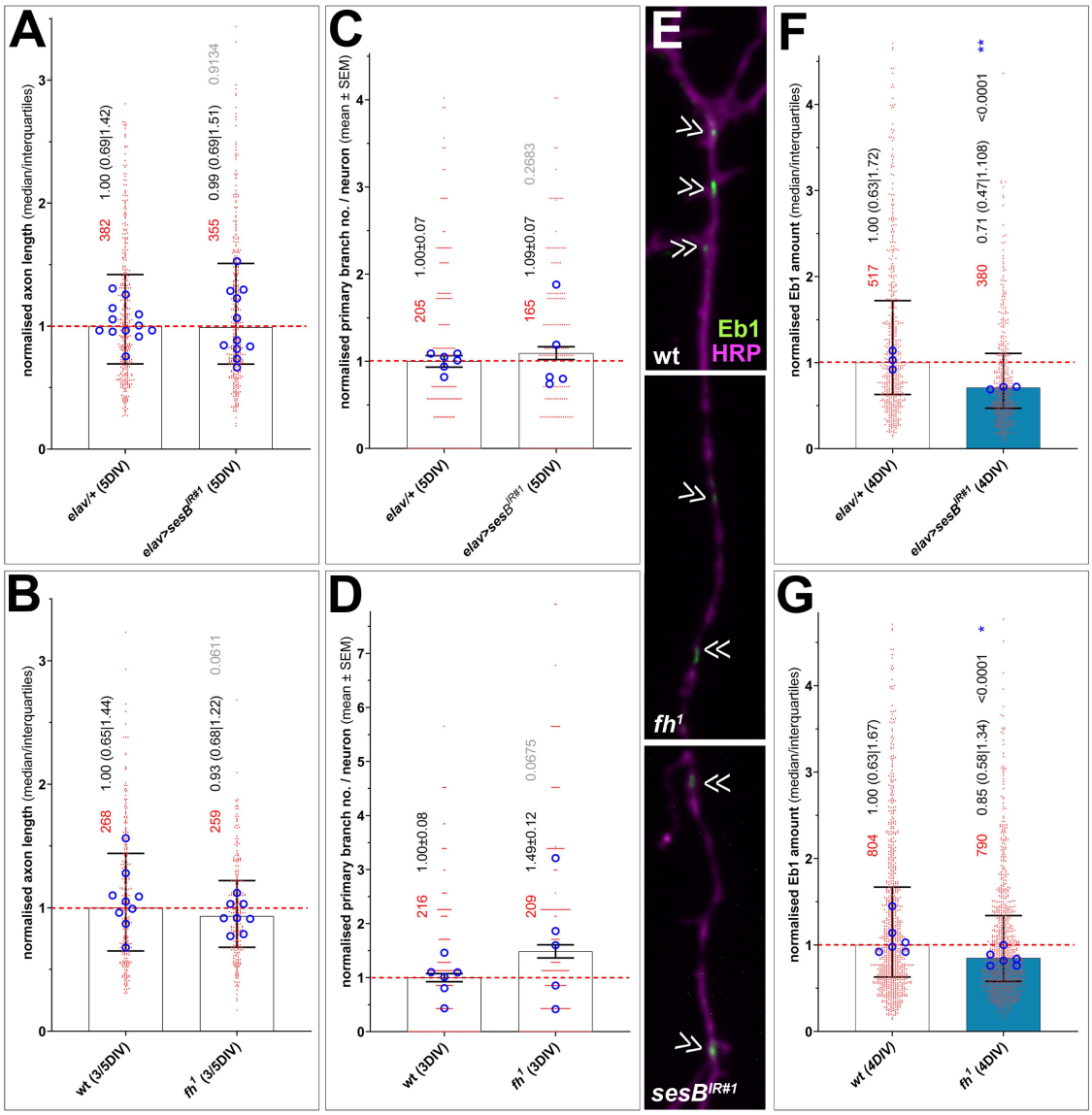

FIGURE 6
Functional losses of Fh and SesB do not affect neuronal morphology but trigger loss of Eb1 from polymerizing MT tips. Quantification of axon growth **(A, B)**, axon branching (number of primary neurites; **C, D)** and Eb1 amounts at MT plus ends **(E-G)** in neurons with loss of SesB (top) or Fh (bottom); images in E show axonal segments (stained for Eb1 in green and HRP in magenta); chevrons indicate Eb1 comets. In the graphs, bars indicate the median with quartile ranges or mean ± SEM (numerical values shown in black font); data are normalized to internal wild-type controls of each experiment (stippled line); single data points are shown as red dots (p values relative to controls established by Mann-Whitney tests are shown in black or grey) and the means of replicates (independent coverslips from usually two experimental repeats) are shown as blue circles with their statistical significance established using *t-tests* indicated as blue asterisks (*$P \leq 0.05$; **$P \leq 0.01$); numbers of assessed neurons are shown in red; the dark-blue bar colour reflects the degree of significance.

mitochondria might be able to cause MT-curling (see section "Introduction"). Since MT bundles provide the lifelines of axons (see section "Introduction"), potential pathological links from mitochondria to MTs might provide new mechanistic explanations for neurodegeneration. We chose the *Drosophila* primary neuron model because it has proven instrumental in the past when addressing other complex cell biological phenomena of axon biology, and was successfully used to demonstrate the fundamental principal that ROS causes MT-curling (Gonçalves-Pimentel et al., 2011; Hahn et al., 2021; Liew et al., 2025; Prokop et al., 2013; Qu et al., 2022; Shields et al., 2025; Voelzmann et al., 2024). Echoing this tradition, also our current approach delivered meaningful results:

Firstly, deficiencies of 5 out of 13 genes caused MT-curling, and all five conditions could be rescued by Trolox suggesting involvement of harmful ROS. Whilst being in line with the generally accepted view that certain mitochondrial aberrations can cause oxidative stress, they also clearly confirm that this impacts MT-bundles as a down-stream effect (Figure 7B). MT bundle deterioration provides therefore a potential mechanism that links mitochondrial dysfunction to axon degeneration. The consistency of our findings might also suggest MT-curling as a complementary,

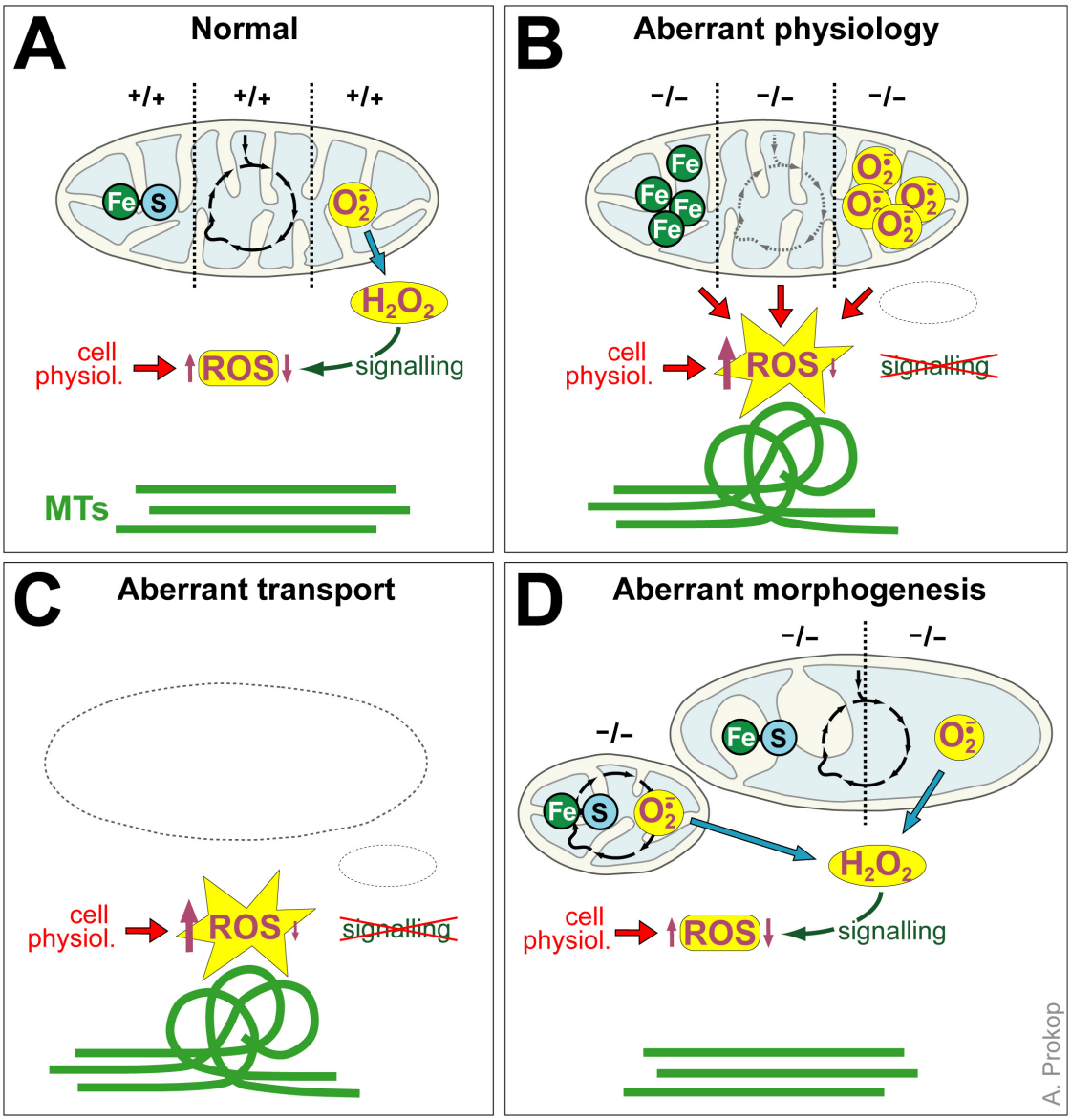

FIGURE 7
Potential interpretations of our findings. **(A)** In wild-type neurons, mitochondria generate iron-sulphur clusters (Fe-S), perform Krebs cycle metabolism (black circle of arrows) and superoxide is metabolised by Sod2 into hyperoxide which triggers signaling in the cytoplasm that helps keeping ROS in balance (up- and down-arrows) and leaves MT bundles unaffected (straight green lines). **(B)** Mutations that affect iron-sulphur production (leading to iron accumulation), that derail Krebs cycle metabolism (potentially leading to aberrant ETC function) or prevent the break-down of hyperoxide may all lead to harmful ROS in the cytoplasm surrounding mitochondria which can cause local MT-curling. **(C)** If mitochondria are absent from axons or wrongly positioned, Sod2-mediated hyperoxide signaling is absent, thus destabilizing ROS homeostasis. **(D)** Contorted mitochondria (here shown fragmented mitochondria from lack of fusion on the left, mitochondria with enlarged or missing cristae on the right) seem well protected and maintain key physiological properties that uphold ROS homeostasis in the cytoplasm.

easy-to-access readout for harmful ROS generation, although further validation including direct ROS measurements in the narrow axonal cytoplasm would be required – but achieving this goal will be a highly challenging task.

Secondly, the results were surprising in that 8 out of the 13 gene deficiencies showed no obvious increases in MT-curling (Ogdh, QIL1, Opa1, Marf, Drp1, ATPsynC, mt:ATPase6, YME1L; Figure 3) despite their established lethality or severely debilitating effects *in vivo*. It was even more surprising that, except Ogdh and YME1L, all these factors are morphogenesis regulators of mitochondria and/or their cristae. Our finding suggests therefore that morphogenetic aberration is a condition that mitochondria can compensate for (at least for several days), potentially through the various stress-response mechanisms that are being reported (Figure 7D; Patergnani et al., 2022; Picca et al., 2023).

We feel that the approach taken here has delivered promising results, and the genetically amenable *Drosophila* neurons used provide the means to extend the study to an even wider scope of mitochondrial genes. Using primary neurons for this study has the advantage that we can deduce cell-autonomous effects directly caused within neuron, but it cannot cover for effects that are more indirect, for example originate in glia cells; this can only be explored

in *in vivo* or co-culture studies. It will also be important to test whether our findings apply to vertebrate neurons, capitalizing on the fact that mitochondrial genes and MT regulators are highly conserved across species (Supplementary Material 2; Brischigliaro et al., 2023; Prokop et al., 2013), and the established knowledge that MT-curling occurs in mouse neurons where it can also be induced by oxidative stress (Liew et al., 2025; Smith et al., 2023).

However, as will be discussed in the next section (see also Supplementary Table 1), our mining of the literature regarding the knowledge about the 13 genes in *Drosophila in vivo* as well as their orthologues in vertebrate and human cells, often revealed inconsistent results as to whether ROS is involved in their pathological phenotypes. These contradictions are likely due to the highly diverse cellular models used which often have very different metabolic footprints, such as liver cells with extreme metabolism rates or cancer cells which tend to switch off oxidative phosphorylation. Findings are therefore difficult to extrapolate between cellular models, and their integration into conceptual understanding is limited. This is different for our results which were all obtained in one standardized neuron model where consistent findings can establish the necessary confidence to deduce concepts. It is then even more ensuring that reports in the literature can be found for all 13 genes that support our findings, as will be discussed in the following.

Potential mechanisms that cause harmful ROS production upon loss of the 5 identified genes

We find ROS-induced MT-curling upon loss of Pdha1, although ROS seems not to feature in the current Pdha1-related *Drosophila* literature. However, ROS was reported from mammalian studies. Skin fibroblasts derived from PDHA-mutant patients showed increased mitochondrial but not cytoplasmic ROS levels (Glushakova et al., 2011), whereas increased cytoplasmic ROS levels were observed in PDHA1-deficient rat fibroblasts where Krebs cycle activity was partially maintained by glutamine-derived α-ketoglutarate (Figure 1 > A–E/g; Wang et al., 2019). In studies of PDH-deficient mouse skeletal muscle, the ETC displayed low efficiency which was compensated for by increased ETC activity - a potentially powerful constellation to cause electron leakage and generate ROS (Gopal et al., 2023). In our culture model, loss of Pdha1 might have even stronger impact on Krebs cycle activity and ETC because the loss of PdhA-derived pyruvate cannot be replenished from β-oxidation (Figure 1 > *C/f*), since fatty acids are virtually absent from the culture medium (Else, 2020; Prokop et al., 2012; Schneider, 1964). Another potential mechanism for ROS generation could be the build-up of lactate (Figure 1 > *A/e*) which causes harmful hyper-lactylation of proteins (Chapp et al., 2021; Wang et al., 2024; Yang et al., 2024). However, build-up of lactate is highly unlikely in *Drosophila* primary neurons which are grown in lactate-free culture medium (Schneider, 1964). In our view, impacts of Krebs cycle aberration on the ETC are the most likely reason for harmful ROS generation upon PDHA deficiency.

SdhA-linked pathology in *Drosophila* has been linked to ROS *in vivo*, which agrees with our findings. For example, antioxidants could reduce synapse loss in $SdhA^{1110}$ and $SdhA^{1404}$ mutant photoreceptors (Mast et al., 2008), and SdhA seems to be part of the Cnc/Nrf2-mediated oxidative stress response pathway (Tsakiri et al., 2019a). For vertebrates, we found reports of ROS elevation for loss of subunits B, C and D (Goffrini et al., 2009; Guzy et al., 2008; Hadrava Vanova et al., 2020; Ishii et al., 2011; Li et al., 2019; Owens et al., 2012), but there were also arguments against ROS. For example, SDHB-deficient chromaffin cells had reduced oxygen consumption (Kuèková et al., 2020), SDH was suggested to act as a ROS sensor dampening Krebs cycle and ETC activity upon elevated H_2O_2 levels (Nulton-Persson et al., 2003; Nulton-Persson and Szweda, 2001). Furthermore, complex II is often missing from respirasome super-complexes where complex I can directly reduce ubiquinone ("Q" in Figure 1 > *EF/cd;* Enriquez and Lenaz, 2014; Hadrava Vanova et al., 2020; Waltz et al., 2024). Regarding the mechanisms of ROS production, Krebs cycle aberration is not a very likely cause since there are various compensatory pathways. For example, SDHB loss in mouse chromaffin cells causes depletion of malate (Figure 1 > *D/d*), but Krebs cycle activity is upheld by pyruvate carboxylase-derived oxaloacetate ("Pcb" in Figure 1 > C/de; Lussey-Lepoutre et al., 2015). The malate-aspartate shuttle (Figure 1 > B/cd; Broeks et al., 2021; Koch et al., 2024), which seems present in *Drosophila* (Curcio et al., 2020; Karp et al., 2017), could even ensure malate dehydrogenase-mediated NADH production ("Mdh2" in Figure 1 > *D/d*). In our view, the most attractive explanation is the suggestion that SDHA loss impairs SDH complex formation in ways that leave the iron-sulphur clusters of SDHB exposed, thus providing a potential source for harmful ROS-production (Hadrava Vanova et al., 2020; Lemarie et al., 2011).

Our results for SesB align with *in vivo* reports for *Drosophila* that its loss causes elevated peroxide levels (Celotto et al., 2006; DeVorkin et al., 2014; Terhzaz et al., 2010; Vartiainen et al., 2014). This is even clearer from mammalian studies. For example, ANT1/2-deficient mouse myoblasts displayed ETC dysfunction as well as reduced glutathione levels and mitochondrial peroxidase activities (Flierl et al., 2022). Knock-down of ANT2 in MCF-7 cells caused significant increase in ROS (Kretova et al., 2014), isolated mitochondria from ANT1-deficient mice had significantly increased hydrogen peroxide production (Esposito et al., 1999), ANT1 overexpression in rat heart protects from ROS damage (Klumpe et al., 2016), and ETC components and activities are upregulated in ANT1/2 double-mutant mouse liver cells (Kokoszka et al., 2004). This said, the ETC was downregulated in ANT1-deficient mouse muscle fibers (Graham et al., 1997) and ANT1 loss was even suggested to be beneficial for cell stresses (Lee et al., 2009), but these studies did not address the potential redundancy of ANT genes. In our view, the most likely cause of ROS production is the dysfunctional ETC in combination with the reduced ability of mitochondrial uncoupling and mPTP impairment, of which the latter is likely to cause calcium stress (Chen Y. et al., 2023) with expected knock-on effects on mitochondrial metabolism including ETC dysregulation (Rossi et al., 2019).

As mentioned before, the potential involvement of ROS in *fh*-linked phenotypes in *Drosophila* is controversially reported and debated (Marelja et al., 2018), but our data clearly demonstrate oxidative stress upon loss of Fh function. Various mechanisms could explain potential ROS increase upon loss of Frataxin. Firstly, the failure of proper iron-sulphur cluster formation will impact on many mitochondrial proteins especially of the ETC which

might cause increased electron leakage, hence ROS production. Secondly, Frataxin-deficient mitochondria displayed increased iron levels (Chen et al., 2016; Navarro et al., 2015) which, in turn, inactivated Sod2 with the respective knock-on effects on MTs (Marelja et al., 2018). High iron levels also trigger ferrotopsis-related ROS-inducing processes including the Fenton reaction which generates highly reactive hydroxyl radicals, also observed in Friedreich's ataxia (Anderson et al., 2008; Costa et al., 2023; La Rosa et al., 2021). In our view, likely all these mechanisms may contribute to our findings.

Our results for Sod2 appear the easiest to explain and align well with very recent data generated in the same *Drosophila* primary neuron system (Shields et al., 2025). Mitochondria of mice lacking SOD2 display reduced respiration, a sensitized transition pore, increased proton leakage, as well as reduced expression of Krebs cycle and ETC enzymes (including SDH and ACONITASE; "Acon" in Figure 1 > *D/f*); knock-out mice display, oxidative stress and genomic DNA damage (Kokoszka et al., 2001; Li et al., 1995; Melov et al., 1998). Increased cytoplasmic ROS levels upon SOD2 loss are likely the consequence of risen intra-mitochondrial superoxide which fails to convert to H_2O_2 and reacts with iron-sulphur clusters (Figure 1 > *CD/c*), thus damaging ETC components and enhancing electron leakage (Palma et al., 2020). Further impact may derive from SOD2-mediated production of H_2O_2 which is far more diffusive and long-lived than superoxide and a known signaling molecule diffusing to the surrounding cytoplasm (Figure 1 > *A/f*); since SOD2 activity is regulated by the metabolic and redox state of mitochondria it could act as an integrating sensor, and its H_2O_2-mediated signaling might help to maintain ROS homeostasis in the surrounding cytoplasm (Palma et al., 2020; Figure 7A).

Taken together, the five MT-curl-inducing conditions (Pdha1, SdhA, Fh, SesB) seem to cause oxidative stress through pathological aberrations of intra-mitochondrial processes leading to leakage of harmful ROS which, in turn, affects MTs in their surrounding. However, of these, Sod2's potential role as an inducer of beneficial H_2O_2-mediated signaling to the surrounding cytoplasm (Palma et al., 2020) might mean that not only Sod2 deficiency, but also the absence of entire mitochondria would deprive axons of this signaling and destabilize ROS homeostasis (Figure 7C). In our view, Sod2 is therefore a potential candidate that might explain MT-curling in the absence of mitochondrial transport (see section "Introduction") (Liew et al., 2025).

Non-curl-inducing conditions

Eight out of 13 gene deficiencies failed to induce MT-curling. Functional loss of ATPsynC or YME1L even showed signs of reduced MT-curling (Figures 3G,H) suggesting a potential drop in ROS levels below baseline as was discussed.

In agreement with our findings for Ogdh1, also OGDHL-deficient human neuroblastoma cells were shown to reduce oxygen consumption (Yap et al., 2021a). OGDH loss affects the Krebs cycle with downstream effects like causing mTORC1 activation (Yoon et al., 2017), although cycle activity can be partly maintained: for example via import of malate (Figure 1 > AB/c; Allen et al., 2016; Yoon et al., 2017) or via methionine catabolism to generate succinyl-CoA (not shown; Yap et al., 2021a). Notably, OGDH was shown to be inactivated through glutathionylation in response to heightened H_2O_2 levels, thus acting as a ROS sensor that can down-regulate NADH production and OXPHOS (Applegate et al., 2008; Nulton-Persson et al., 2003; Nulton-Persson and Szweda, 2001). Loss of Ogdh1 might mimic this silencing effect, expected to cause a reduction or at least no increase in ROS production, as seen in our studies.

Consistent with the lack of MT-curling we observed upon QIL1 knock-down, also MICOS13-deficient cells were reported to display reduced oxygen consumption (Guarani et al., 2015; Kishita et al., 2020) and no studies seem to suggest ROS involvement (Gödiker et al., 2018; Guarani et al., 2016; Russell et al., 2019; Zeharia et al., 2016).

Matters are less clear for the fission and fusion factors. In *Drosophila*, Drp1 loss decreases lifespan (Rana et al., 2017), but there seem to be no reports of ROS-induced neurodegeneration; instead Drp1 deficiency was shown to rescue longevity in a proteasome-deficient model (Tsakiri et al., 2019b), and dominant-negative Drp1 was beneficial in ALS models (Altanbyek et al., 2016). The $Opa1^{s3475}$ mutant allele was shown to cause elevated ROS, shorter lifespan and necrosis of support cells in the fly eye (Tang et al., 2009; Yarosh et al., 2008), and Opa1 knock-down caused axon degeneration (Cao et al., 2017). In other reports, $Opa1^{s3475}$ reduced toxicity in a *Drosophila* Alzheimer model (DuBoff et al., 2012), and *Opa1* knock-down in muscles increased lifespan and improved locomotor activity (Tapia et al., 2021). Similarly, functional loss of Marf caused precocious axon degeneration (Cao et al., 2017), enhances decline in spastic paraplegia models (Fowler and O'Sullivan, 2016), triggers ROS-increase in nephrocytes (Zhu et al., 2024) and leads to ER stress and fragmentation (Debattisti et al., 2014). But it was also shown to rescue frataxin-induced glial degeneration (Edenharter et al., 2018), alleviate ROS-mediated rhabdomere degeneration in Huntington's disease models (Campesan et al., 2023), reduce pink/parkin-induced ER stress in a fly Parkinson model (Celardo et al., 2016; but see Basso et al., 2018) and increase lifespan and locomotor activity (Rana et al., 2017; Tapia et al., 2021). Also in mammalian studies, some report ROS increase upon mitochondrial fragmentation caused by OPA1 or MFN loss (Ježek et al., 2018; Millet et al., 2016; Zhang et al., 2017), whereas others report that DNM1L loss causes proton leakage and a reduction in ROS (Galloway et al., 2012; Kolac et al., 2023), that OPA1-deficient mouse embryonic fibroblasts and hepatocytes have reduced ROS (Lee et al., 2023; Liang et al., 2024), that MFN1-deficient myocytes display normal mitochondrial physiology and improved ROS tolerance (Papanicolaou et al., 2012), and that loss of MFN2 in macrophages have decreased ROS production (Tur et al., 2020). Our experiments for all these factors consistently show lack of MT-curling arguing against ROS increase. We propose that local maintenance mechanisms including mitochondrial protease systems and mitochondria-derived vesicles might be able to keep mitochondrial physiology in balance for an extended period (Misgeld and Schwarz, 2017).

Lack of MT-curling upon functional deficiency of ATP synthase aligns with findings in the literature. For example, blocking mouse ATP synthase affects respirasome assembly and metabolically protects neurons against cellular stresses (Formentini et al., 2014; García-Aguilar and Cuezva, 2018), and cells carrying the dystonia-linked $ATP5MC3^{N106K}$ mutation display reduced ATP production

and oxygen consumption (Neilson et al., 2022). The surprising survival of *mt:ATPase6*^{1} mutant individuals into adult flies was explained by mitochondrial uncoupling (Demine et al., 2019) and glycolytic ATP production (Figure 1 > A/de; Celotto et al., 2011), none of which would suggest ROS production. However, ROS overproduction has been reported for neurodegeneration-linked human MT-ATP6 mutations (Dautant et al., 2018; Galber et al., 2021), in one case involving overproduction of SOD1 and 2 (Geromel et al., 2001). Such effects might relate to roles of the ATP synthase in forming the high-conductance mitochondrial permeability transition pore (mPTP; Bonora et al., 2022) causing late-onset effects not covered by our experimental schedule.

As explained in the Results section, also loss of Yme1L causes late onset of ROS and neurodegeneration in fly and mouse alike, whereas we find that loss of Yme1L causes a strong reduction of MT-curling at 5 DIV. Our results are perhaps best explained by findings in yeast, where loss of *Yme1* causes a severe drop in levels of various ETC components expected to decrease activities of complexes II, III and IV (Kan et al., 2022) and, hence, reduce ROS production. We propose therefore that the loss of Yme1 protease activity causes reduced processing of ETC components leading to early ROS reduction, whereas its roles in mitochondrial quality control, i.e., to remove protein aggregates, become more relevant over a longer time period, potentially masked by other maintenance mechanisms including the shedding of mitochondria-derived vesicles (Held and Houtkooper, 2015; Mattedi et al., 2023; Misgeld and Schwarz, 2017), thus explaining late-onset ROS not seen in our cultures.

Conclusions and future directions

The approach taken here was clearly able to answer the posed questions regarding the ROS-mediated impact of mitochondrial dysfunction on MT bundles. This suggests axonal MT bundles as potential downstream targets in mitochondrial pathology, that would provide a mechanism for axon degeneration. Our data might also suggest Sod2 as a potential candidate explaining MT bundle deterioration upon mitochondrial absence. Further thought-provoking observations were made, such as the absence of MT-curling when affecting mitochondrial morphogenesis, or the strong reduction of curling upon loss of QIL1. Importantly, the *Drosophila* primary neuron system is well-suited to validate findings and test deduced hypotheses efficiently, for example using double- or triple-mutant conditions to clarify compensatory metabolic pathways. Furthermore, findings can be easily validated *in vivo* using the same genetic tools as in culture. This provides promising means to refine our understanding of the role of mitochondria in axons.

Methods

Genetic strategies and fly lines

The wild-type control used throughout the project was the *Drosophila melanogaster* Oregan R strain. Most mutant or transgenic fly stocks were sourced from the Bloomington *Drosophila* Stock Centre (BDSC stock number provided in brackets). Loss-of-function mutant strains were (in alphabetical order): *ATPsynC*^{KO} (*ATPsynC*^{KG01914}; P-element insertion in non-coding 5' exon generating a protein null; BDSC#13923; Lovero et al., 2018); *ATPsynC*^{Df} (*Df(3R)Exel6218*; uncovering ATPsynC; BDSC#7696; Parks et al., 2004); *ATP6*^{KO} (*mt:ATPase6*^{1}; lethal G116E point mutation; BDSC#95253; Celotto et al., 2006); *Drp1*^{KO} (*Drp1*^{T26}; lethal allele; BDSC#3662; Verstreken et al., 2005); *fh*^{KO} (*fh*^{1}; lethal S136R point mutation; BDSC#67161; Chen et al., 2016); *Marf*^{KO} (*Marf*^{B}; amorphic allele; BDSC#67154; Sandoval et al., 2014); *Ogdh1*^{Df} (*Df(3L)Exel7253*; BDSC#7938; Ryder et al., 2007); *Odgh1*^{KO} (*Mi{Trojan-GAL4.1}Ogdh1*$^{MI06026-TG4.1}$ aka *dOgdh-T2A-Gal4*; BDSC#77497; Yoon et al., 2017); *Opa1*^{KO} (*Opa1*^{s3475} aka *Opa1*^{ex2}; BL #12188; strong loss of function due to P-element insertion; Spradling et al., 1999; Yarosh et al., 2008); *Pdha1*^{KO} (*Pdha1*^{A} aka *l(1)G0334A*; lethal G126E point mutation; BDSC#52370; Yamamoto et al., 2014); *SdhA*^{1404} (lethal V445E point mutation; BDSC#81120; Mast et al., 2008); *SdhA*^{1110} (lethal E288K point mutation; BDSC#51659; Mast et al., 2008); *Sod2*^{KO} (*Sod2*^{n283}; BL#34060; 167bp deletion removing part of the first exon and intron; Duttaroy et al., 2003); *YME1L*^{KO} (*YME1L*^{del}; 2kb deletion removing most of the coding region; BDSC#95273; Qi et al., 2016). Knock-down experiments were performed using the Gal4/UAS system (Elliott and Brand, 2008) employing the second-chromosomal *elav-Gal4* driver (BDSC #8765) in combination with the following transgenic UAS constructs: *fh*^{IR} (*P{UAS-fh.RNAi.A}2*; BDSC#24620; Anderson et al., 2005); *Marf*^{IR} (*HMC03883*; BL#55189; Perkins et al., 2015); *Opa1*^{IR} (*HMS00349*; BDSC#32358; Perkins et al., 2015); *Pdha*^{IR} (*P{TRiP.HMC04032}*; BDSC#55345; Perkins et al., 2015); *QIL1*^{IR} (*P{TRiP.GLC01383}*; expression reduced to about 25%; loss of cristae junctions; BDSC#44364; Guarani et al., 2015; Perkins et al., 2015); *sesB*^{IR1} (*P{TRiP.HMS01549};* BDSC#36661; Perkins et al., 2015); *sesB*^{IR2} (*P{TRiP.JF01528};* BDSC#31077; Perkins et al., 2015). Green balancer chromosomes used to identify mutant or construct-expressing embryos were readily available *FM7*, *CyO or TM3* balancers carrying *twi-Gal4 or Kr-Gal4* in combination with *UAS-GFP* or carrying a *Df-GFP* fusion construct (Casso et al., 2000; Halfon et al., 2002; Le et al., 2006).

Drosophila primary cell culture

Drosophila primary neuron cultures were performed as published previously (Prokop et al., 2012; Voelzmann and Sánchez-Soriano, 2022). In brief, stage 11 embryos were treated for 1 min with bleach to remove the chorion, sterilized for ~30 s in 70% ethanol, washed in sterile Schneider's/FCS, and eventually homogenized with micro-pestles in 1.5 ml centrifuge tubes containing 21 embryos per 100 µl dispersion medium and left to incubate for 5 min at 37°C. Cells were washed with Schneider's medium (Gibco), spun down for 4 mins at 650 g, supernatant was removed and cells re-suspended in 90 µl of Schneider's medium containing 20% fetal calf serum (Gibco). 30 µl drops were placed on cover slips. Cells were allowed to adhere for ~2 hrs on cover slips coated with a 5 µg/ml solution of concanavalin A and then grown as a hanging drop culture for several days at 26°C as indicated in each experiment as days *in vitro* (DIV). To abolish maternal product

deposited by heterozygous mothers in their oocytes (Prokop, 2013), we used a pre-culture strategy (Prokop et al., 2012; Sánchez-Soriano et al., 2010) where cells were kept for 5 days in a tube before they were plated on a coverslip [indicated as "(pre)" in Figure 3].

In some experiments, cells were treated with 100 μM Trolox (Sigma) throughout the entire culture period; to make pre-dilutions, Trolox solid was dissolved in culture medium (using rigorous shaking and warming to 37°C) achieving 10 mM concentration; the pre-dilution was then further diluted 1:100 in culture medium, sterile-filtered and used to culture the cells.

Staining procedures

Cultured neurons on coverslip were fixed for 30 min using a drop of 4% paraformaldehyde (PFA) and 0.05% glutardialdehyde in 0.05M phosphate buffer (PB; pH 7–7.2). Cells were washed with 0.5% Tergitol solution in 0.05M PB for 20 min (one exchange). The cells were incubated for 2 hrs in a 200 μl drop of anti-tubulin primary antibodies diluted 1:500 in PB (clone DM1A, mouse, Sigma or clone YOL1/34, rat, Antibodies.com). Cells were washed with PBS and then incubated for 1.5 h with secondary FITC- or Cy3-conjugated anti-mouse antibodies (donkey, Jackson ImmunoResearch, 1:200 in PBS). To image cell morphology and identify neurons, cells were co-labelled with TRITC-, Alexa647-, FITC- or Atto647N-conjugated anti-HRP (goat, Jackson Immuno Research, 1:100). For Eb1 comet analysis, cells were fixed for 10 min in the freezer with pre-cooled Plus-Tip Fixative (90% methanol, 3% formaldehyde, 5 mM sodium carbonate, pH 9), then washed with PBS and stained as described above with anti-DmEb1 (gift from H. Ohkura; rabbit, 1:500; Elliott et al., 2005). For the visualization of mitochondria, cell cultures were incubated with 1 μM MitoTracker Red CMXRos (Invitrogen; Klionsky et al., 2012) for 30 min at room temperature (RT); stock solutions were prepared from 50 μg lyophilized solid dissolved in 94 μl of DMSO of which 2 μl were added to 2 ml of growth medium. Following incubation, cultures were then fixed and stained following the procedures below. Specimens were embedded in ProLong Gold Antifade mounting medium (Invitrogen) on microscope slides. The embedded slides were left to dry overnight in the dark before imaging.

Imaging and data analysis

Neurons were visualized using a compound fluorescence microscope (BX50WI or BX51; Olympus) and images of single neurons were captured using nijiBlueBox and the MatrixVision mvBlueFox3-M2 2124G camera at 100x magnification. Images were analysed using the FIJI/ImageJ 1.54p software. To determine the degree of MT disorganization in axons we used the "MT disorganization index" (MDI; Qu et al., 2017): the axon length (from cell body to tip of the most distant microtubule) was measured using the segmented line tool; area of disorganization was measured using the freehand selection tool; this value was then divided by the the product of axon length multiplied with 0.5 μm (arbitrary axon diameter, thus approximating the expected area of the axon if it were not disorganized); for axon branching data, primary neurites containing a microtubule core of at least 10 μm and branching off the longest neurite were counted. For the Eb1 comet analysis, length and mean intensity of the Eb1 comets were measured using the line tool in FIJI; Eb1 amount was calculated by multiplying comet length with mean intensity (Hahn et al., 2021). All data were normalized against their respective controls. In each experiment, usually three slides per genotype were analysed aiming to image ~30 isolated neurons per slide. Experiments were repeated at least once, data pooled. MDI data were usually not normally distributed but nevertheless plotted as mean ± SEM to avoid misleading median values of zero. Most experiments had only two groups and were assessed using Mann–Whitney Rank Sum tests, experiments with more then two group using Kruskal–Wallis one-way ANOVA with *post hoc* Dunn's test. Means of single slides were used to generate super-plots (Lord et al., 2020) and assessed using standard *t-tests*. Statistical analyses were performed with Graphpad 10.2.2. The data used for our analyses will be made available on request. Image plates were generated with Photoshop CS6 and illustrations with Illustrator CS.

Author contributions

SM-C: Data curation, Formal analysis, Investigation, Writing – review & editing. MO: Data curation, Formal analysis, Investigation, Writing – review & editing. Y-TL: Data curation, Formal analysis, Investigation, Writing – review & editing. MD: Data curation, Formal analysis, Investigation, Writing – review & editing. WC: Data curation, Formal analysis, Investigation, Writing – review & editing. AP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2025.1631752/full#supplementary-material

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