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JB Review

Roles of mitochondrial ubiquitin ligase MITOL/MARCH5 in mitochondrial dynamics and diseases

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Accumulating evidence indicate physiological significance of mitochondrial dynamics such as mitochondrial fusion and division, the dynamic movement of mitochondria along microtubules and interaction of mitochondria with the endoplasmic reticulum. A disruption in mitochondrial dynamics leads to a functional deterioration of mitochondria, resulting in a variety of diseases including neurodegenerative disorders. We previously identified a mitochondrial ubiquitin ligase MITOL/MARCH5, which belongs to the membrane-associated RING-CH E3 ubiquitin ligase (MARCH) family (also called MARCH5). MITOL plays an important role in the regulation of mitochondrial dynamics including mitochondrial morphology, transport and interaction with ER, at least in part, through the ubiquitinations of mitochondrial fission factor Drp1, microtubule-associated protein 1B and mitofusin2, respectively. This review focuses on recent findings that show how MITOL regulates mitochondrial dynamics and which suggest physiological disorders resulting from a failure in such regulation.

Keywords: MAM/MAP1B/mitochondrial dynamics/*S*-nitrosylation/ubiquitin ligase.

Abbreviations: ALS, amyotrophic lateral sclerosis; ER, endoplasmic reticulum; MAM, mitochondrial-associated ER membrane; MAP1B, microtubule-associated protein 1B; MAP1B-LC1, microtubule-associated protein 1B-light chain 1; MEF, mouse embryonic fibroblast; ROS, reactive oxygen species; SOD1, superoxide dismutase 1; UPS, ubiquitin–proteasome system.

Regulation of morphological stability of mitochondria by MITOL via Drp1

The morphological stability of mitochondria is regulated by a balance between fusion and fission. MITOL is an integral mitochondrial outer membrane protein with four membrane spanning segments as shown in Fig. 1A. When MITOL was knocked down using RNAi in HeLa cells, mitochondria rapidly began to divide uncontrollably. Drp1, which is a mitochondrial fission factor was identified as a physiological substrate of MITOL (*1*, *2*). Furthermore, it was revealed that MITOL regulates mitochondrial morphology by polyubiquitinating Drp1, leading to the proteasomal degradation of Drp1 (Fig. 1A). Drp1 was subsequently confirmed as a physiological substrate for MITOL, by using *MITOL*-knockout mouse embryonic fibroblast (MEF) cell line. Immunoblot analysis indicates that Drp1 accumulation was observed in *MITOL*-knockout MEFs and tissues of mice (Nagashima et al unpublished data).

Interestingly, a significant increase in reactive oxygen species (ROS) was observed in the MITOL-knockdown cells (*3*). We confirmed the high level of mitochondrial ROS generation in *MITOL*-knockout MEFs. As Drp1 accumulation has been shown to induce mitochondrial dysfunction and ROS production, we speculated that Drp1 accumulation plays a role in ROS production in MITOL-knockdown cells. However, Drp1 accumulation and mitochondrial fragmentation in *MITOL*-knockout MEFs became milder than that in rapid MITOL-knockdown cells. This result suggests that some compensatory mechanisms might be involved in the regulation of mitochondrial morphology and that Drp1 accumulation by *MITOL* knockout has a partial effect on mitochondrial ROS generation. Thus, the mechanism of mitochondrial ROS generation by *MITOL* knockout remains to be clarified.

On the other hand, some studies reported contrary results that MITOL is required for Drp1-dependent mitochondrial division (*3*, *4*). They showed that MITOL inhibition induced mitochondrial fusion. It is possible that MITOL-dependent Drp1 regulation varies by cell conditions such as cell cycle phase or nutritional status. This issue is at present still controversial and should be determined by further studies.

Degradation of denatured proteins by MITOL

Denatured and aggregated proteins tend to accumulate in mitochondria and interfere with mitochondrial function (*5*). This mechanism is thought to have a potential association with the development of degenerative neurological disorders. For example, superoxide dismutase 1 (SOD1) has a possible link to amyotrophic lateral sclerosis (ALS). The aggregation and accumulation of mutated SOD1 in mitochondria has been shown to be involved in the pathological mechanisms

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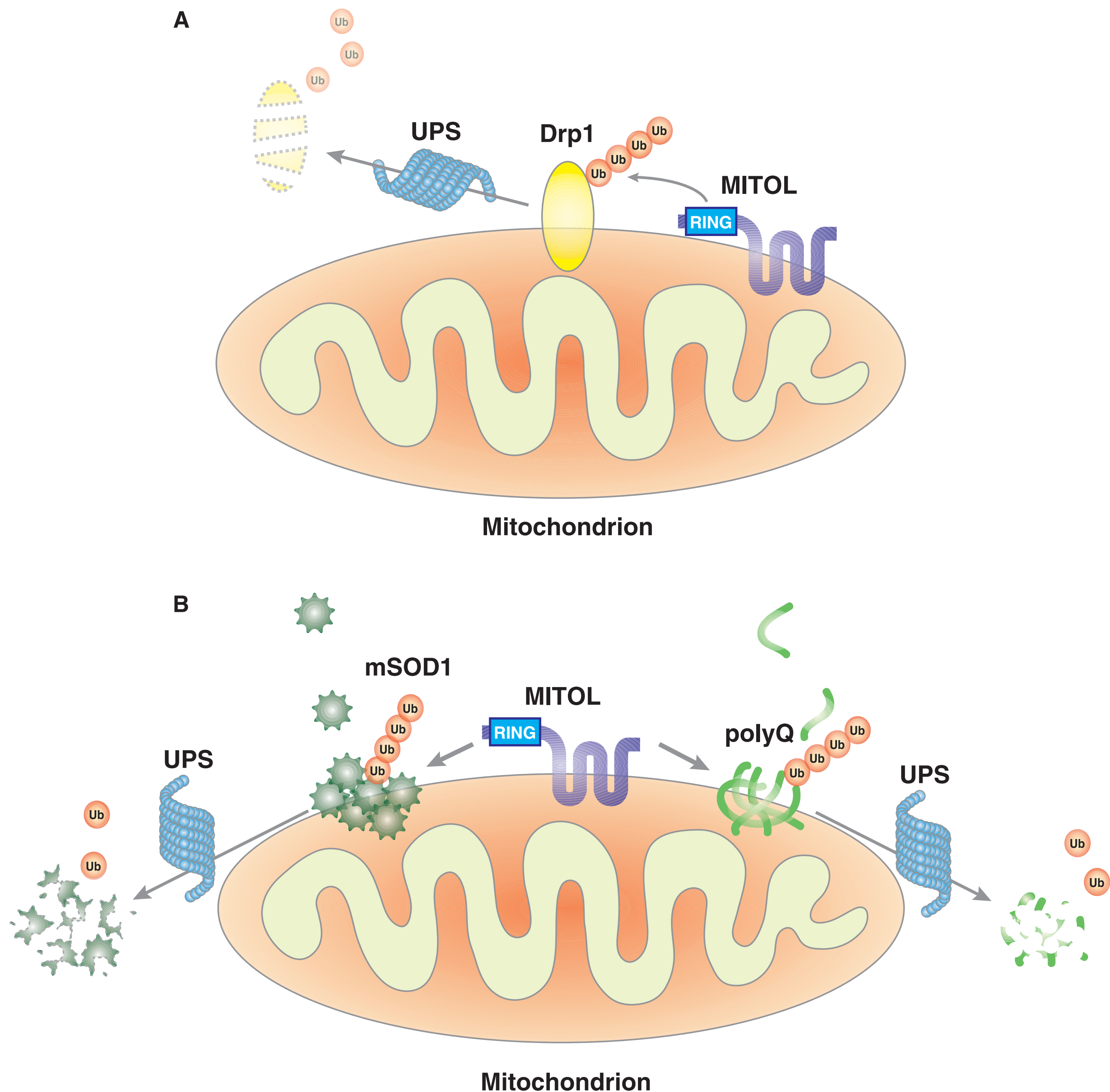

Fig. 1 Roles of MITOL in mitochondrial morphology and misfolded protein accumulation in mitochondria. (A) MITOL is a mitochondrial ubiquitin ligase that has four transmembrane domains within the mitochondrial outer membrane and regulates mitochondrial morphological stability via the fission factor Drp1 degradation by UPS. (B) MITOL specifically recognizes and binds to denatured proteins such as mutated SOD1 or conformationally abnormal proteins with expanded glutamine chains through the intrinsic disordered domain at its C-terminus and thus promotes their degradation by UPS.

underlying ALS (*6*). Similarly, in polyglutamine disease, a typical disorder of genetic spinocerebellar degeneration, the accumulation of conformationally abnormal proteins with elongated glutamine chains (polyQ) in mitochondria is considered a possible cause of neurotoxicity (*7*). We have found that MITOL specifically recognizes and binds to mutated SOD1 and expanded polyQ and promotes their degradation through the ubiquitin–proteasome pathway (UPS) (Fig. 1B) (*8*, *9*). We have also confirmed that a functional decrease in MITOL results in the accumulation of denatured proteins in mitochondria and neuronal cell death. This observation strongly suggests that MITOL plays a role in the degradation of denatured protein molecules in mitochondria and in neuronal cell survival. In terms of neurodegenerative diseases, it is possible that functional disorders of MITOL caused by any reason may lead to the accumulation of denatured proteins in mitochondria and the exacerbation of neurological disorders. Interestingly, our preliminary results indicated that a significant decrease in MITOL activity was observed to be associated with ageing, suggesting that decreased MITOL expression in the elderly may lead to the accumulation of denatured proteins in mitochondria and a subsequent increase in the risk of developing neurological disorders.

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Therefore, MITOL may be a potential therapeutic target for neurodegenerative diseases. We are currently analysing neuron-specific *MITOL*-knockout mice. The relationship between MITOL dysfunction and neurodegenerative diseases would be clarified in the near future.

How does MITOL specifically recognize denatured proteins? MITOL has not been reported to bind to chaperone molecules such as HSP70. Interestingly, the C-terminus of MITOL is a disordered domain that is characterized by a lack of stable tertiary structure (*10*). In our preliminary result, mutated forms of MITOL, wherein the domain has been deleted, fail to associate with and ubiquitinate mutated SOD1 and so do not induce the degradation of mutated SOD1. Therefore, it is possible that MITOL recognizes denatured proteins via the disordered domain at its C-terminus. Detailed mechanisms underlying the recognition of denatured proteins by MITOL remain to be clarified.

As described earlier, denatured proteins tend to accumulate in mitochondria. The inverse scenario, whereby the mitochondria accumulate around denatured proteins, may also be true. Mitochondria may efficiently supply ATP to proteasomes, which also accumulate around denatured proteins. As denatured proteins are predisposed to translocate to microtubules, mitochondria moving on microtubules may recognize these denatured proteins and degrade them by using MITOL. This may be one of the reasons why mitochondria are moving on microtubules. Mitochondria may be important for microtubule maintenance through MITOL.

Regulation of microtubule stability by MITOL and protective mechanisms against nitrosative stress

Time-lapse imaging analysis suggested that mitochondrial movement was significantly attenuated following MITOL knockdown (Yanagi et al unpublished data), but the exact underlying mechanisms are still obscure. As microtubules were highly stabilized by MITOL knockdown, we hypothesized that excessive microtubule stabilization induced by MITOL knockdown inhibits mitochondria movement. In a yeast two-hybrid screen, microtubule-associated protein 1B-light chain 1 (MAP1B-LC1) was identified as a physiological substrate of MITOL. MAP1B is a protein complex that consists of a heavy chain and light chain. MAP1B and its homologue are reported to inhibit retrograde transport of mitochondria and to induce mitochondrial dysfunction and cell death (*11*, *12*). We found that MITOL specifically binds to and ubiquitinates MAP1B-LC1 to promote proteasomal degradation, thus regulating MAP1B-LC1 concentration (*13*). As the suppression of MITOL induces the accumulation of MAP1B-LC1 and results in mitochondrial aggregation and dysfunction, it has been suggested that MITOL protects mitochondria from MAP1B-LC1 toxicity. Furthermore, a novel recognition mechanism of MAP1B-LC1 ubiquitination by MITOL has been demonstrated. MAP1B-LC1 has been shown to be activated through *S*-nitrosylation by nitric oxide (NO) (*14*). Interestingly, MITOL specifically ubiquitinates MAP1B-LC1 in an *S*-nitrosylation-dependent manner. *S*-nitrosylation induces a conformational change in MAP1B-LC1 molecules and exposes the ubiquitinated sites that are otherwise hidden (Fig. 2A). That *S*-nitrosylation of MAP1B-LC1 acts as a signal for ubiquitination, is a new concept and may contribute significantly to the understanding of the pathological mechanisms of neurological diseases.

Various external stimulations of neuronal cells lead to the activation of neuronal NO synthase and subsequent NO production. Although physiological amounts of this gas are neuroprotective, whereas higher concentrations are clearly neurotoxic (*15*). It is possible that after *S*-nitrosylation by NO, active MAP1B-LC1 binds to, not only its counterpart MAP1B heavy chain to stabilize microtubules, but also motor proteins such as dynein to induce a transient arrest of retrograde transport of mitochondria or of vesicle transport on the microtubules. Thus, *S*-nitrosylation of MAP1B-LC1 may transmit transient signals about cellular emergencies caused by NO production, enabling the cells to resist nitrosative stress. However, as prolonged stabilization of microtubules may result in the discontinuation of mitochondrial movement or vesicle transport, leading to cell death. MITOL promotes MAP1B-LC1 degradation to prevent the excessive stabilization of microtubules, enabling restart of mitochondrial transport as well as vesicle transport. Thus, it is considered that one of the reasons for the active movement of mitochondria on microtubules is to control the stability of microtubules as well as to degrade denatured proteins (Fig. 2B).

In many neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease, mitochondrial dysfunction caused by oxidative stress is suspected to underlie pathological mechanisms (*16*). A recent study reported that *S*-nitrosylation of Drp1 mediates β-amyloid-related mitochondrial fission and neuronal injury (*17*), indicating that excess NO production disrupts mitochondrial dynamics through *S*-nitrosylation of Drp1, thereby possibly contributing to the pathogenesis of Alzheimer's disease. In addition, MAP1B was reported to be a component of neurofibrillary tangles in Alzheimer's disease brains (*18*). Considering that both Drp1 and MAP1B are substrates for MITOL, MITOL is thought to play a protective role against nitrosative stress-mediated disruption of mitochondrial dynamics such as morphological stability and transport of mitochondria (Fig. 3). However, when excess NO inactivates MITOL by direct *S*-nitrosylation, it may cause disease progression. Indeed, we found that *MITOL*-knockout MEFs are highly vulnerable to nitrosative stress. Elucidation of the role of MITOL in these pathological conditions is needed for the development of therapeutic approaches.

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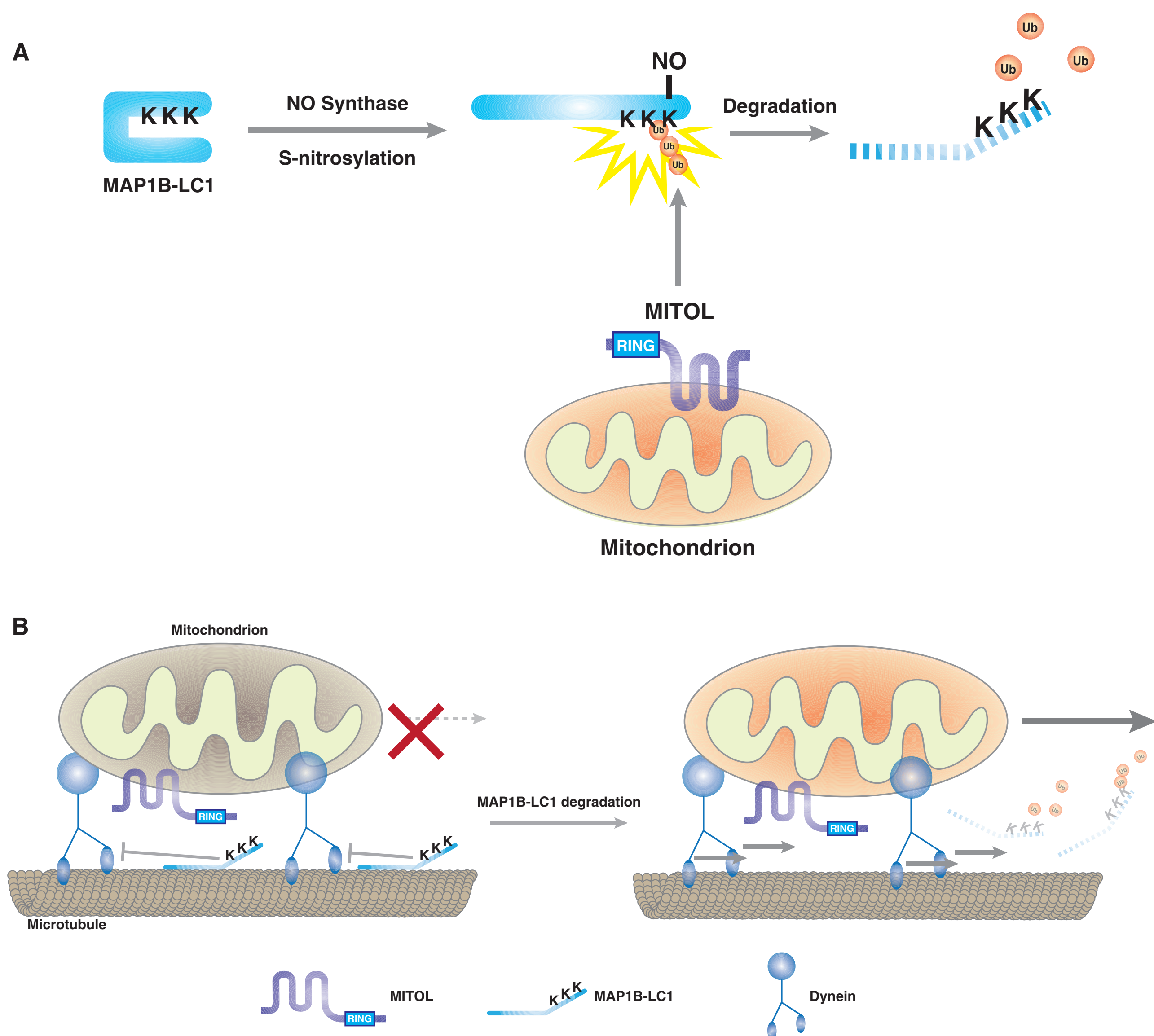

Fig. 2 MITOL controls microtubule stability and mitochondrial transport via regulation of MAP1B-LC1. (A) Mechanisms of MAP1B-LC1 ubiquitination by MITOL. A conformational change is induced in MAP1B-LC1 after *S*-nitrosylation by NO that exposes the ubiquitination region, enabling MAP1B-LC1 to be ubiquitinated by MITOL. (B) Regulation of mitochondrial transport by MITOL via MAP1B-LC1. *S*-nitrosylated and activated MAP1B-LC1 moves quickly to microtubules and suppresses the activities of motor proteins such as dynein to arrest temporarily the transport of mitochondria. MITOL promotes the degradation of MAP1B-LC1 to inhibit excessive stabilization of microtubules.

MITOL activates Mfn2 and regulates the attachment of mitochondria and endoplasmic reticulum

The mitochondrion, an energy-generating organelle, plays an important role in exchanging Ca^{2+} or metabolizing lipids by positioning at sites situated close to the endoplasmic reticulum (ER), which is called mitochondria-associated ER membrane (MAM). Mitofusin2 (Mfn2) is a mitochondrial fusion factor which is localized in the ER as well as in mitochondria and play a role in the attachment of these organelles to each other. Although a previous study indicates that Mfn2 is required for MAM formation, the mechanisms regulating this process remain unknown (*19*). Since we found that MITOL is also localized at MAM and it was previously reported to bind to Mfn2 (*2*), we have analysed the binding mechanism between MITOL and Mfn2. As a result, it was demonstrated that MITOL interacts with and ubiquitinates mitochondrial Mfn2 but not ER-associated Mfn2 (*20*). Importantly, MITOL specifically mediates polyubiquitination of Mfn2 but not its proteasomal degradation. MITOL mediates the addition of a polyubiquitin chain to lysine-63 in Mfn2 and upregulates Mfn2 binding to GTP, resulting in the formation of oligomers based on the GTPase activity of Mfn2.

When MITOL expression was knocked down by RNAi in HeLa cells, the number of attachment sites between the ER and mitochondria was significantly decreased. When MITOL-knockdown cells were stimulated with histamine, the mitochondrial Ca^{2+} concentration, which increases under normal conditions, was significantly lower than that in the control cells.

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From this observation, it was thought that, in MITOL-knockdown cells, the failure of Mfn2 to oligomerize resulted in incomplete attachment between the ER and mitochondria and therefore mitochondria failed

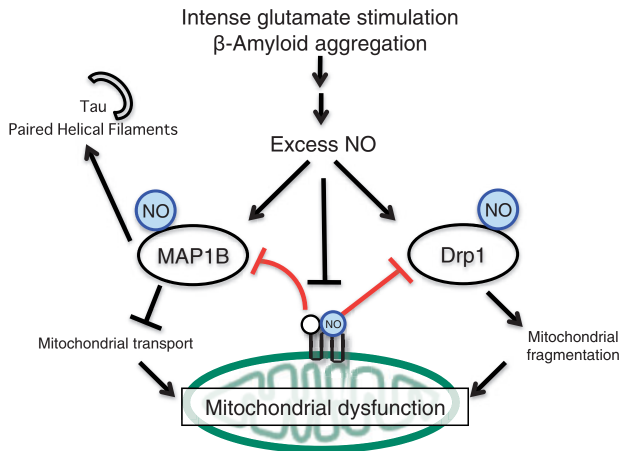

Fig. 3 MITOL protects against NO-induced disruption in mitochondrial dynamics. External stimulations of neuronal cells such as intense glutamate stimulation or β-amyloid aggregation induce excess NO production. NO in excess mediates mitochondrial fission or fragmentation via accumulation of *S*-nitrosylated Drp1 and also inhibits mitochondrial transport via accumulation of *S*-nitrosylated MAP1B, which is a component of neurofibrillary tangles. Thus, NO induces disruption in mitochondrial dynamics, resulting in mitochondrial dysfunction. On the other hand, MITOL protects against disruption in mitochondrial dynamics through control of Drp1 and MAP1B levels by ubiquitinating them. However, when excess NO inactivates MITOL by direct *S*-nitrosylation, it may cause disease progression.

to incorporate Ca^{2+} from ER. Based on our observations, we present a novel hypothesis that MITOL regulates ER tethering to mitochondria by activating Mfn2 (Fig. 4). MITOL binds to mitochondria-localized Mfn2, specifically at polyubiquitinated lysine-192. The polyubiquitinated Mfn2 is then activated, leading to an increase in guanosine triphosphate (GTP)-binding ability and associates with the ER-localized Mfn2, resulting in the oligomerization of Mfn2 and attachment of mitochondria to the ER.

As mutations in *Mfn2* are the cause of Charcot–Marie–Tooth type 2A hereditary neuropathy, elucidation of the control of Mfn2 function has been anticipated. The above findings show promise for the clarification of the mechanisms underlying Mfn2 activation and the pathology underlying Charcot–Marie–Tooth disease. Furthermore, the attachment sites between mitochondria and the ER play an important role in cell functions such as lipid biosynthesis and the transfer of Ca^{2+}(*21*, *22*). As reported by numerous researchers, failure of MAM function results in the development of pathological mechanisms related to a variety of neuronal diseases including Alzheimer's disease (*23*, *24*). In Alzheimer disease, both MAM function and ER–mitochondrial connectivity are significantly increased (*25*). It is at present unknown whether the increased MAM is the cause for disease such as toxicity triggered by calcium overload to mitochondria from ER or the side-effect as a result of weakening mitochondrial damage. We support the latter hypothesis because neuron-specific *MITOL*-knockout mice, whose MAM is severely impaired, show similar phenotypes with Alzheimer model

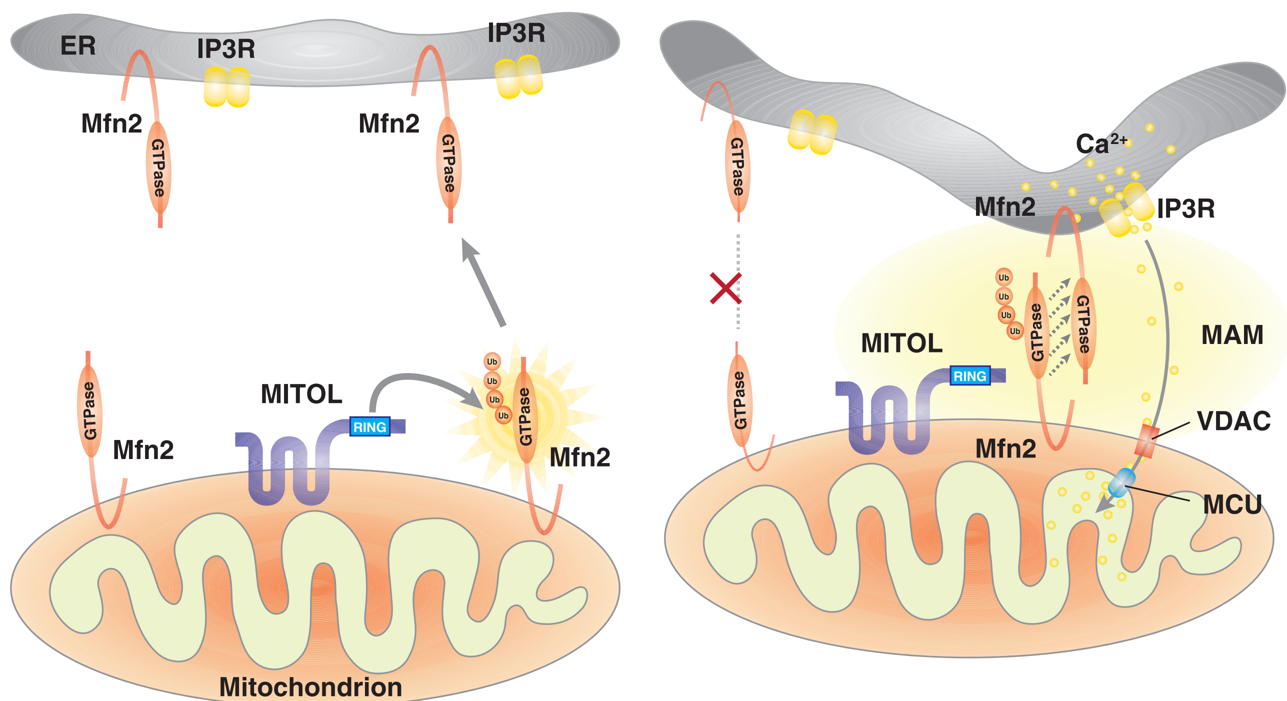

Fig. 4 A model of the mechanism by which MITOL controls interactions between mitochondria and ER. MITOL ubiquitinates mitochondrial Mfn2, thereby activating it. The activated Mfn2 binds to Mfn2 in the ER membrane, resulting in oligomerization of Mfn2 and interaction of the ER with mitochondria.

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mice. We therefore predict that MAM inhibition results in worsening of symptoms of Alzheimer model mice. We are currently mating neuron-specific *MITOL*-knockout mice with Alzheimer model mice. In the near future, we would answer the question by analysing the mice. By elucidating the mechanisms underlying the attachment between mitochondria and the ER, improvements in the understanding and treatment of a variety of neuronal diseases can be expected.

Perspective

When tamoxifen-inducible MEFs derived from the *MITOL*-knockout mice were treated with tamoxifen to knock out the *MITOL* gene, most of the MEFs became enlarged and their cell cycle was terminated, resulting in death within 48 h. However, a few cells survived and then grew actively to a level nearly indistinguishable from that of the wild-type MEFs. This observation indicates that MITOL is not necessarily essential for the survival of cells. However, the mitochondria of these apparently normal *MITOL*-knockout MEFs showed an increase in ROS levels. We are currently investigating cerebral cortex/hippocampus-, myocardium- or keratinocyte-specific *MITOL*-knockout mice and have found a variety of severe pathological phenotypes in all of the animals. As significantly decreased expression of MITOL occurs in response to ageing in normal tissues, MITOL may control ageing by regulating the production of ROS in mitochondria. These observations confirm the importance of mitochondria and further studies on MITOL would help in a deeper understanding of mitochondria-related diseases.

Conflict of Interest
None declared.

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