Mitochondrial membrane proteins and VPS35 orchestrate selective removal of mtDNA.

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Abstract

Integrity of mitochondrial DNA (mtDNA), encoding several subunits of the respiratory chain, is essential to maintain mitochondrial fitness. Mitochondria, as a central hub for metabolism, are affected in a wide variety of human diseases but also during normal ageing, where mtDNA integrity is compromised. Mitochondrial quality control mechanisms work at different levels, and mitophagy and its variants are critical to remove dysfunctional mitochondria together with mtDNA to maintain cellular homeostasis. Understanding the mechanisms governing a selective turnover of mutation-bearing mtDNA without affecting the entire mitochondrial pool is fundamental to design therapeutic strategies against mtDNA diseases and ageing. Here we show that mtDNA depletion after expressing a dominant negative version of the mitochondrial helicase Twinkle, or by chemical means, is due to an exacerbated mtDNA turnover. Targeting of nucleoids is controlled by Twinkle which, together with the mitochondrial transmembrane proteins ATAD3 and SAMM50, orchestrate mitochondrial membrane remodeling to form extrusions. mtDNA removal depends on autophagy and requires the vesicular trafficking protein VPS35 which binds to Twinkle-enriched mitochondrial subcompartments upon mtDNA damage. Stimulation of autophagy by rapamycin selectively removes mtDNA deletions which accumulated during muscle regeneration *in vivo*, but without affecting mtDNA copy number. With these results we unveil a new complex mechanism specifically targeting and removing mutant mtDNA which occurs outside the mitochondrial network. We reveal the molecular targets involved in a process with multiple potential benefits against human mtDNA related diseases, either inherited, acquired or due to normal ageing.

38 INTRODUCTION

39 The accumulation over time of mutations in the mitochondrial genome (mtDNA) is a common
40 process which has been shown to occur in many tissues ¹ and is probably one of the hallmarks
41 of aging ². MtDNA is present in thousands of copies per cell, hence, impairment of mitochondrial
42 function is observed only when the percentage of mutated mtDNA molecules surpasses a
43 specific threshold³.

44 Cells possess a plethora of quality control mechanisms to survey the intactness of DNA, RNA
45 and proteins, but also of entire organelles. In addition to bulk autophagy, which is responsible
46 for the continuous and non-selective turnover of cellular material activated during nutrient
47 shortage, specific mechanisms are initiated to remove malfunctioning organelles upon damage.
48 The process of mitophagy has been investigated extensively in recent years as an important
49 salvage pathway to remove dysfunctional mitochondria ⁴. The ongoing fission and fusion events
50 of the dynamic mitochondrial network are important processes in order to survey mitochondrial
51 quality by predisposing those parts of the network with impaired function to degradation.
52 Mitochondrial fission is a requirement to initiate mitophagy of damaged mitochondria ⁴, whereas
53 knockout of key players of mitochondrial fusion has been shown to induce mtDNA instability,
54 either by causing a rapid accumulation of mtDNA alterations over time ⁵ or by mtDNA depletion
55 ⁶.

56 Recently, a process with a high level of specificity involving mitochondrial derived vesicles
57 (MDVs) was shown to remove not the complete organelle, but rather mitochondrial fragments
58 containing specific cargo^{7,8}. This mechanism requires the coordination of mitochondrial
59 dynamics, mitophagy and also the vacuolar protein sorting (VPS) or retromer complex. In this
60 process, changes in the mitochondrial membrane potential and the oxidation state of
61 mitochondrial subcompartments induce the curvature of the membrane which is followed by
62 recruitment of PINK1 and PARKIN ⁹. The retromer complex, formed by VPS26, VPS29 and
63 VPS35, provides the force to generate a vesicle which is then delivered to lysosomes or
64 peroxisomes in a process which is independent of the autophagy proteins ATG5 or LC3 ¹⁰⁻¹². In
65 summary, mitophagy and its variants are crucial pathways to degrade parts of the mitochondrial
66 network, thus maintaining cellular fitness.

67 Many inherited forms of neurodegenerative diseases are examples for insufficient mitochondrial
68 quality control. Mutations of specific receptors involved in targeting dysfunctional organelles to
69 mitophagy like PINK1 and PARKIN, but also malfunction of lysosomal proteins ATP13A2 and
70 LAMP3 among many others, as well as mutations in the retromer component *VPS35* ¹³ cause
71 familial forms of Parkinson's disease. Parkinson's disease is caused by the specific
72 degeneration of dopaminergic neurons which have been comprehensively shown to be a
73 hotspot for the accumulation of large scale mtDNA deletions during normal aging ^{14,15} making
74 mitochondrial quality control mechanisms especially important in these cells. Unfortunately,
75 these mechanisms are not sufficient to counteract the often progressive phenotype of patients
76 suffering from PD and other diseases caused by either maternally inherited or by acquired
77 mtDNA alterations, the latter due to mutations in proteins essential for mtDNA replication and

maintenance¹⁶. Thus, therapeutic approaches to increase mitochondrial quality control and counteract the progression of mtDNA related diseases have been attempted^{17,18}, but a lack of specificity and activation of undesirable effects are still a hallmark¹⁹. Therefore, understanding the specific mechanisms governing mtDNA turnover is pivotal to develop new therapeutic strategies against these syndromes.

Expressing the mitochondrial helicase Twinkle bearing dominant negative mutations causing mitochondrial disease in patients induces mtDNA instability²⁰. Expression of the disease related *PEO1* Twinkle mutation K320E (from now on K320E) in mouse models accelerates the accumulation of mtDNA deletions in postmitotic tissues like heart and muscle^{21,22} during aging and induce mtDNA depletion in proliferating cells such as epidermis and cartilage^{23,24}. Recently, it has been shown that muscles from those mice accumulated deletions, but the main alterations were gene duplications, leading to a vast variety of different enlarged and rearranged molecules²⁵. Using a combination of *in vivo* and *in vitro* approaches, we have now identified the proteins involved in a new mechanism for targeting and specific degradation of mtDNA to avoid the accumulation of such mutations. Expression of K320E induces the formation of mitochondrial extrusions, much larger than MDVs, which are consecutively engulfed by autophagosomes and fused with lysosomes. Elimination of altered mtDNA molecules is preceded by the relocation of nucleoids to the poles of mitochondria, a process controlled by the interaction between the mitochondrial inner membrane protein ATAD3 and the nucleoid protein Twinkle. The translocase protein SAMM50 and VPS35 are essential to provide the required selectivity and specificity for mtDNA elimination. We show that stimulation of autophagy by rapamycin *in vivo* is sufficient to specifically remove deleted mtDNA, but without affecting the total mtDNA copy number. Thus, modulation of autophagy *in vivo* can be used as an approach to counteract the accumulation of mutations in mtDNA observed in several mitochondrial pathologies and during aging.

RESULTS

Accumulation of mtDNA mutations in skeletal muscles *in vivo* does not induce mitophagy

In a previous work we have found that, in extraocular muscles, which are first affected in *Peo1* patients, mtDNA alterations preferentially accumulated in fast twitch in contrast to slow oxidative fibers, indicating important differences in mitochondrial quality control mechanisms in different muscle fiber types²². To study the nature of these mechanisms surveillant mtDNA integrity, we first analysed fast-twitch M. tibialis anterior (TA) and slow-oxidative M. soleus (SOL), muscles both rich in fibers with a high mitochondrial content in mice, but with a preferentially glycolytic (TA) vs. oxidative metabolism (SOL), respectively. As shown before by deep sequencing²⁵, both muscles showed an accumulation of many mtDNA alterations in 24 months old animals (Fig. 1a). K320E mutant mice carry a wide variety of reorganized mtDNA molecules²⁵, causing an inefficient amplification reaction leading to a smear of products, however there were no changes

118 in total mtDNA copy number (Fig. 1b). Noteworthy, TA from aged wt mice also showed many
 119 mtDNA alterations, while only few were found in SOL. By conventional PCR, we analysed the
 120 presence of deletions common in aged mice²² and selected a deletion covering about 4000bp
 121 (*Mus musculus* mtDNA- $\Delta^{983-4977}$), which was present in both mutant muscles (Fig. S1a).
 122 Considering that mtDNA copy number is on average 20% higher in SOL than in TA (Fig. S1b),
 123 we performed qPCR quantification using the D-Loop region as a reference and found that
 124 indeed, this deletion was on average 20 times more abundant in TA compared to SOL (Fig. 1c).
 125 Steady state levels of common mitochondrial autophagy markers showed that in SOL of
 126 K320E^{skm} mice, the general autophagy adaptors p62 and LC3 were significantly decreased
 127 while levels were similar in TA (Fig. 1d, e). Levels of the specific mitophagy adaptor Optineurin
 128 (OPT) and the mitochondrial marker TOM20 were similar in all samples. *In situ*
 129 immunofluorescence of LC3 and p62 confirmed reduced puncta of those proteins (Fig. S1c-f).
 130 Since such a reduction could reflect both an increase or a decrease of autophagy flux, we
 131 blocked autophagy flux by chloroquine, and analysed the conversion of LC3-I to LC3-II (Fig. 1f,
 132 g). We did not observe an increased autophagy flux in K320E mice compared to control mice.
 133 However, in SOL for K320E mice, chloroquine was not inducing a change in the LC3 ratio
 134 suggesting that, in this muscle, autophagy flux was already at maximum level in steady state.
 135 We therefore hypothesized that oxidative fibres, which are most dependent on intact
 136 mitochondria, possess a faster mitochondrial turnover, hence maintaining mutated mtDNA
 137 molecules more efficiently below a pathogenic threshold. To visualize protein synthesis *in vivo*,
 138 we fed our mice with heavy lysine (¹³C₆-lysine) for two weeks in order to get an estimate for
 139 mitochondrial turnover rates²⁶. Here, we selected SOL and M. extensor digitorum longus (EDL)
 140 for analysis, since the latter contains almost exclusively glycolytic fibers. We first confirmed that
 141 heavy Lysine incorporation was equal in all animals (Fig. S2a, b). We analysed H/L (heavy/light)
 142 ratios of detected proteins (1286 and 1224 for SOL, 1042 and 1018 for EDL in wild-type and
 143 K320E^{skm} mice, respectively) and filtered the mitochondrial proteins using Mitocarta 3.0
 144 (Supplementary Table 1; Fig. S1c). Surprisingly, no mitochondrial protein was showing
 145 differential incorporation of heavy lysine comparing K320E mutant and wild-type animals,
 146 neither in EDL nor in SOL, suggesting that bulk mitochondrial turnover, here measured as an
 147 increase of mitochondrial biogenesis, is not enhanced in muscles bearing mtDNA alterations
 148 (Fig. 1h).

149 **Autophagy is required for depletion of mtDNA following oxidative damage**

151 To further dissect the molecular pathways activated upon mtDNA instability, we generated
 152 stable C2C12 myoblast cell lines expressing tagged versions of Twinkle and K320E.
 153 Colocalization of these variants with mitochondria (outer membrane marker TOM20, Fig. S3a,
 154 b) as well as colocalization with mtDNA and the mtDNA binding protein TFAM was confirmed
 155 (Fig. S3c, d), showing that Twinkle is enriched in mitochondrial nucleoids.
 156 To investigate if K320E expression leads to activation of autophagy or mitophagy in these cells,
 157 C2C12 myoblasts were additionally transfected with plasmids encoding LC3-GFP-mCherry or

158 Fis1p-GFP-mCherry. Expression of K320E induced the accumulation of autolysosomes marked
159 by LC3-GFP-mCherry (Fig. 2a, b; magenta signal). In agreement with our *in vivo* pSILAC
160 results, expression of Fis1p-GFP-mCherry, a mitophagy reporter, showed no activation of acute
161 mitophagy (Fig. 2c, d). Previous studies have demonstrated that the *in vitro* expression of
162 several Twinkle missense mutations often leads to accumulation of mtDNA replication
163 intermediates, producing mtDNA depletion²⁷. Consistently, expression of K320E leads to
164 mtDNA depletion (Fig. 2e), but this was not related to a decrease in mtDNA replication rate, as
165 observed by analysis of mtDNA replication foci in BrdU labelled cells (Fig. S4a, b). We
166 hypothesized that accumulation of lysosomes and mtDNA depletion after expressing K320E
167 was reflecting a novel selective mtDNA degradation process. Thus, we blocked lysosomal
168 activity using chloroquine and showed that, indeed, under these conditions, mtDNA levels
169 remained at control levels (Fig. 2f). Finally, we analysed the spatial relationship between
170 Twinkle foci and autophagy structures, using LC3 as an autophagosome marker and Lamp1 as
171 a late endosomal marker, respectively. As expected, most wt Twinkle foci distributed in a
172 pattern reflecting the mitochondrial network and were independent of autophagy markers (Fig.
173 2g-j). However, colocalization with LC3 and LAMP1, respectively, was observed with K320E.
174 The increased mtDNA degradation flux in K320E cells suggests that expression of this
175 missense mutation harms mtDNA by unknown means. To visualize *in situ* mtDNA damage, we
176 searched for the presence of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a specific base
177 modification induced by reactive oxygen species²⁸. We could not observe any specific staining
178 with α -8-OHdG in steady state levels (Fig. S4c, d), however when cells were incubated in
179 presence of chloroquine, we detected a specific accumulation of 8-OHdG decorating the
180 mitochondrial network only in K320E expressing cells (Fig. 2k, l). In summary, our data indicate
181 that K320E induces mtDNA depletion in a lysosome-dependent manner without involving acute
182 mitophagy, pointing to a more specialized path.

183 To further analyse the contribution of autophagy to mtDNA turnover, we turned to Atg5 KO
184 MEFs and expressed Twinkle variants (Fig. 3a), since Atg5 has been shown to be important in
185 quality control after mitochondrial damage²⁹. Analysis of mitochondrial nucleoids showed that
186 K320E expression decreased nucleoid foci number in Atg5 wt cells (Fig. 3b). In contrast, in Atg5
187 KO cells, foci number was not altered by K320E expression (Fig. 3c). Nevertheless, expression
188 of K320E reduced mtDNA copy number in both Atg5 WT and Atg5 KO cells (Fig. 3d, e).
189 Analysis of mtDNA replication by BrdU labelling showed that, in contrast to wt cells, expression
190 of K320E in autophagy deficient cells led to a reduced number of foci replicating mtDNA within
191 the mitochondrial network (Fig. 3f, g). Chloroquine treatment also restored mtDNA copy number
192 in Atg5 WT cells expressing K320E but not in Atg5 KO cells (Fig. 3h). Our data suggest that in
193 contrast to wt cells where K320E induces mtDNA depletion linked to an increased nucleoid
194 turnover rate, in autophagy deficient cells, mtDNA depletion is caused by reduced mtDNA
195 replication, presumably to avoid accumulation of excessive mtDNA damage.

196 Therefore, to evaluate the role of autophagy in mtDNA depletion, we induced mtDNA alterations
197 by growing these cells for 7 days in medium containing Ethidium Bromide (EtBr), which we also

found to provoke the accumulation of 8-OHdG within the mitochondrial network (Fig. S4c, e). Morphological analysis of mitochondrial nucleoids showed that EtBr decreases foci number in Atg5 WT cells but not in Atg5 KO (Fig. 3i-k). In line with this, Atg5 control cells showed consistent mtDNA depletion, whereas in Atg5 KO cells mtDNA copy number remained unchanged (Fig. 3l). This EtBr-induced depletion could not be recovered by overexpression of wt Twinkle, indicating again a prominent role of autophagy in mtDNA depletion in this case. All these data together confirm that mtDNA damage induces activation of a mtDNA turnover mechanism, which is dependent on autophagosome formation and specifically degrades mtDNA but it does not activate an acute mitophagy response.

mtDNA instability induces the formation of mitochondrial protrusions prior to lysosomal degradation

One of the most surprising features we noticed while studying cellular localization of Twinkle was that TOM20, a mitochondrial outer membrane marker, does not consistently overlap with Twinkle, a mitochondrial matrix protein (Fig. 4a and S3a, b). Live imaging microscopy using C2C12 clones expressing Twinkle-mCherry showed that occasionally, Twinkle moved to Mitotracker Green-negative poles of the network releasing a small particle which was also Mitotracker-negative (Fig. 4b, arrows). These particles were more evident in K320E expressing cells, where most of K320E was residing in such Mitotracker-negative structures (Fig. 4c). As Mitotracker green is independent of the inner membrane potential, we ruled out the possibility that these regions were depolarized areas of the mitochondrial network but mitochondrial regions with different cardiolipin composition.

To determine Twinkle localisation with high resolution, we used Twinkle-APEX2 fusion versions in C2C12 cell lines. APEX2 generates a black precipitate in the presence of DAB, which is visible by electron microscopy. Twinkle-APEX2 distribution in the matrix was heterogeneous and the organization of mitochondria and other cellular organelles was undisturbed by its expression (Fig. 4d). K320E-APEX2, however, accumulated in poles of the network, sometimes close to abnormal cristae structures (Fig. 3d, red arrows) and also inside lysosomes (Fig. 4d, red stars). Such multi-membrane structures were also observed in cells expressing a mitochondrial matrix targeted APEX2, but in that case, they did not show DAB precipitate (Fig. 4d, red stars). A very recent study demonstrated that mitochondrial fission occurring at mitochondrial poles is followed by mitophagy of mitochondrial compartments containing non-replicative mtDNA, and that this is preceded by mitochondrial cristae reorganization³⁰. Indeed, electron tomography of K320E expressing cells revealed that these structures are derived from the inner mitochondrial membrane and that they are formed by protrusions containing reorganized mitochondrial cristae (Fig. 4e).

Twinkle interacts with membrane proteins to facilitate mtDNA removal

We speculated that mtDNA targeting prior to degradation is controlled by specific protein-protein interactions. Thus, as Twinkle follows mtDNA positioning and degradation through lysosomes,

we sought to investigate how nucleoids containing Twinkle distribute in the mitochondrial matrix by analysing the Twinkle interactome. We performed immunoprecipitation of wt Twinkle fused to a V5-APEX2 epitope followed by mass spectrometry analysis. As expected, the majority of proteins interacting with Twinkle were related to mtDNA replication, transcription and translation (Supplementary Table 2), but only a few interactions were found to be significant (Fig. 5a, blue dots), one of them being ATAD3, an AAA-ATPase previously linked to anchoring and distribution of mtDNA to the inner membrane³¹. Direct co-immunoprecipitation and immunofluorescence confirmed the physical interaction between ATAD3 and Twinkle (Fig. 5b; Fig. S5a) enlightening Twinkle as an important part of the link between mitochondrial nucleoids and the inner membrane.

To evaluate the role of these proteins in mtDNA turnover, we generated constitutive *Atad3* knock-down (KD) clones (shAtad3_1+2, shAtad3_3; Fig. S5b, c). *Atad3* KD clones showed steady state levels of mtDNA copy number comparable to control lines (Fig. 5c, d) and no changes in mitochondrial morphology (Fig. S5d). After one week of mild treatment with EtBr, control cells showed a persistent decrease of mtDNA copy number. However, this depletion was not observed in *Atad3* KD clones (Fig. 5d). Number of nucleoid foci per cell was also higher in downregulated clones than in control cells upon EtBr treatment (Fig. 5e). These data suggest that nucleoid binding to the mitochondrial inner membrane through a Twinkle-ATAD3 interaction is essential for mtDNA elimination upon mtDNA damage.

257

258 **mtDNA turnover requires coordination of the retromer complex in a process independent** 259 **of MDVs**

We initially hypothesized that selective removal of mitochondrial fragments containing mtDNA was carried out through MDVs⁷. The E3 Ubiquitin ligase MAPL has been shown to direct mitochondrial cargo to peroxisomes³², while the endosomal adaptor Tollip specifically divert MDVs to lysosomes⁸. In both cases, the VPS35-retromer complex initiates the force to generate a vesicle¹⁰. Thus, in order to investigate if the specific mtDNA degradation we observed follows the MDV or a new pathway, we studied VPS35 and MAPL localization in Twinkle-mCherry expressing cells. Consistent with previous studies, expression of MAPL-GFP induced mitochondrial fission and formation of structures excluding TOM20 (Fig. S6a)³². However, Twinkle was also excluded from those particles. Chloroquine treatment for 4h was sufficient to induce a rapid accumulation of Twinkle positive structures excluding TOM20 and MAPL, suggesting that the fate of those particles is the lysosomal compartment (Fig. S5b). In addition, we also corroborate that Twinkle colocalizes with VPS35 but excludes MAPL (Fig. S6c, d).

Next, we treated these cells as well with EtBr to induce mtDNA damage. One week of treatment was sufficient to reduce mtDNA copy number (Fig. S7a). Image quantification revealed that the percentage of VPS35 containing Twinkle was strongly increased upon EtBr treatment (Fig. 6a-c), even when the overall steady state levels of VPS35 structures did not change (Fig. 6d). Importantly, VPS35 also colocalized with dsDNA (Fig. 6e).

278 We then generated Vps35 KO clones in MEFs and selected two monoclonal lines targeting
279 exon 4 (*Vps35_Ex4*) and exon 5 (*Vps35_Ex5*) (Fig. S6b, c). Vps35 KO cells showed no
280 changes in mtDNA foci number but reduced levels of mtDNA copy number in steady state (Fig.
281 6f-h) and mitochondrial fragmentation (Fig. S7d). After 7 days of EtBr treatment, the number of
282 mtDNA foci was further decreased (Fig. S7e, f) and mtDNA depletion was enhanced (Fig. S7g,
283 h). We analysed the activation of canonical mitophagy in steady state by expressing Fis1p-
284 GFP-mCherry (Fig. 6i) and found that, indeed, in Vps35 KO cells, acute mitophagy was
285 activated (Fig. 6j). Moreover, VPS35 protein level was shown to be affected by lysosomal
286 function, as chloroquine induced VPS35 accumulation in K320E cells (Fig. S7i, j) without
287 interfering with late endosomes or mitochondrial content (Fig. S7i-n).

288 We hypothesize that VPS35 is necessary to fine-tune the elimination of mutated mtDNA without
289 activating acute mitophagy and sought to investigate how VPS35 is recruited to mitochondria
290 upon mtDNA damage. Thus, we performed VPS35 IP followed by MS analysis under basal
291 conditions and in cells treated 7 days with EtBr (Fig. S8a). As expected, in basal medium the
292 protein profile of VPS35 IP revealed poor interaction with mitochondrial proteins (Fig. 7a, red
293 dots). Upon EtBr treatment, however, the association of mitochondrial proteins was markedly
294 increased (Fig. 7b, c). Among them, we noticed the presence of mitochondrial membrane
295 proteins such as VDAC1 and VDAC3, TIMMDC1 or TOMM40 as well as SAMM50, which
296 interaction was confirmed by coimmunoprecipitation (Fig. 7d). The comparison of IP protein
297 profiles of cells in basal vs. EtBr medium highlighted also a strong enrichment of the mAAA
298 protease SPG7 (Fig. S8b). Remarkably, comparison between the IP protein profiles from
299 Twinkle and K320E revealed also a strong enrichment of SAMM50 upon K320E
300 immunoprecipitation (Fig. 7e). SAMM50 belongs to the sorting and assembly machinery in the
301 mitochondrial intermembrane space and has important functions in the biogenesis of respiratory
302 complexes, cristae morphology and mitochondrial shape³³. It was also described as a regulator
303 of PINK1-Parkin mediated mitophagy but excluding the mtDNA for degradation³⁴.
304 Immunoprecipitation of Twinkle confirms that, after mtDNA damage with EtBr, Twinkle can
305 physically interact with SAMM50 (Fig. 7f).

306 Thus, we hypothesize that SAMM50, a protein located in the mitochondrial outer membrane,
307 could serve as a platform to recruit VPS35 and eliminate mitochondrial subcompartments
308 containing damaged mtDNA. Consequently, we generated SAMM50 shRNA KD clones and
309 expressed Twinkle-mCherry (Fig. 7g; Fig. S8c, d). EtBr does not affect the overall population of
310 VPS35 but enhances recruitment to Twinkle (Fig. 7g-i). However, the percentage of VPS35-
311 Twinkle contacts were reduced in absence of SAMM50 (Fig. 7i). To decipher if VPS35
312 recruitment shows Twinkle specificity, we examined VPS35 colocalization with LRPPRC, a
313 native mitochondrial matrix protein (Fig. S8e). Again, the overall number of VPS35 particles
314 was unchanged by EtBr treatment (Fig. S8f) and in this case, we observed that VPS35 contacts
315 with LRPPRC were not increased and therefore, there was no effect following Samm50
316 downregulation (Fig. S8g).

317 In summary, our data thus demonstrate that VPS35 is required for mitochondrial quality control
318 in the presence of mtDNA alterations. We show that a mechanism to specifically remove
319 mtDNA is activated when mtDNA damage is induced both genetically or chemically by K320E or
320 by EtBr, respectively. ATAD3 is required for specific membrane localization of nucleoids,
321 SAMM50 confers nucleoid specificity and VPS35 is involved in fine-tuning of this selective
322 process, removing specific parts of mitochondria containing affected nucleoids, thus avoiding
323 the activation of acute mitophagy which would affect the functional mitochondrial pool.

324

325 **Rapamycin eliminates mtDNA alterations without affecting copy number**

326 Since our data highlight that autophagy, together with a specific nucleoid extraction mechanism,
327 plays an important role in maintaining mtDNA fitness *in vitro*, we aimed to test whether this is
328 also relevant *in vivo*. We have previously shown that expression of K320E in skeletal muscle
329 leads to the accumulation of mtDNA alterations, unfortunately only in very old animals, making
330 this model not very convenient ²² (Fig. 1). However, expression of K320E in muscle satellite
331 cells (Pax7-Cre^{ERT}; K320E^{msc}), followed by cardiotoxin-induced muscle damage and one week
332 of regeneration, shows a rapid accumulation of mtDNA alterations, leading to newly generated,
333 cytochrome-c-oxidase (COX) negative fibers (stained blue, Fig. S8a, b), while mtDNA copy
334 number remained stable (Fig. S8c). In several mitochondrial disease models, it has been shown
335 that rapamycin, an activator of autophagy through the specific inhibition of mTORC1, is able to
336 revert mitochondrial dysfunction and ameliorate disease progression. This probably occurs by
337 stimulating mitochondrial turnover mechanisms ³⁵, and, *in vitro* it was also shown to direct
338 selection against mtDNA mutations ³⁶. Interestingly, genetic induction of autophagy was shown
339 to reduce mtDNA deletions in *Drosophila* ³⁷. Thus, to test if activation of autophagy can purify
340 mtDNA alterations also in mammals *in vivo*, we used this muscle regeneration paradigm in
341 combination with rapamycin treatment. Regenerated muscles from vehicle treated K320E^{msc}
342 mice showed a prominent accumulation of COX negative fibers indicating mitochondrial
343 dysfunction (Fig. 8a). In contrast, mice treated with rapamycin showed much less COX deficient
344 cells in the regenerated area (Fig. 8a-c). Consistently, qPCR analysis revealed that mtDNA
345 copy number remained unchanged (Fig. 8d), while mtDNA alterations were absent and thus had
346 been purified (Fig. 8e). We noticed that COX staining was much lighter in rapamycin treated
347 animals, suggesting a change in mitochondrial OXPHOS activity. Vehicle treated mice showed
348 a predominant accumulation of glycolytic fiber type 2b in both wt and K320E^{msc} mice (Fig. S9d),
349 while in rapamycin treated wt mice, fiber type staining showed a predominant shift towards
350 mitochondria rich type I fibers (Fig. S9e). Interestingly, in K320E^{msc} mice regenerated fibers
351 showed a mixed myosin heavy chain pattern after one week of regeneration. These data
352 indicate that rapamycin can be used as a modulator of mitochondrial turnover which specifically
353 purifies mutated mtDNA species, thus ameliorating mitochondrial dysfunction, albeit changing
354 muscle fiber type composition.

355

356 **Discussion**

Autophagy and specifically mitophagy and its variants are well-established pathways for mitochondrial turnover, which is essential to maintain mitochondrial fitness⁴. Loss of mitochondrial quality control mechanisms, either by specific mutations of key players or by reduced autophagy activity, strikingly correlates with the acquirement of mtDNA mutations³⁸. However, an exacerbated activation of mitophagy may lead to a serious reduction of the mitochondrial pool, thus affecting cellular energy supply³⁹. Therefore, the fine-tuned regulation of mitochondrial quality control mechanisms is essential to maintain cellular energy homeostasis.

Although toxic substances can cause mitochondrial damage, the most prevalent reason is the accumulation of alterations in mtDNA due to replication errors. Mutations in genes encoding proteins involved in mtDNA replication, like the helicase Twinkle, and in mtDNA maintenance cause mutations leading to mitochondrial diseases, with brain and skeletal muscle being regularly affected. In addition, somatic mutations in mtDNA accumulate during the normal aging process in many organs in humans³, leading to a tissue mosaic where few cells with mitochondrial dysfunction, caused by high mutation loads, are embedded in normal tissue⁴⁰.

In general, tissues most depending on mitochondrial function are most severely affected when carrying mtDNA mutations. Paradoxically, we found that expression of the dominant negative K320E mutation of Twinkle in extraocular muscle shows a remarkable differential vulnerability of muscle fiber types, with mitochondrial dysfunction especially affecting fibers with a glycolytic metabolism²². In agreement with these results, we found less mtDNA alterations in aged SOL, a muscle rich in type I fibers which mostly rely on mitochondrial ATP production, compared to the TA mostly composed of fast twitch, glycolytic fibers (Fig. 1). Noteworthy, different muscles rich in oxidative vs. glycolytic fibers show notable differences in the expression of genes involved in mitochondrial dynamics⁴¹, making oxidative muscles more resistant to ageing related dysfunction⁴². In fact, our data shows that SOL expressing K320E already has an increased flux in steady state but this was not related to increased mitochondrial turnover. Nevertheless, mitophagy, understood as the specific removal of the entire damaged organelle, does not provide the required selectivity to remove only mutated mtDNA. Hence, the existence of a specific turnover mechanism has been postulated, but not been proven yet⁴³.

In contrast to terminally differentiated muscle of aged mice, proliferating cells in culture did not accumulate mtDNA alterations upon expression of K320E, instead this led to mtDNA depletion. Both K320E expression or mild treatment with EtBr induced the accumulation of mtDNA damage. EtBr intercalates between base pairs and slows down mtDNA replication. It is accepted that EtBr, when used at low concentration, also induces frame shift mutations and deletions⁴⁴ and we demonstrated that it also triggers oxidative damage in the mtDNA. Our data show that mtDNA depletion induced by damage is caused by a specific exacerbated mtDNA turnover which is Atg5 dependent and thus requires autophagosome formation and lysosomal degradation.

Interestingly, K320E localizes preferentially in specific mitochondrial regions. K320E localization does not overlap completely with the mitochondrial outer membrane protein TOM20

and is present in areas that are also not stained by Mitotracker Green, demonstrating that nucleoids containing Twinkle can be specifically localized in a unique mitochondrial subcompartment. Indeed, mtDNA has been shown to attach preferentially to cholesterol-rich membrane structures and Twinkle, as a nucleoid protein, has been found to be enriched in these areas as well ⁴⁵. The onion-like structures we observed in our cell model have been detected in the muscle of patients with mitochondrial myopathy caused by mtDNA mutations ⁴⁶ and very recently also in ρ^0 -cells lacking mtDNA ⁴⁷. Here we show that mtDNA damage induces cristae remodelling in poles of the mitochondrial network and that these structures containing Twinkle are delivered to lysosomal compartments, which are therefore specifically involved in mtDNA removal.

Three proteins present in three mitochondrial compartments are responsible for mtDNA distribution and selective turnover: Twinkle, in the mitochondrial matrix; ATAD3, in the mitochondrial inner membrane and SAMM50, in the mitochondrial outer membrane. Twinkle arises as the link between nucleoids and the inner membrane through interaction with ATAD3, a protein controlling several aspects of mitochondrial membrane dynamics ⁴⁸. Interestingly, the human ortholog ATAD3B has been recently found to be a mitophagy receptor for mtDNA damage induced by oxidative stress ⁴⁹. In the other side, SAMM50, which resides in the mitochondrial outer membrane, interacts with the MICOS complex, and organizes membrane architecture ⁵⁰. Interestingly, both ATAD3 and SAMM50 have been described as regulators of Pink1-Parkin as well ^{34,51}, thus providing the link between nucleoid localization and specific degradation of mtDNA.

Furthermore, we show that upon mtDNA damage, VPS35, an endosomal protein involved in mitochondrial quality control, increases contacts to mitochondrial subcompartments containing Twinkle. Interestingly, VPS35 deficient cells showed a persistent activation of mitophagy with mtDNA depletion. The fact that *Samm50* depletion induces mitophagy, but excludes mtDNA degradation ³⁴, suggests that SAMM50 confers the required selectivity and VPS35 the specificity to remove only fragments containing mtDNA.

Removal of mitochondrial fragments has been shown to take part through a specialized pathway called Mitochondrial Derived Vesicles (MDVs) ⁷. Currently, only a few proteins have been found to determine cargo and vesicle fate. While MAPL generates vesicles which divert oxidized cargo to peroxisomes ³², Tollip, an endosomal organizer, synchronizes Parkin-dependent MDVs directed to the lysosomal compartment ⁸. Canonical MDVs are generated in the mitochondria in an Atg5 and LC3 independent manner ³², however, mtDNA damage by K320E expression showed accumulation of LC3 autophagosomes and mtDNA depletion which is Atg5 dependent. Additionally, electron microscopic pictures of our cells never showed vesicles resembling MDVs, which are much smaller than the mitochondrial extrusions and autolysosomes containing Twinkle we observed. Furthermore, we unequivocally showed that Twinkle particles are directed to the lysosomal compartment and that VPS35 associates with mitochondria upon mtDNA damage. We also showed that VPS35 recruitment is SAMM50 dependent and, upon mtDNA damage, such recruitment is specific to Twinkle containing

regions. Upon EtBr damage, we confirmed that VPS35 precipitates with mitochondrial outer membrane proteins, such as SAMM50, TOMM40, TIMMDC1 and VDAC1 and VDAC3 and it is in this environment when Twinkle, a mitochondrial matrix protein, interacts with SAMM50. mtDNA removal exemplifies a specialized mitophagy trail. Delivery of specific mitochondrial cargo to quality control pathways through VPS35 was previously shown to be highly regulated. If the selected cargo is diverted into peroxisomes, VPS35 associates with MDVs by interacting with MAPL in a Drp1 independent manner¹⁰. However, if the MDVs are directed to lysosomes the association is dependent on Drp1⁵². On the other hand, SAMM50 modifies mitochondrial structure by interacting with Drp1⁵³ and with the Pink1-Parkin machinery for selective mitophagy³⁴. Hence, SAMM50 works as a platform where mitochondrial fission, membrane architecture, and mitophagy components orchestrate membrane protrusions and facilitate VPS35 recruitment. Very recently, SAMM50 has been linked also to piecemeal mitophagy, independent of MDVs, through direct interaction with the p62 adaptor⁵⁴. Hence, we conclude that specific mtDNA degradation does not follow the MDV pathway, despite the fact that they share some components, and that it is more likely that mitochondrial protrusions containing nucleoids are engulfed in autophagosomes in a specialized mitophagy pathway. VPS35, which has been extensively linked to neurological diseases such as Parkinson's and Alzheimer disease, appears as a regulator of mtDNA quality control necessary to maintain mitochondrial intactness. Modulation of *VPS35* expression has been evaluated as a potential approach against Parkinson's disease⁵⁵ and, in *Drosophila*, *Vps35* overexpression can rescue an LRRK2-induced Parkinson's phenotype⁵⁶. iPSC derived neurons from *LRKK2*-PD patients showed accumulation of mtDNA damage⁵⁷. Nonetheless, the ability of VPS35 to eliminate such molecules in a human disease related model needs to be further explored as a potential therapeutic strategy. Enrichment of Spg7 upon VPS35 IP in EtBr treated cells also suggests the involvement of mitochondrial proteases. Mitochondrial matrix proteases (mAAAs) represent a group of enzymes related to mitochondrial quality control and mitochondrial membrane remodeling upon proteolytic cleavage of Opa1 and Oma1. Spg7 has been found to copurify with Prohibitin participating in the formation of the permeability transition pore^{58,59}. Mutations in the *SPG7* gene are the cause for Hereditary Spastic Paraplegia Type 7 but also a Progressive External Optalmoplegia-like syndrome with accumulation of mtDNA deletions⁶⁰. Hence, it is tempting to propose that Spg7 works as a regulator of mtDNA turnover as well. However, further studies are needed to reveal its specific role and regulation of the process. Finally, our data demonstrate that specific removal of mtDNA is linked to lysosomal activity. Lysosomal degradation of bulk autophagy is regulated by the serine-threonine protein kinase mTORC1 which resides on the lysosomal surface. mtDNA replication defects activate mTORC1 and the integrated mitochondrial stress response in a cascade of effects with wide downstream consequences⁶¹. It is well known that mTORC1 activation inhibits autophagy by influencing both the formation of autophagosomes but also lysosomal acidification⁶². In order to prove the selectivity of this process *in vivo*, we used a mouse model where mtDNA alterations rapidly

accumulate and indeed, activation of the mTORC1 pathway by rapamycin was able to eliminate abnormal mtDNA molecules and thus reduces the accumulation of cells with mitochondrial dysfunction. Rapamycin has been described as a potential treatment against mitochondrial diseases^{18,63}, however, since we observed a fiber-type shift, mTORC1 inhibition might activate other signalling pathways with undesirable effects. Nonetheless, by using Rapamycin we demonstrated that elimination of mutant mtDNA without affecting total mtDNA copy number is possible.

In conclusion, we unveil a new complex mechanism with physiological relevance for mitochondrial fitness. Twinkle mediates nucleoid binding to the mitochondrial inner membrane through ATAD3 interaction, which is responsible for nucleoid organization. SAMM50 provides the required specificity to eliminate mtDNA while VPS35 supplies the selectivity. Interestingly, mutations in *TWINK*, *ATAD3A* and *VPS35* have been linked to several severe mitochondrial diseases having in common mtDNA instability⁶⁴⁻⁶⁶, therefore representing a cluster of proteins involved in specific mtDNA turnover. Fine tuning the activity of such proteins could be used as a therapeutic strategy against mtDNA related diseases, either inherited, acquired or due to normal ageing.

493

494 **Methods**

495

496 ***In vivo* experimental approaches**

497 K320E transgenic mice were generated by crossing R26-K320EloxP/+ mice (point mutation K320E; Rosa26-Stop-construct; downstream EGFP) with mice expressing Cre recombinase under the control of the skeletal muscle-specific MLC1f- promoter or satellite cells Pax7-Cre^{ERT}. 500 All mice used for experiments were housed in a standard animal facility maintained at 23°C, 501 12:12h light-dark cycle, with free access to water and standard rodent chow. Autophagy flux 502 was tested by intraperitoneal injection of 50mg/kg chloroquine 4h prior euthanasia. All 503 procedures and experimentation with mice were performed according to protocols approved by 504 the local authority (LANUV, Landesamt für Natur, Umwelt und Verbraucherschutz NRW, 505 approval number: 2019-A090). Activation of Pax7-Cre^{ERT} promotor was performed by injecting 506 daily, for 5 days, intraperitoneal 2mg tamoxifen dissolved in mygliol. For muscle regeneration 507 experiments, 2 days after the last tamoxifen injection, mice were anesthetized with 2%Xylazin, 508 10% Ketamine in NaCl 0.9% and 10µM Cardiotoxin (*Naja Pallida*, Latoxan) was injected inside 509 the fascia. After 2 days of rest, 2mg/kg rapamycin dissolved in mygliol was injected 510 intraperitoneally daily for 5 days. The day after the last injection, mice were treated as explained 511 below.

512

513 **Molecular biology and vector generation**

514 *Twink* ORF was amplified from plasmids pJet2-Twinkle and pROSA-K320E⁶⁷ and cloned in 515 pmCherry-N1. For retroviral vector generation, Twinkle and Twinkle-mCherry, ORF was 516 amplified and subcloned into pLenti-CMV Puro DEST (Addgene #17452). All ORFs were

subcloned into pBabe-Puro vector, kindly provided by Dr. Bernhard Schermer, and verified by Sanger sequencing. pLX304-TWINKLE-APEX2 vector was kindly provided by Dr. Alice Ting. To generate *Mus musculus* *Twink* vectors, *Homo sapiens* *TWINK* was replaced by *Twink* ORF and subcloned into pBabe-Puro vector. Mitochondrial matrix APEX2 control vector (Addgene #72480) was also subcloned into pBabe-Puro. For generation of Tfam-GFP, total RNA from mouse liver was isolated and converted into cDNA using TRIzol (Thermo Fisher) and RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) using PolyT as feeder. *Tfam* cDNA was cloned into pEGFP-N1. Atad3 KD and Samm50 KD clones were generated by transducing MEFs with the vector pMKO1-GFP (Addgene #10676) containing a shRNA (Table M1). Empty vector and a scramble containing vector were used as a control. For CRISPR Cas9 KO generation two gRNA directed to exon 4 and exon 5 (Table M1) were cloned in pSpCas9 (BB)-2A-Puro V2.0 (Addgene #62988).

529

530 **Generation and culture of cell lines**

C2C12 cell line was purchased from ATTC and grown in DMEM 4.5 g/L Glucose + GlutaMax, 20% FBS, 1x Pen/Strep. Immortalized mouse embryonic fibroblast Atg5WT and Atg5KO MEFs⁶⁸ and immortalized MEFs line were maintained as described⁶⁹. Stable cell lines were generated by transducing C2C12 cells or MEFs with pBabe-Puro retroviruses. Briefly, 2.5x10⁶ HEK293 cells were plated in 10cm² dish transfected with pCL-ECO (5 µg) and pBabe-Puro (10 µg) vectors using PEI (40 µl). After 48h and 72h, medium containing viruses was harvested, filtered through 45 µm, mixed with 8µg/ml Polybrene and added to 250.000 cells previously plated. 48h post transduction, positive clones were selected by adding Puromycin 2.5 µg/ml to the medium in C2C12 and 5µg/ml to MEFs, which was maintained during all the experiments. shRNA clones were generated by transducing MEFs with pMKO.1-GFP vectors. Prior to all experiments, transduction rate was verified to be higher than 99% of GFP expressing cells by Flow cytometry. For generation of Vps35 CRISPR Cas9 KO clones, MEFs were transiently transfected with the vector containing gRNA and selected with 3 µg/ml puromycin for 4 days. Single clones were plated independently using cloning cylinders, analyzed by western blotting. Genomic DNA was isolated from VPS35-negative cells and genomic DNA modification was verified by Sanger sequencing. Exon 4 and Exon 5 were amplified (Table M1) and cloned using pJET1.2 cloning kit (Thermo Fisher) before sequencing.

Transient transfection was achieved transfecting the corresponding plasmids using Lipofectamine 3000 following manufacturer instructions. Plasmids use in this work were: LC3-GFP (Addgene #21073), Lamp1-GFP (Addgene #34831), Fis1p-Cherry-GFP⁷⁰, LC3-Fis1p-Cherry (kindly provided by Dr. Terje Johansen) and MAPL-GFP from CECAD Imaging Facility³².

For mtDNA depletion experiments, cells were maintained as described before for 7 days but media was supplemented with 50 ng/ml Ethidium and 50 µg/ml Uridine. Lysosomal function was blocked using 10 µM Chloroquine for 24h.

556

557 **mtDNA amplification**

558 Total DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's
559 instruction. 25 ng of total DNA was used for analysis of threshold amplification differences
560 between mtDNA and nuclear DNA (delta C(t) method with specific primers (Table M1). Long
561 range PCR was used to screen for the presence of mtDNA alterations. 14 Kb of mtDNA was
562 amplified using Rabbit Bioscience Long Range kit with oligos described in table M1.

563

564 **Western blot and co-immunoprecipitation**

565 Cells pellets were lysed with RIPA-buffer (150 mM NaCl, 1% Triton-X1000, 0.1% SDS, 50 mM
566 Tris-HCl pH 8, 0.5% Na-deoxycholate) containing protease inhibitor (Roche) and protein
567 concentration measured using the Bradford assay. Proteins were transferred after
568 electrophoresis to a PVDF-membrane previously activated with methanol. Membranes were
569 blocked (5% milk in TBS-0.1% Tween-20) and incubated overnight with primary antibodies.
570 Antibodies used in this work are: monoclonal α -V5 (Abcam), polyclonal α -V5 (Thermo
571 Scientific), polyclonal α -TOM20 and monoclonal α -VPS35 (Santa Cruz), monoclonal and
572 polyclonal α -SAMM50 (Abnova and Abcam respectively), polyclonal α -ATAD3, polyclonal α -LC3
573 and polyclonal α -p62 (Proteintech), polyclonal GAPDH (Novus Biologicals). Secondary goat
574 anti-mouse, goat anti-rabbit and goat anti-chicken HRP (Jackson Laboratory). Images were
575 acquired using the ECL Advanced Chemiluminescence kit (GE Healthcare Life Sciences®, UK)
576 according to manufacturer's protocols and visualized using a LAS500 CCD camera.

577 For immunoprecipitation, cells expressing Twinkle-APEX2-V5 were pelleted and solubilized in
578 IP Buffer (500 mM HEPES KOH pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 1% Triton-X1000 and
579 Protease inhibitor (Roche). 500 μ g of total protein extract were used to IP with either 2.5 μ g V5
580 rabbit polyclonal antibody or VPS35 mouse monoclonal over night at 4°C and recovered after
581 incubating for 6h at 4°C in a rotator with equilibrated Agarose Protein-G beads (Abcam).
582 Immunoprecipitation followed by MS analysis was performed as described but using magnetic
583 Protein G beads (Thermo Scientific). Samples were washed 5 times with washing buffer
584 (10 mM HEPES KOH pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 0.2% Triton-X1000) and once with
585 PBS. Prior to analysis by MS, samples were washed 3x with ammonium-bicarbonate (ABC)
586 buffer, denatured with 50 μ l of urea buffer (6 M urea, 2 M thiourea) and followed by disulfide-
587 bridge reduction using dithiothreitol (DTT) at a final concentration of 5 mM for 1 hour at room
588 temperature. To alkylate oxidized cysteines, 2-Iodoacetamide (IAA) was added to the samples
589 until a concentration of 40 mM was reached and incubated for 30 min in the dark. Lys-C was
590 added in a ratio of 1:100 (0.1 μ g enzyme for 10 μ g protein) and incubated for 2-3 hours.
591 Samples were finally diluted with ABC buffer to reach 2M urea concentration. Protein digestion
592 was performed overnight with trypsin 1:100. Samples were acidified with 1% formic acid and
593 desalted using a modified version of the previously described Stop and Go extraction tip
594 (StageTip) protocol⁷¹.

595

596 **Pulsed SILAC labeling in mice and in-solution digestion**

For pulsed SILAC labeling mice, 30-40 weeks old mice for K320E; Mlc1 line (C57BL/6J) were fed a $^{13}\text{C}_6$ -lysine (Lys6)-containing mouse diet (Silantes) for 14 days to monitor newly synthesized proteins by comparing the incorporation of Lys-6 with the naturally occurring Lys-0⁷². Mice were sacrificed at the end of day 14 and tissues dissected and snap-frozen in liquid nitrogen. Samples were grinded and proteins extracted and denatured by the addition of 4% SDS in PBS. To remove residual SDS proteins were precipitated overnight in 4x ice-cold acetone (v:v). On the next day after centrifugation at 16,000 g for 10 min the protein pellets were dissolved in urea buffer (6 M urea / 2 M thiourea). The following protein digestion was performed as described previously but instead of overnight tryptic digestion proteins were only digested with Lys-C (1:100 enzyme-to-protein ratio) for both pre-digestion (2h at RT) and overnight digestion after dilution of urea using ABC buffer.

Liquid chromatography – mass spectrometric analysis

Both affinity-enriched and pulsed SILAC proteomics samples were analyzed in positive mode using data-dependent acquisition (DDA) either by an Easy-nLC 1000 – Q Exactive Plus or an Easy-nLC 1200 – Orbitrap Eclipse tribrid system (all Thermo Fisher). On-line chromatography was directly coupled to the mass spectrometric systems using a nanoelectrospray ionization source. Peptides were separated by reversed-phase chromatography with a binary buffer system of buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in 80% acetonitrile) using a 60 min chromatographic gradient for IP samples and 120 min for pulsed SILAC samples. Separation was performed on a 50 cm long in-house packed analytical column filled with 1.9 μM C18-AQ Reprosil Pur beads (Dr. Maisch). Using the 60 min chromatographic gradient peptide separation based on their hydrophobicity was performed by linearly increasing the amount of buffer B from initial 13% to 48% over 35 min followed by an increase of B to 95% for 10 min. The column was washed for 5 min and initial column conditions were achieved by equilibrating the column for 10 min at 7% B. Full MS spectra (300 to 1750 m/z) were acquired with a resolution of 70,000, a maximum injection time of 20 ms and an AGC target of 3e6. The top 10 most abundant peptide ions were isolated (1.8 m/z isolation windows) for subsequent HCD fragmentation (NCE = 28) and MS/MS recording at a resolution of 35,000, a maximum injection time of 120 ms and an AGC target of 5e5. Peptide ions selected for fragmentation were dynamically excluded for 20 seconds.

Using the 120 min chromatographic gradient peptides were separated by linearly increasing B from initial 4% to 25% over 96 min followed by an increase of B to 55% over 14 min. After a steep increase of B to 95% over 2 min the analytical column was washed for 8 min at 95% B. Full MS spectra (375 to 1500 m/z) were acquired with a resolution of 60,000, a dynamic injection time and an automated AGC target. The top 20 most abundant peptide ions (charge state 2 – 7) were isolated (1.2 m/z isolation windows) for subsequent HCD fragmentation (NCE = 30) and MS/MS recording at a resolution of 15,000, a maximum injection time of 22 ms and an automated AGC target. Peptide ions selected for fragmentation were dynamically excluded for 60 seconds.

637

638 **Data processing and analysis**

639 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
640 Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository ⁷³
641 with the dataset identifier PXD023939. All recorded RAW files were processed with the
642 MaxQuant software suite (1.5.3.8 for IP data, 1.6.14 for pSILAC) ⁷⁴. For peptide identification
643 and scoring MS/MS spectra were matched against the mouse Uniprot database (downloaded
644 08/15/2019) using the Andromeda search algorithm ⁷⁵. For the affinity-enriched samples
645 multiplicity was set to one and trypsin/P was selected as digestive enzyme.
646 Carbamidomethylation was set as a fixed modification and methionine oxidation or N-terminal
647 acetylation was selected as variable modification. Peptides were identified with a minimum
648 amino acid length of seven and a false-discovery rate (FDR) cut-off of 1% on the peptide level.
649 Proteins were identified with FDR < 1% using unique and razor peptides for quantification.
650 Label-free quantification was performed using the standard settings of the maxLFQ algorithm.
651 Match between runs was activated. pSILAC data were analyzed with the same settings with
652 some modifications. Multiplicity was set to two with Lys6 as heavy isotope label. Lys-C/P was
653 selected as digestive peptide and LFQ quantification was deactivated.
654 Statistical analysis and visualization were done with the Perseus (1.6.5) and InstantClue
655 software suits ^{76,77}. LFQ intensities of the IP samples were log₂-transformed and filtered for
656 proteins identified in all replicates of at least one condition. Missing values were imputed by
657 random drawing of values from 1.8 standard deviations (SD) downshifted, 0.3 SD broad normal
658 distribution to simulate the lower detection limits of the mass spectrometer. To evaluate
659 principal components responsible for the variances between samples we performed a principal
660 component analysis. Further, we performed a two-sided Student's *t*-test to identify significantly
661 regulated proteins. We proceeded similarly for the pulsed SILAC data but used the heavy-to-
662 light (H/L) ratios for statistical testing. We used the same filtering-criteria but did not imputed
663 missing values. We performed a 1D annotation enrichment to identify enriched categorical
664 terms in the different conditions ⁷⁸.

665

666 **Histology, Immunofluorescence and Microscopy**

667 For tissue histology, mice were sacrificed by cervical dislocation, muscles dissected, mounted in
668 cork with OCT (Tissue-Tek), snap frozen in isopentane and stored at -80°C till needed. 10 µm
669 thick sections covering the injured area were produced using a cryostat maintained at -20°C
670 (Leica CM 3050s, Techno-med). To assess the integrity of mitochondrial function the sections
671 were sequentially stained for COX and SDH activities. Frozen sections were incubated 20 mins
672 at 37°C in COX solution (20 mg/ml catalase, 74 mg/ml sucrose, 2 mg/ml cytochrome c, and
673 1mg/ml DAB in 50mM Na₂HPO₄ pH 7.4). After 3 PBS washes, sections were then incubated for
674 30 min at 37°C in SDH staining solution (2 mg/ml NBT, 0.2M Sodium succinate, 50mM MgCl₂,
675 50mM Tris-HCl, pH 7.4), washed 3 times with milliQ water, and mounted in Glycerol gelatin
676 medium (Sigma).

For immunofluorescence of autophagy markers LC3 and p62 and mitochondrial TOM20, mice were perfused with PFA 4% in PBS prior to muscle collection. Samples were equilibrated in 15% sucrose for 6h and 30% sucrose overnight before frozen in OCT medium. For LC3 IF, samples were preincubated with 0.1% SDS for 5min. Antibody specificity was determined in muscle sections from LC3-GFP transgenic mice (kindly provided by Dr. Evangelos Kondilis). Cryosections were blocked for 1h with 1% Western blocking reagent (Roche) containing 0.1% Triton in PBST, antibodies incubated overnight at 4°C and secondary antibodies at room temperature for 1h in blocking buffer. Samples were mounted in Fluoromount G containing DAPI. Fiber type staining was performed as described previously²⁶. Images were obtained with Leica SP8 with 63x/1.40 oil PL Apo objective.

For *in vitro* analysis, cells were fixed in 4% PFA/PBS, permeabilized with PBS-0.2% Triton-X1000 for 30 min and blocked for 1 hour at RT in blocking buffer (5% fat free milk powder, 10% FBS, 1% BSA, 0.1% Triton-X100 in PBS). Primary antibodies were incubated in blocking buffer over night at 4°C and secondary antibodies for 1 hour at RT. mtDNA replication rate was determined by pulse BrdU labelling. Briefly, cells were incubated with 20 µM BrdU (Sigma) for 6h, fixed with 4% PFA/PBS for 30 minutes and directly permeabilize with 0.5% Triton X-100 on ice for 5 min. To allow access to mtDNA, cells were incubated with HCl 2N for another 30 min prior to immunofluorescence. Coverslips were mounted using DAPI-Fluoromount G. Images were acquired using a spinning-disk confocal microscope (Ultra View VoX; PerkinElmer) with a Plan-Apochromat total internal reflection fluorescence 60Å-/1.49 NA oil DIC objective, Leica SP5 microscope controlled by Las AF 3 with 2.5x extra magnification and Leica SP8 with 63x/1.40 oil PL Apo objective. Microscopy Live imaging was performed in spinning-disk confocal microscope (Ultra View VoX; PerkinElmer). Cells were seeded onto glass plates and loaded with 500nM Mitotracker green for 30 minutes. Videos were recorded taking 1 picture/min at 37°C and 5% CO₂.

Antibodies used for immunofluorescence were: rabbit polyclonal α-V5 (Thermo); rabbit polyclonal α-TOM20, mouse monoclonal α-Vsp35 and mouse monoclonal α-8-OHdG (Santa Cruz); goat polyclonal α-Vsp35 and mouse monoclonal α-dsDNA (Abcam); rabbit polyclonal α-ATAD3, rabbit polyclonal α-LC3, rabbit polyclonal α-p62, rabbit polyclonal LRPPRC (Proteintech); and monoclonal α-BrdU (BD Bioscience). Fluorescence secondary antibodies goat α-mouse, α-rabbit Alexa Fluor-488, 555 and 647 and rabbit α-goat-647 were used accordingly to the primary antibodies. Additionally, α-mouse IgM Alexa Fluor 488, α-mouse IgG Alexa Fluor 555 and α-mouse IgG2b Alexa Fluor 647 were used for fiber type triple staining.

710

711 **Electron microscopy**

For electron microscope cells were grown on small discs of aclar foil and fixed for 1 h in 2% Glutaraldehyde with 2 %Sucrose in HEPES buffer pH 7.4. After washing two times with 0.1M Cacodylate buffer, free aldehyde groups were quenched with 0.1M Glycin in 0.1M Cacodylate buffer for two times 20 min. After a short wash with 0.1M Cacodylate buffer, cells were incubated in 0.5mg/ml Diaminobenzidine in 0.1M Cacodylate buffer and, after 10 min, a final

concentration of 0.03% H₂O₂ added and incubated for 30min. Finally, cells were washed three times with 0.1M Cacodylate buffer and incubated with 1% Osmiumtetroxid and 1.5% Potassium hexacyanoferrat for 30 min at 4°C. After 3x5min wash with ddH₂O, samples were dehydrated using ascending ethanol series (50%, 70%, 90%, 100%) for 5 min each and infiltrated with a mixture of 50% Epon/ethanol overnight at 4°C and with pure Epon for two times 2 h. Samples were embedded into TAAB capsules and cured for 48 h at 60°C.

For electron tomography Ultrathin sections of 200 nm were cut using an ultramicrotome (Leica, UC7) and incubated with 10 nm protein A gold (CMC, Utrecht) diluted 1:25 in ddH₂O. Sections were stained with 2% Uranyl acetate for 20 min and Reynolds lead citrate solution for 3 min. Images and Tilt series for 1nm thickness were acquired from -65° to 65° with 1° increment on a JEM-2100 Plus Transmission Electron Microscope (JEOL) operating at 200kV equipped with a OneView 4K 32 bit (Gatan) using SerialEM (Mastronarde, 2005). Reconstruction was done using Imod (Kremer et al.,1996).

Image analysis

All image analysis was performed in FIJI (NIH, Bethesda). LC3 and p62 puncta quantification were performed with the “counting cells” internal plugin for particles bigger than 2 pixels to exclude background. mtDNA foci quantification, VPS35-Twinkle analysis, 8-OHdG and BrdU analysis were performed with a self-created macro. Briefly, threshold was set for the different channels. Nuclear signal was selected and removed for the analysis. The signal corresponding to the mitochondrial network was selected and only the particles from the other channel bigger than 1 pixel inside the mitochondrial network were considered to the analysis. For VPS35, the minimum size was determined to be 3 pixels. After thresholding, all VPS35 particles were summarized and the ones in contact with Twinkle or LRPPRC were used to get a percentage. Manders’ coefficient was obtained using JaCOP plugin. (Bolte & Cordelieres, 2006).

Table M1. Oligonucleotides used in this study.

shRNA name	Sequence 5’-3’
shAtad3_1	AGCCTGTATAGGAACGTTCTTCTCGAGGAACGTTCTATACAGGCTTT TTTTTG
shAtad3_2	GCCTGTATAGGAACGTTCTTCTCGAGAGAACGTTCTATACAGGCTT TTTTTG
shAtad3_3	GCAGTTTGATTGGGCTATCTTCTCGAGGATAGCCCAATCAAACCTGCTT TTTTTG
shScramble	CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAG GTTTTTG
shSamm50	GAGGAGATGTGAGCTTCATTTCAAGAGAATGAAGCTCACATCTCCTCT TTTTTG
mVps35-ex4-104fw-s	CACCGTATGAACTTGACAGTACGC
mVps35-ex4-104fw-as	AAACGCGTACTGTACAAGTTCATAC
mVps35-ex5-91fw-s	CACCGATTTGGTAGAAATGTGCCG

mVps35-ex5-91fw-as	AAACCGGCACATTTCTACCAAATC
mtDNA qPCR Forward	CCTATCACCCCTTGCCATCAT
mtDNA qPCR Reverse	GAGGCTGTTGCTTGTGTGAC
nucDNA qPCR Forward	ATGGAAAGCCTGCCATCATG
nucDNA qPCR Reverse	TCCTTGTTGTTTCAGCATCAC
mitoLongRange Forward	GTTCAACGATTAAAGTCCTACGT
mitoLongRange Reverse	GTTGTTTGATCCTGTTTCGTG
Del983-4977 Forward	TCGTAACAAGGTAAGCATACTG
Del983-4977 Reverse	CTCGCGGACTAGTATATCCT
Vps35_Ex4 Forward	CTGAGCCAGGAGATCATGAATTC
Vps35_Ex4 Reverse	GCTTCCACTACTGAGCTAGATCAC
Vps35_Ex5 Forward	GTCTAGACACAACCTACTGACACC
Vps35_Ex5 Reverse	GTAGTGTGTTTGAATACAGTCAAG

744

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751

752 Author contributions

753 Funding Acquisition, DPM and RJW; Conceptualization, DPM and RJW; Investigation and
754 Formal Analysis, DPM, AS, SK, KM, JH; Resources JN; Analysis of MS Data, SK; Visualization,
755 DPM; Writing, DPM and RJW; Writing-Review & Edit, DPM, RJW, SK, MK; Supervision, DPM
756 and MK.

757

758 Conflict of interest

759 The authors declare that they have no conflict of interest.

760

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Figure 1. *In vivo* expression of Twinkle-K320E induces differential accumulation of mtDNA alterations in muscles. (a) Long range PCR analysis, (b) quantification of mtDNA copy number and, (c) qPCR quantification of deletion mtDNA-Δ983-4977 in M. soleus and M. Tibialis anterior from 24 months old control and K320E^{SKM} mice. (d, e) Western blot analysis and quantification of the indicated proteins in muscle extracts from 24 months old mice. M. soleus, control: n=3; Twinkle-K320E: n=4. M. tibialis anterior, control: n=4; Twinkle-K320E: n=4. (f, g) Western blot analysis and quantification of LC3 flux (LC3-II/LC3-I ratio) in muscle extracts from mice treated with saline or 50mg/kg chloroquine 4 hours before euthanasia. n=4 mice per treatment and genotype. Unpaired Students' T-test. Mean ± SEM. (h) Volcano plots for mitochondrial and non-mitochondrial proteins detected in the muscles analysed for *in vivo* Pulse SILAC. Red dots: mitochondrial proteins; dark red: mitochondrial proteins with significant difference; blue dots: non mitochondrial proteins with significance difference. (p-value < 0.05 and a H/L ratio fold change > 1.5). n=5.

Figure 2. Twinkle-K320E triggers mtDNA damage and induces autolysosome accumulation independent of bulk mitophagy. (a-e) C2C12 expressing untagged Twinkle constructs transiently expressing the autophagy reporter LC3-GFP-mCherry or (c) the mitophagy reporter Fis1p-GFP-mCherry. Red signal shows lysosomal localization. (b, c) Manders' coefficient Red / Green quantification of transfected cells. n=3. A decrease in Manders' coefficient indicates autolysosome or mito-lysosome accumulation, n=3, 10-15 cells per replicate. Cells treated overnight with 10 μ M Antimycin/Oligomycin were used to induce canonical mitophagy. (e, f) mtDNA copy number in C2C12 cells stably expressing Twinkle or Twinkle-K320E (K320E), respectively, vs. empty vector (pBABE). In (f), mtDNA levels were recovered by treating cells with the lysosomal inhibitor chloroquine for 24h, n=3. (g-h) Confocal images and quantification of C2C12 expressing mCherry tagged Twinkle transfected with plasmids encoding the autophagosome marker LC3-GFP and (i, j) lysosome marker Lamp1-GFP. Manders' colocalization coefficient was used to confirm Twinkle colocalization with autophagic organelles, n=3 (>10 cells per experiment). (k) mtDNA oxidative damage detected by immunofluorescent labelling with α -OHdG and α -TOM20 in cells expressing Twinkle and treated with chloroquine (CQ) for 24h. (l) Relative intensity quantification of α -OHdG signal inside the mitochondrial network. Scale Bar, 10 μ m. (c-f and l) ANOVA, Tukey multiple comparison. **, p<0.01; ***, p<0.001. (h and j) Unpaired Student's T-test. *, p<0.05. Mean $\pm$ SEM.

Figure 3. Autophagy is required for mtDNA clearance after mtDNA damage. Atg5 WT and KO cells were transduced with Twinkle-APEX-V5 plasmids. **(a)** α -DNA and α -V5-tag immunofluorescence confirming localization of Twinkle in mtDNA containing nucleoids. Scale bar 5 μ m. **(b, c)** Quantification of mtDNA foci number in Atg5 WT and Atg5 KO cells. $n=3$ (>25 cells per experiment). **(d, e)** Steady state mtDNA copy number in Atg5 cells. **(f, g)** α -BrdU and α -TOM20 immunofluorescence and quantification of mtDNA replicating foci detected by treating the cells for 6h with 20 μ M BrdU. $n=3$, >20 cells per replicate **(h)** Quantification of mtDNA copy number ratio in Atg5 cells treated with Chloroquine for 24h. $n=3$. **(i-k)** α -DNA and α -TOM20 immunofluorescence and mtDNA foci quantification in steady state and in cells treated with 50ng/ μ l EtBr for 7 days. $n=3$, >20 cells per replicate. **(l)** mtDNA copy number analysis for steady state and in cells treated for 7 days with EtBr. $n=3-4$. Scale Bar, 10 μ m. **(b-e, h and l)** ANOVA, Tukey multiple comparison. **, $p < 0.01$; ***, $p < 0.001$. **(g, j and k)** Unpaired Student's T-test. *, $p < 0.05$. Mean $\pm$ SEM.

Figure 4. Twinkle-K320E localizes to specialized mitochondrial regions. (a) Immunofluorescence of C2C12 cells expressing Twinkle-APEX-V5 variants probed with α-TOM20 and α-V5 antibodies. Arrow shows TOM20 negative particle containing Twinkle-K320E. Bar, 10 μm (b, c) Live imaging of C2C12 cells expressing tagged Twinkle-m-Cherry variants counterstained with mitotracker green. Insets represent a 1 min time lapse series of the selected area. Bar, 10 μm (d) Transmission electron microscopic pictures of C2C12 cells expressing APEX2-V5 clones. APEX2 generates a black precipitate in presence of DAB. Red arrows indicate multimembrane structures, asterisks indicate autophagosomes. Bar, 1 μm. (e) Electron tomography in C2C12 cells expressing Twinkle-K320E illustrating extrusion of mitochondrial fragments containing multimembrane structures. Bar, 0.5 μm

Figure 5. Twinkle mediates interaction of nucleoids with the inner mitochondrial membrane protein ATAD3. (a) Volcano plot showing proteins enriched after immunoprecipitation of Twinkle-APEX2-V5 in C2C12 cells. Differentially enriched proteins compared with cells transfected with empty vector (p-value < 0.05 and FC > 2 or < -2) are highlighted in blue (Peo1 = Twinkle). (b) Co-immunoprecipitation of V5-tagged Twinkle and ATAD3. (c) α-TOM20 and α-dsDNA immunofluorescence in Atad3 KD MEFs in steady state and grown for 7 days in presence of 50ng/ml EtBr. Bar, 10 μm. (d) Quantification of mtDNA copy number in steady state and EtBr treated cells. n=4-5. and (e) mtDNA foci quantification for Atad3 KD in steady state and EtBr treated cells. n=3, >20 cells per replicate. ANOVA, Tukey multiple comparison. *, p<0.05, **, p<0.01. Mean ± SEM.

Figure 6. VPS35 is required for autophagy-dependent mtDNA removal. Immunofluorescence of C2C12 cells expressing Twinkle-mCherry variants labelled with α-VPS35 and α-TOM20 and grown in (a) basal medium or (b) treated for 7 days with 50ng/ml EtBr. Arrows indicate colocalization. Bar, 10μm. (c, d) Quantification of VPS35 particles in contact with Twinkle. n=3 (e) Immunofluorescence of C2C12 wild type cells labelled with α-dsDNA and α-VPS35. Arrows indicate colocalization between VPS35 and dsDNA puncta. Bar, 10μm. (f) α-TOM20 and α-dsDNA immunofluorescence of Vps35 KO MEFs. Bar, 10μm. (g) mtDNA foci analysis (n=3) and (h) mtDNA copy number quantification of Vps35 KO cells. n=5. (i) Control and Vps35 KO cells transfected with Fis1p-GFP-mCherry plasmid to detect mitophagy. Red signal represents mitolysosomes. Bar, 10μm. (j) Manders' coefficient quantification of transfected cells. n=3. A decrease in Manders' coefficient indicates process activation. n=3 (20 images per replicate). ANOVA, Tukey multiple comparison. *, p<0.05; **, p<0.01; ***, p<0.001. Mean ± SEM

Figure 7. SAMM50 is a mitochondrial receptor for VPS35 recruitment into mitochondrial subcompartments containing nucleoids. (a) Volcano plot showing proteins enriched after immunoprecipitation of VPS35 in MEFs. Only differentially enriched proteins are highlighted (adj. p-value < 0.05 and FC > 2 or < -2). Blue, non-mitochondrial proteins; Red, mitochondrial proteins; Green, retromer proteins (b) Comparison of VPS35 IP profiles of cells grown in normal medium or 1 week treated with 50ng/ml EtBr. n=3. (c) Violin Plots showing enrichment of mitochondrial proteins upon pull down of VPS35 in presence of EtBr. Numbers indicate p-value. Students' T-test. (d) Co-Immunoprecipitation of VPS35 and SAMM50 in steady state and EtBr treated cells. IgG from mouse was used as a control. (e) Comparison of interactome profiles of Twinkle and K320E. n=3. (f) co-Immunoprecipitation of Twinkle and SAMM50 in steady state and in cells treated with EtBr for 1 week. (g) Immunofluorescence of control and Samm50 KD MEFs transduced with Twinkle-mCherry constructs and labelled with α -VPS35 in basal and 7 days treated with 50ng/ml EtBr. Bar, 10 μ m. (h, i) Quantification of VPS35 particles and VPS35 in contact with Twinkle. n=3, >20 cells per replicate. ANOVA, Tukey multiple comparison. **, p<0.01. Mean $\pm$ SEM

Figure 8. Rapamycin eliminates mtDNA deletions without affecting copy number *in vivo*. COX-SDH staining of regenerated TA muscle from Pax7-K320E mice (K320E^{msc}). After cardiotoxin induced injury, mice were injected for five days either with (a) vehicle or (b) 2 mg/Kg Rapamycin. Bar, 500µm. (c) Quantification of COX negative cells (blue) in the injured area. (d) mtDNA quantification by qPCR or (e) Long-range PCR in regenerated muscle from K320E^{msc} mice treated with vehicle or with Rapamycin. n=5-6 mice per condition, genotype and treatment. (c and d) Unpaired Students' T-test. Mean ± SEM.

Figure S1. (a) Conventional PCR with specific oligonucleotides flanking the deletion mtDNA- Δ 983-4977. (b) mtDNA copy number in muscles from 24 months old control and Twinkle-K320E mice. This graph shows a comparison between samples analysed in Fig 1b. (c-f) *In situ* immunofluorescence and image quantification showing autophagic markers LC3 and p62 in cryosections of M. soleus (c, d) and M. tibialis anterior (e, f). 5 random pictures with 4 fibers per picture were analysed per animal to obtain averaged values. Control: n=4; Twinkle-K320E: n=5. Bar, 20 μ m. (b, c, e, h and i) Unpaired Students' T-test. Mean $\pm$ SEM.

Figure S2. (a) Component analysis of *In vivo* Pulse SILAC in M. extensor digitorum longus (EDL) and M. soleus. (b) Box plot and Violin plots showing Lys-6 incorporation in muscles from Twinkle mice. (c) Box plot analysis for mitochondrial and non-mitochondrial proteins detected in the muscles analysed for *in vivo* Pulse SILAC. n=5.

Figure S3. (a, b) α -TOM20 immunofluorescence of clones expressing (a) m-Cherry and (b) APEX2-V5 tagged Twinkle variants. (c, d) Immunofluorescence of (c) m-Cherry tagged clones transiently expressing Tfam-GFP and (d) APEX2-V5 tagged clones probed with α -dsDNA antibody showing nucleoid localization (see magnifications in lower panels). Bar, 10 μ m.

Figure S4. (a, b) Analysis of mtDNA replicating foci by α -BrdU and α -TOM20 immunofluorescence of C2C12 cells expressing Twinkle plasmids treated with 20 μ M BrdU. Only BrdU foci inside the mitochondrial network were analysed. (c, d) α -OHdG and α -TOM20 immunofluorescence and quantification of the average intensity inside the mitochondrial network of C2C12 in steady state. (e, f) α -OHdG and α -TOM20 immunofluorescence and quantification of the average intensity inside the mitochondrial network in C2C12 treated 24h with 50 ng/ul EtBr. Students' T-test. Mean $\pm$ SEM. *, $p < 0.05$. Scale Bar, 10 μ m.

Figure S5. (a) Immunofluorescence of C2C12 cells expressing Twinkle-mCherry stained with α-ATAD3 antibody. Scale bar, 10 μm. (b) Analysis of Atad3 expression by RT-qPCR and (c) western blot upon shRNA transduction. n=3-4 (d) Quantification of Mitochondrial morphology in shATAD3 cells. n=3, >30 cells per replicate. (b and d) ANOVA, Tukey multiple comparison. **, p<0.01; ****, p<0.0001. Mean ± SEM.

Figure S6. (a-c) Immunofluorescence of C2C12 cells expressing Twinkle-mCherry transiently transfected with MAPL-GFP and stained with α-TOM20 antibody (a, b) or α-VPS35 antibody (c, d). In (b, d) Cells were treated 4h with 10μM Chloroquine. Bar, 10 μm.

Figure S7. (a) mtDNA copy number in C2C12 cells treated for 7 days with 50ng/ml EtBr. n=3. (b) Western blot analysis of MEFs after CRISPR Cas9 mediated KO of *Vps35*. (c) Schematic representation of DNA modification in *Vps35* Exon 4 and Exon 5 triggered by CRISPR-Cas9. (d) Quantification of mitochondrial morphology in *Vps35* KO clones. n=3, >30 cells per replicate. (e) mtDNA foci quantification in control and (f) *Vps35* KO clones treated with EtBr. n=3. (g) qPCR mtDNA copy number analysis of control and (h) *Vps35* KO clones treated with EtBr. Students' T-test. **, p<0.01; ***, p<0.001. n=5. (i) Western blot analysis of autophagy markers and VPS35 levels in C2C12 cells expressing Twinkle-APEX2-V5 variants (WT, KE) or matrix targeted APEX2 protein, treated 24h with 10μM Chloroquine. n=3. (j-n) Intensity quantification of the indicated proteins relative to empty vector pB (pBABE). (a, g, h and j-n) ANOVA analysis of variance. Tukey multiple comparison test. Mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001. (d) Two-way ANOVA. Control lines were compared with KO lines. **, p<0.01; ****, p<0.0001.

Figure S8. (a) Component analysis of VPS35 IP. (b) Comparison of VPS35 IP profiles from cells in basal medium or treated with 50ng/ml EtBr for 1 week. Only significantly different proteins are highlighted. Blue, cytosolic proteins; Red, mitochondrial proteins; Green, retromer proteins. (c) mRNA quantification and (d) western blot analysis of *Samm50* upon shRNA transduction. *Gapdh* mRNA and GAPDH was used as an internal control for both experiments. (e) Immunofluorescence of Control and *Samm50* KD MEFs labelled with α -VPS35 and α -LRPPRC in basal and 7 days treated with 50ng/ml EtBr. Scale bar, 10 μ m. (f, g) Quantification of VPS35 particles and VPS35 in contact with LRPPRC. n=3. Mean $\pm$ SEM. ANOVA analysis of variance. Tukey multiple comparison test.

Figure S9. (a) Representative image for COX-SDH staining in a regenerated area from the M. Tibialis anterior from control and K320E-Twinkle^{msc} mice. Bar, 500 μ m. (b) Long Range PCR for mtDNA deletions and, (c) mtDNA copy number analysis in control and Twinkle-K320E mice. n=5. Muscle regeneration was induced with intramuscular injection of 10 μ M Cardiotoxin (*Naja Pallida*). Students' T-test. (d) Fiber type and COX-SDH serial analysis of regenerated muscles in vehicle and (e) rapamycin treated mice. Mean $\pm$ SEM. Bar, 250 μ m.