ATP13A2-mediated endo-lysosomal polyamine export counters mitochondrial oxidative stress

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Recessive loss-of-function mutations in ATP13A2 (PARK9) are associated with a spectrum of neurodegenerative disorders, including Parkinson’s disease (PD). We recently revealed that the late endo-lysosomal transporter ATP13A2 pumps polyamines like spermine into the cytosol, whereas ATP13A2 dysfunction causes lysosomal polyamine accumulation and rupture. Here, we investigate how ATP13A2 provides protection against mitochondrial toxins such as rotenone, an environmental PD risk factor. Rotenone promoted mitochondrial-generated superoxide (MitoROS), which was exacerbated by ATP13A2 deficiency in SH-SY5Y cells and patient-derived fibroblasts, disturbing mitochondrial functionality and inducing toxicity and cell death. Moreover, ATP13A2 knockdown induced an ATF4–CHOP-dependent stress response following rotenone exposure. MitoROS and ATF4–CHOP were blocked by MitoTEMPO, a mitochondrial antioxidant, suggesting that the impact of ATP13A2 on MitoROS may relate to the antioxidant properties of spermine. Pharmacological inhibition of intracellular polyamine transport with α-difluoromethylornithine (DFMO) also increased MitoROS and ATF4 when ATP13A2 was deficient. The polyamine transport activity of ATP13A2 was required for lowering rotenone/DFMO-induced MitoROS, whereas exogenous spermine quenched rotenone-induced MitoROS via ATP13A2. Interestingly, fluorescently labeled spermine uptake in the mitochondria dropped as a consequence of ATP13A2 transport deficiency. Our cellular observations were recapitulated in vivo, in a Caenorhabditis elegans strain deficient in the ATP13A2 ortholog catp-6. These animals exhibited a basal elevated MitoROS level, mitochondrial dysfunction, and enhanced stress response regulated by atfs-1, the C. elegans ortholog of ATF4, causing hypersensitivity to rotenone, which was reversible with MitoTEMPO. Together, our study reveals a conserved cell protective pathway that counters mitochondrial oxidative stress via ATP13A2-mediated lysosomal polyamine export.

Significance

Mutations in ATP13A2 cause a spectrum of related neurodegenerative disorders. ATP13A2 is a lysosomal exporter of polyamines that stabilizes mitochondrial function and inhibits oxidative stress. The late endo-lysosomal transporter ATP13A2 strongly contributes to the total cellular polyamine content via a two-step process: Firstly, polyamines are transferred from the late endo-lysosome to the cytosol. Secondly, ATP13A2 transport provides cellular protection by lowering reactive oxygen species (ROS), which may relate to the antioxidant properties of polyamines. Consequently, dysfunctional ATP13A2 sensitizes cells to oxidative stress, which impairs mitochondria, and induces toxicity and cell death. ATP13A2-mediated polyamine transport represents a conserved pathway that protects against mitochondrial oxidative stress. The comprehensive impact of ATP13A2 on lysosomal health and mitochondrial oxidative stress may explain why ATP13A2 exerts potent neuroprotective effects.
enter the cell via endocytosis and subsequently, polyamines are transported by ATP13A2 into the cytosol (9). This process complements polyamine biosynthesis via the ornithine decarboxylase (ODC) pathway (9). Importantly, ATP13A2’s polyamine transport function is crucial for its neuroprotective effect, since it prevents lysosomal polyamine accumulation and subsequent lysosomal rupture, while improving lysosomal health and functionality (9). Moreover, when activated by its two regulatory lipids—phosphatidylinositol-3,5-biphosphate [PI(3,5)P2] and phosphatidic acid (PA)—ATP13A2 exerts a cell protective effect against the mitochondrial neurotoxin rotenone (16), an environmental risk factor for PD (17). Rotenone is a mitochondrial complex I inhibitor, which leads to high levels of reactive oxygen species (ROS), promoting protein aggregation and damaging organelles. However, how ATP13A2’s polyamine transport function exerts a cell protective effect against rotenone, or other mitochondrial neurotoxins, is not yet clear.

Interestingly, the transported substrates spermine and spermidine reduce oxidative stress (14, 15). Spermine is a potent free radical scavenger (18) and a biologically important antioxidant (19–23). We therefore hypothesize that ATP13A2-mediated polyamine transport may counteract oxidative stress (16, 24) and preserve mitochondrial health (11, 12). Here, we demonstrate in complementary human cell models and Caenorhabditis elegans that lysosomal polyamine export by ATP13A2 effectively lowers ROS levels and modulates mitochondrial health and functionality, pointing to a lysosomal-dependent cell protective pathway that may be implicated in ATP13A2-related neurodegenerative disorders.

Results
ATP13A2 Protects Cells and Mitochondria against Mitochondrial Toxins. We previously validated stable human neuroblastoma (SH-SY5Y) cell models with either ATP13A2 knockdown (sh-ATP13A2/kd) or wild-type ATP13A2 overexpression (WT-OE), and control cell lines with overexpression or shRNA-mediated knockdown of firefly luciferase (Fluc or sh-Fluc, respectively) (16). ATP13A2 expression offered protection, whereas knockdown exacerbated toxicity against rotenone (17) (Fig. 1 A and B), but also against other mitochondrial neurotoxins such as 1-methyl-4-phenylpyridinium (MPP⁺) and 6-hydroxydopamine hydrobromide (6-OHDA) (25, 26) (SI Appendix, Fig. S1 C–F). The increased rotenone toxicity in kd cells was associated with a drop in mitochondrial membrane potential (MMP) (Fig. 1B) and ATP production (Fig. 1C), whereas ATP13A2 overexpression prevented the reduction in MMP (Fig. 1B). Interestingly, fibroblasts isolated from patients with HSP and KRS that harbor homozygous loss-of-function mutations (31) demonstrated that ATP13A2 kd cells underlies the ATF4-driven stress response. No effect was observed on ATFS and C/EBPβ expression, highlighting the specificity of the ATF4 stress response as a consequence of MitoROS accumulation (SI Appendix, Fig. S5).

The mitochondrial phenotype and stress response observed in ATP13A2 kd cells are most likely caused by the induction of MitoROS, rather than as a consequence of cell death. Indeed, kd cells already presented a clear increase in MitoROS (SI Appendix, Fig. S3) and initiation of the MitoROS-dependent stress response (Fig. 3A) as early as 6 h post rotenone exposure, resulting in a decreased MMP, even though cell death was not yet significantly increased (SI Appendix, Fig. S6).

Taken together, ATP13A2 reduces MitoROS accumulation in rotenone conditions preventing the activation of a MitoROS-induced stress pathway and cell death initiation.

Spermine Transported by ATP13A2 Reduces MitoROS. Next, we tested whether the impact of ATP13A2 on MitoROS depends on the polyamine transport function of ATP13A2, rather than on its transport-independent scaffold function (33). Indeed, WT-OE strongly reduced MitoROS in the presence of rotenone, whereas expression of a transport-inactive ATP13A2 mutant (D508N-OE) (9) had no effect (Fig. 4A), despite comparable expression of D508N-OE and WT-OE (16). Moreover, pharmacological inhibition of the formation of the regulatory lipids PI(3,5)P2 and PA, which activate ATP13A2 transport (9, 16, 24), abolished the impact of ATP13A2 on ROS and MitoROS (SI Appendix, Fig. S7), further demonstrating that the transport function of ATP13A2 is required for reducing the MitoROS levels.

We previously reported that ATP13A2 activity critically contributes to the native polyamine content as well as the cellular uptake of BODIPY-labeled polyamines spermidine and spermine (9, 24), reflecting the biochemical activity of ATP13A2 (9). This explains why Fluc cells exhibited higher cellular uptake of the BODIPY-spermine and -spermidine as compared to ATP13A2 kd (SI Appendix, Fig. S8). Interestingly, extracellular administration of a physiological concentration of 1 μM unlabeled spermine (35) fully abolished the rotenone-induced MitoROS increase in Fluc cells to similar levels as observed in WT-OE cells that are maximally protected (Fig. 4B), but had no significant impact on ATP13A2 kd cells (Fig. 4B). Together, ATP13A2 promotes uptake of extracellular spermine to counter the accumulation of MitoROS.
Inhibition of Polyamine Synthesis in ATP13A2-Deficient Cells Causes a MitoROS Response. Since impaired polyamine transport induced a MitoROS response, inhibition of polyamine synthesis may also elevate MitoROS in ATP13A2 kd cells. Indeed, treating ATP13A2 kd cells with α-difluoromethylornithine (DFMO), a compound that blocks ODC (a rate-limiting enzyme of polyamine synthesis), resulted in higher MitoROS levels (Fig. 4 B) and ATF4-CHOP up-regulation (Fig. 4D), which was accompanied by a significant increase in cell death (Fig. 4E). As expected from the reduced ATP13A2 activity, this could not be prevented by exogenous spermine, whereas MitoTEMPO completely prevented the initiation of a stress response and cell death in ATP13A2 kd cells subjected to DFMO (Fig. 4 D and E). In Fluc cells, DFMO resulted in a nonsignificant increase of MitoROS that was completely prevented by exogenous spermine addition, whereas no MitoROS accumulation was observed in WT-OE cells (Fig. 4C). Thus, DFMO treatment mimics the rotenone phenotype in ATP13A2 kd cells, pointing to a direct effect of the transported polyamines on MitoROS, the stress response, and cell death.

Importantly, we independently recapitulated both the rotenone and DFMO phenotypes (SI Appendix, Fig. S9) in CRISPR/Cas9-mediated ATP13A2 knockout cells (KO) that were recovered with either wild-type ATP13A2 (KO/WT) or the transport dead D508N mutant (KO/D508N) (9). The ATP13A2 transport deficiency in KO/D508N cells caused an increased MitoROS and ATF4-stress response in rotenone or DFMO conditions that was only rescued by MitoTEMPO, but not spermine administration (SI Appendix, Fig. S9 B–D).

Thus, ATP13A2-mediated spermine transport protects mitochondria by countering mitochondrial oxidative stress and complements ODC-dependent polyamine synthesis.

ATP13A2 Promotes Cellular and Mitochondrial Polyamine Uptake. Next, we examined whether the documented lysosomal dysfunction (8–10) may be responsible for the MitoROS phenotype in ATP13A2-deficient cells. While lysosomal dysfunction in ATP13A2 KO and KO/D508N cells can be restored by administration of acidic nanoparticles that enter the cells via endocytosis (9, 36), these nanoparticles had no impact on MitoROS (Fig. 5A), indicating that lysosomal dysfunction is not the underlying cause of the MitoROS phenotype. Instead, MitoROS levels may be a direct consequence of a reduced polyamine content, which we examined here via metabolomics. As observed before (9), the native polyamine levels in KO/D508N cells versus KO/WT were significantly decreased in untreated conditions (SI Appendix, Fig. S9E), however, without evoking a significant MitoROS response (SI Appendix, Fig. S9B). Putrescine and spermidine levels were further reduced in KO/D508N cells exposed to DFMO (Fig. 5B), in line with higher MitoROS (SI Appendix, Fig. S9B). DFMO had no significant effect on spermine levels (Fig. 5B), as observed before (37). In KO/WT cells, the combined polyamine pool (SI Appendix, Fig. S9E), and also MitoROS levels (SI Appendix, Fig. S9B), were not significantly affected by DFMO, indicating that ATP13A2-mediated polyamine uptake activity adequately compensated for the inhibition of polyamine synthesis in these cells, possibly due to the relatively higher ATP13A2 expression in KO/WT versus control cells (9). Moreover, adding exogenous spermine in DFMO conditions
led to a significantly higher polyamine content in DFMO-treated KO/WT cells (SI Appendix, Fig. S9E), but not in KO/D508N cells, indicating again that KO/WT cells can maintain their polyamine levels by taking up spermine, in contrast to KO/D508N cells.

To further examine spermine uptake and subcellular distribution, we turned to confocal microscopy to detect BODIPY-labeled spermine in ATM13A2 KO cell models. ATM13A2 promoted BODIPY-spermine transport to the mitochondria, as evidenced by the significantly higher basal colocalization between BODIPY-spermine and the mitochondrial marker TOMM22 in KO/WT cells versus KO/D508N cells (Fig. 5C). Moreover, the mean fluorescent intensity of BODIPY-spermine within the mitochondrial network was significantly higher in KO/WT cells (Fig. 5D).

Thus, spermine transported by ATM13A2 is redistributed to the mitochondria, where it may locally counteract MitoROS accumulation and protect mitochondrial function irrespective of lysosomal deficiency.

The MitorOS Protective Response via ATM13A2 Is Highly Conserved and Relevant In Vivo. Finally, we validated whether the antioxidant effect of ATM13A2 and the subsequent prevention of a stress response are conserved among species and may be relevant in vivo. Therefore, we turned to a C. elegans strain that is deficient in catp-6, one of three related nematode orthologs that has the largest similarity with ATM13A2 because of its late endolysosomal localization (38, 39). The C. elegans catp-6(ok3473) animals were hypersensitive to rotenone (Fig. 6A). Moreover, a reduced MMP was already evident under basal conditions in the catp-6(ok3473) strain, which was further aggravated in the presence of rotenone (Fig. 6B and SI Appendix, Fig. S10A) and partially rescued by MitoTEMPO (Fig. 6C and SI Appendix, Fig. S10B). Moreover, MitoROS levels were constitutively elevated in catp-6(ok3473) animals, and further increased by rotenone exposure (Fig. 6D and SI Appendix, Fig. S10C). MitoTEMPO diminished MitoROS accumulation (Fig. 6D and SI Appendix, Fig. S10C) and lethality (Fig. 6E) in response to rotenone. The reexpression of wild-type catp-6 rescued the MitoROS phenotype, while the catalytically inactive mutant catp-6(D465N) did not, demonstrating the requirement of transport activity (Fig. 6D).

With a Pshelf-60::GFP reporter, a marker for hsp-60 expression (40), we observed that catp-6 mutant worms presented a constitutively induced mitochondrial stress response (Fig. 6F and SI Appendix, Fig. S10D). This response could be partially rescued by reexpression of wild-type catp-6 (Fig. 6F and SI Appendix, Fig. S10D) or by the addition of MitoTEMPO (Fig. 6G and SI Appendix, Fig. S10D). Knockdown of atfs-1, the closest ortholog to mammalian ATF4, down-regulated the expression of the Pshelf-60::GFP reporter (Fig. 6H and SI Appendix, Fig. S10D), indicating that atfs-1 is at least partially responsible for the hsp-60 up-regulation in the catp-6(ok3473) animals. Of the three C. elegans orthologs, catp-6 appears the most relevant isoform that phenocopies mammalian ATM13A2 (39), since the triple catp-7(0) catp-6(0); catp-5(0) null mutant displayed a similar MMP phenotype to the catp-6(ok3473) animals (SI Appendix, Fig. S10E).

In conclusion, like ATM13A2 in human cells, catp-6 counters mitochondrial oxidative stress in C. elegans, which prevents the up-regulation of an ATF4/atfs-1-dependent response.
Discussion

Lysosomal Spermine Export via ATP13A2 Promotes Lysosomal and Mitochondrial Health. Our current study offers compelling insights into the physiological role of ATP13A2 as a polyamine transporter placed at the intersection of lysosomes and mitochondria. We previously reported that ATP13A2 maintains healthy and functional lysosomes by preventing toxic lysosomal accumulation of polyamines (9). In addition, a reduced ATP13A2 activity lowers the cellular polyamine content and impairs cellular polyamine distribution (9). Here, we showed that this contributes to downstream problems. Our findings show that ATP13A2-mediated lysosomal polyamine export lowers mitochondrial-derived ROS and improves mitochondrial function (81). The dual impact of ATP13A2-mediated polyamine transport on lysosomal and mitochondrial health may explain why ATP13A2 exerts a strong neuroprotective effect and why ATP13A2 deficiency is associated with neurological disorders hallmarked by lysosomal and mitochondrial dysfunction (41).

Lysosomal Spermine Export Exerts an Antioxidant Response That Protects Mitochondria. The phenotypes we observe in our ATP13A2/catp-6-deficient models are clearly MitoROS driven. The increased lethality, high (Mito)ROS levels, aberrant MMP, and stress response induction can all be reversed by the MitoROS scavenger MitoTEMPO or a functional ATP13A2/catp-6, but not by a transport inactive mutant. We showed that polyamines provide the basis of the protective effect toward mitochondrial complex I inhibition and subsequent MitoROS accumulation. The impact of ATP13A2’s transport function on MitoROS can be explained by the antioxidant properties of spermine and spermidine, which are potent free radical scavengers (14, 15, 18–23).

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scavenger inside mitochondria (49). ATP13A2 potently reduces MitoROS, indicating that polyamines may quench superoxide in or near the mitochondria (49). ATP13A2-mediated polyamine transport may also regulate MMP, as evidenced by the higher MMP in our WT-OE cells, or mitophagy, explaining the higher mitochondrial volume consistently reported in cells with ATP13A2 deficiency (11, 12). Additional studies are under way to further dissect the role of ATP13A2 in mitochondrial lysosomal cross-talk, interactions, and polyamine transfer.

**Synergy between ATP13A2-Mediated Polyamine Transport and Polyamine Metabolism.** A dynamic interplay between polyamine uptake and metabolism has been repeatedly reported, but the relative contribution of polyamine uptake versus synthesis may be cell-type dependent. Various cell models present different DFMO sensitivities, and our observations indicate that ATP13A2 activity modulates DFMO sensitivity (9). We found that ATP13A2 plays a prominent role in controlling the cellular polyamine content and synergizes with the ODC pathway for polyamine synthesis. Indeed, deletion of ODC and ATP13A2 orthologs are synthetically lethal in Saccharomyces cerevisiae (50) and C. elegans (51). Also, ATP13A2 KO and KO/D508N cells display a strong reduction in polyamine levels, indicating that ODC activity is unable to compensate for the loss of polyamine uptake, despite an up-regulation of ODC mRNA expression (SI Appendix, Fig. S9F). DFMO treatment elevates ROS levels and triggers an ATF4-dependent stress response specifically in ATP13A2-deficient cells. Based on these observations, we speculate that consequences of ATP13A2 dysfunction may be strongest in cell types that are more reliant on polyamine uptake than on polyamine synthesis to maintain the endogenous polyamine pool. DFMO treatment caused a reduction in putrescine and spermidine in KO/D508N cells, but not spermine, as reported before (37), whereas spermine administration affected putrescine and spermidine levels, but not spermine content, indicating that the spermine pool is most strictly regulated. Due to polyamine interconversions and redistribution, it remains difficult to correlate changes in MitoROS levels to local changes in specific polyamine types. Future studies will be needed to determine the contribution of other enzymes in

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**Fig. 4.** Spermine transported by ATP13A2 provides protection toward oxidative stress, thereby preventing the activation of a stress response. SH-SY5Y cells stably overexpressing Fluc (control), ATP13A2 (WT-OE), catalytically inactive D508N ATP13A2 (D508N-OE), or sh-ATP13A2 (kd) were treated with rotenone (Rot, 1 μM, 24 h; A and B) or DFMO (0.1 mM, 48 h; C–E) with or without spermine (SPM) (1 mM; B–E; last 24 h) or MitoTEMPO (1 μM; D and E; last 24 h). Subsequently, superoxide levels were measured with the MitoSOX probe (A–C), protein levels of stress response markers ATF4 and CHOP were assessed via immunoblotting (D), or cell death readout was performed by means of a propidium iodide assay with flow cytometry (E). Data are the mean of a minimum of three independent experiments ± SEM. MFI, mean fluorescence intensity. ***P < 0.001; ****P < 0.0001; ns, nonsignificant versus respective untreated unless depicted otherwise, ANOVA post hoc Tukey’s multiple comparison test.
polyamine metabolism, while following the subcellular polyamine content, interconversions, and distribution.

**ATP13A2 and Polyamines Are Implicated in Oxidative Stress Responses.**

ATP13A2 and its transported substrates have been reported to protect against conditions of oxidative stress and oxidative stress pathways (52–54). ATP13A2’s potent antioxidant effect may be key to confer mitochondrial protection to various ROS-inducing neurotoxins such as rotenone, 6-OHDA, and MPP+. Conversely, ATP13A2 activity promotes paraquat toxicity, which as a polyamine homolog may be taken up in cells more efficiently, possibly explaining the increased toxicity (55). Excess ROS is a main contributor of neuronal cell death, which is related to the high susceptibility of neurons to oxidative damage (56, 57). Oxidative stress in neurodegenerative disorders may arise due to a combination thereof. Subsequently, oxidative stress pathways (52–54) have been suggested to improve dopaminergic neuron survival (53).

MitoROS accumulation in ATP13A2-deficient cells also drives an ATF4/CHOP/HSP60-dependent stress response (32), a modulator of mitochondrial quality control (64–66). ATF4 has a major role as cell fate decision maker (67), so the ATF4-CHOP axis may exert a proapoptotic (68, 69) or prosurvival (70, 71) role in our models, which remains to be investigated.

**Fig. 5.** Spermine transported by ATP13A2 is redistributed to the mitochondria. SH-SYSY neuroblastoma cells with endogenous ATP13A2 levels (control) or with ATP13A2 KO overexpressing WT ATP13A2 (KO/WT), or a catalytically dead mutant ATP13A2 (KO/D508N) were exposed to DFMO (1 mM, 48 h; A and B), nanoparticles (180 ng/mL, 1 h incubation before DFMO addition) (A), spermine (SPM, 1 μM, 6 h; B), BODIPY-labeled spermine (BODIPY-SPM, 1 μM, 90 min; C and D), or a combination thereof. Subsequently, superoxide levels were assessed with the MitoSOX probe (A), polyamine levels were determined via metabolomics of total cell lysates (B), or cells were fixed and stained for TOMM22 to analyze colocalization (yellow) of BODIPY-SPM (green) and TOMM22 (red) (C) or BODIPY-SPM mean fluorescence intensity within the TOMM22-stained mitochondrial network (D, yellow borders represent mitochondria). Representative images are shown, boxed areas are enlarged in the Inset. For the analysis in C, images were taken with settings optimized for the individual cell lines. For the analysis in D, images were taken with equal settings to enable a comparison of mitochondrial BODIPY-SPM mean fluorescence intensity between the cell lines (Scale bar, 5 μm). Data are the mean of a minimum of three independent experiments ± SEM. In each experiment, data are gathered of two isogenic cell lines (for control, KO, KO/WT, and KO/D508N) of which the average is displayed. MFI, mean fluorescence intensity. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns, nonsignificant versus respective untreated (A and B), or KO/WT (C and D) unless depicted otherwise, ANOVA post hoc Tukey’s or Dunnett’s multiple comparison test (A and B, respectively), Mann–Whitney U test (C), or two-tailed unpaired t test (D).
In conclusion, ATP13A2-mediated polyamine export from the late endo/lysosome into the cytosol reduces ROS generated by mitochondria, protecting mitochondrial health and preventing cell death. This highly conserved pathway counters mitochondrial oxidative stress and may contribute to ATP13A2’s neuroprotective effect.

Materials and Methods

A more detailed description of the materials and methods can be found in SI Appendix, Supplementary Materials and Methods.

Cell Culture Conditions. SH-SYSY neuroblastoma cell lines stably overexpressing firefly luciferase or sh-firefly luciferase (Fluc or sh-Fluc, depending on comparison with either overexpression or knockdown cell lines, respectively), Homo sapiens wild-type ATP13A2 (WT-OE), catalytically deficient ATP13A2 (D508N-OE) or sh-ATP13A2 (kd) were generated via lentiviral transduction as described previously (16). We made use of two independent polyclonal lines with ATP13A2 knockdown (kd1 and kd2, since two different shRNAs targeting ATP13A2 were used), of which the mean is reported in the results. Immunoblots show the independent clonal cell lines. KO of ATP13A2 (D508N-OE) or sh-ATP13A2 (kd) were generated via lentiviral transduction as described previously (16). We made use of two independent polyclonal lines with ATP13A2 knockdown (kd1 and kd2, since two different shRNAs targeting ATP13A2 were used), of which the mean is reported in the results. Immunoblots show the independent clonal cell lines. KO of ATP13A2

Fig. 6. The ATP13A2 ortholog catp-6 exerts a mitochondrial protective antioxidant function in vivo in C. elegans, thereby preventing the activation of a stress response. WT (control) C. elegans and strains carrying a loss-of-function mutation (α3473) in the PS8-ortholog catp-6, either rescued or not by overexpression of wild-type catp-6 (WT) or a catalytically inactive mutant (D465N), were exposed to rotenone (Rot, 10 μM) (A, B, D, and E) or analyzed under basal conditions (C, F, G, and H) in absence (A, B, F, and H) or presence (C, D, E, and G) of MitoTEMPO (10 mM). Subsequently, we measured (A and E) lethality (60 h and 72 h Rot exposure, respectively), (B and C) MMP (16 h Rot exposure), (D) superoxide levels (16 h Rot exposure), or (F-H) expression level of the Phsp60::GFP reporter. In H, animals were treated either with mock(RNAi) or with atfs-1(RNAi). For representative pictures of each panel, please see SI Appendix, Fig. S10. Data are the mean of a minimum of three independent experiments ± SEM. MFI, mean fluorescence intensity. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns, nonsignificant versus respective untreated unless otherwise indicated; **P < 0.01, ****P < 0.0001 versus rotenone-treated control; ANOVA or Kruskal–Wallis with post hoc Tukey’s (A, B, and E) or Dunn’s (C, D, F, G, and H) multiple comparison test, respectively.
SDS/PAGE and Immunoblotting. After saponin treatment, cells were incubated in Dulbecco’s Modified Eagle Medium high-glucose culture medium supplemented with 1% glutamine (Life Technology), 1% penicillin/streptomycin (Sigma), 15% fetal calf serum, and 0.1 mM gentamycin (Gibco), and selection antibiotic (5 μg/ml blasticidin or 2 μg/ml puromycin [InvivoGen]). All treatments were performed in the same medium, but without selection antibiotic and with 5% fetal calf serum instead of 15%. Fibroblasts were maintained in Basal Medium Eagle media (Sigma), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Spermine was administered in heat-inactivated fetal calf serum to inactivate serum polyamine oxidase activity.

Determination of Intracellular ATP Level. The cells were treated as indicated. Intracellular ATP was determined after saponin treatment by luciferase conversion, following manufacturer’s instructions. Bioluminescence was assessed by optical top reading via a Flex Station 3 microplate reader (Molecular Devices Inc.).

SDS/PAGE and Immunoblotting. Cells were seeded at 3 × 10^4 or 2 × 10^4 cells/10-cm dish, depending on whether the subsequent treatment would take 24 or 48 h, respectively. After treatment for the indicated time periods, cells were harvested and subsequently lysed with radio-immunoprecipitation assay buffer (89900, Thermo Fisher Scientific) supplemented with protease inhibitors (S8820, Sigma). Next, protein concentration was determined by means of a bicinchoninic acid protein assay. SDS/PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and immunoblotting were performed as described in (24).

Analysis of BODIPY-spermine Uptake and Redistribution via Immunocytochemistry. Cells were seeded at 50,000 cells/well in a 12-well plate with coverslips. Next day, cells were treated with 1 μM BODIPY-labeled spermine for 90 min at 37 °C. Afterward, cells were processed for immunocytochemistry as described in ref. 9. Samples were then mounted and images were acquired using an LSM780 confocal microscope (Zeiss) with the following settings optimized for the individual cell lines. Both for KOWT and KO/D508N, >75 images were used for analysis to analyze BODIPY-spermine mean fluorescence intensity within the mitochondrial network. (Fig. S4), images were taken with equal settings to enable a comparison of mitochondrial BODIPY-spermine intensity between the cell lines. Both for KOWT and KO/D508N, >145 individual cells were used for analysis.

Cell Viability Assay. Cell viability was assessed by means of a 4-methylumbelliferyl heptanoyl (MUH, M2514, Sigma) assay. Cells seeded at a density of 10,000 cells/well in a 96-well plate were washed with PBS (without Ca^2+ and Mg^2+) (Sigma) followed by incubation with 0.1 mg/ml MUH (30 min, 37 °C). End-point measurement was assessed using a Flex Station plate reader (Molecular Devices). Briefly, emission at 460 nm was measured upon excitation at 355 nm. 455 nm was taken as a cutoff value.

Determining Cell Death, MMP, MitoxANs, and Polyamine Uptake via Flow Cytometry. When measuring cell death, cells were collected, briefly centrifuged (450 × g, 5 min), resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS without Ca^2+ and Mg^2+, supplemented with 1% wt/vol bovine serum albumin) (BS5.4, Roche) and subsequently incubated with 2 μg/ml propidium iodide or 5 nM SYTOX Red (10 min, 4 °C) before quantifying the mean fluorescent intensity with either the Attune Cytometer (Life Technologies) for one-dye experiments or the Canto II AIG equipped with BD FACSDiva software version 6 (BD Bioscience) for simultaneous detection of two dyes. When analyzing MMP, MitoxANs, MitoxANs, or BODIPY-labeled polyamine uptake, cells were treated with 0.1 μM tetrathiomolybdate methyl ester (35 min, 37 °C), 10 μM DCFDA (35 min, 37 °C) (CM-H2DCFDA, Thermo Fisher Scientific), 3 μM MitoxANs (35 min, 37 °C) (M36008, Thermo Fisher Scientific), or 5 μM BODIPY-labeled polyamine (2 h, 37 °C), respectively. Next, cells were immediately collected and further processed for flow cytometry analysis as described above, with (Fig. S6) or without the SYTOX Red incubation step.
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