Neurodegenerative Stress Related Mitochondrial Proteostasis

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List of Abbreviations

AAA+····· ATPase associated with various cellular activities
Amyloid beta ·····Aβ
AD ·····Alzheimer's disease
ADOA ·····Autosomal dominant optic atrophy
ALS ·····Amyotrophic lateral scleroses
ATP ····· Adenoid triphosphate
CCCP ····· Carbonyl cyanide m-chlorophenylhydrazone
CMT2A ·····Charcot-Marie-Tooth disease 2A
CO_2 ·····Carbon dioxidide
DAT ·····Dopamine transporter
DR ····· Dopamine receptor
Drp1 ·····Dynamin-related protein-1
DUB ····· Deubiquitylation enyzme
ER ····· Endoplasmic reticulum
ERAD ·····ER-associated degradation
ETC ····· Electron transport chain
FAD ····· · Flavin adenine dinucleotide
FTD ····· Frontotemporal dementia
Fzo1 ····· Fuzzy onions homolog
HD ····· Huntington's disease
HDAC6 ·····Histome deacetylase 6
HETC domain ·····Homologous to the E6AP C-Terminus
HTG ····· High-tension glaucoma
IBM ·····Inclusion Body Myopathy
IBMPFD ·····Inclusion Body Myopathy with Paget Disease of Bone and / or Frontotemporal Dementia
IMM ·····Inner mitochondria membrane
IOP ·····Intraocular pressure
MAP ·····Microtubule-associated protein
MAPL ·····Mitochondrial-anchored protein ligase
MARCH5 ·····Membrane-associated RING-CH
MEFs ····· Mouse embryonic fibroblasts
Mff ····· Mitochondrial fission factor
Mfn2 ····· Mitofusin2
MiD49 ····· Dynamics protein 49kD
MiD51 ····· Mitochondrial dynamics protien 51kD
MitoYFP ····· Mitochondria-targeted yellow fluorescent protein
MOMP ····· Mitochondrial outer membrane permeabilization
MPP ····· Mitochondrial processing peptidase

MtDNA ····· Mitochondrial genome
MtHSP60 ····· Heat shock protein 60kDa
MtHSP70 ····· Heat shock protein 70kDa
MtPA-GFP ·····Mitochondria-targeted photoactivatable-GFP
MULAN ·····Mitochondrial ubiquitin ligase activator fo NF-κB
NADH ····· Nicotinamide adenine dinucleotide
NO ····· Nitric oxide
NTG ····· Normal-tension glaucoma
O_2 ····· Oxygen
OMM ····· ·· Outer mitochondria membrane
OMMAD ····· Outer mitochondrial membrane associated degradation
OPA1 ·····Optic atrophy tpye-1 (autosomal dominant)
OXPHOS ····· Oxidative phosphorylation
PolyQ ····· Poly glutamine chains
PolyUb ····· Polyubiquitin
PARL ····· Presenilin associaterhomboid-like protease
PD ····· Parkinson's disease
PINK1 ····· PTEN-induced kinase 1
Q ····· Ubiquinone
RGC ····· Retinal ganglion cells
RNS ····· Reactive nitrogen species
ROS ····· Reactive oxygen species
SDS ····· Sodium dodecyl sulfate
SIHM ····· Stress-induced mitochondrial hyperfusion
SOD ····· Superoxide dismutase
STS ····· Staurosporine
TCA ····· Tricarboxylic acid cycle
TDP-43 ····· TAR DNA binding protein-43
TH ····· Tyrosine hydroxylase
TMRE ····· Tetramethylrhodamine, ethyl ester
TOM ····· Translocase of the OMM complex
UPS ····· Unbiquitin-proteasome system
VADC ····· Voltage-dependent anion channel
VCP ····· Valosin-containing protein
6-OHDA ····· 6-hydroxidopamine
7-AAD ····· 7-aminoactinomycin D

1 Summary

1.1 Background

Mitochondria are the main site of energy production in most cells. Furthermore, they are involved in a multitude of other essential cellular processes, such as regulating the cellular calcium pool, lipid metabolism and programmed cell death [1]. Healthy and functional mitochondria are critical to meet the fundamental needs for almost all cell types, which makes mitochondrial quality control (QC) very important [2]. Given the high energy demand of neuronal cells, their vulnerability to endo- and exogenous stressors, and their post-mitotic status, mitochondrial QC plays an important role in neuronal survival with failing mitochondrial quality control linked to many neurodegenerative diseases such as Alzheimer's disease and many others [3-6].

Several well-orchestrated mechanisms of mitochondrial QC are in place to maintain mitochondrial function and to prevent cellular damage through dysfunction of mitochondria. Depending on the severity of mitochondrial damages, mitochondrial QC takes place on the molecular level, organelle level, and/or cellular level [7, 8]. Through those mechanisms damaged proteins are degraded, damaged mtDNA is repaired, and depolarized mitochondria are recycled. However when the first two mechanisms fail to reverse the damage, cellular level QC in form of programmed death or apoptosis takes place to limit further damage resulting from dysfunctional mitochondria [9]. Mitochondria are highly dynamic organelles that consistently undergo fusion and fission processes [10, 11]. Mitochondrial dynamics is a central part of mitochondrial quality control as mitophagic recycling of damaged mitochondria and as execution of cell death relies on the functional mitochondrial fission machinery [12, 13].

Ubiquitin-dependent proteasome-mediated protein degradation was recently implicated in mitochondrial QC as well as in the regulation of mitochondrial morphology [14, 15]. As part of the ubiquitin-proteasome system, (Membrane-Associated-Ring-CH 5) RING finger (Really-

Interesting-New-Gene)-domain containing transmembrane ubiquitin E3 ligase MARCH5 was shown to localize to the outer mitochondrial membrane (OMM) [16]. In addition, a role for MARCH5 in regulating mitochondrial dynamics was demonstrated through the ubiquitin-modification of dynamin-related-protein 1 (Drp1) and some others [17]. Interestingly, inactivation of MARCH5 resulted in mitochondrial elongation consistent with a role of MARCH5 in the regulation of Drp1-mediated mitochondrial fission. This feature correlates mitochondrial QC with the ubiquitin-proteasome-system (UPS) [18]. Furthermore, MARCH5 was shown to be involved in the degradation of the amyotrophic lateral sclerosis-related protein mSOD1 and the Joseph-Machado-disease protein ataxin 3 [19, 20]. Thus, MARCH5 function is likely involved in maintaining mitochondrial health in neuronal cells.

In order for proteasomal degradation of mitochondrial proteins to take place, protein retrotranslocation from the mitochondrion to the cytosol where the proteasome resides is needed. Recently, the AAA-ATPase p97/VCP/Cdc48, an essential part of the ubiquitin-proteasome system known to facilitate the retrotranslocation of ubiquitylated proteins for further degradation [21, 22], was shown to be involved in mitochondrial QC. While the role of p97 in mitochondrial protein QC is far from being understood, mutations in p97 were reported to be involved in Inclusion Body Myopathy with Paget Disease of Bone and/or fronto-temporal Dementia (IBMPFD) [23].

1.2 Aims of the study

The main aim of this study was to further explore and elucidate mechanisms of ubiquitin proteasome system-mediated mitochondrial proteostasis and quality control. Despite a lot of progress in the last several years, the involvement of outer mitochondrial membrane- localized ubiquitin ligases such as MARCH5, and their involvement in OMM proteins degradation is still not well understood. Thus, a particular aim of the study was to further characterize the involvement of MARCH5 in mitochondrial maintenance. Next to ubiquitin ligases, protein retrotranslocation was proposed to be important for mitochondrial maintenance with the AAA-

ATPase p97 recently connected to this process. Thus, the characterization of potential mitochondrial roles of p97 has been the focus of the next goal, in order to investigate the potential capacities of this multifunctional AAA-ATPase in mitochondrial membrane associated protein degradation. A last aim was to further connect mitochondrial quality control to mitochondrial function in neuronal-like cells and to better understand the role of ubiquitylation-dependent protein degradation in maintaining the health of neuronal-type cells especially under low stress conditions. To this end we investigated mitochondrial behavior and functional alternations in neuron-like cell lines in response to inactivation of MARCH5- and p97-dependent mitochondrial QC mechanisms.

1.3 Methods

As model for neuronal cells, two different neuron-like cell lines were used during the experiments, namely RGC5 (mouse neuron-like cell) [24, 25] and SH-SY5Y (human neuroblastoma) cells [26]. In order to analyze the ubiquitin-mediated, proteasome-dependent degradation of mitochondrial proteins under normal and low stress conditions, the activity of two proteins, MARCH5 and p97, shown to be involved in mitochondrial protein degradation was blocked. To this end, cell lines expressing dominant-negative mutants of MARCH5 ($MARCH5^{H43W}$) [16] or p97 ($p97^{QQ}$) [27]were generated by transfection and selection for stable expression. In order to model low level neurodegenerative stress to neuronal cells below the mitophagic and apoptotic threshold but likely to induce mechanisms of mitochondrial proteostasis, neuron-like cells were exposed to conditions of elevated pressure or hypoxia/re-oxygenation or were treated with the known neurotoxic agents 6-hydroxydopamine [28], the mitochondrial complex I inhibitor rotenone [29], or the Alzheimer's related Aβ peptide [30]. Confocal microscopy was used to acquire high solution images of mitochondrial morphology, while quantification of mitochondrial fusion and fission dynamics was performed by using a mitochondrial matrix-targeted photoactivatable-GFP (mtPA-GFP)-based fusion assay [31]. In addition, confocal life cell imaging and flow cytometry was employed to analyze other mitochondrial functional parameters, such as mitochondrial membrane potential,

mitochondrial reactive oxygen species (ROS) production, and cellular survival. A modified western blot technique (OyxBlot) was applied to demonstrate oxidized protein levels on isolated mitochondria. Unpaired, two-tailed Student's *t*-test, and paired t-test with Holm adjustment for multiple comparisons were used to analyze the data according to different specific data sets. For a detailed description of the employed methods please refer to the material and methods section of the included manuscripts.

1.4 Results

Neuron-like RGC5 cells exposed to neurodegenerative stress in form of increased hydrostatic pressure, hypoxia-reoxygenation, and electron transport chain inhibition following rotenone treatment showed increased mitochondrial fragmentation rate, increased cytochrome *c* release and cell death. Expression of MARCH5^{H43W} alleviated the mitochondrial damage following exposure to stressors, by significantly ($p<0.05$) reducing mitochondrial fragmentation, inhibiting mitochondrial cytochrome *c* release, and preventing RGC5 cell death, in comparison to MARCH5 expressing cells. These effects were shown to be similar to Drp1 inactivation, which was previously shown to be involved in mitochondrial fission and the execution of cell death. (Published in *PLoS One* 2012;7(12):e52637)

Furthermore, neuron-like SH-SY5Y cells exposed to neurotoxic stressors such as rotenone, 6-hydroxydopamine, and amyloid-beta peptide were employed to investigate the role of MARCH5-mediated QC in neuronal cells. As for SH-SY5Y cells, expression of MARCH5^{H43W} significantly inhibited mitochondrial fragmentation induced by these stressors evidenced by more fused and interconnected mitochondrial networks ($p<0.05$) compared to MARCH5 expressing cells. In addition, MARCH5^{H43W} expression significantly ($p<0.05$) prevented mitochondrial membrane potential depolarization, which is considered a decisive event of mitochondrial failure. At last, intracellular ROS levels were evaluated by using CellROX-DeepRed staining and quantitative confocal imaging. Consistently, MARCH5^{H43W} expression was able to

lower the ROS production significantly following neurotoxic treatments (Published in *Front Cell Neurosci* 2013 Oct 10;7:176.).

To further investigate the role of ubiquitin-dependent proteasome-mediated mitochondrial QC in neuronal cells, the impact of p97 inactivation in SH-SY5Y cell treated with neurodegenerative stressors such as rotenone, 6-hydroxydopamine, and amyloid-beta peptide (25-35) was analyzed. Compared to p97 expressing cells, $p97^{QQ}$ expressing cells displayed increased mitochondrial fragmentation, with additive effects observed during neurotoxic stress ($p<0.01$). Mitochondrial membrane potential level, as detected by TMRE (tetramethylrhodamine, ethyl ester) and evaluated by flow cytometry was significantly decreased following $p97^{QQ}$ expression. Again additive effects were found under neurotoxic stress conditions ($p<0.05$). ROS production was evaluated using the mitochondrial specific dye MitoSOX. A significant increase of mitochondrial ROS production was found in $p97^{QQ}$ expressing cells with or without treatments as compared to p97. In parallel, OxyBlot further confirmed increased levels of oxidized mitochondrial proteins in $p97^{QQ}$ expressing cells ($p<0.05$).

Targeted mitochondrial autophagy or mitophagy occurs when mitochondrial damage cannot be dealt with by the molecular repair capacity degrading the entire, damaged organelle for recycling. Mitochondrial translocation of the ubiquitin ligase Parkin is an important step in this process. Thus, Parkin mitochondrial translocation assays were used to evaluate potential induction of mitophagic degradation under mild stress conditions and to further support the involvement of the proteasomal degradation system as part of mitochondrial QC (submitted to *Front Cell Neurosci* – under review).

1.5 Conclusions

Healthy and steady mitochondrial dynamics and QC machinery maintain mitochondrial function and subsequently support neuronal cell survival during endo- and/or exogenous neurotoxic stresses. Thus, understanding mitochondrial QC and its failing will yield important insight into the mechanisms of neurodegeneration.

2 Introduction

2.1 Mitochondria

One of the first descriptions of mitochondria is from more than one hundred twenty years ago. There, Altmann described them as independent, intracellular structures likely involved in energetic processes [32]. Structure analysis showed mitochondria are subcellular double membrane, highly dynamic and partially independent intracellular organelles existing in most eukaryotic cells and containing their own genomic DNA (mtDNA). Mitochondria are most widely known as the power plant of the cell due to their production of adenosine triphosphate (ATP) through electron transport chain and oxidative phosphorylation (OXPHOS) reactions [33]. Besides their role in energy production, mitochondria play various roles in regulating cellular metabolic signals, programmed cell death known as apoptosis, and calcium balancing [34].

2.1.1 Mitochondrial structure

As shown in Figure 2.1, mitochondria are comprised of an outer mitochondria membrane (OMM) and an inner mitochondria membrane (IMM). Those two membranes enclose the intermembrane space and separate the mitochondrial matrix which harbors the mitochondrial DNA from the cytosol [35]. The outer mitochondria membrane has a similar protein-to-phospholipid ratio as the eukaryotic plasma membrane. The inner mitochondrial membrane where the protein complexes of the electron transport chain (ETC) are located is highly folded to forming numerous so called cristae. The intermembrane space contains a different molecular content as the cytosol, since the OMM allows small molecules like glucose to pass though freely, whereas larger proteins depend on specific transporters or membrane channels to pass this membrane. Cytochrome *c* is a soluble protein which is localized in the intermembrane space as well. In addition to mtDNA and ribosomes, the mitochondrial matrix contains a high density of proteins, mostly enzymes involved in pyruvate oxidation, fatty acid β-oxidation and tricarboxylic acid cycle (TCA cycle), also known as Krebs cycle. Mitochondrial DNA, which

contains approximately 16,600 base pairs, is coding for 37 genes including 13 peptides, 22 tRNA and 2 rRNA unique for mitochondrial structure and function [36]. Mutations of mtDNA, either in the coding or the noncoding region, heteroplasmic or homoplasmic, have been linked to many human diseases [37].

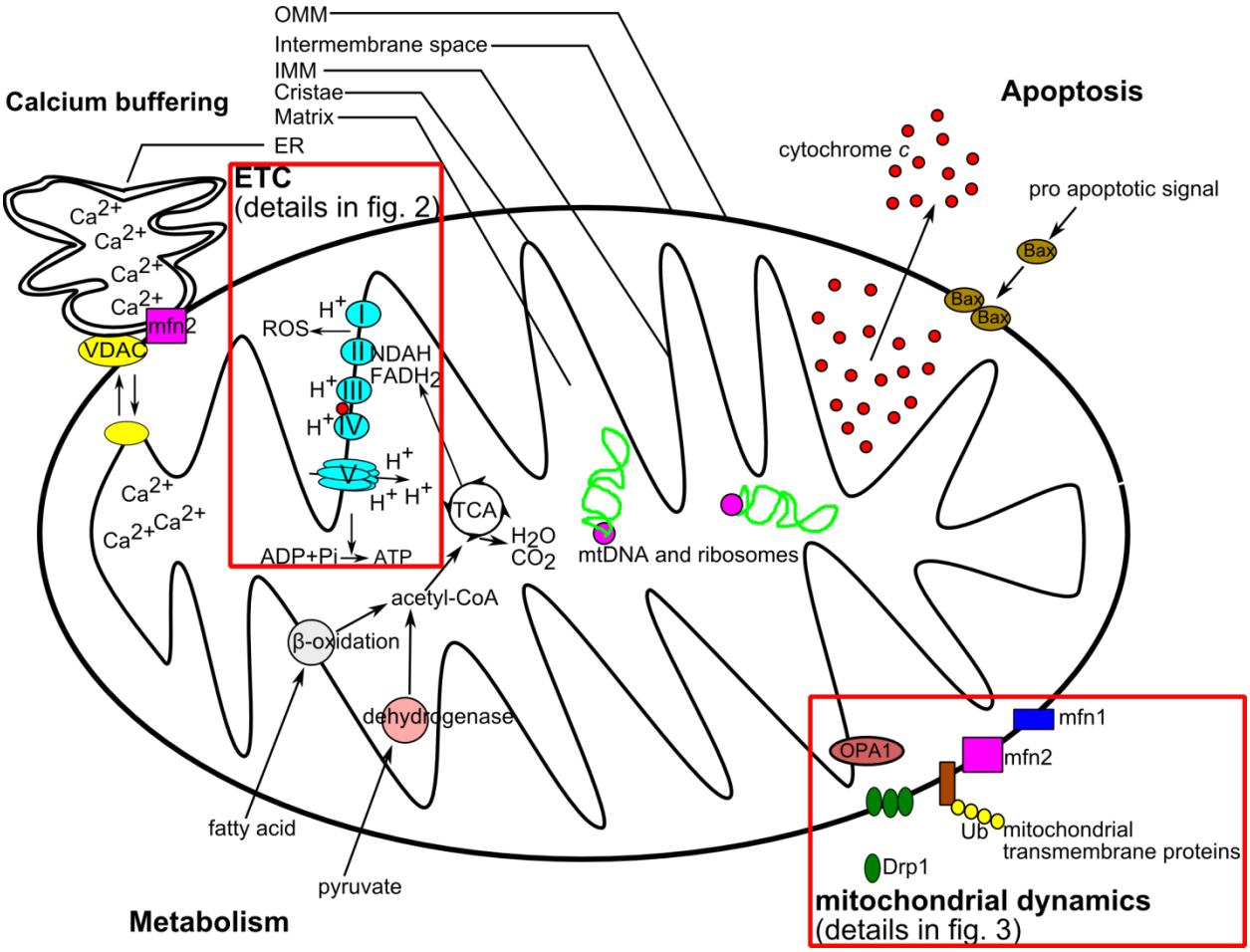

Figure 2.1 Overview of mitochondrial structure and functions

Mitochondria are double membrane-bound organelles with an outer (OMM) and inner mitochondrial membrane (IMM). The mitochondrial matrix is the inner most part of mitochondria harboring mtDNA and also the enzymatic machinery for the tricarboxylic acid cycle (TCA cycle). The IMM is highly folded forming so called cristae in order to expand the membrane surface for the components of the electron transport chains (ETC). The ETC is generating the mitochondrial membrane potential ultimately used for the production of ATP by complex V or the F_0F_1-ATPase. Mitochondria are involved in many different functions. Mitochondrial morphology is governed by mitofusin1 and 2 (Mfn1/2) and dynamin related protein 1 (Drp1) localized on the OMM, while optic atrophy type 1 (OPA1) is found in the intermembrane space and on the IMM. Ion transporters such a voltage dependent anion channel (VDAC) are involved in Ca^{2+} buffering. Cytochrome *c* is a small protein localized in intermembrane space as part of the ETC. Release of cytochrome *c* following certain apoptotic signals, like Bcl-2-associated X protein (Bax) cytosolic to mitochondria translocation, is a key trigger of intercellular apoptosis pathway.

2.1.2 Mitochondria and energy production

Mitochondria are best known for their role in cellular energy conversion from food stuff to ATP, a form of chemical energy usable for almost every cellular reaction. During this process, multistep biochemical reactions are carried out mostly in the matrix. Firstly, pyruvate is oxidized to produce acetyl-CoA, and then citric acid cycle is applied to oxidize acetyl-CoA into water and carbon dioxidide (CO_2). In the meantime NAD^+ and FAD (flavin adenine dinucleotide) are reduced into NADH (nicotinamide adenine dinucleotide) and $FADH_2$, respectively, to store the energy for later use. Finally, oxidative phosphorylation (OXPHOS), which is carried out by the electron transport chain , is responsible for transferring the energy from NADH and $FADH_2$ to ATP, the universal 'petroleum' of cells [38].

The ETC consists of five large protein complexes: complex I, II, III, IV, and V, which together perform OXPHOS (Figure 2.2) [39]. In detail, complex I (NADH dehydrogenase, or NADH ubiquinone oxidoreductase) transfers the electrons from NADH to ubiquinone to form ubiquinol, thereby translocating protons (H^+) across the membrane from the mitochondrial matrix into intermembrane space generating a proton gradient. Complex I is one of the main site of electron leakage happens and reactive oxygen species (ROS) produces, such as superoxide [40], especially when its function been inhibited by specific blocker like rotenone. Complex II (succinate dehydrogenase) is a parallel electron transport pathway to complex I, in which additional electrons are transferred to quinone (QH_2) from $FADH_2$. However, no proton transmembrane process is conducted in this step. Complex III (ubiquinol-cytochrome c oxidoreductase) catalyzes the oxidation of ubiquinone and reduction of cytochrome *c* (from Fe^{3+} to Fe^{2+}), and further translocates protons out to contribute the H^+ gradient [41]. If complex III is functionally jeopardized, e.g. by antimycin A toxicity, electrons will leak to oxygen and contribute to ROS production as well. Complex IV (cytochrome *c* oxidase) removes four electrons from cytochrome c to oxygen (O_2) to produce two molecules of water (H_2O), and again more protons are transferred out from matrix to the intermembrane space. F_0F_1-ATP synthase performs the final step of ETC, in which F_0 acts as an ion channel that allows protons

flux back into the mitochondria matrix [42]. The reflux of H^+ releases free energy generated from NADH oxidation, which subsequently been used by F_1 to catalyzes ATP synthesis [43, 44]. The coupling of oxidative phosphorylation with electrochemical gradient release is the key step of ATP production [45].

In brief description, mitochondria ETC transports the electrons from donors (NADH and QH_2) to final acceptor O_2 through series redox reactions (see Figure 2). The energy released from the chemical reactions is used by the proton pumps (complex I, III, IV) to transmembranely transfer the H^+ and to generate proton gradient. Finally the gradient potential is released via H^+ reflux and utilized to synthesize ATP [46].

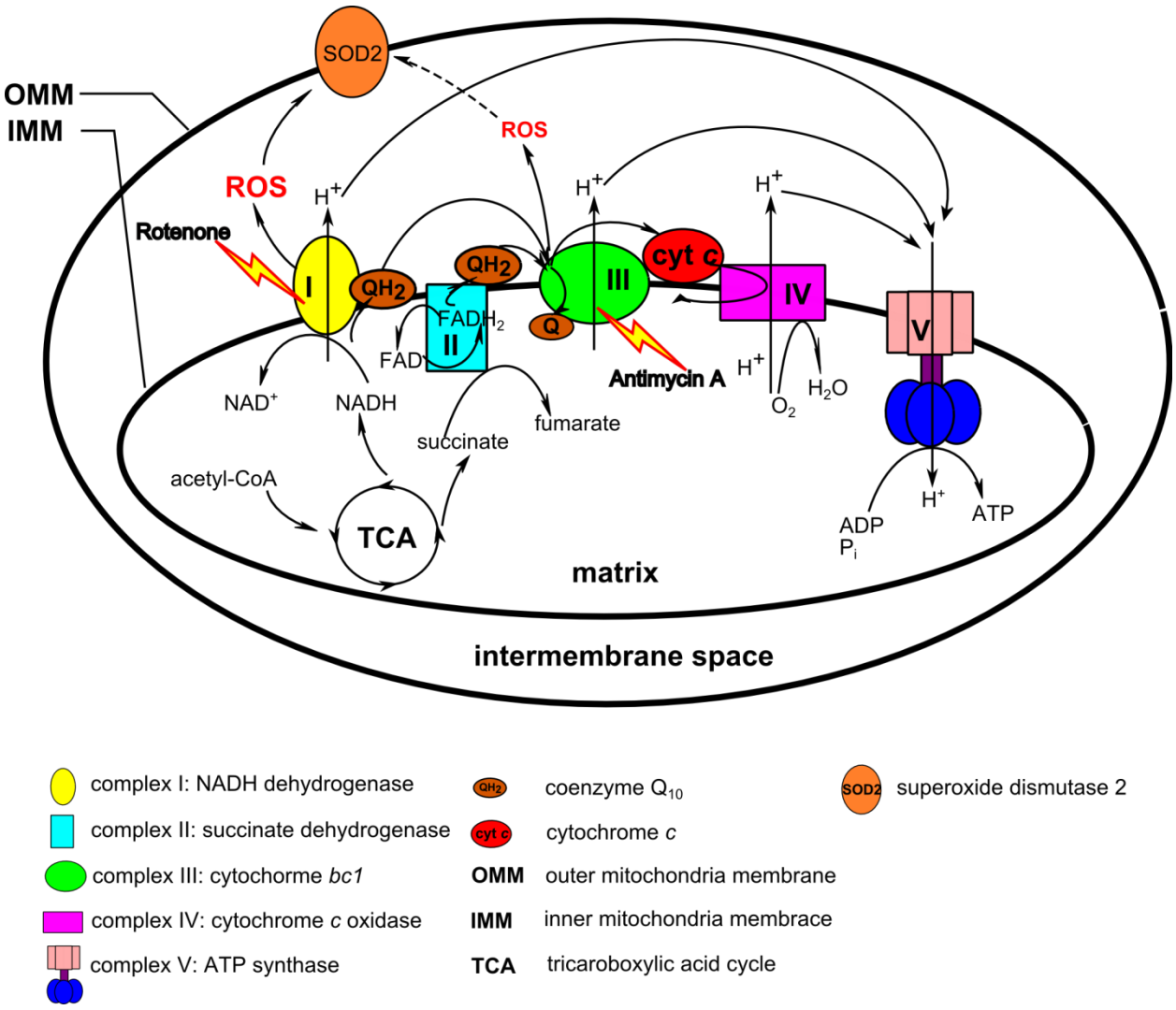

Figure 2.2 Electron Transport Chain (ETC)

The electron transport chain is composed of five complexes (complex I - V). The substrates NADH and succinate generated by the tricaroboxylic acid cycle - pass electrons through the ETC (I - IV) to O_2 generating H_2O, meanwhile protons (H^+) are transferred out of the matrix into the intermembrane space generating an electro-chemical potential across the IMM to store the energy. At last,H^+ flow back through complex V, also known as F_0F_1-ATPase, driving ATP production. As byproduct of OXPHOS, the ETC is involved in ROS production, which can normally be neutralized by local antioxidant, such as superoxide dismutase 2 (SOD2). However, overwhelming ROS production triggered by certain stresses will lead to oxidative stress. Complex I and complex III are the two major sites of ROS production. For example, rotenone specifically inhibits complex I, while antimycin A acts on complex III inducing ROS production.

2.2.3 Mitochondria as source for reactive oxygen species (ROS)

Reactive oxygen species describe a variety of molecules and free radicals derived from oxygen, which are extremely reactive oxidants. ROS include free radicals such as the superoxide anion ($O2\cdot^-$), or the hydroxyl radical (·OH), but also non-radical oxidants such as superoxide, hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) [47]. The extremely reactive and therefore short-lived superoxide anion is the product of a one-electron reduction of oxygen and the precursor of most other cellular ROS. Detoxification or dismutation of the superoxide anion (either spontaneously or catalyzed through superoxide dismutase) leads to the generation of H_2O_2, which is either fully reduced to water or partially reduced to the hydroxyl radical (·OH), one of the strongest oxidants in nature [48]. ·OH as one of the most potent ROS and readily reacts with cellular components is responsible for oxidative damage to lipids, DNA, and proteins. Additionally, $O_2\cdot^-$ may react with other radicals, including nitric oxide (NO·) [49], leading to the generation of reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$), another highly reactive oxidant capable of reacting with cellular components.

ROS are a common byproduct of the cellular metabolism. As ROS are generated during oxygen metabolism and as mitochondria are the main cellular oxygen sink, it is no surprise that mitochondria are the major cellular source for ROS. Under normal physiological conditions it is estimated that more than 90% of the consumed oxygen is reduced to H_2O by mitochondrial electron transport chain. While the ETC is highly efficient, nonetheless a small portion of this oxygen is only partially reduced leading to ROS production [50].

ETC-linked ROS production was first reported a half century ago. It was observed that antimycin A-treated isolated mitochondria are producing hydrogen peroxide [51]. Further studies identified more mitochondrial components responsible for ROS production, including complex I, complex III, and other mitochondria-localized redox systems.

Complex I is recognized as a major source of ROS in mitochondria [40]. However under normal conditions, the production of superoxide by complex I is negligible. But under pathological

conditions, large amounts of ROS are produced by complex I. It is estimated with 40% of all mitochondrial disorders are related to mutations of complex I subunits [52]. Parkinson's disease (PD) is one of the typical examples. PD is characterized with a progressive loss of dopaminergic neurons and cell bodies of the substantia nigra pars compacta and nerve terminals in the striatum. ROS are considered as one of the main pathogenesis factors based on dopamine oxidation-related metabolic pathways. Under physiological circumstance, oxidative deamination of dopamine by monoamine oxidase produces hydrogen peroxide [53]. In the pathological pathway, dopamine can be oxidized non-enzymatically by superoxide forming dopamine quinone which will be reduced by mitochondrial complex I to generate semiquinone followed by a transfer of its electron to molecular oxygen to form superoxide, completing a vicious oxidative cycle [54]. Both somatic and mitochondria DNA point mutations might cause complex I dysfunction, thus subsequently linking ROS-mediated damage to neurodegenerative disorders such as Leber's hereditary optic neuropathy (LHON), Leigh's syndrome (LS), and mitochondrial encephalomyopathy, lactic acidosis, and stroke like episodes (MELAS) [40].

Complex II does not seem to be a the main contributor to mitochondrial ROS generation [55], partly because succinate levels, the substrate of complex II, are relatively low in tissue. However, isolated mitochondria oxidizing succinate at high concentration did produce H_2O_2 under *in vitro* conditions [56]. Under pathological conditions, evidence points to a correlation of complex II deficiency and increased ROS production. During drug-induced apoptosis, intracellular pH environment becomes significantly acidic impairing complex II activity and resulting in ROS generation [57]. On the molecular level this likely results from the dissociation of SDHA (flavoprotein subunit) and SDHB (iron-sulfur protein-containing part) subunits from the membrane-bound components of complex II causing single-electron reduction of oxygen by these reduced iron-sulfur clusters [58]. In addition, complex II dysfunction is one hallmark of Huntington's disease (HD). Interestingly, treatment with 3-Nitropropionic acid, an irreversible inhibitor of complex II activity, induces HD-like symptoms along with increased ROS generation [59, 60].

Complex III accepts reduction equivalents originating from complex I and complex II and processes them with the Q-cycle mechanism. In brief, the cycle starts with ubiquinol releasing its proton to the IMS and donating one electron to an iron-sulfur protein, producing one semiquinone on the outer side of the IMM. Semiquinone (Q-·) continuously passes these electrons to hemes of cytochrome b_L, and then to cytochrome b_H. Cytochrome b_H reduces ubiquinone to generate ubisemiquinone followed by its further reduction by a second electron and protonation [61]. Under physiological conditions, the fast oxidation rate of $Q^{-\cdot}$ gives it less chance of losing electrons and thus contributing to ROS production. However, if the flow of electron through the complex III is stalled e.g. following application of complex III inhibitors such as antimycin A, myxothiazol, or stigmatellin, semiquinone levels are elevated resulting in more opportunities to donate single-electron to reduce oxygen [62-64].

Aside of complex I and III as the major production site of mitochondrial ROS, also complex IV is able to generate ROS [65]. Complex IV, also called cytochrome c oxidase, is a protein-phospholidpid complex containing four redox centers (Cu_A, cyt. a, cyt. a_3 and Cu_B) involved in electron transport and the conversion of oxygen to water. During this process, several 'peroxyl' and 'ferryl' intermediates are produced, which are considered as potential sources of free radicals [65]. However, sophisticated defense mechanisms have been developed to protect the complex IV, including regulating ROS generation and removal [66]. In vitro mitochondrial ischemia/reperfusion experiment showed significant increase of ROS production, and complex IV has been suggested to contribute around 30-35% of total superoxide [67].

2.1.4 Other mitochondrial functions

In addition to metabolic process and energy production, mitochondria play important roles in many other cellular events. First of all, activated by various signal molecules and modulated by proteins of the Bcl-2 family, mitochondria release cytochrome *c* from the IMM into the cytosol through the opening of certain channel on the outer mitochondria membrane. This step of cytochrome *c* release is considered the step-of-no-return in the induction of apoptosis [68, 69].

Secondly, mitochondria have the ability of storing calcium in their matrix compartment, thus serving as a major calcium buffering system of the cell. Furthermore, mitochondria are the major source of ROS, thus, playing an important role in ROS-related signaling events [70].

2.2 Mitochondrial dynamics

The word mitochondrion stems from the ancient Greek mitos (meaning thread) and chondrion (meaning granule). This name aptly describes the primary observation of the heterogeneous morphologic appearance of these organelles - sometimes bean-shaped or granule-like while other times elongated, thread-like [71]. These early insightful observations of the polymorphic nature of mitochondria are nicely confirmed by modern live cell imaging techniques using mitochondria-targeted fluorescent proteins to follow mitochondrial morphological changes in vivo. Mitochondrial morphology is less static as previously appreciated based on electron microscopic analyses, with mitochondria continuously changing their shapes through fission and fusion [3]. Thus, mitochondria are highly dynamic organelles constantly changing in sizes, shape, and numbers. And interestingly, dynamic behavior of mitochondria is linked to the function and also dysfunction of these organelles [72, 73] .

2.2.1 Molecular machineries of mitochondrial dynamics in mammalian cells

Mitochondrial morphology is governed by two distinct processes, mitochondrial fission or division of mitochondrial tubules and the fusion of mitochondria. Balanced mitochondrial fission and fusion together result in a steady-state morphology. Fusion of mitochondrial tubules necessitates the fusion to two sets of membrane without jeopardizing organellar integrity. As shown in figure 2.3A, three large GTPases are responsible for mitochondrial fusion. Mfn1 and Mfn2, two large transmembranane GTPases located on the OMM, are necessary for outer mitochondrial membrane fusion [74, 75]. Mouse gene knock out models have been established as strong supportive evidence. Single knockout mouse embryonic fibroblasts (MEFs) lacking either Mfn1 or Mfn2 demonstrated significantly higher fragmented mitochondria as compared to control MEFs, while double knock out of both Mfn1 and Mfn2 resulted in the completed loss

of mitochondrial fusion representing small fragmented mitochondria [76]. Interestingly and in addition to mitochondrial fusion, Mfn2 is involved in the formation of mitochondria – endoplasmic reticulum (ER) membrane contact sites [77, 78]. While fusion of the OMM is governed by the mitofusins, fusion of the IMM is under control of optic atrophy tpye-1 (OPA1), also a large GTPase located in the intermembrane space and on the IMM. OPA1 was identified through human genetic studies, as its mutation results in autosomal dominant optic atrophy (ADOA), a disease associated with retinal ganglion cells degeneration resulting in optic nerve atrophy and ultimately blindness [79-82]. In OPA1 knock down cells, highly fragmented mitochondria have been observed, as well as severe disorganization of mitochondrial cristae. In addition, loss of OPA1 results in serious impairment of mitochondrial function, such as reduced respiratory capacity, but also increased sensitivity to apoptosis [83, 84]. Experiments on skeletal myocytes and cerebellar Purkinje cells have showed that lacking of OPA1 or mitofusins demonstrated severe decline in respiration functions [85, 86].

Mitochondrial fission on the other hand is mediated by dynamin-related protein-1 (Drp1) [87]. Upon mitochondrial fission, Drp1 is recruited from a large cytosolic pool of Drp1 to mitochondria. The recruitment of Drp1 to future scission sites is under control of a group of outer mitochondrial membrane proteins, such as the mitochondrial fission factor (Mff), mitochondrial dynamics protein 49kD (MiD49) and mitochondrial dynamics protein 51kD (MiD51), as well as hFis1 [88, 89]. These OMM-anchored proteins serve as receptors of Drp1 on out mitochondria membrane, similar to yeast Fis1 [90]. The analyses of dominant negative mutants of Drp1 ($Drp1^{K38A}$) and RNA interference experiments strongly support the function of Drp1 in mitochondrial fission. Loss of Drp1 activity results in elongated and entangled mitochondrial morphology, and confers resistance to mitochondrial fragmentation stimuli, such as treatment with the mitochondrial uncoupler CCCP (carbonyl cyanide m-chlorophenylhydrazone) [91]. Also, loss of Drp1 activity greatly increases resistance to apoptotic stimuli further connecting mitochondrial morphology to the execution of apoptosis. Drp1 is the target of several regulatory mechanisms, as it is crucial for maintaining a balanced

mitochondrial network. Drp1 activity is impacted by several post-translational modifications: Phosphorylation by cyclin B1-CDK1 activates Drp1 during cell mitosis; nitric oxide (NO) activates Drp1 through S-nitrosylation [92]; mitochondrial-anchored protein ligase (MAPL) can regulate Drp1 activity by SUMOylation [93]; and membrane-associated RING-CH (MARCH5) as well as Parkin are both ubiquitin ligases which can regulate Drp1 activation and degradation through ubiquitylation [16, 17, 94, 95]. (Fig. 2.3B)

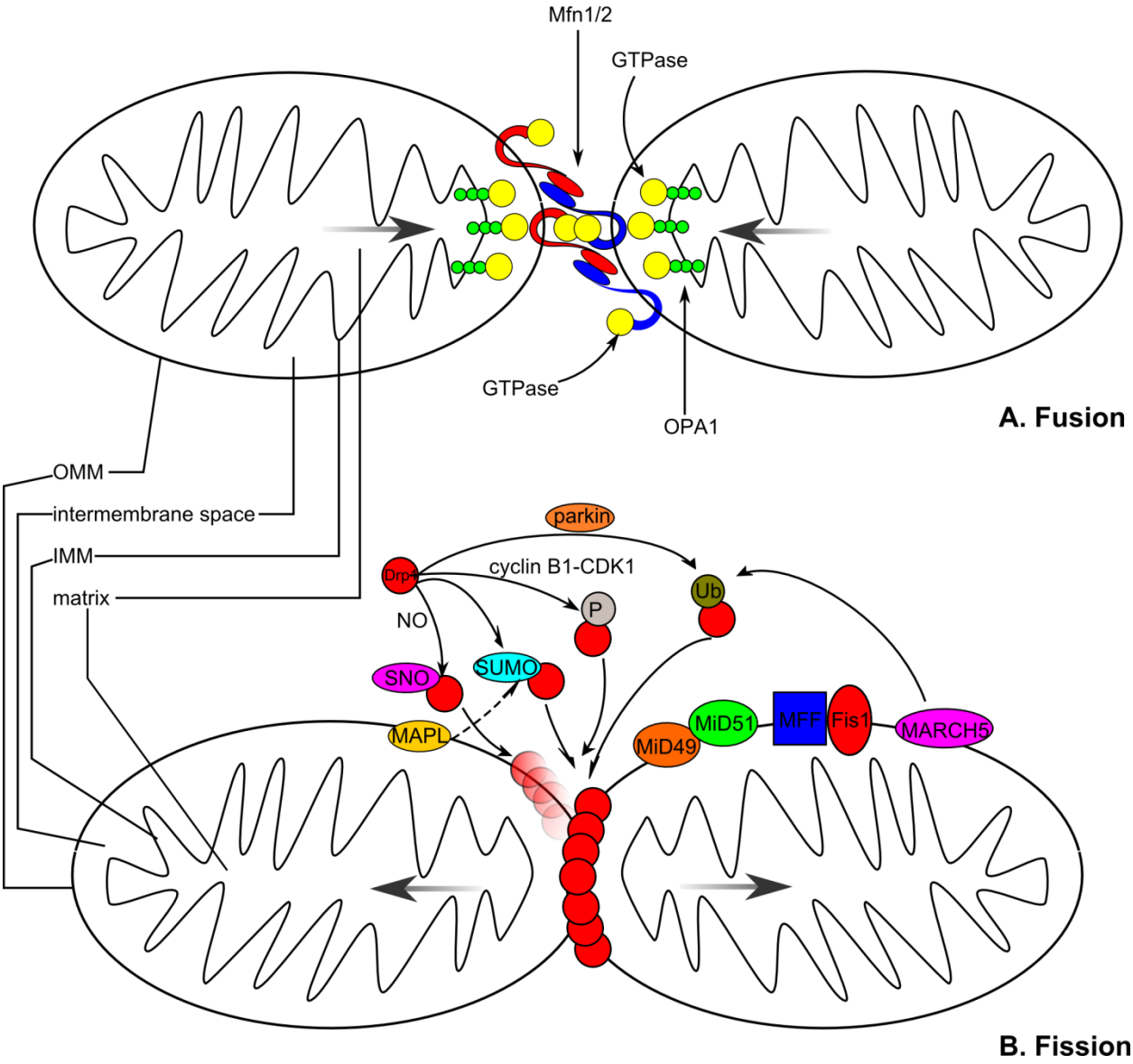

Figure 2.3 Machinery of mitochondrial dynamics

Mitochondrial fusion (A) is mediated by three large membrane GTPases. Mfn1and Mfn2 both located on outer mitochondrial membrane (OMM) mediate fusion of the OMM, while OPA1 governs fusion of the inner mitochondrial membrane (IMM). Mitochondrial fission (B) is depended on recruitment of Drp1 from the cytosol to the OMM by a group of cofactors, including mitochondrial fission factor (Mff), mitochondrial dynamics protein 49kD (MiD49), mitochondrial dynamics protein 51kD (MiD51), or hFis1. Posttranslational modifications of Drp1, such as phosphorylation by cyclin B-CDK1, ubiquitylation by Parkin and membrane-associated RING-CH 5 (MARCH5), or SUMOlytion by mitochondrial-anchored protein ligase (MAPL) further regulate the activity of Drp1 and thus modulate the mitochondrial fission rate.

2.2.2 Importance of mitochondrial dynamics

Mitochondrial fusion and fission are two opposing but exquisitely balanced processes appropriately maintaining the shape, size and number of mitochondria. Beyond simply maintaining mitochondrial morphology, fusion and fission processes are considered extremely critical to many functional features of mitochondria.

First of all, continuously mitochondrial fusion and fission promotes content mixture effectively between individual mitochondrial subunits. Considering the relative instability of mtDNA and the heterogenic pool of mitochondria, the frequent exchange of content promoted by frequent mitochondrial fusion provides opportunities for repairing mutated mtDNA via complementation or for compensating of respiratory capacity by supplying functional mitochondrial proteins to damaged mitochondria. Thus, fusion of a damaged with a functional mitochondrial subunit provides a chance to repair mitochondrial function [96-98]. And indeed it was shown, that blocking mitochondrial fusion results in loss of mitochondrial fidelity in mammalian cells and complete loss of cristae structure and respiratory capacity in budding yeast [99-101] . Just as mitochondrial fusion is essential for maintaining mitochondrial function, the fission process is also involved in mitochondrial maintenance. Mitochondrial fission promotes the segregation of functional from damaged mitochondrial contents, and facilitates the degradation of damaged mitochondria beyond repair [102, 103]. Taken together, mitochondrial dynamics aids mitochondrial quality control system by enabling rescue and repair of mitochondrial through fusion-mediated complementation and also by promoting segregation of damaged mitochondria from the healthy mitochondrial network.

Connected to its function in mitochondrial complementation and repair, mitochondrial dynamics promotes adaptation to certain types of stresses. In cellular experiments, nutritious starvation or exposure to UV light induces highly elongated, interconnected mitochondria, a phenomenon described as stress-induced mitochondrial hyperfusion (SIMH) [104]. Interestingly, SIMH is accompanied by elevated mitochondrial membrane potential and

increased ATP production [104]. SIMH depends on the function of Mfn1 and OPA1, and confers a certain resistance to additional stress-related insults [105, 106].

Also connected to the maintenance of mitochondrial network integrity is the mitophagic destruction of damaged mitochondrial subunits. Enhanced mitochondrial fission as result of a decreased fusion rate due to Parkin-mediated degradation of mitofusin promotes mitophagy (see 2.4 for further detail). On the other hand, mitochondrial elongation interferes with mitophagy [107-109]. Decreased translocation of Drp1 to mitochondria has been identified during starvation, resulting in a lower rate of fission pushing the balance towards fusion, therefore protecting mitochondria from excessive starvation induced mitophagy [110].

Besides the maintenance of mitochondrial function, mitochondrial dynamics is also involved in the initiation of programmed cell death or apoptosis. The release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol is one of the key steps of the intrinsic apoptotic pathway [111], as cytosolic cytochrome *c* is an essential component of the apoptosome involved in caspase activation. Cytochrome *c* is released as result of mitochondrial outer membrane permeabilization (MOMP) induced by pro-apoptotic members of the Bcl-2 family, e.g. Bax and Bak. Morphological analyses showed that increased mitochondrial fission and fragmentation occurs closely before or during cytochrome *c* release and the induction of apoptosis [112]. Furthermore, Drp1 and Bax translocation to mitochondria are connected, further supporting the relationship between mitochondrial fission and apoptosis [113, 114]. Interestingly, inhibition of mitochondrial fission interferes with cytochrome *c* release and slows-down the induction of apoptosis [115, 116] while excessive mitochondrial fragmentation as a result of insufficient mitochondrial fusion is promoting cytochrome c release and, thus, apoptosis.

2.3 The mitochondria associated ubiquitin-proteasome system

The degradation of damaged or superfluous proteins through proteolytic processes is the most critical mechanism for maintaining of cellular protein homeostasis. The most important protein

quality control mechanism is the ubiquitin-proteasome system (UPS) as it is responsible for targeted degradations of proteins residing in nucleus, the cytosol, and endoplasmic reticulum [117] and, as recent evidence suggests, the mitochondria [118].

2.3.1 The ubiquitin-proteasome system

The ubiquitin-proteasome system (UPS) is a highly specific proteolytic machinery existing in eukaryotic cells whereby the small protein-modifier ubiquitin is transferred to a substrate protein destined for degradation by the large, cytosolic proteasome. The UPS is commonly recognized as the major route of intracellular proteostasis, where the unfolded, misfolded, or abnormally aggregated proteins are selectively targeted and degraded.

Ubiquitylation is a post-translational protein modification, which requires three enzymatic activities to work in concert. The highly conserved ubiquitin protein is in a first step activated by the ubiquitin-activating-enzyme (E1), then transferred to one of about 80 ubiquitin-conjugating-enzymes (E2), until in a third step, ubiquitin is transferred to the substrate protein via a so called E3 enzyme or ubiquitin-ligase [119, 120]. The specificity of this process is guaranteed by the many hundreds of distinct ubiquitin-ligases that can recognize particular substrates. There are two main classes of E3 ligases classified according to the catalytic domains they contain: HECT domain enzymes and RING-finger domain enzymes. HETC domain (Homologous to the E6AP C-Terminus) E3 ligases generate Ub-thiolester-intermediate prior to establishing an isopeptidic bond between the C-terminus of Ub and an amino group on the substrate protein, while RING-finger E3 ligases catalyze the transfer of activated Ub directly from E2 to the substrate [121, 122]. Multiple repetitions of this process, through ubiquitylation of ubiquitin itself on lysine residues 48 (Lys48), will elongate the polyUb tails (to least four Ub molecules) on the target protein. Upon formation of a polyUb chain on the substrate, recognition and degradation of the ubiquitylated substrate protein by the 26S proteasome ensues. Besides the well-known Lys48-linked polyubiquitylation, other forms of polyubiquitylation occur and impact a diverse array of cellular functions [123] (Figure 2.4).

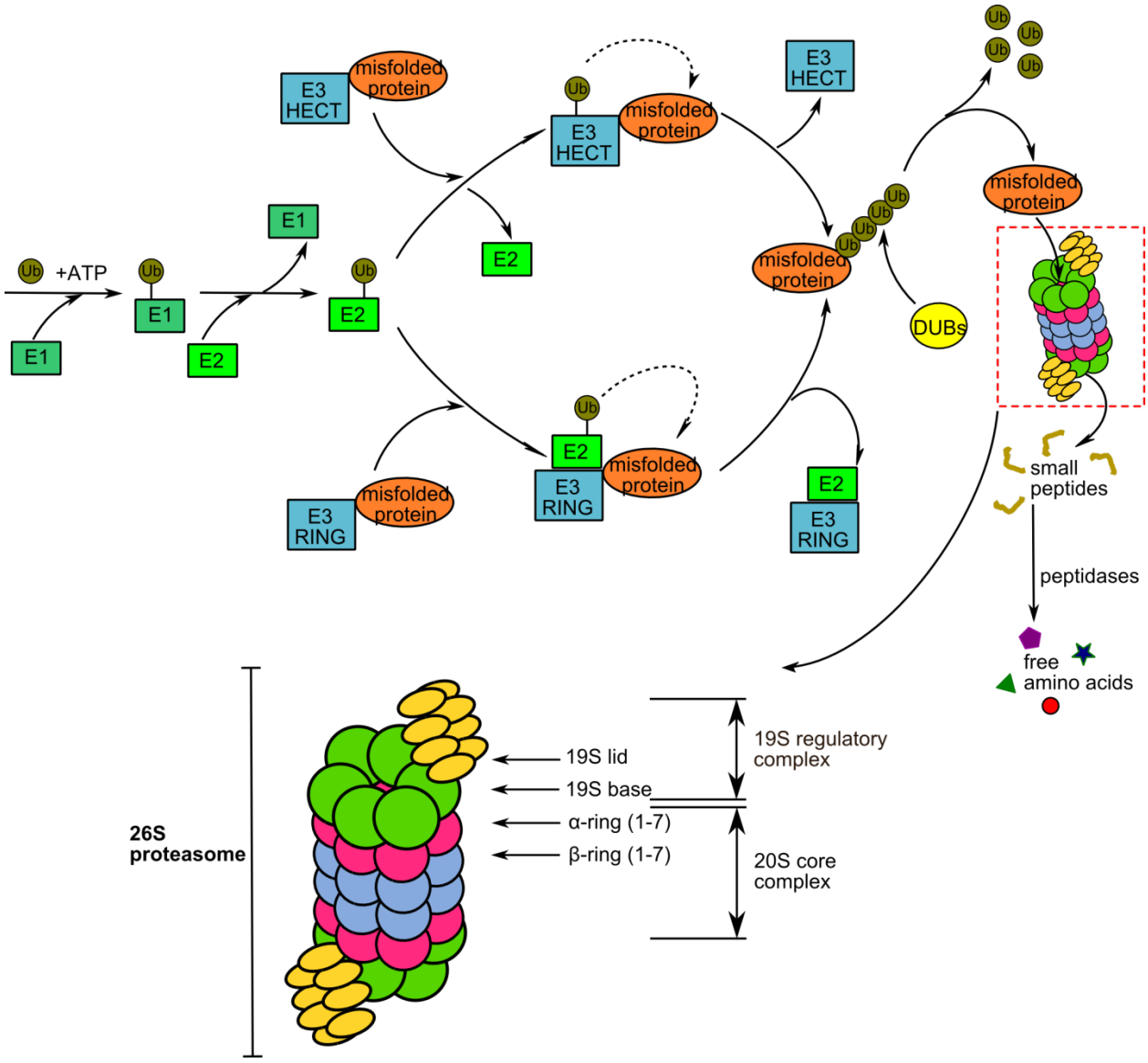

Figure 2.4 Ubiquitin proteasome system (UPS)

The ubiquitin-activating-enzyme (E1) activates ubiquitin (Ub), transfers it to an ubiquitin-conjugating-enzyme (E2) before final transfer of activated ubiquitin with the help of an ubiquitin-ligase (E3) occurs. Due to catalytic domain difference, E3 are categorized into HETC domain (Homologous to the E6AP C-Terminus) E3 and RING-finger (Really interested new gene) E3. Poly-ubiquitylated proteins are recognized and degraded by the 26S proteasome. As part of the proteasomal degradation process deubiquitylation enyzmes (DUB) will release Ub for reuse.

2.3.1.1 UPS and organellar protein quality control

The UPS plays an important role not only in cytosolic proteins turnover, but is also essential for organellar proteostasis. The endoplasmic reticulum (ER) is the organelle that proteins pass through before entering the Golgi, the plasma membrane, or vacuoles. As part of the quality control mechanism called ER-associated degradation or ERAD, abnormal and misfolded proteins in the ER are ubiquitylated and retro-translocated to the cytosol for degradation by the proteasome [117]. In addition to dedicated E2 and E3 enzymes, ERAD employs different co-factors to achieve cytosolic protein degradation. As the substrate proteins originate from a membrane confined organelle, additional factors such as the AAA-ATPase Cdc48/p97 are required for extraction from the ER (more details discussed later).

Similar to the ER, mitochondria were recently linked to UPS in form of outer mitochondrial membrane-associated degradation or OMMAD [124]. Evidence showed that proteasome inhibitors can increase the levels of ubiquitylated mitochondrial proteins, indicating the potential role that proteasome plays on mitochondrial protein degradation. Additionally, outer mitochondrial membrane proteins such as Mfn1, Mfn2 and Mcl-1 were found to be poly-ubiquitinated and degraded by the proteasome. Also, several specific E3 ligases were found to localize to the OMM and were shown to be involved in the ubiquitylation of mitochondrial proteins, including MULAN, Parkin, MARCH5, RNF185, and IBRDC2[15, 125, 126]. Among many others, RING-E3 ligases Parkin, MULAN and MARCH5 are widely studied together with their potential mitochondrial substrates (mitofusins, Drp1, Mutated SOD1, etc), while the latter one is part of our research subjects [27] (Tab. 2.1). Interestingly and analogous to ERAD, p97 is also involved in promoting the extraction of PolyUb-proteins from the mitochondrial membrane and transport to the cytosolic proteasome [14]. Previous research has proved that mutated mitochondrial proteins like mtSOD1 are selectively degraded through UPS, which support the connections between UPS and mitochondrial membrane protein QC directly [19]. Described by many studies, several mitochondrial dynamics regulators (mitofusins, Drp1) are involved in ubiquitylation processes [127]. Thus, by affecting mitochondrial fission and fusion machinery

functions, the UPS is certainly connected to mitochondrial dynamics, therefore participating in mitochondrial maintenance. The UPS is also through the ubiquitin E3 ligase Parkin, which serves to initiate mitophagy, connected to mitophagic QC [128].

Table 2.1 Mitochondrial E3 ligases and known substrates

Mitochondrial E3 Ligase	**Localization**	**Mitochondrial Substrates**
Parkin (Ub)	Cytoplasm→Mitochondria	Mcl-1, Mfn1/2, Drp1
MULAN (Ub) or MAPL (SUMO)	Mitochondria Mitochondria	Omi/HtrA2 ? Drp1
MARCH5 (Ub)	Mitochondria	Drp1, Mfn1/2, MuSOD1, ataxin-3, polyQ
IBRDC2	Cytosol and Mitochondria	Bax

Abbreviations:

MULAN: Mitochondrial Ubiquitin Ligase Activator of NF-kB, MARCH5: membrane-associated RING-CH 5, MAPL: mitochondrial-anchored protein ligase, Mcl-1: induced myeloid leukemia cell differentiation protein, Mfn: mitofusin, Drp1: dynamin-related protein 1, Omi/HtrA2: Serine protease HTRA2-mitochondrial, SOD: superoxide dismutase, PolyQ: Polyglutamine.

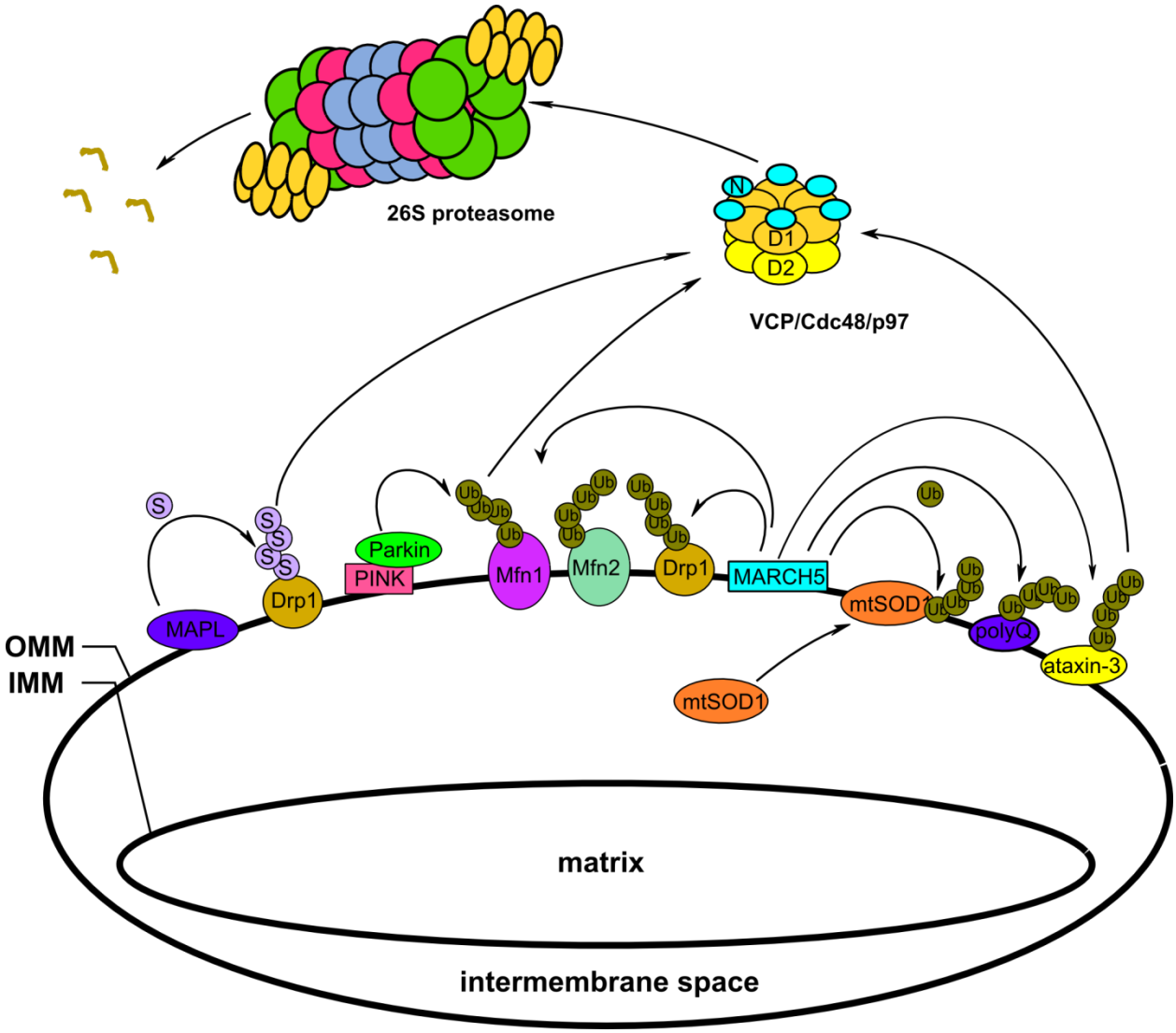

Figure 2.5 Outer mitochondrial membrane associated degradation

Outer mitochondrial membrane associated degradation or OMMAD refers to the protein quality control machinery localized on the outer mitochondrial membrane, in which multiple ubiquitin ligases namely MARCH5, IBRDC2, RNF185, MULAN/MAPL and Parkin are involved. The influence of OMMAD is not only restricted to protein quality control, regulation of other mitochondrial functions is connected to this process. Abbreviations: IMM: inner mitochondrial membrane, MARCH5: membrane-associated RING-CH 5, MAPL: mitochondrial-anchored protein ligase, Mfn: mitofusin, Drp1: dynamin-related protein 1, mtSOD: mutated superoxide dismutase, PolyQ: polyglutamate.

2.3.2 MARCH5 and mitochondrial quality control

Membrane-associated ring finger-CH 5 (MARCH5) is an RING-domain ubiquitin E3 ligase found to localized to the OMM and to have a regulatory role in mitochondrial morphology and function [129]. Structure analysis demonstrated that MARCH5 is a protein of 278 amino acids, containing one short N-terminal Really Interesting New Gene (RING) finger domain and 4 C-terminal transmembrane domains. Previous work showed that loss of function of RING-domain MARCH5 by either point mutation ($MARCH5^{H43W}$) or RNAi interfering resulted in mitochondrial morphology alternations, characterized by excessive interconnected and elongated mitochondria [16]. Further experiments demonstrated that MARCH5 affects mitochondrial morphology via influencing the docking of Drp1 to prospective mitochondrial fission sites. Additionally, MARCH5 was shown to ubiquitylate Drp1 localized on outer mitochondrial membrane leading to Drp1 degradation by proteasome [16]. Also, MARCH5 was described to be involved in Mnf1 degradation and related mitochondrial functions [130]. Considering the mutual expression of Mfn2 on both mitochondria and ER, MARCH5 has also been demonstrated to control the intracellular tethering between those two organelles via activating Mnf2 with ubiquitin modification [131]. Thus, ER-mitochondrial crosstalk is connected to MARCH5-dependent mechanisms. Beside mitochondrial dynamics, MARCH5 plays an essential role in mitochondrial protein QC. MARCH5 was shown to be involved in the removal of mutated or otherwise abnormal proteins, such as ataxin-3-polyQ, or mutated SOD1, through ubiquitylation and UPS degradation [19, 20]. Both of them are the cause of two neurodegenerative disorders, Machado-Joseph disease and amyotrophic lateral scleroses (ALS), respectively.

2.3.3 P97/VCP/Cdc48

Valosin-containing protein (VCP) or p97 in mammals, or CDC48 in yeast and *Caenorhabditis elegans* is a 92kDa protein, which is a well conserved chaperone belonging to AAA (ATPases associated with diverse cellular activities) -ATPase family of proteins [23]. In eukaryotic cells, p97 accounts for approximately 1% of the total protein content. Studies have confirmed the

role of p97 in molecular level of protein quality control as a 'molecular gearbox' and 'segregation machinery' in ubiquitin proteasome pathway, especially in endoplasmic reticulum associated protein degradation (ERAD) [132, 133]. Besides these functions, a variety of cellular processes is impacted by p97 function, including transcriptional and metabolic regulation, DNA damage response, chromatin remodeling, selective autophagy, cell cycle progression as well as cell death [22, 134-136].

P97 is a homo-hexameric complex whereby the six subunits are arranged into a ring-like structure with a central pore. Each subunit of p97 contains a mobile N-terminal domain and two conserved AAA-ATPase domains on the C-terminus, named D1 and D2 [137]. Therefore, the p97 complex contains 12 AAA-ATPase in total. However, these ATPase domains are not equival in function. The D2 domains play the major activity of p97 in physiological environment, whereas D1 is a heat-activated ATPase with a maximum activity at 60 °C. D1 is considered to be involved in hexamerization and probably contributes to p97 functioning under heat shock conditions [138]. Several co-factors are involved in the many functions of p97. These co-factors can be categorized into substrate-recruiting factors and substrates-processing factors, providing binding specificity and substrate recognition to p97 complexes [139]. These co-factors contain protein-protein interaction motifs including N-domain-interacting UBX domain, UBX-like domain, SHP box, VCP-binding motif, and VCP interacting motif (VIM), as well as PUB, PUL domains [140-142]. During ERAD, p97 interacts with its co-factors Ufd1 and Npl4, forming a p97-Ufd1-Npl4 complex [143, 144]. The resulting complex recognizes and binds to ubiquitylated ER membrane proteins, subsequently segregates them from the ER and helps with their translocation, and together with additional downstream factors (eg. Dsk2, Rad23) guides substrate proteins toward proteasome for degradation [139]. Recently, p97 was connected to mitochondrial protein degradation during stress conditions in budding yeast. Here, VCP/Cdc48-associated mitochondrial stress-responsive protein 1 (Vms1) was shown to participate in p97-dependent mitochondrial maintenance [145]. In yeast, a Cdc48-Vms1-Npl4 complex specifically is established under mitochondrial stress, followed by the degradation of yeast mitofusin Fzo1

(yeast Mfn1/Mfn2 ortholog) which had been marked by ubiquitylation [146]. In another connection between p97 and mitochondria, Parkin-dependent degradation of mitofusion was shown to be p97-depended. Also, mutation of p97 is connected to hereditary inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD), a severe degenerative disorder [147, 148]. IBMPFD was found to be accompanied by impaired ERAD, mitochondrial dysfunction, and formation of ubiquitin-tagged protein aggregates [149]. These examples support the notion that p97 plays important roles in mitochondrial physiology [27].

2.3.4 Maintaining mitochondrial function

2.3.4.1 Levels of mitochondria maintenance / quality control

Based on the high volume of biochemical processes involving oxygen in mitochondria, the complexity and diversity of mitochondrial structure and components is under constant threat. Various harmful molecules with the potential to damage mitochondrial components are produced during mitochondrial metabolism, as redox reactions inside the electron transport chain are the main source of free radicals and ROS generation, such as superoxide anion (O_2·-) [150]. Generally, the deleterious effects of ROS to mitochondrial health are due to damage to mtDNA and subsequent accumulation of mutations, oxidation of polyunsaturated fatty acids in lipids, and oxidation of amino acids in proteins resulting in the inactivation of specific enzymes by oxidative modifications [151, 152]. This mitochondrial damage is likely directly linked to cell death and neurodegeneration. There is direct evidence linking ROS to neuronal apoptosis or necrosis, as it was shown that excessive amount of ROS exposure is correlated with neurodegenerative diseases including Alzheimer's disease with diminished memory capabilities, cognitive dysfunction, and motor neuron malfunction [153, 154]. As mitochondrial damage is the inevitable result of ROS production and as loss of mitochondrial function has serious consequences, mitochondrial quality control mechanisms are in place to eliminate harmful ROS, as well as its downstream byproducts and effects, and to maintain mitochondrial functions.

These different molecular mechanisms act on various levels namely, the molecular, organelle and cellular level [7, 155, 156]. (Figure 2.6)

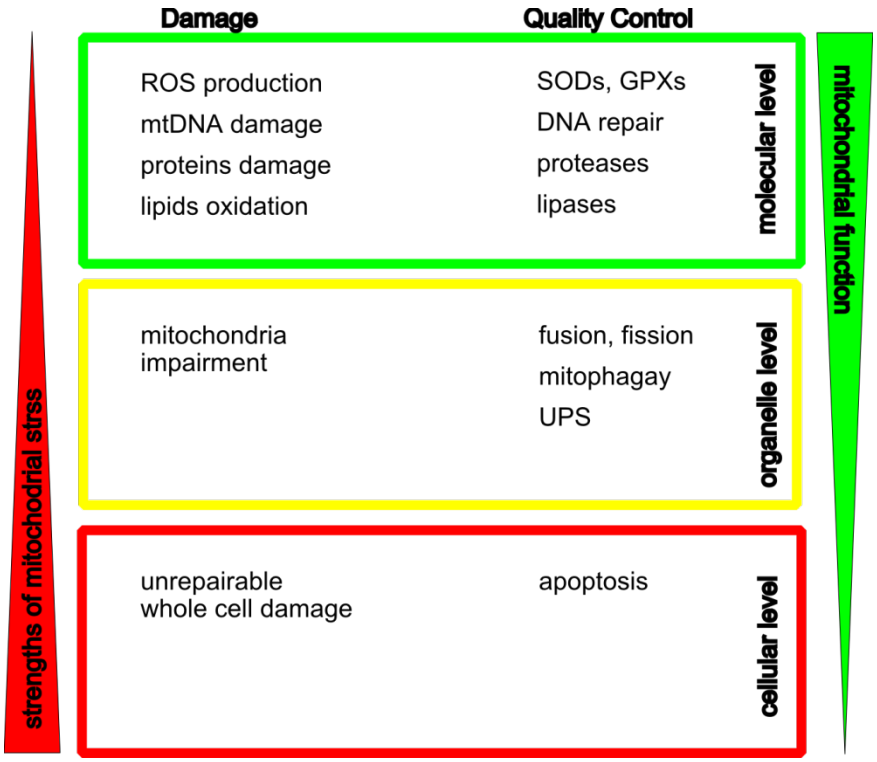

Figure 2.6 Levels of mitochondrial quality control

Mitochondrial quality control (QC) is a system that mitochondria developed against different levels of stresses, and can be categorized into three levels based on the involving mechanisms and the stress severity that is being dealt with. Molecular level QC is the front line of mitochondrial defense in which many antioxidants like SODs, glutathione peroxidase (GPXs), and other detoxifying enzymes participate. Also proteases and DNA-modifying enzymes can be considered as part of the molecular level of mitochondrial maintenance. These deal with molecular damage caused by mild stresses, such as mtDNA damage, proteins and lipid oxidations. Along with the increased levels of stress, mitochondrial function gets partially impaired. Organelle level QC takes charge to preserve mitochondrial functions. This organellar level QC involves processes involving mitochondrial dynamics and mitophagic processes, to restore the function of the entire mitochondrial network. However, once stress levels are high enough to cause devastating damage beyond the capacity of the molecular and organellar QC to salvage, cellular level QC will finally launch the apoptotic program to restrict the damage from spreading beyond the individual cells to the tissue level.

2.4 Mechanisms of mitochondrial quality control

2.4.1 Molecular level of mitochondrial quality control

The activities of the molecular level mitochondrial quality control constitute a rapid response to mitochondrial stress. As first line of defense is the mitochondrial antioxidant system in place. Consisting of antioxidant enzymes such as superoxide dismutase (SOD) peroxidases and catalase, the antioxidant system catalyzes the breakdown of superoxide anion (O_2·-) into oxygen (O_2) and hydrogen peroxide (H_2O_2). Afterward, H_2O_2 is broken down by preoxidases and catalase (GPX) into H_2O and O_2. The importance of the antioxidant defense is underlined by the consequences of loss of function of the mitochondrial isozyme SOD2 as experiment showed that mice lacking SOD2 die shortly after birth [157].

Apart from the ROS scavenging antioxidant system as the first line of defense, several other mechanisms exist as second line of mitochondria quality control on the molecular level. Efficient DNA repair pathways are found to exist in the mitochondrial matrix, including base excision repair, direct reversal and mismatch repair. The dynamic DNA repair system can respond effectively to mtDNA damage accumulation [158]. Also, lipases are ready to deal with lipid-peroxidation by digesting and repairing oxidized lipids maintaining membrane function [159].

Accumulation of unfolded, misfolded or otherwise damaged proteins disturbs mitochondrial proteostasis. Mitochondrial protein homeostasis is maintained by balanced protein turnover or protein degradation and novo protein synthesis and import into mitochondria. Unlike for DNA, only limited mechanisms are in place to directly repair protein damage. One example is the methionine sulfide reductase system, consisting of MsrA and MsrB, which can reduce oxidized methionine moieties back to methionine. In humans, MsrA localizes both to the cytoplasm/nucleus and to mitochondria [75]. As for misfolded proteins, chaperones, such as heat shock protein 70kDa (mtHSP70) and heat shock protein 60kDa (mtHSP60), are able to guide the refolding of misfolded proteins into native, correct three-dimension structures [160].

Beyond these very limited protein repair mechanisms, degradation of damaged proteins is the main pathway for maintaining mitochondrial proteostasis. Different mitochondrial proteases are located in mitochondrial matrix, intermembrane space, and the inner mitochondrial membrane. ATP-dependent proteases that belong to the AAA+ super-family are important components of mitochondrial protein quality control. Both i-AAA and m-AAA proteases are located on inner mitochondrial membrane, but oriented oppositely towards the intermembrane space or the matrix, respectively [161]. These proteases maintain the electron transport chain through their chaperone and protease functions. The function of i-AAA and m-AAA appears to be vital for mitochondrial biogenesis and maintenance as several hereditary neurodegenerative diseases among them spastic paraplegias are connected to mutations in these proteases [162]. Also, two AAA+ serine proteases, ClpXP and Lon, exist in the mitochondrial matrix, both of which play important roles in soluble matrix protein maintenance [163, 164].

Besides these specialized mitochondrial proteases, recent evidence has shown that also the ubiquitin-proteasome system is involved in mitochondrial membrane protein quality control [14]. Several ubiquitin ligases have been discovered localizing on or in association with the outer mitochondrial membrane, namely mitochondrial ubiquitin ligase activator of NF-κB (MULAN), Parkin, RNF185 [125] in between-RING finger domain protein (IBRDC2) [126] and MARCH5 [118]. Similar to endoplasmic reticulum (ER) associate degradation, outer mitochondrial membrane associated degradation (OMMAD) is named for mitochondrial protein quality control [124]. (Please see 2.3 for a more in-depth description of the UPS and its involvement in mitochondrial quality control)

2.4.2 Organelle level of mitochondrial quality control

In case of moderate insults causing damage to mitochondria, which cannot reversed on the molecular level, mechanisms relying on the dynamic nature of the organelles are taking over. Mitochondrial fusion process governed by Mfn1/2 and OPA1, can support the

complementation of impaired mitochondrial functions by promoting the mixture of mitochondrial contents, thus diluting damaged components below a damage-inducing threshold [165]. But also mitochondrial fission is involved in quality control. In contrast to fusion, mitochondrial fission is independent of the mitochondrial potential, causing the segregation of depolarized mitochondrial subunits by the fission process from the mitochondrial network either to be recovered or turned over by mitophagy [166].

Mitophagy is a specialized type of organelle autophagy, by which depolarized mitochondrial subunits beyond repair are selectively tagged, isolated and expelled from an otherwise healthy mitochondrial network, engulfed by autophagosomes and subsequently digested in the lysosomal compartment [167]. Mechanisms of mitophagy in mammalian cells have not yet been fully elaborated. During the past years, specific mitophagic mechanisms have been identified: First of all, the process of erythrocyte maturation from reticulocyte is accompanied by the elimination of all intracellular organelles, including mitochondria. Crucial for this mitophagy process are the mitochondria outer membrane protein Nix and the microtubule-associated protein light chain 3 (LC3), both of which are principle autophagosome-associated proteins [168]. Another mitophagy mechanism deals with damaged mitochondria and is under the control of two Parkinson's disease related genes: PINK1 (PTEN-induced putative protein kinase 1) and Parkin [169]. PINK1 and Parkin were identified through genetic studies connecting loss-of-functional mutations in cases of early onset familial Parkinson's disease (PD) [170]. PINK1 is a serine/threonine kinase containing a mitochondrial targeting sequence, while Parkin is an E3 ubiquitin ligase [171]. Studies in Drosophila showed loss-of-function of both PINK1 and Parkin result in similar defects of mitochondria [172, 173]. On polarized, and thus functional, mitochondria, PINK1 levels on the outer mitochondrial membrane are low, due to the continuous internalization of PINK1 and further cleavage by inner membrane presenilin-associated rhomboid-like protease (PARL) and mitochondrial-processing protease (MPP) [174, 175]. However, upon the collapse of the mitochondrial membrane potential due to excessive mitochondrial damage, PINK1 internalization and degradation is interrupted resulting in its fast

accumulation on the outer mitochondrial surface. In turn, PINK1 acts as a recruiter for Parkin from the cytosol to mitochondria, which as the acts as ubiquitin E3 ligase and ubiquitylates numerous outer mitochondrial membrane proteins [176]. As result, the docking site for ubiquitin-binding adaptor SQSTM1/p62 is established, which accumulates on mitochondria and facilitates the recruitment of damaged mitochondrial to autophagosome by binding to LC3 [177]. Upon Parkin translocation, phosphorylated-Mfn2 might be the potential receptor of Parkin to stabilize it on OMM [178]. Based on its E3 ligase activity, the translocation of Parkin increases the ubiquitylation status of OMM-localized proteins, many of which are considered as substrates of Parkin. For example, the mitofusins Mfn1 and Mfn2 were identified as Parkin substrate. Ubiquitylation of mitofusins by Parkin results in their degradtion by the proteasome and subsequently inhibits mitochondrial fusion to favor the uneven segregation of depolarized mitochondria from healthy ones [179]. Apart from Mfn1/2, many other Parkin substrates have been identified, including voltage-dependent anion channel (VDAC), translocase of the OMM (TOM) complex (TOM70, TOM40, and TOM20), pro-apoptotic factor Bax, and other[180]. Also, ubiquitylated OMM proteins seem to serve as a signal facilitating autophagosome formation [181].

Taken together, mitophagy as organellar level of mitochondrial quality control helps cells to selectively eliminate and recycle damaged and uncoupled mitochondria that are beyond repair, while preserving healthy mitochondria functioning above a certain threshold, consequently preventing further damage leading to cell death. However, the existence of PINK1/Parkin mitophagy mechanism in neuron is plausible although the treatment of CCCP induces the Parkin translocation in neuron mitochondria [182]. Given the fact that neurons cannot survive on exclusive glycolytic metabolism, equally means that they cannot afford with the completely loss of mitochondria. As such we hypothesize that limited mitochondria quality control might take place in neurons, in order to establish a relatively steady metabolic environment, by the ways of selective degradation of unwanted protein or organelles instead of whole organelle recycling in a short term [183].

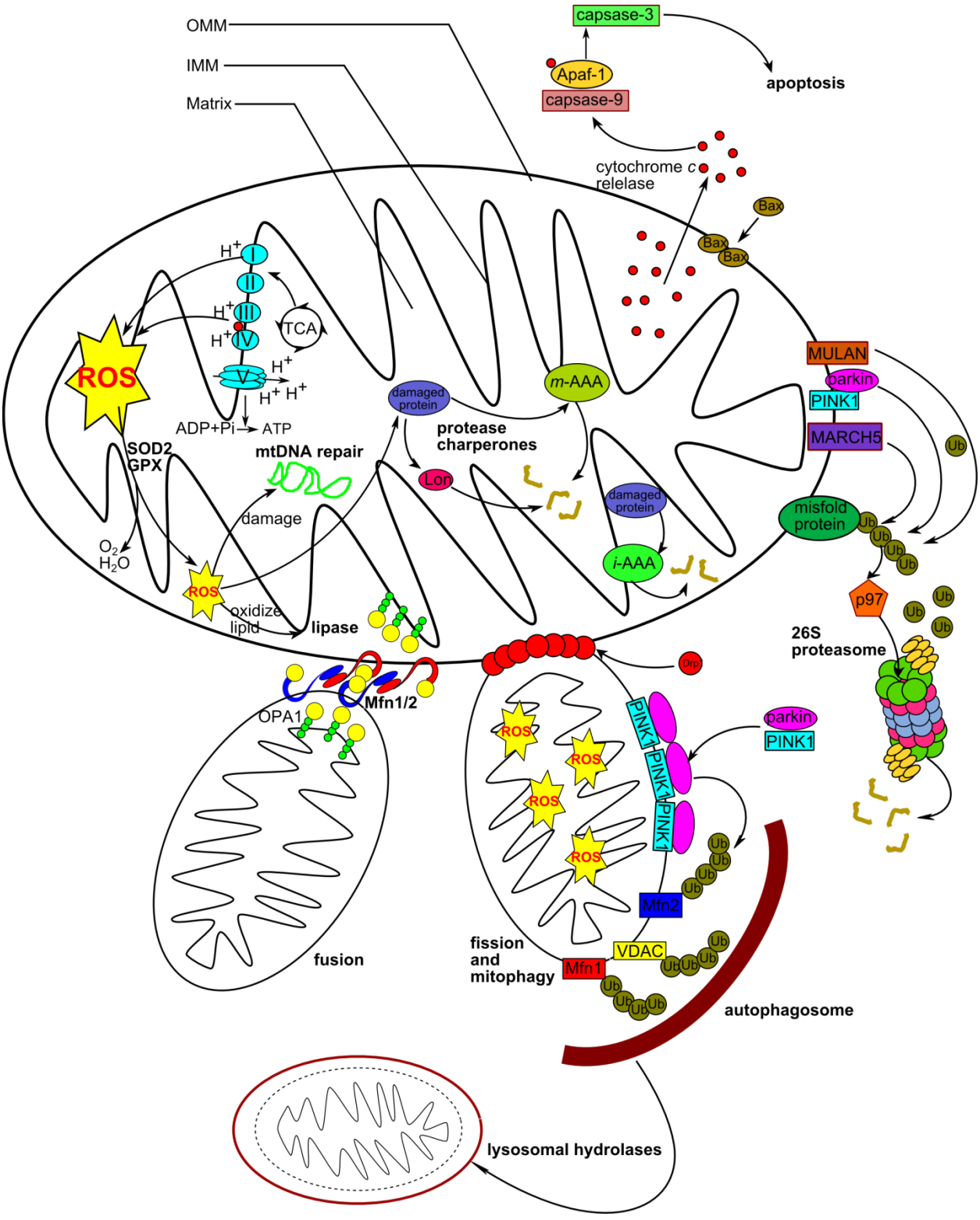
OMM
IMM
Matrix
capsase-3
apoptosis
Apaf-1
capsase-9
cytochrome c
relelase
Bax
Bax
Bax
H+
I
II
III
IV
V
TCA
ROS
ADP+Pi
ATP
SOD2
GPX
mtDNA repair
damage
damaged protein
protease charperones
m-AAA
Lon
damaged protein
i-AAA
MULAN
parkin
PINK1
MARCH5
Ub
misfold protein
p97
26S proteasome
O2
H2O
ROS
oxidize lipid
lipase
Drp
Mfn1/2
OPA1
ROS
ROS
ROS
ROS
PINK1
PINK1
PINK1
PINK1
parkin
PINK1
Mfn2
VDAC
Mfn1
fusion
fission and mitophagy
autophagosome
lysosomal hydrolases

Figure 2.7 Mechanisms of mitochondrial quality control

Although mitochondrial QC can be categorized into three different levels as described in figure 4, there is no clear boundary between them. For mitochondrial QC, molecular, organellar, and cellular mechanisms work together to maintain sufficient mitochondrial functions and perusing cellular survival. **Abbreviations:** ROS: reactive oxygen species, SOD2: superoxide dismutase-2, GPX: glutathione peroxidase, TCA: tricaroboxylic acid cycle, i/m-AAA: intermembrane space/matrix-ATPase associated with various cellular activities, MULAN: mitochondrial ubiquitin ligase activator of NF-kB, MARCH5: membrane-associated RING-CH 5, PINK: PTEN-induced putative kinase, Ub: ubiquitin, VDAC: voltage dependent anion channel, Mfn: mitofusin, OPA1: optic atrophy type 1, Drp1: dynamin-related protein 1, Apaf-1: Apoptotic protease activating factor 1.

2.4.3 Cellular level of mitochondrial quality control

Above a certain stress level, molecular and organellar QC mechanisms are overwhelmed and are no longer able to repair or contain mitochondrial damage. As mitochondrial impairment accumulates to a certain level where neither the existing repair systems can sufficiently preserve mitochondrial function, putting the mitochondrial network beyond rescue. At this point the cellular level of quality control takes effect and commits the entire mitochondrial network and therefore the corresponding to apoptosis. One of the first steps in this process is the translocation of the fission molecule Drp1 and the pro-apoptotic factor Bax to the outer mitochondrial membrane, simultaneously inducing extensive mitochondrial fragmentation paired with membrane depolarization and opening of the mitochondrial membrane permeability pore, through which cytochrome *c* is released from the intermembrance space into the cytosol [184]. There, cytochrome *c* interacts with cytosolic Apaf-1 and caspase-9 to establish the apoptosome leading to the activation of caspase-3, thus irreversibly starting the intrinsic caspase cascade and leading to apoptotic cell death. Apoptosis is considered to be the last line of defense against mitochondrial damage to eliminate the threats that possess mitochondrial dysfunction to the whole organism [185].

2.5 Mitochondria and neurodegeneration

2.5.1 Neurodegeneration

Neurodegeneration describes the loss of neuronal cells that accompanies degenerative neurological disorders, as the fundamental feature of these disorders is the gradual loss of selective neuron resulting in the progressive decline of cognitive and/or motor function. Typical examples of neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) [186]

Many important triggers and risk factors are linked to neurodegenerative diseases and the associated neuronal cell death. Genetic predisposition as well as genetic mutations are considered as one of the main risks for the development of neurodegeneration [187, 188]. For example, loss of function mutations in PTEN-induced putative kinase 1 (PINK1) or Parkin were identified as causative for familial PD [189]. Furthermore, mutations in the β-amyloid (Aβ) precursor protein cause AD [190] or mutations in microtubule-associated protein Tau are the reason for frontotemporal dementia (FTD) with Parkinson symptoms [191]. In another example, mutations in SOD1 are one of the frequent reasons for familial ALS [192]. Also, protein misfolding and abnormal aggregation is observed in many neurodegenerative diseases, whether these changes in protein behavior are either cause or consequence is still controversial, but they definitely contribute to the vicious circle of neurodegeneration [193, 194]. For example, hyperphosphorylated Tau protein is the main component of neurofibrillary tangles in AD brain biopsy samples [195]; while accumulation of Aβ aggregates is responsible for the senile plaques; and α-synuclein can aggregate to form pathological Lewy bodies connected to cases of familial AD [196]. It is assumed that abnormal proteins accumulations can induce neuronal loss and further neuronal network dysfunction. On the other hand, strategies that reduce the production, enhance the elimination, or assist the neutralization of abnormal proteins have been shown to counteract neurological disabilities effectively [197].

2.5.2 Mitochondrial dysfunction and neurodegeneration

Neuronal cells have a high energy demand. The brain consumes at nearly 2% of body mass on average 20% of the total energy of the body. This makes the neuronal cells almost entirely dependent on glucose oxidation for ATP production [198]. Thus, a proper functional mitochondrial network with sufficient and persistent ATP production is essential to neuronal survival and function. Additionally, highly dynamic calcium fluctuations are happening inside neuronal, which also require the steady buffering capacity of mitochondria for calcium ions. Despite of the heterogeneous feature of neurodegenerative diseases, the overwhelming consensus is that mitochondria are at the heart of neurodegeneration.

Many neurodegenerative disorders are age related, which is also the most important risk factors for such diseases like AD, PD, and ALS. And interestingly, mitochondrial function declines with aging. It is assumed that mitochondria accumulate mtDNA mutations and, thus, non-functional proteins during the lifespan of the organism, thus contributing to the process of aging as well as neurodegeneration due to insufficient ATP production. In addition, mitochondria are the trigger of intracellular apoptosis responsible for the final loss of neuron cell numbers [199, 200]. Also, extensive literature point at oxidative stress as the key perpetrator for neurodegeneration further linking mitochondria to the demise of neurons as the main source of ROS [201]. As oxidative stress causes mitochondrial dysfunction and as failing mitochondria producing even more ROS [202], a vicious cycle progresses in which more oxidative stress induces more structural and metabolic damages (nucleic acid breakdown, enzymatic proteins inactivation, lipid peroxidation), resulting in even more severe mitochondrial dysfunctions (ATP synthesis impairment, mitochondrial fragmentation, mitochondrial membrane depolarization, etc.). [203].

Aside the more general concept of oxidative stress and age-related mitochondrial dysfunction leading to neurodegeneration, additional links between mitochondrial maintenance or dysfunction and neurodegeneration are described. It was shown that around 60% of cases of

autosomal dominant optic atrophy (ADOA) are caused by a loss of function mutation of OPA1, a known key regulator of mitochondrial inner membrane fusion. Due to impaired mitochondrial fusion caused by OPA1 insufficiency, ADOA displays gradually vision loss up to completely blindness as result of irreversible retinal ganglion cell damage [84]. In another example, Charcot-Marie-Tooth disease 2A (CMT2A) is a heredity autosomal dominant disease characterized by primary axonal peripheral neuropathy, sometimes associated with visual and hearing impairment. It was shown that mutations of the mitofusin Mfn2 are causative for the disease [204]. This is likely due to mitochondrial fusion deficiency caused by Mfn2. However, detailed mechanistic insight is still missing, but ADOA and CMT2A strongly link unbalanced mitochondrial dynamics to neurodegenerative processes.

Beyond mitochondrial dynamics, dysfunction of mitochondrial protein degradation is also directly linked to neurodegeneration. Multiple studies have investigated the function of MARCH5 in mitochondrial protein degradation, and the possible connection to neurodegeneration. The degradation of at least three different mitochondrial proteins relies on the E3 ligase activity of MARCH5. MARCH5 is able to ubiquitylate mutant SOD1 on mitochondria and attenuate the corresponded mitochondria dysfunction. As SOD1 mutation is one of the main causes of human ALS [19], MARCH5 function is directly linked to this neurodegenerative disease. Further, MARCH5 is responsible for the degradation of mitochondria-localized polyQ aggregation in an ubiquitin-proteasome dependent manner, and as previous studies indicated that PolyQ toxicity leads to mitochondrial dysfunction and consequently polyQ diseases, including Machado–Joseph disease, Huntington's disease or several types of spinocerebellar ataxias [20]. And recently the research on MARCH5 demonstrated that it is involved in the degradation of NO-stressed mitochondrial protein S-nitrosylated microtubule-associated protein 1B-light chain 1 (LC1), and prevented the neuron cytotoxicity [205]. LC1 S-nitrosylation has been implicated with several human neurological disorders, such as giant axonal neuropathy, fragile-X syndrome, spinocerebellar ataxia type 1, and Parkinson disease [205]. Another example is hereditary spastic paraplegia, a human axonal degeneration caused by one

m-AAA protease (paraplegin) mutation. The sufficient function of paraplegin is critical for inner mitochondrial membrane proteostasis, especially responsible for the surveillance of proper status of electron transport chain complexes which directly links to mitochondrial energy production [206, 207].

In summary, mitochondria are central to cellular survival due to their many different functions. Neuron cells, characterized by their high energy demand, distinct metabolic style and long life span, are extremely sensitive to mitochondrial insufficiency. Therefore, properly functioning mitochondrial maintenance preserving mitochondrial function at high fidelity are essential for neuronal health and survival, as the failure of mitochondrial quality control leads to such irreversible neurodegeneration.

3 Inactivation of MARCH5 prevents mitochondrial fragmentation and cell death in a neuronal cell model

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Short title: MARCH5 activity modulates neuronal cell death

Keywords: mitochondria, MARCH5, Drp1, mitochondrial fission, mitochondrial fusion

3.1 Abstract

Purpose: To study the impact of the mitochondrial ubiquitin ligase MARCH5 on mitochondrial morphology and induction of apoptosis using an *in vitro* model of neuronal precursor cells exposed to glaucoma-relevant stress conditions.

Methods: RGC5 cells transfected with expression constructs for MARCH5, MARCH5^{H43W}, Dpr1^{K38A} or vector control were exposed to either elevated pressure of 30 mmHg, oxidative stress caused by mitochondrial electron transport chain (ETC) inhibition, or hypoxia-reoxygenation conditions. Mitochondrial morphology of RGC5 cells was analyzed following staining of the mitochondrial marker cytochrome *c* and photoactivatable GFP (PAGFP) diffusion assay. Induction of apoptotic cell death in these cells was determined by analyzing the release of cytochrome *c* from mitochondria into the cytosol and flow cytometry.

Results: Exposure of RGC5 cells to oxidative stress conditions as well as to elevated pressure resulted in the fragmentation of the mitochondrial network in control cells as well as in cells expressing MARCH5. In cells expressing inactive MARCH5^{H43W} or inactive Drp^{K38A}, mitochondrial fragmentation was significantly blocked and mitochondrial morphology was comparable to that of control cells under normal conditions. Exposure of RGC5 cells to elevated pressure or oxidative stress conditions induced apoptotic cell death as assessed by cytochrome *c* release and DNA staining, while expression of dominant-negative MARCH5^{H43W} or Drp1^{K38A} did significantly delay cell death.

Conclusion: Preventing mitochondrial fragmentation through interference with the mitochondrial fission machinery protects neuronal cells from programmed cell death following exposure to stressors physiologically relevant to the pathogenesis of glaucoma.

3.2 Background

Death of retinal ganglion cells (RGCs) is responsible for vision loss in glaucoma patients. The exact mechanisms causing the demise of RGCs are still under investigation. Different triggers in the various forms of glaucoma probably lead to the observed neurodegenerative process. Elevated intraocular pressure (IOP) is involved in RGC death associated with high-tension glaucoma (HTG) [1], while vascular dysregulation and associated ischemia-reperfusion injury is linked to normal-tension glaucoma (NTG) [2]. Irrespective of the actual trigger and the glaucoma subtype, at its heart, glaucoma is a slowly progressing neurodegenerative disorder. RGC5 cells were used as cellular model. These cells are murine neuronal precursor cells and display certain features such as the expression of specific neuronal marker upon differentiation with various compounds [3].

As mitochondrial dysfunction is generally accepted to be one unifying theme for all neurodegenerative disorders [4], mitochondria and failing mitochondrial function connect the different glaucoma subtypes. Due to the complex architecture of mitochondria and their endosymbiotic origin [5], diverse systems are in place to maintain mitochondrial fidelity [6]. These systems include bacterial type proteases dealing with oxidatively damaged mitochondrial matrix proteins, but also inner mitochondrial membrane-anchored proteases involved in protein processing and protein degradation. Recently, we and others described an important role for the ubiquitin-proteasome system (UPS) and ubiquitin-dependent protein degradation in mitochondrial maintenance [7]. Membrane-anchored ubiquitin ligases such as MULAN/MAPL [8-9], RNF185 [10] and MITOL/MARCH5 [11-13] were shown to impact mitochondrial physiology. Furthermore, MARCH5 was demonstrated to promote the degradation of mSOD1 [14], a protein linked to amyotrophic lateral sclerosis, and of polyQ-extended ataxin-3 causative for Machado-Joseph disease [15]. In addition, MARCH5 was connected to the degradation of nitrosylated proteins suggesting a role for this ubiquitin ligase in mitochondrial quality control [16]. Besides the degradation of damaged or superfluous proteins, mitochondrial maintenance critically depends on balanced mitochondrial morphology. Mitochondria form a dynamic

network constantly reshaped by the fission and fusion of mitochondrial tubules [17]. MARCH5 was implicated by us and others in the regulation of mitochondrial morphology with inactivation of MARCH5 causing massive mitochondrial elongation due to a block in mitochondrial fission [12]. Mitochondrial fusion is mediated by the mitofusins Mfn1 and Mfn2 that together with Opa1 perform the coordinated fusion of outer and inner mitochondrial membranes. Interestingly, mutations in fusion components are linked to neurodegenerative disorders with Opa1 mutations causative for dominant optic atrophy [18] and mutations in Mfn2 linked to Charcot-Marie-Tooth type 2A disease, a peripheral neuropathy sometimes accompanied by optic degeneration and hearing loss [19]. Division of mitochondria is performed by the dynamin-related protein Drp1 together with hFis1, Mff and MiD49/51[20-22]. In a rare case, mutation of Drp1 caused premature death accompanied by microcephaly, persistent lactic acidemia as well as optic degeneration [23], strongly pointing to an underlying mitochondrial etiology. Thus, dynamically balancing and adapting the organelles morphology is an integral part of mitochondrial maintenance and essential for neuronal survival. This is especially true for RGCs, most likely due to their highly specialized anatomy involving non-myelinated parts, their exposure to UV stress, and their - even for neuronal cells - exceptional energy demand [1]. This integration of mitochondrial morphogens into cellular physiology is mirrored in their connection to programmed cell death [24]. Interference with mitochondrial fusion and fission dynamics modulates cell death thresholds with excessive fission sensitizing and blocked fission desensitizing cells to apoptotic stimuli [25-26]. Consistent with glaucoma being a neurodegenerative disorder and a protective role of mitochondrial fusion in most experimental paradigms, increased expression of the OPA1 fusion protein is protective for RGCs in a mouse model of glaucomatous nerve damage [27].

To investigate the role of the mitochondrial ubiquitin ligase MARCH5 and mitochondrial maintenance during neuronal cell stress, we studied mitochondrial dynamics and induction of cell death in neuronal cells with altered mitochondrial maintenance under physiologically relevant stress conditions.

3.3 Results

Exposure of differentiated RGC5 cells to 30 mmHg elevated pressure for three days, to the mitochondrial complex I inhibitor rotenone or to hypoxia-reoxygenation conditions resulted in the fragmentation of the mitochondrial network (Figure 3.1). While the mitochondrial network in cells kept under ambient pressure displayed normal tubular mitochondrial morphology, in about 60 % of cells exposed to elevated pressure mitochondria switched to a fragmented phenotype. For oxidative stress conditions, treatment with rotenone resulted in about 80 % of RGCs in mitochondrial fragmentation, while reoxygenation was responsible for fragmented mitochondria in about 30 % of cells.

As shown in Figure 3.2, expression of MARCH5 in comparison to control cells did not interfere with pressure-induced mitochondrial fragmentation. Interestingly, expression of a RING-deficient, dominant-negative MARCH5^{H43W} significantly blocked pressure-induced mitochondrial fragmentation and was able to maintain normal mitochondrial morphology in about 75 % of cells (Figure 3.2A). Measuring mitochondrial interconnectivity using a photoactivatable GFP (PAGFP) diffusion assay in cells expressing MARCH5, MARCH5^{H43W} or YFP as control confirmed that expression of MARCH5 did not block pressure-induced changes in mitochondrial interconnectivity, while inactive MARCH5^{H43W} prevented the pressure-induced alteration of the mitochondrial network (Figure 3.2B). To evaluate the specificity of pressure-induced mitochondrial fragmentation in RGC5 cells, HeLa cells were exposed to identical pressure conditions, and no mitochondrial fragmentation was observed (data not shown). Blocking of mitochondrial fission through expression of dominant-negative Drp1^{K38A} was used to assess the specificity of MARCH5^{H43W} action on mitochondrial morphology following exposure to stress conditions. As shown in Figure 2C, mitochondrial fragmentation was not blocked in RGC5 cells expressing Drp1 following exposure to elevated pressure, while Drp1^{K38A} was capable of blocking organelle fragmentation in about 70 % of cells under these conditions.

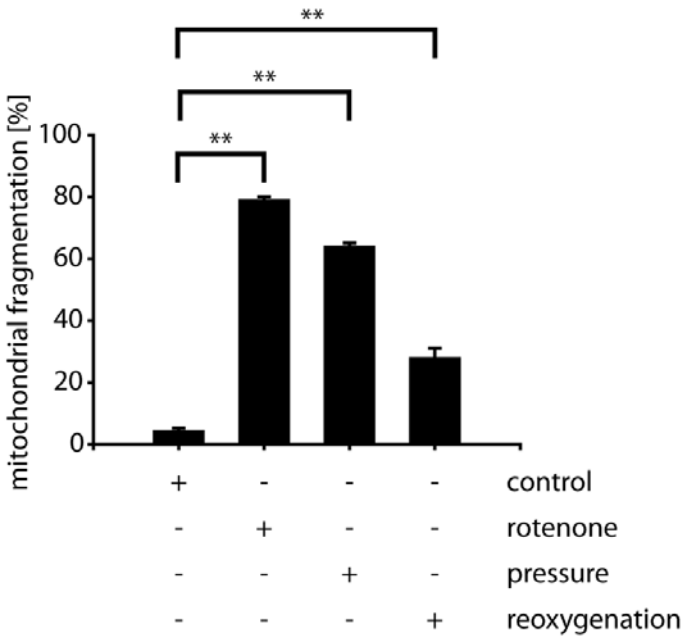

Figure 3.1: Stress-induced mitochondrial fragmentation in RGC5 cells

Differentiated RGC5 cells were exposed to 0.25 µM rotenone for 12 hours, 30 mmHg elevated pressure for 72 hours or hypoxia-reoxygenation (24 hours 1 % oxygen, 2 hours normoxia), fixed and stained using anti-cytochrome *c* antibodies. Mitochondrial morphology was scored visually. Shown are the averages of three independent experiments (>200 cell counted/condition) with error bars representing SEM and * representing $p<0.05$ and ** representing $p<0.01$ (Student's t-test).

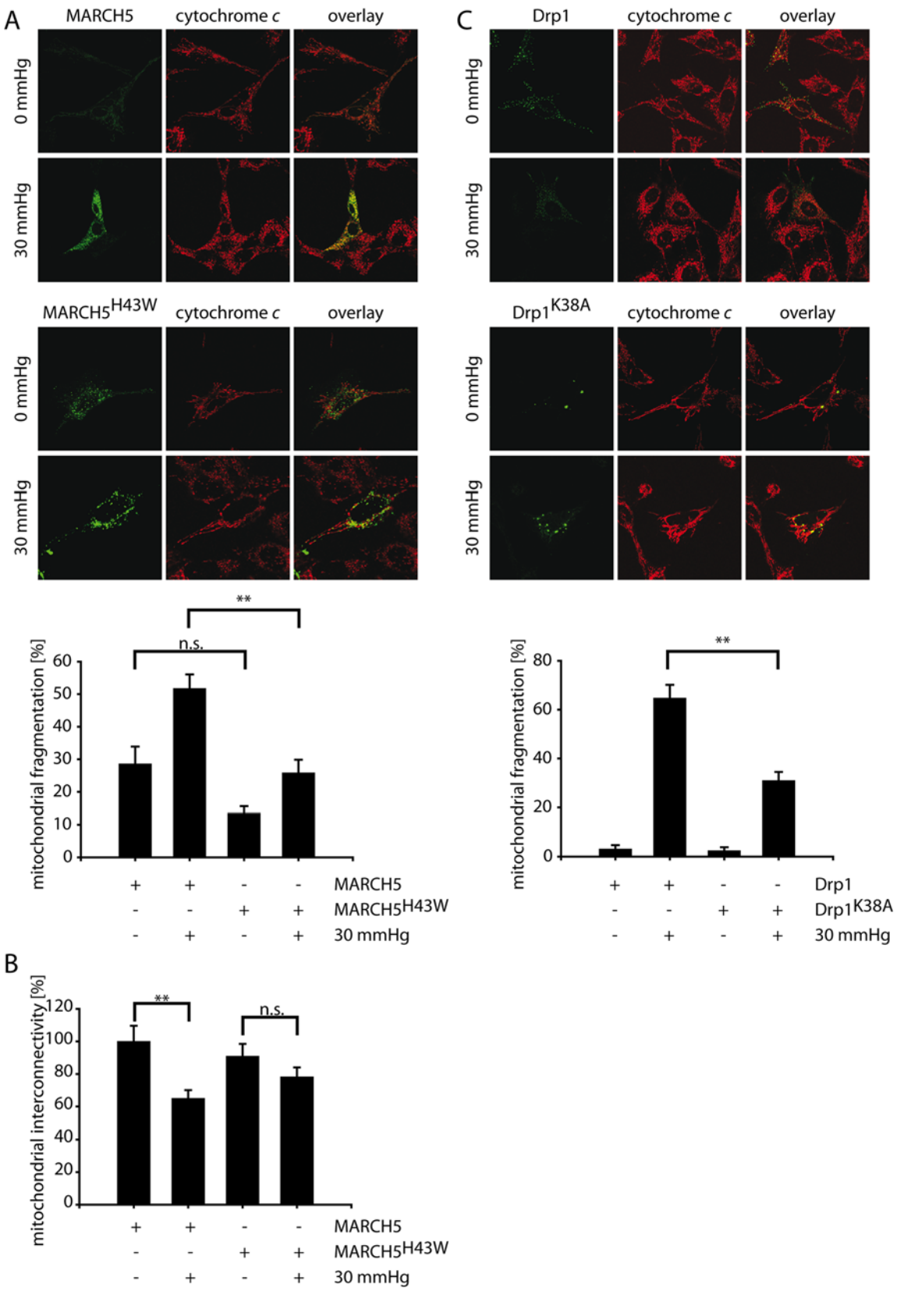
A
MARCH5
cytochrome c
overlay
0 mmHg
30 mmHg
MARCH5H43W
cytochrome c
overlay
0 mmHg
30 mmHg
**
n.s.
mitochondrial fragmentation [%]
60
50
40
30
20
10
0
+ + - - MARCH5
- - + + MARCH5H43W
- + - + 30 mmHg
B
mitochondrial interconnectivity [%]
120
100
80
60
40
20
0
**
n.s.
+ + - - MARCH5
- - + + MARCH5H43W
- + - + 30 mmHg
C
Drp1
cytochrome c
overlay
0 mmHg
30 mmHg
Drp1K38A
cytochrome c
overlay
0 mmHg
30 mmHg
**
mitochondrial fragmentation [%]
80
60
40
20
0
+ + - - Drp1
- - + + Drp1K38A
- + - + 30 mmHg

Figure 3.2: Inactivation of MARCH5 and Drp1 blocks pressure-induced mitochondrial fragmentation.

(A) Differentiated RGC5 cells transfected with expression constructs for MARCH5^{YFP} or MARCH5$^{H43W\text{-}YFP}$ were exposed for 72 hours to 30 mmHg elevated pressure or left untreated as control. Mitochondrial morphology was assessed following cytochrome *c* staining. The bar graph represents three independent experiments (>200 cell counted/condition) with * marking $p<0.05$ and ** marking $p<0.01$ (Student's t-test). Error bars correspond to SEM. (B) RGC5 cells expressing MARCH5 or MARCH5^{H43W} and photoactivatable GFP (PAGFP) were exposed to 30 mmHg for three days or left untreated as control and mitochondrial interconnectivity was measured by PAGFP diffusion after photoactivation and compared to ambient pressure, MARCH5 expressing cells. Analyzed were 20 cells/condition with the error bars representing SEM and ** marking $p<0.01$ and n.s. marking $p>0.05$ (Student's t-test). (C) Differentiated RGC5 cells transfected with expression constructs for Drp1^{YFP} or Drp1$^{K38A\text{-}YFP}$ were treated as described in A.

In an experimental paradigm of oxidative stress, exposure of differentiated RGC5 cells to rotenone, an inhibitor of the complex I of the electron transport chain, did also result in mitochondrial fragmentation (Figure 3.3A+B) as compared to untreated control cells. Control cells or cells expressing wildtype MARCH5 displayed mitochondrial fragmentation under these conditions. However, ectopic expression of MARCH5^{H43W} interfered with rotenone-induced mitochondrial fragmentation with almost 60 % of cells maintaining a tubular mitochondrial network (Figure 3.3B). Measuring mitochondrial interconnectivity using PAGFP diffusion assay, we confirmed the inhibition of rotenone-induced fragmentation of the mitochondrial network by inactive MARCH5^{H43W} but not wildtype MARCH5 (Figure 3.3C). Drp1^{K38A} was able to interfere with mitochondrial fragmentation in differentiated RGC5 cells under oxidative stress conditions. While around 80 % of control or Drp1-expressing cells displayed mitochondrial fragmentation following rotenone treatment, mitochondrial morphology was tubular in around 60 % of Drp1^{K38A}-expressing cells (Figure 3.3A+B).

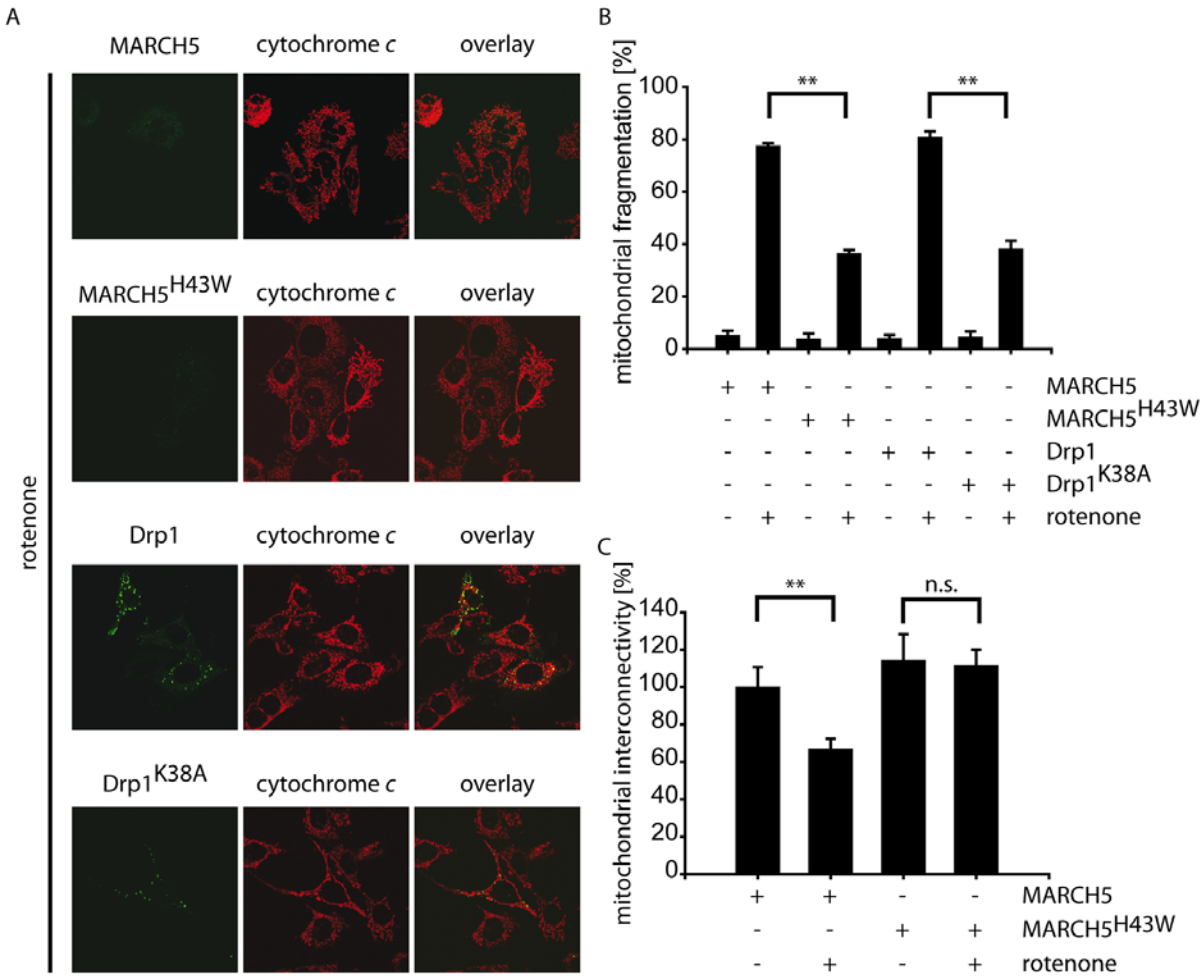

Figure 3.3: Rotenone-induced mitochondrial fragmentation is reduced following MARCH5 or Drp1 inactivation.

(A + B) Differentiated RGC5 cells expressing MARCH5^{YFP}, MARCH5$^{H43W\text{-}YFP}$, Drp1^{YFP} or Drp1$^{K38A\text{-}YFP}$ were exposed to 0.25 µM rotenone for 12 hours prior to fixation and cytochrome *c* staining. Shown is the average of three independent experiments (>200 cell counted/condition), with ** marking $p<0.01$ (Student's t-test) and error bars representing SEM. (C) RGC5 cells expressing MARCH5 or MARCH5^{H43W} and PAGFP were exposed to 0.5 µM rotenone for 4 hours and mitochondrial interconnectivity was assessed by measuring PAGFP diffusion following photoactivation. Analyzed were 20 cells/condition with error bars representing SEM and ** marking $p<0.01$ and n.s. marking $p>0.05$ (Student's t-test).

Re-oxygenation following exposure to low oxygen atmosphere mimicking ischemia-reperfusion conditions induces mitochondrial fragmentation in differentiated RGC5 cells (Figure 3.1 and 3.4). Re-oxygenation-induced mitochondrial fragmentation was not blocked in cells ectopically expressing MARCH5 or Drp1 when compared to transfected control cells (Figure 3.4). Interestingly, ectopic expression of MARCH5^{H43W} or Drp1^{K38A} did completely block mitochondrial fragmentation under these conditions (Figure 3.4A). We confirmed this observation in MARCH5 or MARCH5^{H43W} expressing cells treated with hypoxia-reperfusion using PAGFP diffusion assay (Figure 4B).

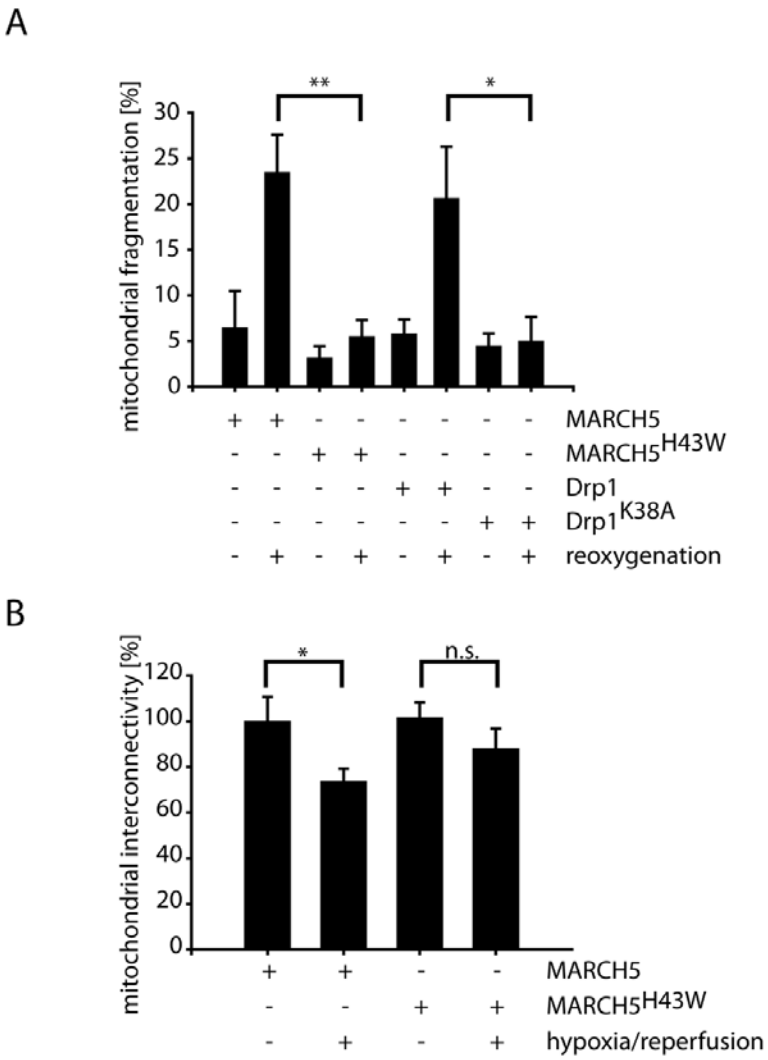

Figure 3.4: Mitochondrial fragmentation following hypoxia-reoxygenation is ameliorated by inactivation of MARCH5 or Drp1.

(A) Differentiated RGC5 cells expressing MARCH5^{YFP}, MARCH5$^{H43W\text{-}YFP}$, Drp1^{YFP} or Drp1$^{K38A\text{-}YFP}$ were cultured in the presence of low oxygen (1 %) for 24 hours followed by normoxia for 2 hours. Mitochondrial fragmentation was analyzed following cytochrome *c* staining in three independent experiments (>200 cell counted/condition). Error bars represent SEM, p-Values for Student's t-test are marked with * ($p<0.05$) or ** ($p<0.01$). (B) RGC5 cells expressing MARCH5 or MARCH5^{H43W} and PAGFP were stressed using hypoxia-reoxygenation and mitochondrial interconnectivity was assessed by measuring PAGFP diffusion following photoactivation. Analyzed were 20 cells/condition with error bars representing SEM and ** marking $p<0.01$ and n.s. marking $p>0.05$ (Student's t-test).

Furthermore, we assessed whether modulation of mitochondrial morphology through expression of Drp1^{K38A} or MARCH5^{H43W} altered the sensitivity of differentiated RGC5 cells to apoptotic stimuli. To this end, RGC5 cells were exposed either to 100 mmHg elevated pressure for one day, rotenone alone, or a combination of elevated pressure and rotenone, and apoptotic induction in the presence of pan-caspase inhibitor was assessed by counting the release of cytochrome *c* from mitochondria. As shown in Figure 5, exposing RGC5 to these stress conditions leads to the induction of apoptotic cell death in control cells and in cells expressing wildtype MARCH5 (Figure 3.5A) or Drp1 (Figure 3.5B). Interestingly, expression of MARCH5^{H43W} (Figure 3.5A) or Dpr1^{K38A} (Figure 3.5B) resulted in significant suppression of apoptotic cell death induction.

To further examine the extent to which inactivation of mitochondrial fission by mutant MARCH5 or mutant Drp1 delays apoptosis, we measured cell death following prolonged exposure to oxidative stress conditions. To this end, cells expressing MARCH5 or MARCH5^{H43W} (Figure 3.5C) or Drp1 or Drp1^{K38A} (Figure 3.5D) or YFP as control were treated for 24 or 48 hours with 1 μM rotenone or left untreated. The amount of accumulating dead cells following this treatment was measured using flow cytometric analysis of 7-aminoactinomycin D (7-AAD) exclusion. Interestingly, expression of wildtype MARCH5 (Figure 3.5C) or wildtype Drp1 did not interfere with the progression of cell death, while MARCH5^{H43W} or Drp1^{K38A} did significantly diminish cell death during 24 hours of rotenone treatment in comparison to control or MARCH5 or Drp1 expressing cells, respectively. However, at 48 hours of rotenone treatment, neither expression of MARCH5^{H43W} nor Drp1^{K38A} did significantly alter the accumulation of 7-AAD-positive cells.

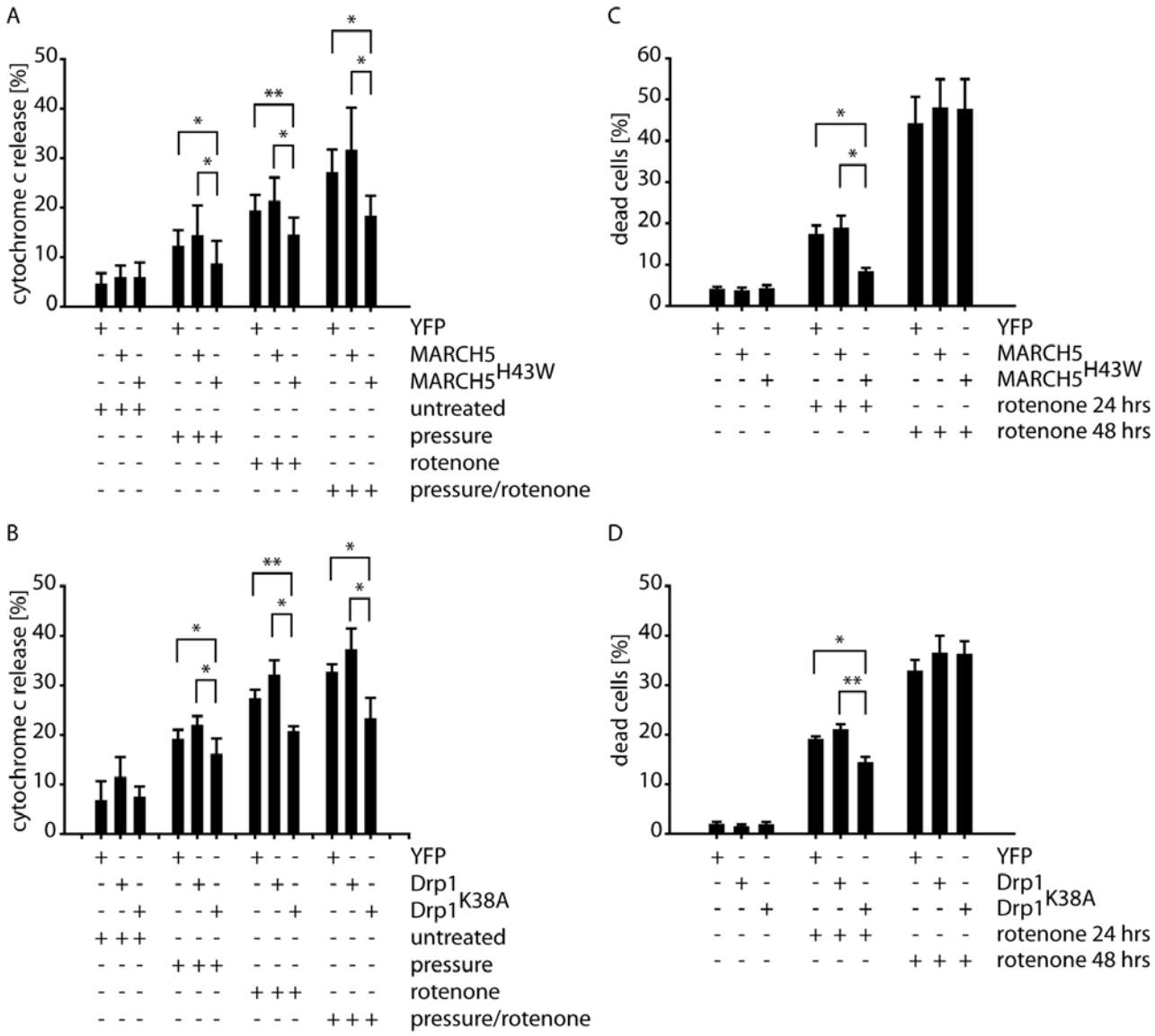

Figure 3.5: Inactivation of MARCH5 or Drp1 delays induction of apoptosis and cell death.

RGC5 cells expressing (A) MARCH5^{YFP}, MARCH5$^{H43W\text{-}YFP}$ or YFP as control or (B) Drp1^{YFP} or Drp1$^{K38A\text{-}YFP}$ or YFP as control were exposed to 100 mmHg for 24 hours, 1 µM rotenone for 6 hours or combined 100 mmHg pressure and 1 µM rotenone in the presence of the pan-caspase inhibitor zVAD-fmk. Following treatment, cells were fixed and cytochrome *c* release from mitochondria into the cytosol was assessed by fluorescence microscopy (>200 cell counted/condition). The bar graphs represent four independent experiments with * marking $p<0.05$ and ** marking $p<0.01$ (Student's t-test). RGC5 cells expressing (C) MARCH5^{YFP}, MARCH5$^{H43W\text{-}YFP}$ or YFP as control or (D) Drp1^{YFP} or Drp1$^{K38A\text{-}YFP}$ or YFP as control were exposed to 1 µM rotenone for 0, 24 or 48 hours and the amount of dead cells was measured by flow cytometry following 7-AAD staining of DNA.

3.4 Discussion

Mitochondrial fidelity is important for neuronal survival, with failing mitochondria and mitochondria-mediated cell death involved in neuronal degeneration. Therefore, maintaining mitochondria in a healthy and functional state is essential for neuronal survival. Mitochondrial surveillance and repair is performed by a multi-tiered system involving specialized mitochondrial proteases, the ubiquitin-proteasome system, properly balanced mitochondrial dynamics, but also cell death mechanisms [4]. The mitochondrial ubiquitin ligase MARCH5 is involved in mitochondrial maintenance through the clearing of mutated, damaged mitochondrial proteins but also through regulating mitochondrial fission [12, 14-16]. Both of these functions of MARCH5 are important for neuronal survival. Degradation of proteins such as mSOD1 or polyQ-ataxin-3 associated with amyotrophic lateral sclerosis or Machado-Joseph disease [15], respectively, by MARCH5 was shown to exert neuroprotective functions. Furthermore, maintenance of a plastic mitochondrial network through balanced mitochondrial fission and fusion was shown to be important for neuronal survival. For example, $mfn2^{-/-}$ mice display severe loss of Purkinje cells likely caused by unbalanced mitochondrial dynamics [28]. Furthermore, in humans, impaired mitochondrial fusion caused by mutations in Opa1 and Mfn2 are associated with dominant optic atrophy or Charcot-Marie-Tooth disease 2A (CMT2A), respectively. We now identified a role for MARCH5 in the death neuronal cells caused by stress conditions that are relevant for glaucoma progression. We found that modulation of mitochondrial morphology through MARCH5 depends on its regulation of Drp1 recruitment to the OMM. For mitochondrial fission to occur cytosolic Drp1 has to assemble into fission complexes on mitochondria in a MARCH5-dependent manner; expression of dominant-negative $MARCH5^{H43W}$ locks Drp1 in fission-incompetent mitochondrial division complexes. The observed block in mitochondrial fission in differentiated $MARCH5^{H43W}$-expressing RGC5 cells following exposure to elevated pressure, oxidative stress or to ischemia-reperfusion conditions is consistent with the known role of MARCH5 in the regulation of mitochondrial morphology [12].

This notion is supported by our findings that expression of dominant-negative Drp1^{K38A} but not of wildtype Drp1 blocks mitochondrial fragmentation following elevated pressure, oxidative stress or re-oxygenation in a comparable manner. This finding is consistent with MARCH5 acting upstream of Drp1 in the regulation of mitochondrial fission. Interestingly, inactivation of MARCH5 blocks mitochondrial fragmentation under the tested stress conditions as effectively as dominant-negative inactivation of Drp1 function. While the exact role of MARCH5 during mitochondrial fission is still unclear, our data suggest a strong dependence of Drp1-mediated fission on MARCH5 activity.

Whereas MARCH5 is neuroprotective under conditions of insufficient protein quality control as seen e.g. in Machado-Joseph disease [15], its expression has no beneficial effect under glaucoma-relevant stress conditions. In contrast, inactivation of MARCH5 function through dominant-negative action of MARCH5^{H43W} exerts anti-apoptotic activity, slows down cell death and exerts a neuroprotective function. Thus, under the conditions tested here, the function of MARCH5 in mitochondrial division is predominant. In addition, elevated pressure, oxidative stress and ischemia-reperfusion do not seem to cause damage to mitochondrial proteins that are under the surveillance of the mitochondrial ubiquitin ligase MARCH5. One might speculate that inhibition of mitochondrial fragmentation through pharmacological inactivation of MARCH5 might be beneficial under certain circumstances, similar to what has been shown for Drp1, where pharmacological inhibition of mitochondrial fission by the small molecule Drp1 inhibitor mdivi-1 proved neuroprotective [29].

3.5 Conclusions

Taken together, the versatile mitochondrial ubiquitin ligase MARCH5 impacts neuronal survival in various ways either through the degradation of damaged mitochondrial proteins or by modulating mitochondrial morphology. In case of glaucoma-related stressors and neuronal cells, the role of MARCH5 as fission regulator outweighs its other roles in maintaining mitochondrial proteostasis.

3.6 Methods

3.6.1 Cell culture

Immortalized neuronal precursor cells (RGC5) were cultured in high glucose DMEM, supplemented with 10 % fetal bovine serum, 2mM L-glutamine, MEM non-essential amino acid (Sigma-Aldrich) and incubated in a humidified incubator at 5 % CO_2 and 37°C. RGC5 cells were received as gift from Neville Osborne (University of Oxford) and tested as of mouse origin by PCR [30] and are thus considered neuronal precursor cells instead of retinal ganglion cells as originally proposed [31]. Where stated, cells were cultured in media containing 1% FBS. For immunocytochemistry, cells were seeded onto sterilized 18mm diameter glass coverslips in 6-well plates at a density of $1x10^5$ cells/well. Cells were transfected using Effectene (Qiagen) at a ratio of 1:10 (DNA:transfection reagent) following manufacturer's instructions.

To induce differentiation of RGC5 cells, cells were seeded into appropriate vessels and allowed to attach for 6h in media containing 10 % FBS before changing to serum-free media for 24 hours. Afterwards, cells were incubated in FBS medium containing *s*uccinyl-concanavalin A (50µg/ml) for 3 days [31].

To expose RGC5 cells to elevated pressure, cells were placed into a custom-made pressure chamber [32] and exposed to 30mmHg (72 h) or 100mmHg (24 h) above ambient pressure. To induce oxidative stress, RGC5 cells were treated with the mitochondrial complex I inhibitor rotenone (Sigma-Aldrich) at the indicated concentrations. To expose RGC5 cells to hypoxia-reoxygenation, cells were cultured at 1% oxygen/5% CO_2 for 24 hours following exposure to normoxia for 2 hours prior to fixation.

To prevent progression of apoptosis following stress treatment, cells were pre-treated with 50 µM of the pan-caspase inhibitor zVAD-fmk (PeptaNova).

3.6.2 Immunocytochemistry

To assess mitochondrial morphology and release of cytochrome *c*, RGC5 cells were fixed using methanol-free electron microscopy grade 4% paraformaldehyde in PBS (Pierce) for 15 minutes at RT, permeabilized for 15 minutes at RT using 0.15 % Trixon X-100 in PBS and blocked for 1 h in 10 % BSA in PBS. To stain for cytochrome *c,* cells were incubated with mouse anti-cytochrome *c* antibody (1:1000, Abcam 556432) and Alexa546-conjugated anti-mouse antibodies (1:500, Invitrogen). Samples were mounted using mounting medium (Vectashield, H-1000) and observed using immunofluorescence microscopy (Olympus BX 61, 60x/1.35 objective) or confocal microscopy (Zeiss Meta710, 63x/1.4 objective).

3.6.3 Analysis of mitochondrial morphology

Mitochondrial morphology was judged visually following cytochrome *c* staining relying on observer experience. Mitochondria were judged "normal" if appearance resembled control cells with mostly middle sized mitochondria and only some smaller fragments. Mitochondria were scored as fragmented if the majority of mitochondria (>90 %) were either round or slightly elongated (length < 2x width). Mitochondria were scored as elongated if only a minor fraction of mitochondria were of "normal" size and organelle continuously extended across the entire length/width of the cell. Each experiment was done independently at least three times and a minimum of 200 cells/condition were counted by an unblinded observer.

To more quantitatively assess mitochondrial morphology, RGC-5 cells were cultured on chambered cover glass and co-transfected in a 1:3 ratio with mitochondria-targeted photoactivatable GFP (mito-PAGFP) and MARCH5 or MARCH5^{H43W} expression constructs. The PAGFP assay was performed on a LSM710 confocal microscope (Zeiss) equipped with a 63x objective using ZEN software. For photoactivation, a 75 pixel wide circle was randomly selected. Activation was performed using the 405nm laser line (100% output) at zoom 4, 100µs pixel dwell time and 2 iterations. One z-stack, both before and after activation, was acquired (5 images at a 0.67µm interval). LSM Image Browser (Zeiss, v. 4.2.0.121) and ImageJ (NIH, v. 1.45s) were used to analyze mitochondrial interconnectivity as follows: the activated area (75 pixels)

was masked and a maximum intensity projection of the z-stack was created using LSM Image Browser. ImageJ was used to measure the total area of fluorescent mitochondria outside the photoactivated area (steps performed in ImageJ: threshold adjustment so that only photoactivated mitochondria were visible, then median filter radius 2 followed by analyze particles size 25-infinity). Twenty randomly selected cells were analyzed for each condition. The measured area was compared between different treatment groups relative to untreated MARCH5-expressing cells.

3.6.4 Flow cytometric analysis of cell death

RGC5 cells expressing MARCH5^{YFP}, MARCH5$^{H43W\text{-}YFP}$ or YFP as control were treated with 1µM rotenone for 24 or 48 hours or left untreated. Attached and floating cells were harvested, stained with 1µg/ml 7-aminoactinomycin D and analyzed by flow cytometry (CyAn ADP, Beckman Coulter). 7-AAD fluorescence (FL4) was measured in YFP positive cells to determine percentage of dead cells.

3.6.5 Statistical analysis

All experiments were performed at least three times. For each experiment a minimum of 200 cells/condition were counted. Statistical analysis was done using unpaired, two-tailed Student's t-test as implemented in Microsoft Excel. A p-Value of <0.05 or smaller was considered statistically significant and is marked with *, while p-Values of <0.01 are marked with **. Error bars represent the standard error of the mean (SEM). For PAGFP diffusion assay, 20 cells per condition were measured and statistical analysis was performed as described above.

3.7 Supplementary References

1. Nickells, R.W., et al., Under Pressure: Cellular and Molecular Responses During Glaucoma, a Common Neurodegeneration with Axonopathy. Annu Rev Neurosci, 2012.
2. Flammer, J. and M. Mozaffarieh, Autoregulation, a balancing act between supply and demand. Can J Ophthalmol, 2008. 43(3): p. 317-21.
3. Schnichels, S., et al., Trichostatin A induces cell death at the concentration recommended to differentiate the RGC-5 cell line. Neurochem Int, 2012. 60(6): p. 581-91.
4. Karbowski, M. and A. Neutzner, Neurodegeneration as a consequence of failed mitochondrial maintenance. Acta Neuropathol, 2012. 123(2): p. 157-71.
5. Frank, S., E.G. Robert, and R.J. Youle, Scission, spores, and apoptosis: a proposal for the evolutionary origin of mitochondria in cell death induction. Biochem Biophys Res Commun, 2003. 304(3): p. 481-6.
6. Baker, M.J., T. Tatsuta, and T. Langer, Quality control of mitochondrial proteostasis. Cold Spring Harb Perspect Biol, 2011. 3(7).
7. Neutzner, A., et al., Role of the ubiquitin conjugation system in the maintenance of mitochondrial homeostasis. Ann N Y Acad Sci, 2008. 1147: p. 242-53.
8. Neuspiel, M., et al., Cargo-selected transport from the mitochondria to peroxisomes is mediated by vesicular carriers. Curr Biol, 2008. 18(2): p. 102-8.
9. Li, W., et al., Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. PLoS One, 2008. 3(1): p. e1487.
10. Tang, F., et al., RNF185, a novel mitochondrial ubiquitin E3 ligase, regulates autophagy through interaction with BNIP1. PLoS One, 2011. 6(9): p. e24367.
11. Nakamura, N., et al., MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology. EMBO Rep, 2006. 7(10): p. 1019-22.
12. Karbowski, M., A. Neutzner, and R.J. Youle, The mitochondrial E3 ubiquitin ligase MARCH5 is required for Drp1 dependent mitochondrial division. J Cell Biol, 2007. 178(1): p. 71-84.
13. Yonashiro, R., et al., A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. EMBO J, 2006. 25(15): p. 3618-26.
14. Yonashiro, R., et al., Mitochondrial ubiquitin ligase MITOL ubiquitinates mutant SOD1 and attenuates mutant SOD1-induced reactive oxygen species generation. Mol Biol Cell, 2009. 20(21): p. 4524-30.
15. Sugiura, A., et al., A mitochondrial ubiquitin ligase MITOL controls cell toxicity of polyglutamine-expanded protein. Mitochondrion, 2011. 11(1): p. 139-46.
16. Yonashiro, R., et al., Mitochondrial ubiquitin ligase MITOL blocks S-nitrosylated MAP1B-light chain 1-mediated mitochondrial dysfunction and neuronal cell death. Proc Natl Acad Sci U S A, 2012. 109(7): p. 2382-7.
17. Westermann, B., Mitochondrial fusion and fission in cell life and death. Nat Rev Mol Cell Biol, 2010. 11(12): p. 872-84.
18. Delettre, C., et al., Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. Nat Genet, 2000. 26(2): p. 207-10.
19. Carelli, V., et al., Retinal ganglion cell neurodegeneration in mitochondrial inherited disorders. Biochim Biophys Acta, 2009. 1787(5): p. 518-28.

20. Chang, C.R. and C. Blackstone, Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1. Ann N Y Acad Sci, 2010. 1201: p. 34-9.
21. Gandre-Babbe, S. and A.M. van der Bliek, The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. Mol Biol Cell, 2008. 19(6): p. 2402-12.
22. Palmer, C.S., et al., MiD49 and MiD51, new components of the mitochondrial fission machinery. EMBO Rep, 2011. 12(6): p. 565-73.
23. Waterham, H.R., et al., A lethal defect of mitochondrial and peroxisomal fission. N Engl J Med, 2007. 356(17): p. 1736-41.
24. Martinou, J.C. and R.J. Youle, Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev Cell, 2011. 21(1): p. 92-101.
25. Lee, Y.J., et al., Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol Biol Cell, 2004. 15(11): p. 5001-11.
26. Frank, S., et al., The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell, 2001. 1(4): p. 515-25.
27. Ju, W.K., et al., Increased optic atrophy type 1 expression protects retinal ganglion cells in a mouse model of glaucoma. Mol Vis, 2010. 16: p. 1331-42.
28. Chen, H., J.M. McCaffery, and D.C. Chan, Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell, 2007. 130(3): p. 548-62.
29. Grohm, J., et al., Inhibition of Drp1 provides neuroprotection in vitro and in vivo. Cell Death Differ, 2012.
30. Van Bergen, N.J., et al., Recharacterization of the RGC-5 retinal ganglion cell line. Invest Ophthalmol Vis Sci, 2009. 50(9): p. 4267-72.
31. Krishnamoorthy, R.R., et al., Characterization of a transformed rat retinal ganglion cell line. Brain Res Mol Brain Res, 2001. 86(1-2): p. 1-12.
32. Xin, X., et al., Meningothelial cells react to elevated pressure and oxidative stress. PLoS One, 2011. 6(5): p. e20142.

4 MARCH5 inactivation supports mitochondrial function during neurodegenerative stress

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4.1 Abstract

Neuronal cell death is accompanied by mitochondrial dysfunction with mitochondrial maintenance critical to neuronal survival. The mitochondrial ubiquitin ligase MARCH5 has dual roles in the upkeep of mitochondrial function. MARCH5 is involved in targeted degradation of proteins harmful to mitochondria and impacts mitochondrial morphology upstream of the fission protein Drp1. In a neuronal cell model, dominant-negative MARCH5 prevents mitochondrial fragmentation during neurodegenerative stress induced by the neuron-specific reactive oxygen generator 6-hydroxydopamine, the complex I inhibitor rotenone or Alzheimer's-releated Aβ peptide. In addition, preservation of mitochondrial function in terms of membrane potential and lower reactive oxygen generation was observed following inactivation of MARCH5. Our findings connect MARCH5 to neuronal stress responses and further emphasize the link between mitochondrial dynamics and function.

4.2 Introduction

Mitochondrial dysfunction is at the heart of neurodegeneration [1], since neuronal cells are especially dependent on high fidelity mitochondria to meet their extraordinary energy demand. Loss of mitochondrial fidelity due to accumulation of damage is thought to be one of the central mechanisms for the death of neuronal cells associated with virtually all neurodegenerative disorders as well as aging. Damage to mitochondria is caused mainly through reactive reaction intermediates of the mitochondrial electron transport chain (ETC) namely reactive oxygen (ROS) and reactive nitrogen species (RNS). Main targets of ROS and RNS are mitochondrial proteins as well as mitochondrial DNA but also mitochondrial lipids. Especially damage to proteins of the ETC and to mitochondrial DNA, which codes mainly for ETC components, impacts mitochondrial health as a subpar ETC gives rise to even more reactive intermediates through electron leakage. Various repair, salvage and maintenance mechanisms are in place to deal with such stresses and to keep mitochondria in a healthy and functional state. On the level of mitochondrial DNA, various repair mechanisms are active, drawing from the large redundancy of mitochondrial DNA with up to 10'000 copies per cell [2]. On the protein level, removal of damaged proteins takes place via specialized proteases in the matrix and inner mitochondrial membrane [3] or through the ubiquitin-proteasome system during outer mitochondrial membrane associated degradation (OMMAD) [4]. On the organellar level, the mitochondrial network is maintained through dynamic fission and fusion of mitochondrial tubules essential for adaption of the network to cellular demand [5]. Furthermore, removal of irreparable mitochondrial subunits by mitophagy, a specialized autophagic process, is essential for maintaining organellar fidelity [6]. Lastly, on the cellular level, irreparable damage to the mitochondrial network causes the induction of apoptosis, thus constituting a complete removal of dysfunctional organelles [1, 7]. In the case of neuronal tissue with its very limited capacity for regeneration, induction of the cell death program is deleterious and results in the irreparable loss of neuronal function leading ultimately to neurodegenerative disease.

The mitochondrial ubiquitin ligase MARCH5/MITOL [8, 9] is involved in maintaining mitochondrial function. MARCH5 was shown to regulate mitochondrial morphology through regulating Drp1 activity [8], thereby impacting cellular senescence [10] and modulating neuronal death [11]. Furthermore, MARCH5 was implicated in the regulation of ER-mitochondrial tethering through ubiquitination of mitofusin Mfn2 [12]. MARCH5 also plays a role in the degradation of mSOD1 associated with amyotrophic lateral sclerosis [13], in the disposal of mutated ataxin-3 causative for Machado-Joseph disease [14] as well as the clearance of S-nitrosylated MAP1B-light chain 1 linked to neuronal degeneration [15].

Here we found MARCH5 to be involved in the mitochondrial answer to neurodegenerative stress evoked by 6-hydroxydopamine, a superoxide generating compound selective for neuronal cells, the mitochondrial poison rotenone or Alzheimer's related amyloid beta (Aβ). While MARCH5 increased mitochondrial effects of neurodegenerative stress, inactivation of MARCH5 reversed stress-induced fragmentation and strongly ameliorated stress-related mitochondrial dysfunction pointing to an active role of MARCH5 in stress response decisions.

4.3 Results

Here, treatment of SH-SY5Y neuroblastoma cells with the dopaminergic and noradrenergic neuron-specific ROS generator 6-hydroxydopamine, the mitochondrial ETC complex I inhibitor rotenone, or the Aβ peptide was employed to study mitochondrial function in a model for neuronal cells under neurodegenerative stress. As revealed by cytochrome *c* staining, following treatment with 6-hydroxydopamine (Figure 4.1 – second panel), rotenone (Figure 4.1A – third panel) or Aβ peptide (Figure 4.1 – fourth panel) the mitochondrial network is considerably fragmented when compared to control cells (Figure 4.1 – first panel). The extent of mitochondrial fragmentation was strongest with 6-hydroxydopamine, still strong following rotenone and less pronounced after Aβ treatment. These data are consistent with mitochondrial fragmentation in neuronal cells in response to neurodegenerative stress evoked

by exogenous ROS, mitochondria-generated ROS as well as mitochondrial dysfunction due to Aβ, respectively.

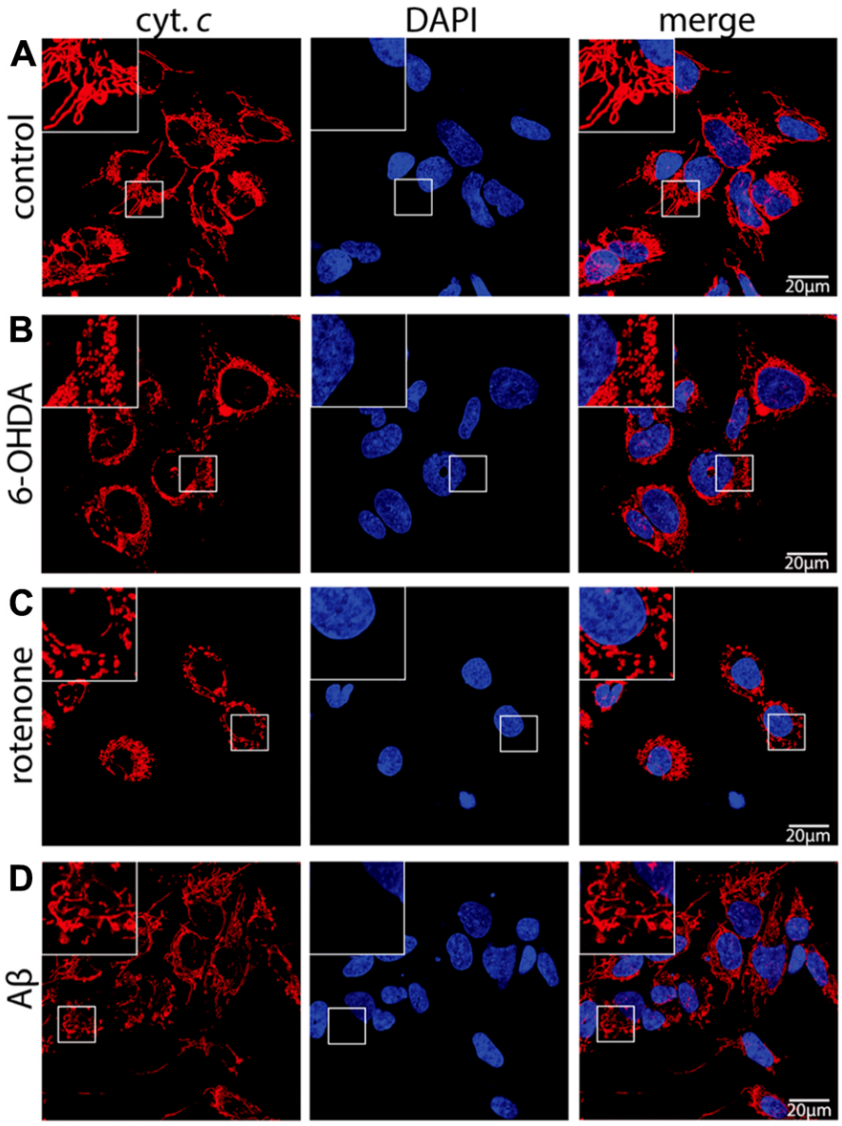

Figure 4.1: Neurodegenerative stress causes mitochondrial fragmentation in neuronal cells.

SH-SY5Y cells mock treated (control) or exposed for 6 hours to 75 µM 6-hydroxydopamine (6-HODA), for 6 hours to 5 µM rotenone (rotenone), or for 24 hours to 25 µM Aβ peptide (Aβ) were fixed and stained using anti-cytochrome *c* antibodies and DAPI to reveal mitochondrial morphology and the nucleus, respectively. Shown are representative pictures of at least three independent experiments.

To study the role of MARCH5 in the mitochondrial response to neurodegenerative stress in neuronal cells, wildtype MARCH5, ubiquitin ligase activity negative MARCH5^{H43W} or mitochondria-targeted yellow fluorescent protein (mitoYFP) as control were stably expressed in SH-SY5Y cells. As shown in Figure 4.2A and consistent with previous observations in HeLa cells [8], expression of MARCH5 in SH-SY5Y cells had no discernible impact on mitochondrial morphology, while expression of dominant-negative MARCH5^{H43W} caused considerable elongation of the mitochondrial network when compared to control cells. In addition and as also reported previously [8], MARCH5^{H43W} localized to distinct sub-mitochondrial foci in SH-SY5Y cells while wildtype MARCH5 localized to mitochondria in a circumscribing fashion.

To test whether MARCH5 activity is necessary for neurodegenerative stress-induced mitochondrial fragmentation, cells stably expressing MARCH5, MARCH5^{H43W} or mitoYFP were treated with 6-hydroxydopamine (Figure 4.2B), rotenone (Figure 4.2C) or Aβ (Figure 4.2D) and mitochondrial morphology was observed following cytochrome *c* staining. While mitochondrial fragmentation was still evident in cells expressing MARCH5 or YFP, expression of MARCH5^{H43W} in SH-SY5Y cells prevented mitochondrial fragmentation evoked by neurodegenerative stress conditions.

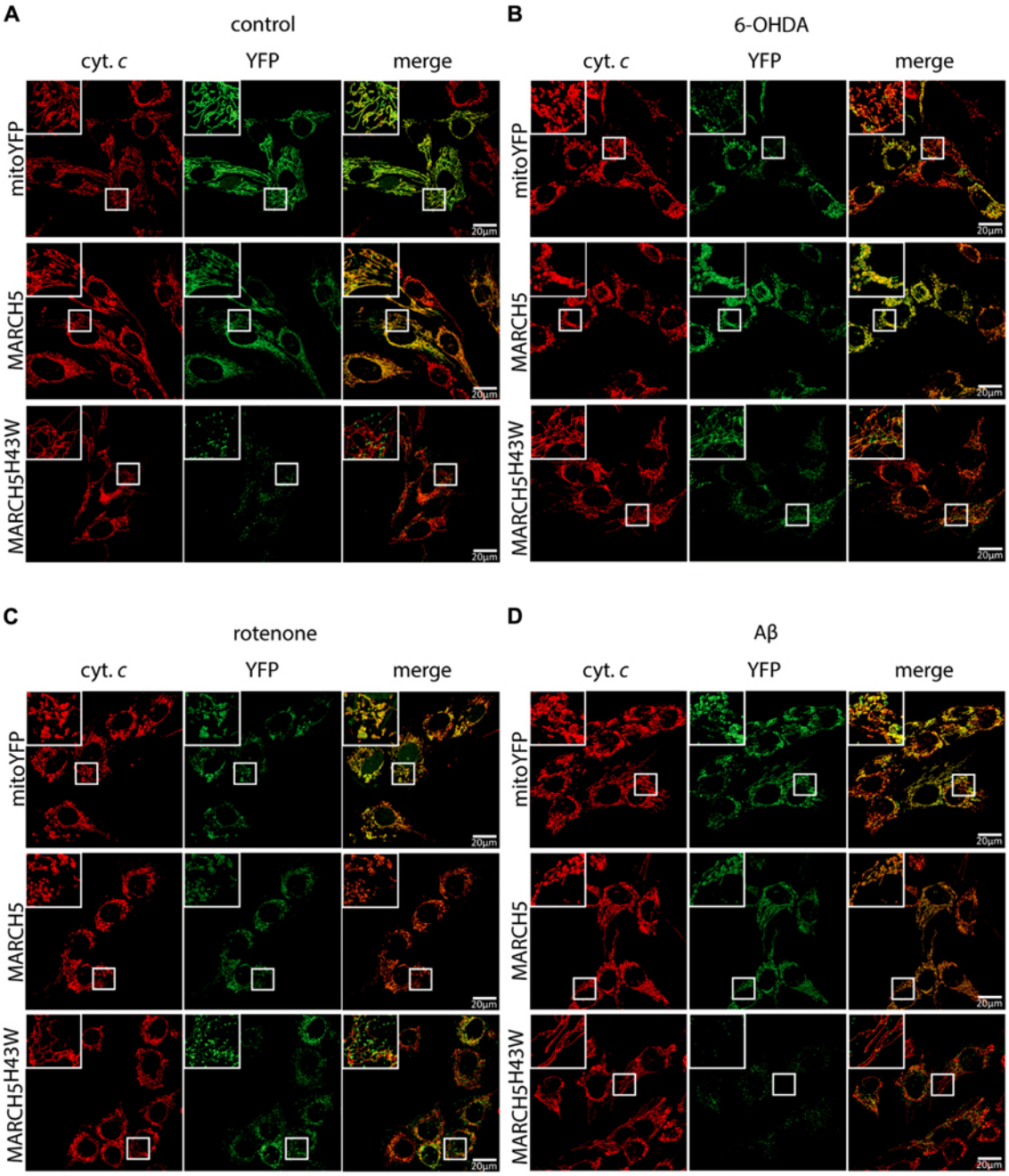

Figure 4.2: Inactivation of MARCH5 prevents stress-induced mitochondrial fragmentation.

SH-SY5Y cells selected to express mitochondria-targeted YFP (mitoYFP), MARCH5-YFP or MARCH5^{H43W}-YFP mock treated **(A)** or treated with 75 µM 6-hydroxydopamine for 6 hours **(B)**, 5 µM rotenone for 6 hours **(C)**, or 25 µM Aβ peptide for 24 hours **(D)** were fixed and stained using anti-cytochrome *c* antibodies and imaged using confocal microscopy (cytochrome *c* – red, YFP – green). Shown are representative pictures of at least three independent experiments.

To quantify the impact of MARCH5^{H43W} on preservation of mitochondrial morphology in SH-SY5Y cells following neurodegenerative stress, mitochondrial interconnectivity was measured. To this end, SH-SY5Y cells co-transfected with expression plasmids for MARCH5 or MARCH5^{H43W} and mitochondria-targeted photoactivatable GPF (PA-GFP) were treated with 6-hydroxydopamine (Figure 4.3A), rotenone (Figure 4.3B) or Aβ (Figure 3C) or mock treated as control and diffusion of PA-GFP was measured following 405 nm laser activation of a small part of the mitochondrial network. The area of PA-GFP of the mitochondrial network covered by activated PA-GFP served as measure for interconnectivity of individual mitochondrial tubules. Treatment of SH-SY5Y cells with 6-hydroxydopamine in the presence of MARCH5 expression caused a loss of mitochondrial connectivity compared to mock treated MARCH5 expressing cells, while treatment with 6-hydroxydopamine of MARCH5^{H43W} expressing cells had no impact on mitochondrial interconnectivity compared to untreated control cells (Figure 4.3A). Using rotenone as inducer of neurodegenerative stress, blocking MARCH5 function through expression of MARCH5^{H43W} was also able to almost completely suppress loss of mitochondrial interconnectivity (Figure 4.3B). As for treatment of SH-SY5Y cells with Aβ, MARCH5^{H43W} was able to suppress mitochondrial fission compared to MARCH5 expressing cells and preserve connectivity of mitochondrial tubules at levels almost comparable to control cells (Figure 4.3C).

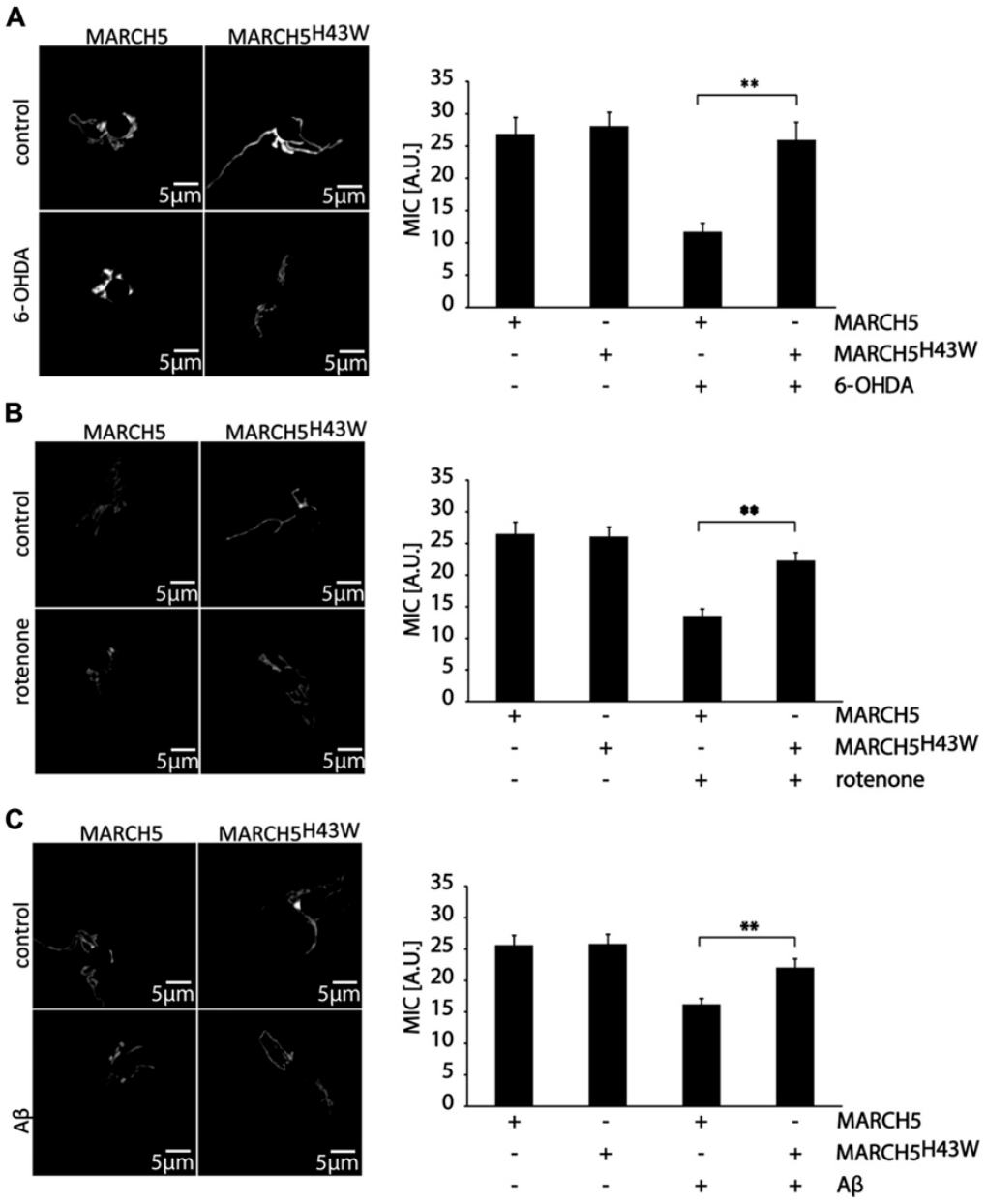

Figure 4.3: Inactive MARCH5 supports mitochondrial interconnectivity under neurodegenerative stress conditions.

SH-SY5Y co-transfected with expression plasmids for PA-GFP and either MARCH5 or MARCH5^{H43W} were treated with 75 µM 6-hydroxydopamine for 6 hours **(A)**, 5µM rotenone for 6 hours **(B)**, or 25 µM Aβ peptide for 24 hours **(C)** and mitochondrial interconnectivity was measured following activation of PA-GFP in a small area of the mitochondrial network. Shown is one representative picture and the average of three independent experiments with 15 cells each/condition. Statistical significance was analyzed using Student's t-test with ** marking $p < 0.01$. Error bars represent SEM.

To evaluate whether inactivation of MARCH5 has impact not only on mitochondrial dynamics, but also on mitochondrial core function under neurodegenerative stress conditions, mitochondrial membrane potential in MARCH5 or MARCH5^{H43W} expressing cells following treatment with 6-hydroxydopamine, rotenone or Aβ was measured (Figure 4.4) with mock treated cells serving as control. To this end, cells were loaded with the mitochondrial membrane sensitive dye tetramethylrhodamine ethyl ester (TMRE) and single cell analysis of confocal images was performed. While expression of MARCH5 had no effect on mitochondrial membrane potential when compared to mitoYFP expressing control cells, expression of MARCH5^{H43W} caused mitochondrial hyperpolarization (Figure 4.4A). Treatment of MARCH5 expressing cells with 6-hydroxydopamine (Figure 4.4B), rotenone (Figure 4.4C) or Aβ (Figure 4.4D) caused significant loss of mitochondrial membrane potential in comparison to untreated MARCH5 expressing cells. Also, treating MARCH5^{H43W} expressing cells with these stressors resulted in a loss of mitochondrial membrane potential compared to untreated MARCH5^{H43W} control cells. However, loss of membrane potential was less pronounced in MARCH5^{H43W} expressing cells between stressed and unstressed conditions compared to wildtype MARCH5 expressing cells. Comparing MARCH5 and MARCH5^{H43W} expressing cells, the membrane potential in stressed MARCH5^{H43W} cells is at levels seen in unstressed MARCH5 expressing cells.

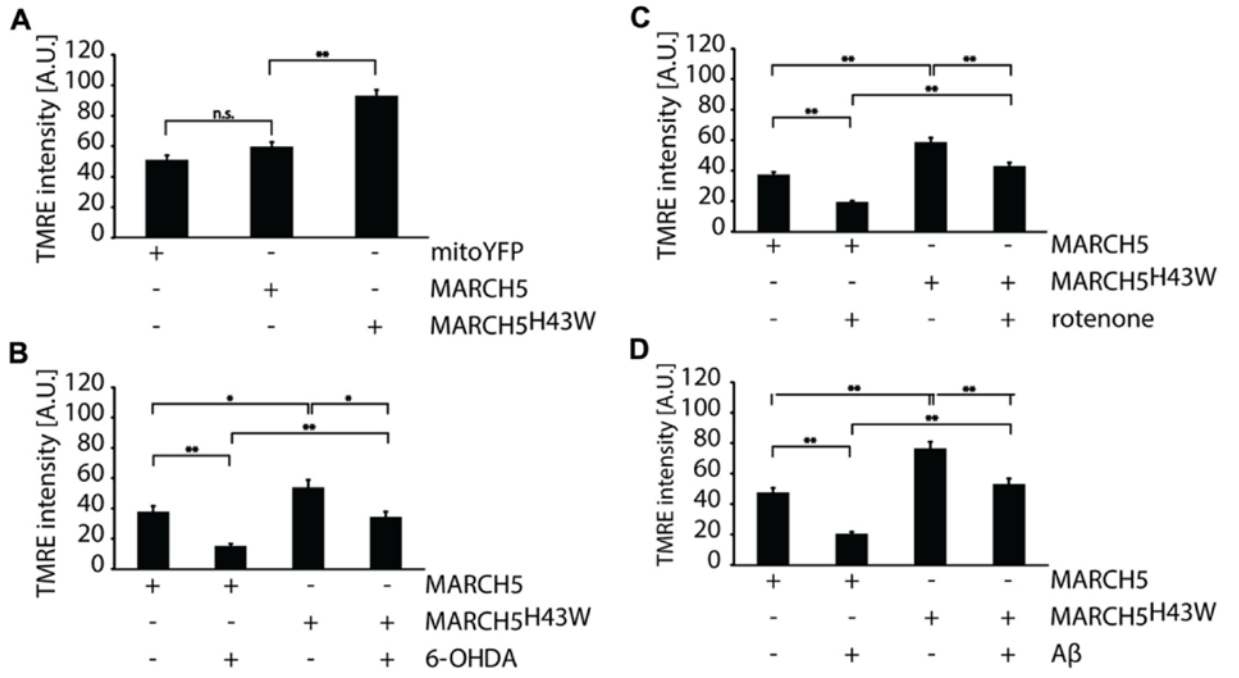

Figure 4.4: Mitochondrial membrane potential under neurodegenerative stress conditions is increased following inactivation of MARCH5.

(A) SH-SY5Y cells expressing mitoYFP, MARCH5-YFP or MARCH5^{H43W}-YFP were stained with the mitochondrial membrane potential sensitive dye TMRE, images were taken by confocal microscopy and TMRE fluorescence as measure for mitochondrial membrane potential was determined using image analysis. SH-SY5Y cells expressing MARCH5-YFP or MARCH5^{H43W}-YFP were treated with 75 µM 6-hydroxydopamine for 6 hours **(B)**, 5µM rotenone for 6 hours **(C)**, or 25 µM Aβ peptide for 24 hours **(D)** and mitochondrial membrane potential was measured as in A. Shown is the average of three independent experiments with 10 cells each per condition. Statistical significance was analyzed using Student's t-test with ** marking $p < 0.01$. Error bars represent SEM.

To further gain insight into the mechanisms responsible for MARCH5^{H43W}-mediated protection from neurodegenerative stress, cellular levels of ROS were assessed using single cell analysis of CellRox fluorescence. To this end, first, ROS levels were measured in cells expressing MARCH5, MARCH5^{H43W} or mitoYFP and no significant difference between either group was detected (Figure 4.5A). Analysis of MARCH5 or MARCH5^{H43W} expressing cells treated with 6-hydroxydopamine revealed an increase in intracellular ROS in MARCH5 cells, while expression of MARCH5^{H43W} prevented this 6-hydroxydopamine mediated spike in ROS almost completely (Figure 4.5B). Similarly, expression of MARCH5^{H43W} was able to blunt ROS production following

treatment with rotenone, while ROS levels were elevated about 2-fold in MARCH5 expressing cells compared to untreated MARCH5 expressing control cells (Figure 4.5C). As for mitochondrial stress evoked by treatment with Aβ, ROS levels in MARCH5^{H43W} expressing cells were about 50% compared to cells producing MARCH5. However, Aβ treatment caused still an increase in ROS even in the presence of inactive MARCH5, albeit to a lower extent compared to control cells.

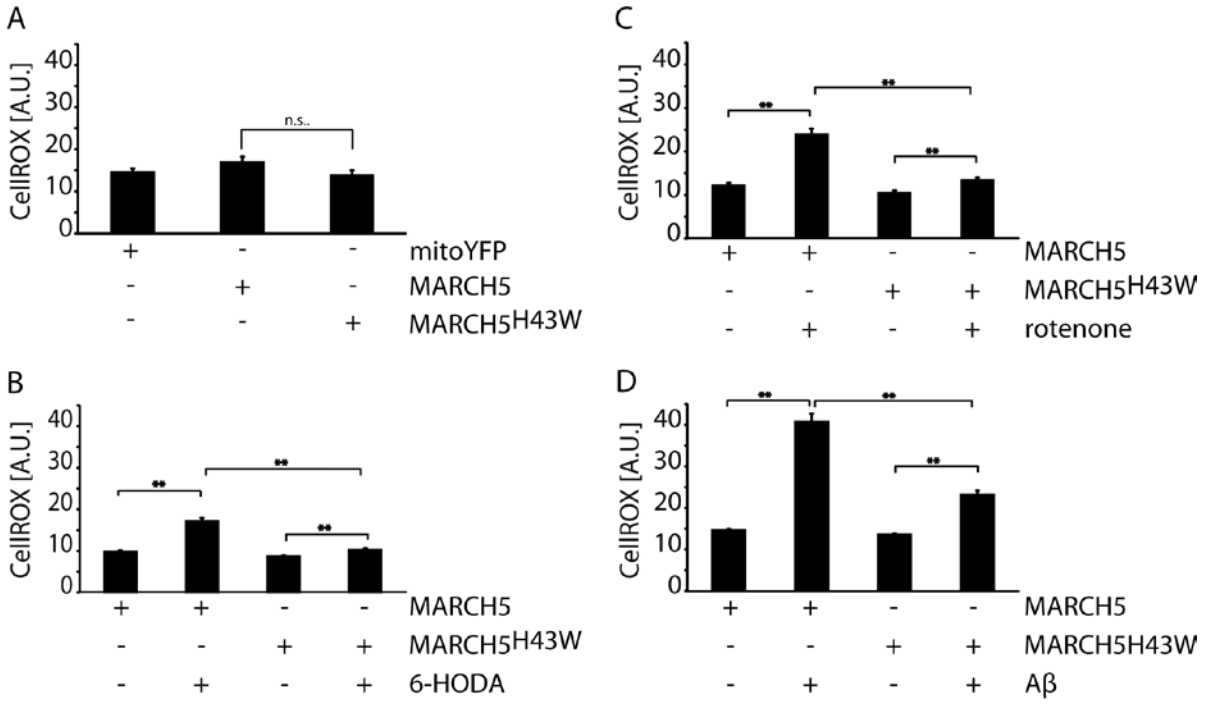

Figure 4.5: Inactivation of MARCH5 blunts ROS production under neurodegenerative stress conditions.

(A) SH-SY5Y cells expressing mitoYFP, MARCH5-YFP or MARCH5^{H43W}-YFP were treated with the ROS-sensitive dye CellROX and intracellular ROS levels were measured using image analysis of confocal pictures. SH-SY5Y cells expressing MARCH5-YFP or MARCH5^{H43W}-YFP were treated with 75 µM 6-Hydroxydopamine for 6 hours **(B)**, 5 µM rotenone for 6 hours **(C)**, or 25 µM Aβ peptide for 24 hours **(D)** and mitochondrial membrane potential was measured as in A. Shown is the average of four independent experiments with 10 cells per condition. Statistical significance was analyzed using Student's t-test with ** marking $p < 0.01$ and n.s. marking no significant difference. Error bars represent SEM.

4.4 Material and methods

4.4.1 Cell culture

Human neuroblastoma cell line SH-SY5Y was purchased from DSMZ. SH-SY5Y cells were cultured in high glucose DMEM, supplemented with 2mM L-glutamine, 1mM sodium pyruvate, and 15% fetal bovine serum (Sigma). Cells were incubated in a humidified incubator at 5% CO_2 and 37 °C.

SH-SY5Y cells were transfected using Effectene (Qiagen) according to the manufacturer's recommendations. To generate stably expressing SH-SY5Y cells, selection was performed using geneticin sulfate (400µg/ml) for 4 weeks. Degree of transfection was established using flow cytometric analysis (CyAn ADP, Beckman Coulter) and found to be around 80%. SH-SY5Y cells were treated with 6-hydroxydopamine (75µM, 6 hours), rotenone (5µM, 6 hours), and Aβ-peptide 25-35 (Sigma A4559, 25 µM, 24 hours).

4.4.2 Microscopy

For immunocytochemistry, cells were seeded in 6-well plates onto glass slides at $1x10^4$ cells/well in 2ml culture medium. Samples were fixed using methanol-free electron microscopy grade 4% paraformaldehyde in PBS for 15 minutes at RT, permeabilized for 15 minutes at room temperature using 0.15% Trixon X-100 in PBS and blocked for 1 hour in 10% BSA in PBS. To visualize mitochondria, samples were then incubated with mouse anti-cytochrome *c* antibody (BD Biosciences 556432, 1:1000) overnight at 4 °C and Alexa546-conjugated anti-mouse antibodies (Invitrogen A11003, 1:500) for 1 hour at RT. Samples were mounted in mounting medium (Vectashield H1000) and observed using a confocal microscope (Zeiss LSM Meta710, 63×/1.4 objective). For life cell imaging, cells were seeded onto chambered coverglass (Nunc Lab-Tek, 154461) at a density of $5x10^3$/well in 1ml culture media. Measurement of mitochondrial interconnectivity was performed as described before [11]. In short, SH-SY5Y cells were transfected with an expression construct for photoactivatable GFP (PAGFP) and

mitochondrial network area following photoconversion of PAGFP employing a 405 nm laser was measured. To measure mitochondrial membrane potential, cells were stained with 100nM TMRE (Invitrogen, T-669) in media for 20 minutes at 37 °C and washed three times with media. Imaging was performed on a LSM710 confocal microscope (Zeiss) equipped with a live cell imaging chamber. Z-stacks (5 images, 1 µm distance) were acquired and image analysis was performed using Imaris v7.0 software (Bitplane Scientific Software). Data are expressed as mean signal intensity of thirty randomly selected cells per treatment group (three independent experiments, 10 cells each). To measure cellular ROS, cells were stained using 5µM CellROX Deep Red Reagent (Invitrogen, C10422) for 30 minutes at 37 °C, washed three times with PBS and fixed using 4% paraformaldehyde before imaging. Z-stacks (5 images at 1µm intervals) were acquired and analyzed using Imaris 7.0. Data are expressed as mean signal intensity of cells (four independent experiments, 10 cells each/group each).

4.4.3 Statistical analysis

Statistical analysis was performed using unpaired, two-tailed Student's t test as implemented in Microsoft Excel. A p-Value of <0.05 or smaller was considered statistically significant and is marked with*, while p-Values of <0.01 are marked with **. Error bars represent the standard error of the mean (SEM).

4.5 Discussion

While MARCH5 is involved in the removal of proteins associated with specific neurodegenerative disorders such as ataxin-3 in Joseph-Machado disease or mSOD1 in amyotrophic lateral sclerosis likely supporting mitochondrial function, MARCH5 activity during general mitochondrial oxidative stress does not seem to confer a protective effect. Mitochondrial fragmentation in response to oxidative insults evoked by external ROS generators such as 6-hydroxydopamine or internal ROS generators such as rotenone or Aβ was greatly diminished in cells expressing MARCH5^{H43W}, while wildtype MARCH5 did not prevent the

remodeling of the mitochondrial network in response to stress. Thus it is conceivable that during neurodegenerative stress, removal of damaged proteins from mitochondria through MARCH5 seems not to be as essential as one might expect from an ubiquitin ligase involved in mitochondrial protein quality control. Rather, the function of MARCH5 as mitochondrial morphogen modulating Drp1 activity might be important in this context. And indeed we previously showed that inactivation of MARCH5 protects neuronal cells from stress-induced cell death [11] likely through the inhibition of Drp1-dependent mitochondrial fragmentation in accordance with previous observations where inhibition of Drp1 activity strongly delayed cell death [16]. Thus, the function of MARCH5 in regulating mitochondrial morphology might also be the main factor in the here observed positive effect on mitochondrial fidelity upon expression of dominant-negative MARCH5^{H43W}. Fragmentation of the mitochondrial network is a response to potentially lethal stress conditions such as increased oxidative stress or loss of membrane potential. As mitochondrial fragmentation is an integral part of the apoptotic program with forced fragmentation sensitizing cells to apoptotic stimuli [17], shortening mitochondria does not seem to have a protective effect on cells but might rather be seen as preparation for starting the cell death program although mitochondrial fission *per se* is not an apoptotic stimulus [5]. In contrast, elongation of mitochondria seems to be protective as evidenced by de-sensitization to apoptotic stimuli following increased mitochondrial fusion [18]. Also during stress-induced mitochondrial hyperfusion (SIMH), enhanced fusion and therefore highly interconnected mitochondrial tubules prove to increase resistance against certain stresses [19]. While most experimental stress conditions induce mitochondrial fragmentation, stress at levels well below the apoptotic threshold induces mitochondrial elongation. The SIMH-associated adaption of mitochondrial morphology is brought about in an Mfn1- and OPA1-dependent but Mfn2-independent manner and is likely not achieved by forced mitochondrial elongation due to inhibition of Drp1 function [19]. The increased interconnectivity during SIMH conditions mitochondria against further stress potentially by boosting their ATP production likely via increased availability of substrates and ETC intermediates in fused mitochondrial reticulum with its extended matrix space. Whether

inactivation of MARCH5 induces SIMH is unclear as mitochondrial elongation in the absence of external stress is evident in MARCH5^{H43W} expressing cells, however, the observed increase of mitochondrial membrane potential in unstressed cells and the blunting of stress-induced ROS production point in this direction. This leaves the question, which additional pathways besides inhibition of Drp1-mediated fission might be influenced by MARCH5^{H43W} as simply inhibiting Drp1 does not seem to evoke SIMH [19]. Based on our results, one might speculate that MARCH5 as upstream regulator of Drp1 is important during cellular stress responses and might modulate the activity of other targets besides Drp1. The notion of MARCH5 regulating other such targets is supported by our observation that expression of wildtype MARCH5 did not preserve mitochondrial function under neurodegenerative stress conditions although no effect on mitochondrial interconnectivity was observed [8]. Importantly, expression of MARCH5 in the absence of stress conditions did not impact mitochondrial membrane potential or ROS production further hinting to a role for MARCH5 during mitochondrial stress. Thus, based on our observations following dominant-negative MARCH5^{H43W} expression and the effects of wildtype MARCH5, it seems conceivable that MARCH5 is involved in the decision for stress-induced fragmentation versus protective mitochondrial elongation. Taken together, our data further support a role of MARCH5 in the modulation of Drp1 activity during mitochondrial fission and implicate MARCH5 in mitochondrial stress response pathways. As the mitochondrial stress response is pathophysiologically significant from diabetes to cardiovascular disease to neurodegeneration, blockage of MARCH5 might be an interesting therapeutic strategy.

4.6 Supplementary References

1. Karbowski, M. and A. Neutzner, Neurodegeneration as a consequence of failed mitochondrial maintenance. Acta Neuropathol, 2012. **123**(2): p. 157-71.
2. Bogenhagen, D. and D.A. Clayton, The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. Quantitative isolation of mitochondrial deoxyribonucleic acid. J Biol Chem, 1974. **249**(24): p. 7991-5.
3. Rugarli, E.I. and T. Langer, Mitochondrial quality control: a matter of life and death for neurons. EMBO J, 2012. **31**(6): p. 1336-49.
4. Neutzner, A., R.J. Youle, and M. Karbowski, Outer mitochondrial membrane protein degradation by the proteasome. Novartis Found Symp, 2007. **287**: p. 4-14; discussion 14-20.
5. Youle, R.J. and A.M. van der Bliek, Mitochondrial fission, fusion, and stress. Science, 2012. **337**(6098): p. 1062-5.
6. Youle, R.J. and D.P. Narendra, Mechanisms of mitophagy. Nat Rev Mol Cell Biol, 2011. **12**(1): p. 9-14.
7. Tatsuta, T. and T. Langer, Quality control of mitochondria: protection against neurodegeneration and ageing. EMBO J, 2008. **27**(2): p. 306-14.
8. Karbowski, M., A. Neutzner, and R.J. Youle, The mitochondrial E3 ubiquitin ligase MARCH5 is required for Drp1 dependent mitochondrial division. J Cell Biol, 2007. **178**(1): p. 71-84.
9. Yonashiro, R., et al., A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. EMBO J, 2006. **25**(15): p. 3618-26.
10. Park, Y.Y., et al., Loss of MARCH5 mitochondrial E3 ubiquitin ligase induces cellular senescence through dynamin-related protein 1 and mitofusin 1. J Cell Sci, 2010. **123**(Pt 4): p. 619-26.
11. Fang, L., et al., Inactivation of MARCH5 prevents mitochondrial fragmentation and interferes with cell death in a neuronal cell model. PLoS One, 2012. **7**(12): p. e52637.
12. Sugiura, A., et al., MITOL Regulates Endoplasmic Reticulum-Mitochondria Contacts via Mitofusin2. Mol Cell, 2013.
13. Yonashiro, R., et al., Mitochondrial ubiquitin ligase MITOL ubiquitinates mutant SOD1 and attenuates mutant SOD1-induced reactive oxygen species generation. Mol Biol Cell, 2009. **20**(21): p. 4524-30.
14. Sugiura, A., et al., A mitochondrial ubiquitin ligase MITOL controls cell toxicity of polyglutamine-expanded protein. Mitochondrion, 2011. **11**(1): p. 139-46.
15. Yonashiro, R., et al., Mitochondrial ubiquitin ligase MITOL blocks S-nitrosylated MAP1B-light chain 1-mediated mitochondrial dysfunction and neuronal cell death. Proc Natl Acad Sci U S A, 2012. **109**(7): p. 2382-7.
16. Frank, S., et al., The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell, 2001. **1**(4): p. 515-25.
17. Lee, Y.J., et al., Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol Biol Cell, 2004. **15**(11): p. 5001-11.
18. Neuspiel, M., et al., Activated mitofusin 2 signals mitochondrial fusion, interferes with Bax activation, and reduces susceptibility to radical induced depolarization. J Biol Chem, 2005. **280**(26): p. 25060-70.
19. Tondera, D., et al., SLP-2 is required for stress-induced mitochondrial hyperfusion. EMBO J, 2009. **28**(11): p. 1589-600.

5 Mitochondrial functions in neuronal cells depends on p97/VCP/Cdc48-mediated quality control

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Short title: p97 and maintenance of mitochondrial function

Key words: mitochondria, p97/VCP, neuronal cells, quality control

5.1 Abstract

Maintaining mitochondrial function is essential for neuronal survival and offers protection against neurodegeneration. Ubiquitin-mediated, proteasome-dependent protein degradation in the form of outer mitochondrial membrane associated degradation (OMMAD) was shown to play roles in maintenance of mitochondria on the level of proteostasis, but also mitophagy and cell death. Recently, the AAA-ATPase p97/VCP/Cdc48 was recognized as part of OMMAD acting as retrotranslocase of ubiquitinated mitochondrial proteins for proteasomal degradation. Thus, p97 likely plays a major role in mitochondrial maintenance. Support for this notion comes from mitochondrial dysfunction associated with amyotrophic lateral sclerosis and hereditary inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD) caused by p97 mutation. Using SH-SY5Y cells stably expressing p97 or dominant-negative $p97^{QQ}$ treated with mitochondrial toxins rotenone, 6-OHDA, or Aβ-peptide as model for neuronal cells suffering from mitochondrial dysfunction, we found mitochondrial fragmentation under normal and stress conditions was significantly increased upon inactivation of p97. Furthermore, inactivation of p97 resulted in loss of mitochondrial membrane potential and increased production of reactive oxygen species (ROS). Under additional stress conditions, loss of mitochondrial membrane potential and increased ROS production was even more pronounced. Loss of mitochondrial fidelity upon inactivation of p97 was likely due to disturbed maintenance of mitochondrial proteostasis as the employed treatments neither induced mitophagy nor cell death. This was supported by the accumulation of oxidatively-damaged proteins on mitochondria in response to p97 inactivation. Dysfunction of p97 under normal and stress conditions in neuron-like cells severely impacts mitochondrial function, thus supporting for the first time a role for p97 as a major component of mitochondrial proteostasis.

5.2 Introduction

Failing mitochondrial maintenance is at the heart of neurodegeneration and associated neuronal death [1, 2]. To prevent the untimely death of neuronal cells, mitochondria are kept healthy and in shape through the complex interplay of various molecular mechanisms aimed at repairing mitochondrial damage on the molecular level [3] or at removing damaged mitochondrial subunits from the cell [4]. A key component of mitochondrial maintenance is quality control of damaged, dysfunctional proteins through protein degradation [5, 6]. Owed to the complex architecture and the endosymbiotic nature of mitochondria, several protein degradation mechanisms are in place to maintain proteostasis in the various mitochondrial compartments [7]. Recently, we and others described roles for the ubiquitin-proteasome system in maintaining mitochondrial function and proteostatis. E3 enzymes, namely MARCH5/MITOL [8, 9], MAPL/MULAN [10], IBRDC2 [11], RNF185 [12], and Parkin [13] were found to localize to the mitochondrial outer membrane. Furthermore, ubiquitin-dependent protein degradation was shown to modulate mitochondrial morphology [8, 14-16] and impact mitophagy [17]. In addition, quality control of mitochondria-localized poly-Q [18], amyotrophic lateral sclerosis associated mSOD1 as well as S-nitrosylated proteins [19] is performed by the ubiquitin-proteasome system. Analogous to the endoplasmic reticulum (ER) which is quality controlled by ER associated degradation or ERAD [20], mitochondria might be considered to be under control of outer mitochondrial membrane associated degradation or OMMAD [21].

Quality control of proteins localized to a membrane-bound organelle by the cytosolic ubiquitin-proteasome system must involve the protein extraction from the organelle and retrotranslocation into the cytosol for degradation. The AAA-ATPase valosin containing protein VCP/p97/Cdc48 is the central component of this retrotranslocation machinery necessary for proteasomal degradation of organellar proteins [22]. Interestingly, p97 fulfils this function for ERAD [23] and OMMAD [24] alike. As such, p97 is an integral part of proteasomal quality control of ER-localized as well as mitochondrial proteins. While the role of p97 in maintaining ER proteostasis is extensively studied, the connection between p97 and mitochondrial health is

less clear. However, p97 dysfunction was recently linked to some forms of amyotrophic lateral sclerosis and hereditary inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD) and a connection to failed mitochondrial quality control was suspected [25].

To further define the role of p97 in mitochondrial maintenance especially in neuronal-like cells, we studied the influence of p97 inactivation on mitochondrial health and function in SH-SY5Y cells in comparison to known neurotoxic mitochondrial insults. We found that inactivation of p97 negatively impacts mitochondrial function in terms of membrane potential, reactive oxygen production, morphological changes, and accumulation of oxidized proteins comparably to treatment with the electron transport chain inhibitor rotenone, the neurotoxin 6-hydroxydopamine as well as Alzheimer's disease related Aβ peptide. These findings support an important function for p97 in maintaining neuronal health through mitochondrial protein quality control and further strengthen the link between mitochondrial dysfunction and premature neuronal death.

5.3 Results

5.3.1 Mitochondrial fragmentation as result of inactivation of p97

Mitochondria fragment in response to mitochondrial insults such as the complex I inhibitor rotenone, the neurotoxic compound 6-hydroxydopamine (6-OHDA) or the Alzheimer's-related peptide Ab. To assess whether p97 is involved in mitochondrial maintenance and stress protection in neuron-like cells, p97 function was blocked by overexpression of $p97^{QQ}$, a dominant-negative version of p97, under conditions of mitochondrial insult. In order to induce low level mitochondrial insult, toxin concentration and time of insult was selected to minimize impact on cellular viability. To this end, SH-SY5Y neuroblastoma cells stably expressing p97 or dominant-negative $p97^{QQ}$ under control of a tetracycline-inducible promoter were treated with tetracycline in the presence of rotenone, 6-OHDA, or Ab. As shown in Figure 5.1, expression of

$p97^{QQ}$ caused mitochondrial fragmentation in 57.3 +/- 5.9% compared to 5 +/- 2% in control cells expressing p97 and 7 +/- 1% in uninduced $p97^{QQ}$ cells. Treatment of p97-expressing cells with the mitochondrial toxins rotenone, 6-OHDA or Ab resulted in mitochondrial fragmentation in 59.3 +/- 7.6%, 69 +/- 6.2% and 53.3 +/- 6.7%, respectively. Interestingly, expression of $p97^{QQ}$ in the presence of mitochondrial toxins had a strong additive effect and caused mitochondrial fragmentation in 92.3 +/- 3.1% (rotenone), 95 +/- 1.7% (6-OHDA), and 96.7 +/- 2.5% (Ab) of SH-SY5Y cells. These data are consistent with a role for p97 in mitochondrial maintenance during normal as well as under mitochondrial stress conditions.

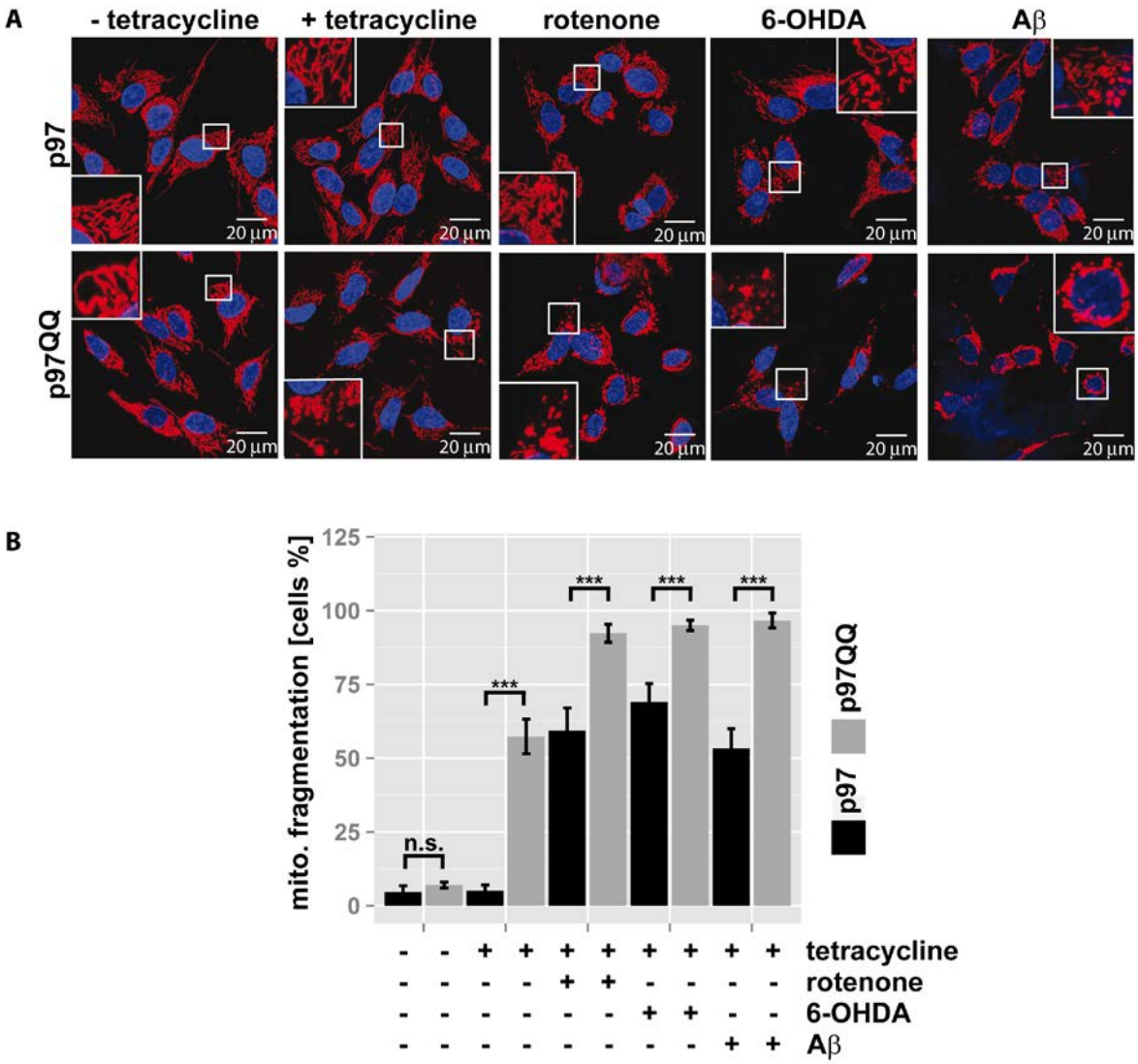


Figure 5.1: Inactivation of p97 increased mitochondrial fragmentation under stress.

(A) SH-SY5Y cells stably expressing p97 or dominant-negative $p97^{QQ}$ under control of the Tet-On promoter were induced with tetracycline for 16 hours or left uninduced and treated with 5 µM rotenone, 75 µM 6-OHDA, or 50 µM Aβ for 6 hours. Cells were fixed and stained using anti-cytochrome *c* antibody and Alexa546-conjugated secondary antibody. Shown are representative pictures from three independent experiments. **(B)** Fragmentation of the mitochondrial network in cells from A was quantified by visual examination. Shown is the average of three independent experiments. Statistical analysis was performed using pair-wise t-tests with p-value adjustment according to Holm. Statistical significance is marked with n.s. for not significant, *** $p < 0.001$.

5.3.2 Inactivation of p97 negatively impacts mitochondrial membrane potential and increases production of reactive oxygen species

Mitochondrial membrane potential is a measure for mitochondrial health with a drop in membrane potential being a sign of mitochondrial dysfunction. Mitochondrial membrane potential was measured in neuron-like cells under conditions of mitochondrial insult during p97 inactivation to further assess the role of p97 in maintaining mitochondrial health. To this end, SH-SY5Y cells stably expressing p97 or $p97^{QQ}$ were treated with rotenone, 6-OHDA, or Ab or left untreated as control and mitochondrial membrane potential was measured using the membrane-potential sensitive dye tetramethylrhodamine ethyl ester (TMRE). As shown in Figure 5.2A, ectopic expression of p97 did not impact mitochondrial membrane potential compared to control, while the potential was diminished to 80.6 +/- 6.0% of control by expression of $p97^{QQ}$. Furthermore, treatment with mitochondrial toxins in addition to $p97^{QQ}$ expression caused a further reduction of mitochondrial membrane potential to 60.9 +/- 8.3% (rotenone), 61.5 +/- 4.6% (6-OHDA), and 62.9 +/- 5.6% (Ab). In addition to mitochondrial depolarization, increased production of reactive oxygen species (ROS) is a hallmark of failing mitochondrial maintenance and subsequent dysfunction. To further analyse the role of p97 in mitochondrial maintenance in neuron-like cells cells, ROS production was measured under mitochondrial stress conditions in the presence or absence of p97 function. Again, SH-SY5Y cells stably containing tetracycline-inducible p97 or $p97^{QQ}$ were induced with tetracycline, treated with rotenone, 6-OHDA, or Aβ or left untreated as control, and mitochondrial ROS levels were analyzed by flow cytometry using the ROS-sensitive dye MitoSox. While ectopic expression of p97 had only a minor influence on ROS levels (114.9 +/ 8.7%) compared to uninduced control cells (Figure 5.2B), expression of $p97^{QQ}$ alone caused ROS levels to increase to 212.7 +/- 18.7%. Additional treatment of p97 expressing cells with mitochondrial toxins further increased ROS levels to 158 +/- 8.3% (rotenone), 167.7 +/- 9% (6-OHDA), and 294.3 +/- 14.1% (Aβ). In response to p97 inactivation under mitochondrial stress conditions ROS levels further increased to 290.4 +/- 55.6% (rotenone), 360.9 +/- 58.9% (6-OHDA), and 611.5 +/- 129.1% (Aβ). The observed

increase of ROS production following p97 inactivation and the significant additive effect on ROS production of inactive p97 during mitochondrial stress strongly support a crucial role for p97-dependent mitochondrial maintenance under normal as well as stress conditions.

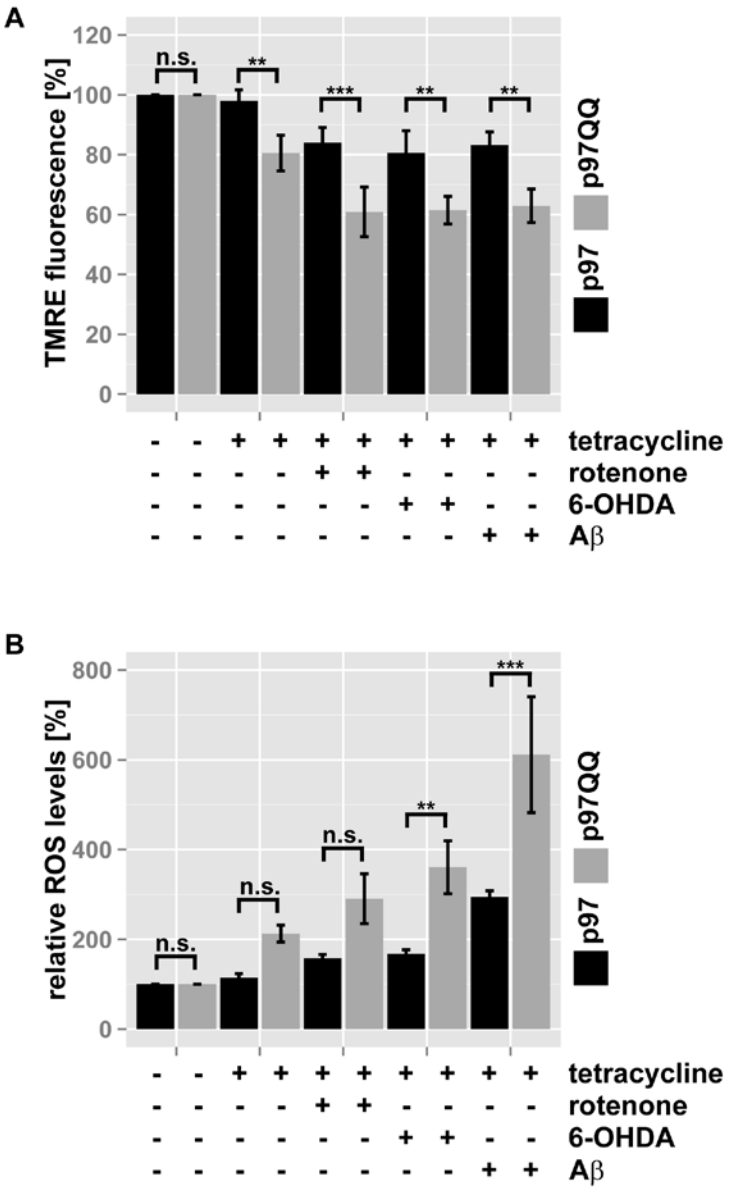
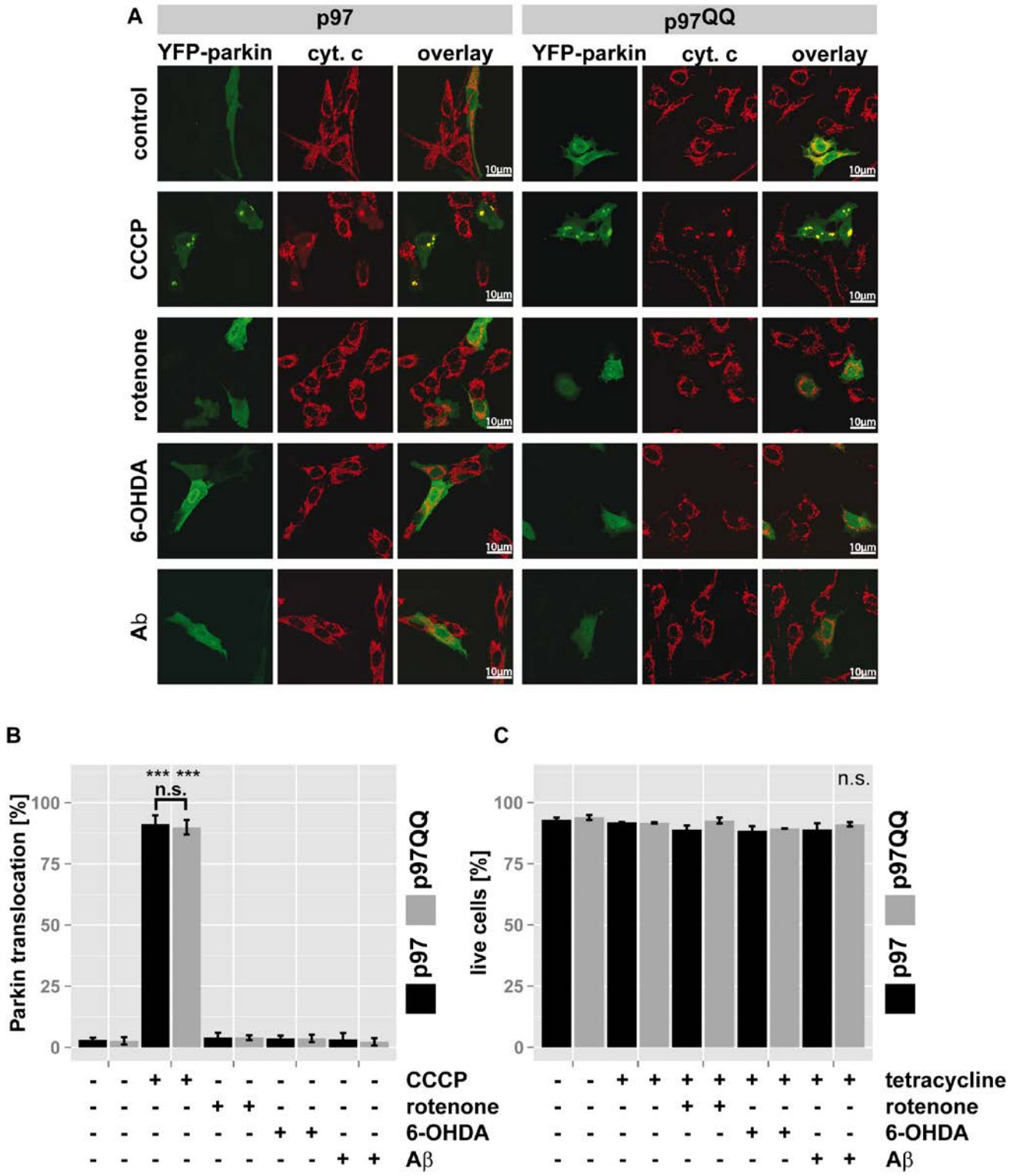


Figure 5.2: Inactivation of p97 impairs mitochondrial function during neurotoxic stress.

(A) SH-SY5Y cells stably expressing p97 or dominant-negative $p97^{QQ}$ under control of the Tet-On promoter were induced with tetracycline for 2 hours or left uninduced and treated with 5 µM rotenone, 75 µM 6-OHDA, or 50 µM Aβ for an additional 6 hours. Cells were stained with the mitochondrial membrane sensitive dye TMRE and analyzed by flow cytometry. **(B)** Cells treated as in A were stained with the ROS-sensitive dye MitoSox and mitochondrial ROS generation was measured using flow cytometry. Statistical analysis was performed using pair-wise t-tests with Holm p-value adjustment. Statistical significance is marked with n.s. for not significant, * $p<0.05$, ** $p<0.01$, *** $p< 0.001$.

5.3.3 Inactivation of p97 impairs mitochondrial maintenance below the mitophagic threshold

Recently, p97 was implicated in the execution of mitophagy [26]. To assess whether the observed mitochondrial dysfunction under the employed stress conditions and p97 inactivation are linked to blocked mitophagy or might be attributed to other roles of p97, Parkin translocation to mitochondria [13] as a marker for mitophagic induction was measured. To this end, SH-SY5Y cells stably expressing p97 or $p97^{QQ}$ were transfected with an expression construct for YFP-tagged Parkin, induced with tetracycline and exposed to mitochondrial insults by treating with rotenone, 6-OHDA, or Ab. Treatment with the protonophore (3-Chlorophenyl)hydrazonomalononitrile (CCCP) capable of complete mitochondrial depolarization and subsequent Parkin translocation served as control. As shown in Figures 5.3A+B, while treatment with CCCP induced Parkin translocation in almost all cells, neither expression of p97 or $p97^{QQ}$, nor neurotoxin treatment at the used concentrations caused significant (< 4%) translocation of Parkin to mitochondria. Consistent with this finding, the employed concentrations of rotenone, 6-OHDA, and Ab also did not lead to cell death as measured by flow cytometry (Figure 5.3C), neither in the presence of ectopic p97 nor following expression of $p97^{QQ}$. Thus, while the employed treatments with neurotoxic substances degraded mitochondrial function, the threshold for mitophagic induction as well as cell death was not reached. Therefore, the observed role for p97 in mitochondrial maintenance under the stress conditions employed by us is also likely on the protein degradation level rather than on the mitophagic or cell death level.


A
p97
p97QQ
YFP-parkin
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Ab
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B
Parkin translocation [%]
*** ***
n.s.
p97QQ
p97
CCCP
rotenone
6-OHDA
Aβ
C
live cells [%]
n.s.
tetracycline

Figure 5.3: p97 is involved in mitochondrial maintenance at damage levels below the thresholds for mitophagy and cell death.

(A) SH-SY5Y cells stably expressing p97 or dominant-negative $p97^{QQ}$ under control of the Tet-On promoter were transfected with an expression construct for YFP-tagged Parkin, induced with tetracycline for 2 hours or left uninduced and treated with 5 µM rotenone, 75 µM 6-OHDA, or 50 µM Aβ for an additional 6 hours. Cells were fixed, stained for the mitochondrial marker cytochrome *c* (cyt. C) and Parkin translocation from the cytosol to mitochondria was visually analyzed using fluorescence microscopy. Shown are representative images of three independent experiments. **(B)** Shown is a quantification of Parkin translocation from the cytosol to mitochondria from A. **(C)** Cells treated as in A were stained with the cell impermeable dye 4',6-diamidino-2-phenylindole (*DAPI) and the percentage of dead cells was determined by flow cytometry.* Statistical analysis was performed using pair-wise t-tests with p-value adjustment according to Holm. Statistical significance is marked with *** p< 0.001. In C no statistical significance was observed.

5.3.4 p97 is involved in clearing oxidatively -damaged proteins from mitochondria

To further support this notion, the ubiquitin- and p97-mediated proteasome-dependent turnover of oxidized mitochondrial proteins under mitochondrial stress conditions was measured. In SH-SY5Y cells ectopically expressing p97, treatment with neither rotenone, nor 6-OHDA or Ab significantly increased levels of oxidized proteins compared to untreated control cells (Figure 5.4, for representative western blots see Figure 5.S1). Interestingly, in $p97^{QQ}$ expressing cells, levels of oxidized mitochondrial proteins were significantly increased ($p < 0.001$) compared to p97 expressing cells confirming a function for p97 in the removal of oxidatively-damaged mitochondrial proteins. Furthermore, levels of oxidized proteins significantly increased in $p97^{QQ}$ expressing cells under mitochondrial stress conditions due to rotenone, 6-OHDA, and Ab treatment compared to $p97^{QQ}$ control cells. Again, these data are consistent with the involvement of p97 in the degradation of damaged proteins to maintain mitochondrial function in neuron-like cells under mitochondrial stress conditions below the threshold of mitophagic induction or cell death.

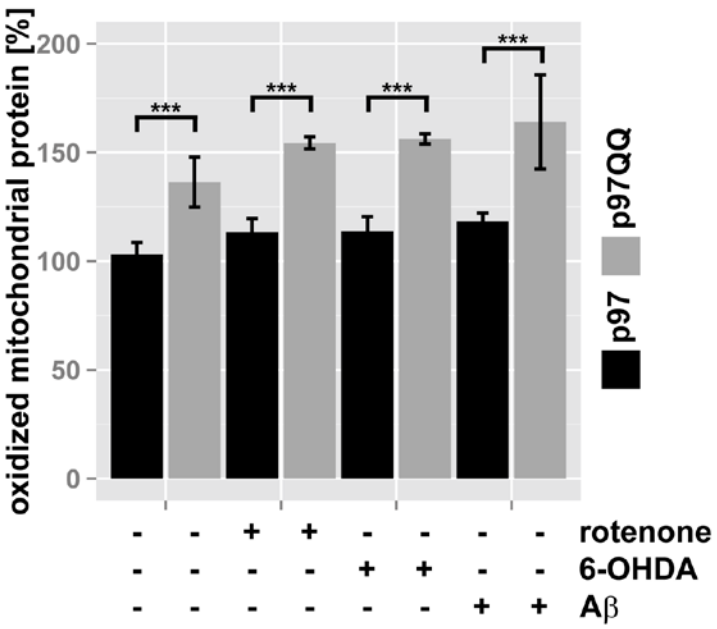


Figure 5.4: Clearance of oxidatively-damaged mitochondrial proteins is impaired following inactivation of p97.

SH-SY5Y cells stably expressing p97 or dominant-negative $p97^{QQ}$ under control of the Tet-On promoter were induced with tetracycline for 2 hours or left uninduced and treated with 5 µM rotenone, 75 µM 6-OHDA, or 50 µM Aβ for an additional 6 hours. Mitochondria were isolated using anti-TOMM22 magnetic beads resulting in highly purified mitochondria. Protein carbonylation as measure for oxidative damage was determined by infrared laser-based quantitative western blotting following derivatization with 2,4-Dinitrophenylhydrazine (DNPH) and detection using anti-DNP antibodies. Shown is the average of three independent experiments. Please see Figure S1 for representative western blot images. Statistical analysis was performed using pair-wise t-tests with p-value adjustment according to Holm. Comparisons shown are tetracycline-induced, untreated cells vs. tetracycline-induced, treated cells. Statistical significance is marked with n.s. for not significant, *** $p< 0.001$.

5.4 Discussion

Mechanisms of mitochondrial maintenance act on the cellular level by removing complete dysfunctional mitochondrial networks through programmed cell death. On the organellar level they act through mitophagic degradation of dysfunctional mitochondrial subunits and finally on the molecular level by repairing and/or removing damaged mitochondrial components such as DNA, lipids or proteins [7]. While programmed cell death is an effective means to rid the body

of damaged and, due to their excessive ROS production, potentially harmful mitochondrial networks, in post-mitotic neurons excessive apoptotic cell death is equivalent to neurodegeneration [27]. Thus, mitophagy and degradation of damaged proteins are likely the first lines of defence against mitochondrial dysfunction in neurons as neuronal death is avoided. Which mechanism, mitophagy or protein degradation is more prevalent in neurons under normal conditions remains unclear. However, it is conceivable that the removal of individual components such as damaged proteins from otherwise functional mitochondria might be preferable to the destruction of whole mitochondrial subunits. Especially under conditions of everyday stress, with slowly accumulating, low overall mitochondrial damage, keeping mitochondrial damage below the mitophagic threshold through the constant removal of damaged proteins is likely to prevent neuronal damage.

Using known mitochondrial toxins such as rotenone, 6-OHDA, and Aβ at concentrations and treatment times below the threshold of cell death and mitophagic induction, we intended to model the above mentioned low level stress and evaluate the importance of p97 and associated protein degradation as well as its influence on mitochondrial fidelity in neuronal cells. Interestingly, inactivation of p97 alone – without additional exogenous stress - for as little as eight hours negatively influenced mitochondrial morphology, membrane potential and ROS production. Although p97 has many cellular functions and pleiotropic effects of p97 inactivation have to be taken into account, these observations are consistent with a direct role for p97 in mitochondrial maintenance under normal conditions in the absence of external mitochondrial stress. This notion is further supported by the accumulation of oxidatively-damaged mitochondrial proteins following p97 inactivation. Even under normal conditions without exogenous mitochondrial stress, a considerable amount of oxidized protein is present in mitochondria and significantly accumulates as consequence of a short period of p97 inactivation. Thus, in the absence of any detectable mitophagic activity, continuous turnover of damaged mitochondrial proteins occurs in a p97-dependent manner. As p97 is an ubiquitin-dependent chaperone, this is consistent with constantly ongoing mitochondrial maintenance in

neuronal cells through the ubiquitin-proteasome system under normal, unstressed conditions. Consistent with this finding, we recently connected p97 and oxidatively-damaged proteins to outer mitochondrial membrane associated degradation or OMMAD, the ubiquitin-dependent, proteasome-mediated degradation mitochondrial proteins (ref: 25062828). Further support to this notion is lent by the effect of p97 inactivation under exogenous stress conditions below the mitophagic threshold. Concurrent stress and p97 inactivation had additive effects on mitochondrial fragmentation, membrane potential, ROS production as well as the accumulation of oxidatively-damaged mitochondrial proteins. These observations again support a function for p97 in dealing with mitochondrial damage to keep dysfunction of mitochondria below the threshold above which mitophagic degradation or even cell death occurs. Thus, we propose that constant repair of mitochondrial damage by the ubiquitin-proteasome system in a p97-dependent manner even under conditions of no or low exogenous mitochondrial stress is critically important for maintaining mitochondrial function under normal conditions. Mitophagic clearance on the other hand might be responsible for maintaining mitochondrial function in response to more drastic insults likely not encountered under normal conditions. Taken together, p97-mediated mitochondrial proteostasis is likely an important mechanism to prevent mitochondrial dysfunction as result of slowly accruing mitochondrial damage under normal conditions and to keep associated neurodegenerative processes at bay.

5.5 Material and methods

5.5.1 DNA constructs

To generate a vector for the one-step generation of stable, tetracycline-inducible human cell lines, the CMV promoter in the AAVS1 donor cloning vector DC-DON-SH01 (Genome-TALER™ human AAVS1 safe harbor gene knock-in kit, GeneCopoeia) was replaced by the hybrid CMV/Tet-On promoter originating from pcDNA5/FRT/TO (Invitrogen) with *Mlu*I/*Pme*I. Afterwards, a stuffer sequence with 5' *Pme*I site followed by *Eco*RV site and 3' *Bst*BI site was inserted using *Pme*I, *Bst*BI to generate pAN2066. To enable tetracycline regulation of the

construct, the *GFP* coding region in pAN2066 was replaced with the coding sequence of the tetracycline repressor. To this end, the bGH poly-A-Ef1α-*GFP* fragment was amplified from DC-DON-SH01 using ATTCGACTCGAGTTCGAATTTAAATCGGATCCCT and ATTCGAGATATCGATCCGGTGGAGCCGGG and cloned into pBluescript SK (+) with *Xho*I/*Eco*RV to create pAN2067. Next, *GFP* was replaced by *TetR* amplified using PCR from pcDNA6/TR (Invitrogen) with ATTCGAAAGCTTGTGAGTTTGGGGACCCTTG and ATTCGAGATATCGCATAAGATCTGAATTCCCGGGA and inserted *Hin*dIII/*Eco*RV to generate pAN2070. Fragment bGH poly-A-Ef1α-*TetR* was released from pAN2070 with *Bst*BI/*Eco*RV and transferred to pAN2066 cut with *Bst*BI/*Nru*I to obtain pAN2071. Then, p97 or $p97^{QQ}$ (gift from S. Fang) was amplified by PCR using GACTCGGATATCATGGCTTCTGGAGCCGATTCAA and TGTAACAACGTTTTAGCCATACAGGTCATCATCATCATT and cloned *Eco*RV/*Bst*BI into pAN2071.

5.5.2 Cell culture and generation of cell lines

SH-SY5Y cells were cultured in 5% CO_2 incubator at 37°C in high glucose DMEM (Sigma, D6546) containing 15% Tet System Approved FBS (Clontech, 631106), supplemented with 2mM L-glutamine (Sigma, G5713), and 1mM sodium pyruvate (Sigma, G7513). To generate stably transfected SH-SY5Y cells expressing p97 or $p97^{QQ}$ under control of the Tet-On promoter, cells were transfected using Effectene (Qiagen, 301425) according to manufacturer's recommendations with two TALEN constructs for the PPP1R2C or AAVS1 "safe harbor" locus and an expression construct containing p97 or $p97^{QQ}$ under control of the Tet-On promoter as well as a puromycin resistance gene to enable selection. For stably transfected cells, 0.75µg/ml puromycin was added to maintain selection (Invivogen, ant-pr-1). Expression of p97 or $p97^{QQ}$ was induced by treatment with 1µg/ml tetracycline (Roth, Hp63.1). Mitochondrial stress was induced by treatment with 5µM rotenone (Sigma, R8857), 75µM 6-hydroxdopamine (Sigma, H8523) or 50µM amyloid-β protein fragment 25-35 (Sigma, A4559).

5.5.3 Flow cytometry

Flow cytometry was performed using a CyAn ADP Analyzer (Beckman Coulter). SH-SY5Y cells grown in 6 well cell culture plates (Sarstedt) and induced and/or treated as indicated were co-stained with DAPI and either 10nM TMRE (Invitrogen) for 30 minutes at 37 °C, or 5 µM MitoSOX^{TM} (Invitrogen) for 10 minutes at 37 °C. Cells were harvested, washed twice in PBS and resuspended in 1 ml PBS containing 0.5 % (w/v) BSA and 50µM EDTA. Flow cytometry was performed immediately afterwards. Data analysis was performed using FlowJo v.10. Sequential gating was performed as follows (with identical gates used for each experiment): cells were gated for using logarithmic forward/sideward scatter axes; doublet discrimination was performed using forward scatter area/forward scatter followed by pulse width/forward scatter dot plots. Dead cells were excluded in DAPI/forward scatter dot plots.

5.5.4 Isolation of mitochondria

The human mitochondria isolation kit (Miltenyi Biotec, 130-094-532) was used according to manufacturer's instructions. Briefly, cells were harvested and either directly processed or stored overnight in liquid nitrogen. All following steps were performed on ice with pre-cooled buffers. Cells were resuspended in 800µl lysis buffer supplemented with protease inhibitors (1µg/ml pepstatin; 1µg/ml leupeptin; 1mM PMSF) and 50µM EDTA. Cells were passed 15 times through a 25 gauge needle. Nine ml separation buffer and 50µl Anti-TOMM22 MicroBeads were added to the cell homogenate before rotating the suspensions for 1 hour at 4 °C. Magnetic separation was performed using a MACS Separator. Purified mitochondria were immediately lysed in RIPA buffer (Thermo Scientific) supplemented with protease inhibitors (1µg/ml pepstatin; 1µg/ml leupeptin; 1mM PMSF) and 50µM EDTA and subjected to five 10 second intervals of sonication at 10 kHz. Total protein content was measured using the Pierce BCA protein assay kit (Thermo Scientific) and was immediately followed by DNPH labeling.

5.5.5 Labeling with DNPH

Labeling with DNPH was performed according to [28] with minor alterations. Briefly, 60µg of total protein in 20µl total volume was added to 20µl 12 % sodium dodecyl sulfate (SDS). 40µl of

20mM DNPH in 2M HCl were added, samples were briefly mixed and incubated for 15 minutes. To control for DNPH-reactive protein carbonyls, samples were reacted with 2M HCl lacking DNPH. 30µl of 2M Tris base, 30 % glycerol and a final concentration of 50mM 1,4-Dithiothreit were added to stop the reaction. Samples were briefly mixed again and immediately used for SDS PAGE.

5.5.6 Western Blot

DNPH-labeled protein lysates were resolved by SDS PAGE (5µg total protein per lane were loaded) and blotted onto nitrocellulose membrane (Whatman). Fast Green FCF (Sigma Aldrich, F7252) was used to evaluate total protein loaded per lane as follows: nitrocellulose membranes were stained for 10 minutes in Fast Green FCF staining solution (0.001 % FCF, 30 % methanol, 7 % acetic acid), followed by 10 minutes in destaining solution (30 % methanol, 7 % acetic acid) and then 10 minutes in water. An infrared laser scanner (LiCor) was used for detection of FCF. Following Fast Green FCF, membranes were blocked for 1 hour in 3% (w/v) Top Block (Lubio science, TB232010) in PBS-Tween 20 (0.05%) and immune-detection using polyclonal rabbit anti-DNP antibodies (D9656 Sigma, 1:2000) and polyclonal goat anti-rabbit Dylight 800 (35521 Pierce, 1:6000) was performed and detected using an infrared laser scanner (LiCor) to obtain quantitative measurement. Each experiment was performed three times independently and each measurement was performed in triplicates. Image analysis was performed using ImageJ [29].

5.5.7 Microcopy

Cells were seeded in 6-well plates onto glass slides at $1x10^4$ cells/well in 2ml culture medium. Samples were fixed using methanol-free electron microscopy grade 4% paraformaldehyde in PBS for 15 minutes at RT, permeabilized for 15 minutes at RT using 0.15% Trixon X-100 in PBS and blocked for 1 hour in 10% BSA (w/v) in PBS. To visualize mitochondria, samples were then incubated with mouse anti-cytochrome *c* antibody (BD Biosciences 556432, 1:1000) overnight at 4 °C and Alexa546-conjugated anti-mouse antibodies (Invitrogen A11003, 1:500) for 1 hour at

RT. Nuclei were stained by incubation with DAPI (Invitrogen, D1306, 1:1000) for 5 minutes right after Alexa546 incubation. Samples were mounted in mounting medium (Vectashield, H1000) and observed using a confocal microscope (Zeiss LSM Meta710, 63×/1.4 objective). Mitochondria fragmentation and Parkin-to-mitochondria translocation were assessed visually and quantified as percentage of control. The extend of mitochondrial fragmentation was judged based on comparison to untreated control cells with mitochondrial networks scored as fragmented if most mitochondria in a cell did no longer exhibit an elongated phenotype. All experiments were performed independently in triplicates.

5.5.8 Statistical analysis

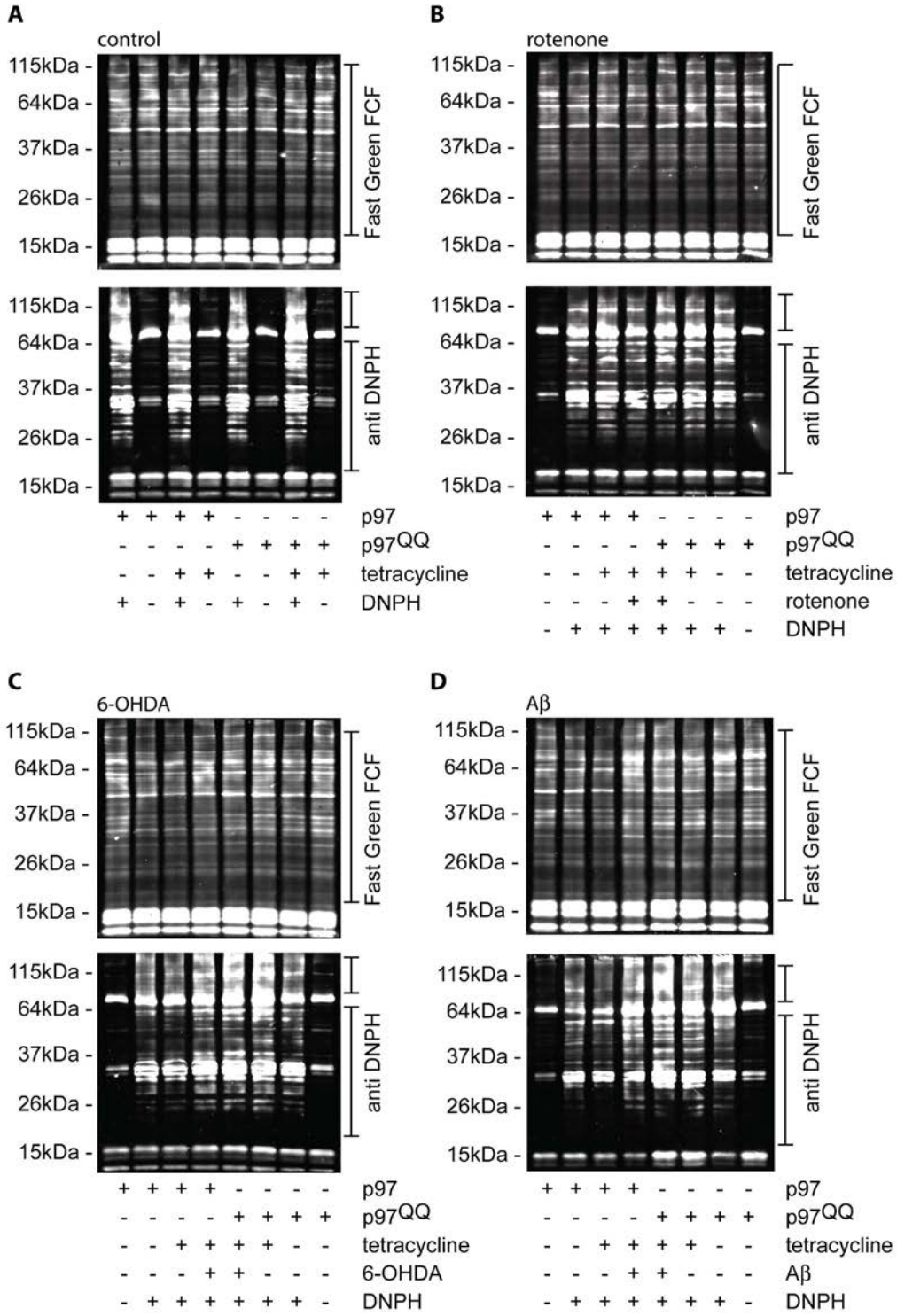
Statistical significance of differences was assessed using pair-wise t-tests with the adjustment for multiple comparisons according to Holmes as implemented in R [30]. Significance is indicated with n.s. $p>0.05$, * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

5.6 Supplementary material

Supplementary Figure 5.S1: Detection of carbonylated mitochondrial proteins.

SH-SY5Y cells stably expressing p97 or dominant-negative $p97^{QQ}$ under control of the Tet-On promoter were induced with tetracycline for 2 hours or left uninduced and treated with **(A)** vehicle, **(B)** 5µM rotenone, **(C)** 75µM 6-OHDA, or **(D)** 50µM Aβ for an additional 6 hours. Mitochondria were isolated using anti-TOMM22 magnetic beads resulting in highly purified mitochondria. Mitochondrial proteins were derivatized using DNPH and analyzed by western blotting. Total protein was determined using Fast Green FCF staining and quantified using an infrared laser-based scanner. Protein oxidation was determined using anti-DNP antibody-based detection of DNPH-derivatized carbonylated proteins and also quantified by using infrared laser scanning. Shown is one representative membrane (three technical and three biological replicates) stained using Fast Green FCF to visualize total protein loading (upper panel) followed by anti-DNP western blotting (lower panel). The bars mark the areas quantified using image analysis.

Figure 5.S1: Representative western blots for detecting carbonylated mitochondrial proteins.



5.7 Supplementary references

1. Karbowski, M. and A. Neutzner, Neurodegeneration as a consequence of failed mitochondrial maintenance. Acta Neuropathol, 2012. **123**(2): p. 157-71.
2. Kornmann, B., Quality control in mitochondria: use it, break it, fix it, trash it. F1000Prime Rep, 2014. **6**: p. 15.
3. Anand, R., T. Langer, and M.J. Baker, Proteolytic control of mitochondrial function and morphogenesis. Biochim Biophys Acta, 2013. **1833**(1): p. 195-204.
4. Youle, R.J. and D.P. Narendra, Mechanisms of mitophagy. Nat Rev Mol Cell Biol, 2011. **12**(1): p. 9-14.
5. Neutzner, A., et al., Role of the ubiquitin conjugation system in the maintenance of mitochondrial homeostasis. Ann N Y Acad Sci, 2008. **1147**: p. 242-53.
6. Escobar-Henriques, M. and T. Langer, Dynamic survey of mitochondria by ubiquitin. EMBO Rep, 2014. **15**(3): p. 231-43.
7. Tatsuta, T. and T. Langer, Quality control of mitochondria: protection against neurodegeneration and ageing. EMBO J, 2008. **27**(2): p. 306-14.
8. Karbowski, M., A. Neutzner, and R.J. Youle, The mitochondrial E3 ubiquitin ligase MARCH5 is required for Drp1 dependent mitochondrial division. J Cell Biol, 2007. **178**(1): p. 71-84.
9. Nagashima, S., et al., Roles of mitochondrial ubiquitin ligase MITOL/MARCH5 in mitochondrial dynamics and diseases. J Biochem, 2014. **155**(5): p. 273-9.
10. Braschi, E., R. Zunino, and H.M. McBride, MAPL is a new mitochondrial SUMO E3 ligase that regulates mitochondrial fission. EMBO Rep, 2009. **10**(7): p. 748-54.
11. Benard, G., et al., IBRDC2, an IBR-type E3 ubiquitin ligase, is a regulatory factor for Bax and apoptosis activation. EMBO J, 2010. **29**(8): p. 1458-71.
12. Tang, F., et al., RNF185, a novel mitochondrial ubiquitin E3 ligase, regulates autophagy through interaction with BNIP1. PLoS One, 2011. **6**(9): p. e24367.
13. Narendra, D., et al., Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol, 2008. **183**(5): p. 795-803.
14. Neutzner, A. and R.J. Youle, Instability of the mitofusin Fzo1 regulates mitochondrial morphology during the mating response of the yeast Saccharomyces cerevisiae. J Biol Chem, 2005. **280**(19): p. 18598-603.
15. Cohen, M.M., et al., Ubiquitin-proteasome-dependent degradation of a mitofusin, a critical regulator of mitochondrial fusion. Mol Biol Cell, 2008. **19**(6): p. 2457-64.
16. Leboucher, G.P., et al., Stress-induced phosphorylation and proteasomal degradation of mitofusin 2 facilitates mitochondrial fragmentation and apoptosis. Mol Cell, 2012. **47**(4): p. 547-57.
17. Narendra, D., J.E. Walker, and R. Youle, Mitochondrial quality control mediated by PINK1 and Parkin: links to parkinsonism. Cold Spring Harb Perspect Biol, 2012. **4**(11).
18. Sugiura, A., et al., A mitochondrial ubiquitin ligase MITOL controls cell toxicity of polyglutamine-expanded protein. Mitochondrion, 2011. **11**(1): p. 139-46.
19. Benischke, A.S., et al., Proteasome-mediated quality control of S-nitrosylated mitochondrial proteins. Mitochondrion, 2014.
20. Ruggiano, A., O. Foresti, and P. Carvalho, Quality control: ER-associated degradation: protein quality control and beyond. J Cell Biol, 2014. **204**(6): p. 869-79.
21. Neutzner, A., R.J. Youle, and M. Karbowski, Outer mitochondrial membrane protein degradation

by the proteasome. Novartis Found Symp, 2007. **287**: p. 4-14; discussion 14-20.
22. Meyer, H., M. Bug, and S. Bremer, Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. Nat Cell Biol, 2012. **14**(2): p. 117-23.
23. Wolf, D.H. and A. Stolz, The Cdc48 machine in endoplasmic reticulum associated protein degradation. Biochim Biophys Acta, 2012. **1823**(1): p. 117-24.
24. Xu, S., et al., The AAA-ATPase p97 is essential for outer mitochondrial membrane protein turnover. Mol Biol Cell, 2011. **22**(3): p. 291-300.
25. Yamanaka, K., Y. Sasagawa, and T. Ogura, Recent advances in p97/VCP/Cdc48 cellular functions. Biochim Biophys Acta, 2012. **1823**(1): p. 130-7.
26. Tanaka, A., et al., Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. J Cell Biol, 2010. **191**(7): p. 1367-80.
27. Radi, E., et al., Apoptosis and oxidative stress in neurodegenerative diseases. J Alzheimers Dis, 2014. **42**(0): p. S125-52.
28. Wehr, N.B. and R.L. Levine, Quantification of protein carbonylation. Methods Mol Biol, 2013. **965**: p. 265-81.
29. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis. Nat Methods, 2012. **9**(7): p. 671-5.
30. R_Development_Core_Team, R: A Language and Environment for Statistical Computing. 2011: R Foundation for Statistical Computing.

6 Conclusions

Due to the energetic sensitivity and structural constraints of neuronal cells, mitochondrial maintenance is thought to be essential for neuronal health. Within the scope of this study, we have investigated the role of MARCH5 and p97 in mitochondrial quality control and their connection to neuronal health through the use of *in vitro* neuronal cell-like models.

6.1 Cellular models

Two cellular models representing cells with neuronal characteristics were used during the course of these studies. While neuron-like cell lines have limitations with regards to their applicability to the *in vivo* situation, they still proved very useful to decipher basing biochemical and cell biological pathways present in neuronal cells. The first cell line employed in our studies is the RGC-5 cell line. RGC-5 was first reported in 2001 [25], as immortalized line derived from rat retinal ganglion cells (RGCs), constituting one of the most promising cellular models for retina ganglion degeneration research. Hundreds of studies have been performed based on the ganglion cells character of RGC-5 cells [24]. Subsequently, further examination of RGC-5 revealed that this cell line was derived from 661W cells, a mouse retinal photoreceptor cell line [208]. While this invalidated RGC-5 cells as *bona fide* model for retinal ganglion cells, RGC-5 are still considered neuron-like cells and demonstrate several features representative for of neuronal cells, especially after certain differentiation stimulus. For instance, RGC-5 express neuron-specific markers, such as Tau, βIII-tubulin, microtubule-associated protein (MAP)-1b, MAP2, and PGP9.5 [209]. In addition, treatment with staurosporine (STS) or trichostatin A can terminally transform RGC-5 leading to a neuron-like phenotype [210].

As a second model for neuron-like cells, the human neuroblastoma cell line SH-SY5Y was employed. SH-SY5Y represents a sub-clonal population derived from SK-N-SH, an isolate from a bone marrow biopsy taken from a four year-old female with neuroblastoma. SH-SY5Y is

reported to steadily express dopaminergic neuronal markers, such as tyrosine hydroxylase (TH), dopamine receptor 2 and 3 subtypes (D2R and D3R), and dopamine transporter (DAT) [211, 212], and SH-SY5Y is also reported to feature acetylcholinergic, glutamatergic and adenosinergic phenotypes [212, 213]. Those features let to the use of SH-SY5Y cells as cellular model neuronal cells and these cells are widely used [214].

6.2 Neurodegenerative stressors

In order to mimic conditions of neurodegeneration in the employed cellular models of neuronal-like cells, exogenous stressors were applied. As neurodegeneration is a slowly progressing process, we reasoned that low stress conditions might most faithfully replicate the *in vivo* situation in terms of mitochondrial dysfunction and levels of oxidative stress. Our hypothesis was that under normal, everyday conditions slowly accumulating mitochondrial damage is likely taken care of by the molecular levels of mitochondrial maintenance rather than mitophagic destruction of entire mitochondrial subunits. To this end, different stressors were applied at concentrations at which it was reasonable to assume that the molecular level of mitochondrial maintenance is engaged. To this end, the levels of mitochondrial stress were chosen based on the lack of induction of mitophagy or even cell death. The following agents with known impact on mitochondrial function were employed. First, the agricultural pesticide, mitochondrial complex I inhibitor rotenone was used. Rotenone is known to inhibit the transfer of electrons from iron-sulfur centers in complex I to ubiquinone, thus interrupting the electron transport chain and negatively impacting ATP production. The block in the ETC in turn leads to the premature transfer of electrons to oxygen leading to ROS production [215]. Animal experiments showed that injection of rotenone into rats induces Parkinson-like symptoms, while cellular studies demonstrated that rotenone treatment induced oxidative damage and cell death in dopaminergic neurons [29, 216]. As another stressor, 6-hydroxidopamine (6-OHDA), a compound designed to selectively enter dopaminergic or noradrenergic neurons through dopamine and noradrenaline reuptake

transporters, known to induce intracellular oxidative stress in neuronal cells [28]. Application of 6-OHDA in animal models elicits mimics Parkinson's disease and serves as PD model to study disease mechanisms and to test potential medications[217]. As third mitochondrial stress, amyloid β (Aβ(1-42)) protein, a major component of Alzheimer's disease-associated amyloid plaques, was employed to induce mitochondrial dysfunction in neuron-like cells [218]. While the exact mode of action for Aβ is unknown, it is accepted that Aβ is crucially involved in the development of AD [219, 220]. However, some potential explanations for Aβ neurotoxic activity are discussed in the literature. A relationship between ubiquitin-proteasome system function and the clearance of Aβ aggregates has been widely discussed [221]. Also, it was postulated that the mitochondrial quality control system is involved in Aβ pathogenesis as well [222]. Aβ (25-35) peptide, one of the most toxic derivatives of Aβ, has lower molecular weight and more water soluble than Amyloid-β (1-42) [223]. Studies showed that Aβ (25-35) peptide originating form extracellular aggregates are able to induce oxidative stress and neuronal cell apoptosis, and induce AD-related neuron functional impairment similar as the full-length Amyloid-β (1-42) [30, 224, 225].

6.3 MARCH5

The mitochondrial ubiquitin ligase MARCH5 is implicated in various mitochondrial processes and seems to fulfill two major functions, namely the degradation of various mitochondrial proteins as well as the modulation of mitochondrial fission. It was shown that MARCH5 is able to modulate mitochondrial morphology by influencing Drp1 activity and therefore fission of mitochondrial tubules [16]. In detail, it was shown that expression of dominant-negative MARCH5^{H43W}, a RING domain mutant of MARCH5, inhibits mitochondrial fission and causes highly fused and interconnected mitochondrial networks. In addition and in contrast to wild type MARCH5, MARCH5^{H43W} co-localizes with Drp1 on mitochondria in a focal fashion. These MARCH5^{H43W}/Drp1 containing mitochondrial foci were speculated to represent blocked mitochondrial fission sites. Thus, MARCH5 is modulator of mitochondrial fission and

therefore potentially involved in the organellar level of mitochondrial maintenance. Also, as fission is strongly connected to the induction of apoptosis, MARCH5 function is likely involved to apoptotic induction with inactive MARCH5 likely acting anti-apoptotic.

Aside its clear function in mitochondrial fission, MARCH5 is also involved in the molecular level of mitochondrial quality control by clearing mutated or damaged protein aggregates in neuronal cells, such as mutated SOD1 and polyglutamine (polyQ) [19, 20]. Mutated SOD1 is a misfolded, mitochondria-localized protein that is involved in the pathology of the neurodegenerative disorder amyotrophic later sclerosis (ALS) [226], while accumulation of polyQ proteins is the cause of polyQ diseases such as Machado–Joseph disease, another neurodegenerative disorder [227]. In addition, excessive nitro oxide (NO) will cause cellular stress through S-nitrosylation of target proteins. It was shown that MARCH5 is involved in the degradation of S-nitrosylated microtubule-associated protein 1B-light chain (MAP1B-LC1 connecting MARCH5 to mitochondria intracellular transport mechanisms [205]. These observations strongly connect MARCH5 to mitochondrial protein degradation pathways and potentially to degradation processes important to the health of neuronal cells. Recently, it was reported that the mitofusin Mfn2 is a substrate of MARCH5 [131]. Aside its function in mitochondrial fusion, Mfn2 is a key factor of mitochondria-endoplasmic reticulum (ER) membrane cross talk involved in calcium buffering. In this case, MARCH5 regulates the function of Mfn2 and is involved in mitochondria-ER cross talk potentially interesting for neurodegeneration as mis-balanced calcium homeostasis is harmful to neuronal cells [228]. Despite the interesting connections between MARCH5-mediated protein degradation and neuronal health and neurodegenerative processes, no direct involvement of MARCH5 in neurodegenerative diseases has been shown to date. Given the two main functions of MARCH5, degradation of toxic mitochondrial proteins and the regulation of mitochondrial fusion, it is not clear which of these functions contributes more to general mitochondrial maintenance under normal conditions. Especially, as these two functions might contribute differently to the outcome of MARCH5-mediated mitochondrial maintenance. MARCH5-

mediated protein degradation is part of the molecular levels of mitochondrial maintenance with the potential to rescue individual mitochondrial subunits, while MARCH5-mediated mitochondrial fission might be involved in organellar-level maintenance or might even promote apoptotic cell death.

In this study, the influence of MARCH5 inhibition on the outcome of neurotoxic stress was assessed in neuron-like *in vitro* models using MARCH5^{H43W} to modulate MARCH5 activity.

Under the stress conditions employed in the present study, neuron-like cells with expression of MARCH5^{H43W} featured inhibited mitochondrial fragmentation compared to control cells where stress-induced mitochondrial fission was evident. The prevention of mitochondrial fragmentation and preservation of an interconnected network served to maintain mitochondrial function, kept excessive ROS production at bay, and reduced cell death. Based on previous studies, the protective effect of MARCH5^{H43W} exerted in our cell model can be attributed to the role of MARCH5 in the regulation of mitochondrial dynamic namely its interaction with the pro-fission protein Drp1 [16, 17, 127]. This conclusion is supported by the observation that blocking Drp1 function under the same stress condition conferred similar protective effects to neuron-like cells. It is likely that the MARCH5^{H43W}-induced highly fused mitochondrial network in neuron-like cells induced strengthens the abilities of these cells to deal with mitochondrial stress. Interestingly, stress-induced mitochondrial hyperfusion [104] also protects cells from further insult supporting the notion that fused mitochondrial networks are more stress resistant than single mitochondrial subunits. As for the function of MARCH5 in the degradation of neuro-toxic proteins, it seems that under the stress conditions employed this function of MARCH5 did not seem to contribute to the observed maintenance of mitochondrial function. It remains unclear whether MARCH5 function in terms of protein degradation is inhibited in cells expressing MARCH5^{H43W} and it therefore remains uncertain to what extent MARCH5-dependent protein degradation on mitochondria is involved in protecting mitochondria against neuro-toxic stressors. However, it is reasonable to assume

that the function of MARCH5 in modulating mitochondrial dynamics plays an important role in protecting neuron-like cells during neuro-toxic insult.

6.4 p97

The AAA-ATPase and chaperone p97 is involved in a plethora of cellular functions among the ubiquitin-dependent protein quality control [229]. IBMPFD or Inclusion Body Myopathy associated with Paget's disease of the Bone and Fronto-Temporal Dementia is caused by mutations in p97 [230, 231]. Interestingly, a mitochondrial component for the progression of IBMPFD is discussed [232, 233]. In addition, *in vitro* studies have shown that certain p97 mutations induce mitochondrial uncoupling and decreased ATP production [232]. Furthermore, p97 was connected to mitochondrial quality control via PINK/parkin-dependent mitophagic pathways, together with the adaptor protein Npl4/Ufd1 [234].

Also recently, p97 was connected to mitochondrial maintenance and degradation of mitochondrial proteins likely as part of the retrotranslocase responsible for the extraction of ubiquitylated proteins from mitochondria. It was shown that p97 is essential for the degradation of mitochondrial Mcl-1 [145, 235] and also damaged proteins such as carbonylated and S-nitrosylated mitochondrial proteins in a proteasome-depended manner preserving mitochondrial functions [236, 237]. In the present study, multiple low dose neuro-toxic treatments were applied to SH-SY5Y cells under conditions of inactive p97. Here, a critical role of p97 in preserving mitochondrial function in neuron-like cells was identified. Especially a role for p97 in clearing carbonylated mitochondrial proteins as part of mitochondrial QC was confirmed. The data presented here clearly supports a role for p97 in mitochondrial maintenance also in neuronal cells. Taken together, it is reasonable to assume that the mitochondrial role of p97 is indeed essential for neuronal survival and that proper functioning of p97 on mitochondria acts neuroprotective. This adds an additional function to the already busy AAA-ATPase p97 and firmly connects this important chaperone involved in

ubiquitin-dependent degradation of damaged or superfluous proteins also to mitochondrial protein degradation and quality control.

6.5 Summary

In this *in vitro* study connections between mitochondrial maintenance and mitochondrial function in neuron-like cells were explored to gain insight into mechanisms of neurodegeneration. Our results confirm the strong link between mitochondrial maintenance and processes impairing the function of neuronal cells. It was shown that effective and sufficient mitochondrial quality control system is critical to neuronal cell survival. Also, our findings strongly support the importance of removing damaged proteins from mitochondria in order to maintain their overall function, a process not well studied and underappreciated in the past. Taken together, novel insight into mechanisms of mitochondrial maintenance is import to better understand mitochondrial dysfunction and its connection to neurodegenerative processes and to devise novel therapeutic approaches to fight these diseases.

7 References

1. Tait, S.W. and D.R. Green, Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol, 2010. **11**(9): p. 621-32.
2. Kornmann, B., Quality control in mitochondria: use it, break it, fix it, trash it. F1000Prime Rep, 2014. **6**: p. 15.
3. Archer, S.L., Mitochondrial dynamics--mitochondrial fission and fusion in human diseases. N Engl J Med, 2013. **369**(23): p. 2236-51.
4. Hroudova, J., N. Singh, and Z. Fisar, Mitochondrial dysfunctions in neurodegenerative diseases: relevance to Alzheimer's disease. Biomed Res Int, 2014. **2014**: p. 175062.
5. Morato, L., et al., Mitochondrial dysfunction in central nervous system white matter disorders. Glia, 2014.
6. Szklarczyk, R., M. Nooteboom, and H.D. Osiewacz, Control of mitochondrial integrity in ageing and disease. Philos Trans R Soc Lond B Biol Sci, 2014. **369**(1646): p. 20130439.
7. Karbowski, M. and A. Neutzner, Neurodegeneration as a consequence of failed mitochondrial maintenance. Acta Neuropathol, 2012. **123**(2): p. 157-71.
8. Andreux, P.A., R.H. Houtkooper, and J. Auwerx, Pharmacological approaches to restore mitochondrial function. Nat Rev Drug Discov, 2013. **12**(6): p. 465-83.
9. Busch, K.B., A. Kowald, and J.N. Spelbrink, Quality matters: how does mitochondrial network dynamics and quality control impact on mtDNA integrity? Philos Trans R Soc Lond B Biol Sci, 2014. **369**(1646): p. 20130442.
10. Chen, H. and D.C. Chan, Mitochondrial dynamics--fusion, fission, movement, and mitophagy--in neurodegenerative diseases. Hum Mol Genet, 2009. **18**(R2): p. R169-76.
11. Itoh, K., et al., Mitochondrial dynamics in neurodegeneration. Trends Cell Biol, 2013. **23**(2): p. 64-71.
12. van der Bliek, A.M., Q. Shen, and S. Kawajiri, Mechanisms of mitochondrial fission and fusion. Cold Spring Harb Perspect Biol, 2013. **5**(6).
13. Cagalinec, M., et al., Principles of the mitochondrial fusion and fission cycle in neurons. J Cell Sci, 2013. **126**(Pt 10): p. 2187-97.
14. Taylor, E.B. and J. Rutter, Mitochondrial quality control by the ubiquitin-proteasome system. Biochem Soc Trans, 2011. **39**(5): p. 1509-13.
15. Ling, Q. and P. Jarvis, Dynamic regulation of endosymbiotic organelles by ubiquitination. Trends Cell Biol, 2013. **23**(8): p. 399-408.
16. Karbowski, M., A. Neutzner, and R.J. Youle, The mitochondrial E3 ubiquitin ligase MARCH5 is required for Drp1 dependent mitochondrial division. J Cell Biol, 2007. **178**(1): p. 71-84.
17. Nakamura, N., et al., MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology. EMBO Rep, 2006. **7**(10): p. 1019-22.
18. Nagashima, S., et al., Roles of mitochondrial ubiquitin ligase MITOL/MARCH5 in mitochondrial dynamics and diseases. J Biochem, 2014. **155**(5): p. 273-9.
19. Yonashiro, R., et al., Mitochondrial ubiquitin ligase MITOL ubiquitinates mutant SOD1 and attenuates mutant SOD1-induced reactive oxygen species generation. Mol Biol Cell, 2009. **20**(21): p. 4524-30.
20. Sugiura, A., et al., A mitochondrial ubiquitin ligase MITOL controls cell toxicity of polyglutamine-expanded protein. Mitochondrion, 2011. **11**(1): p. 139-46.

21. Stolz, A., et al., Cdc48: a power machine in protein degradation. Trends Biochem Sci, 2011. **36**(10): p. 515-23.
22. Meyer, H., M. Bug, and S. Bremer, Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. Nat Cell Biol, 2012. **14**(2): p. 117-23.
23. Yamanaka, K., Y. Sasagawa, and T. Ogura, Recent advances in p97/VCP/Cdc48 cellular functions. Biochim Biophys Acta, 2012. **1823**(1): p. 130-7.
24. Agarwal, N., RGC-5 cells. Invest Ophthalmol Vis Sci, 2013. **54**(13).
25. Krishnamoorthy, R.R., et al., Characterization of a transformed rat retinal ganglion cell line. Brain Res Mol Brain Res, 2001. **86**(1-2): p. 1-12.
26. Constantinescu, R., et al., Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y. J Neural Transm Suppl, 2007(72): p. 17-28.
27. Tanaka, A., et al., Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. J Cell Biol, 2010. **191**(7): p. 1367-80.
28. Grunblatt, E., et al., Potent neuroprotective and antioxidant activity of apomorphine in MPTP and 6-hydroxydopamine induced neurotoxicity. J Neural Transm Suppl, 1999. **55**: p. 57-70.
29. Kotake, Y. and S. Ohta, MPP+ analogs acting on mitochondria and inducing neuro-degeneration. Curr Med Chem, 2003. **10**(23): p. 2507-16.
30. Kowall, N.W., et al., In vivo neurotoxicity of beta-amyloid [beta(1-40)] and the beta(25-35) fragment. Neurobiol Aging, 1992. **13**(5): p. 537-42.
31. Lovy, A., et al., A faster, high resolution, mtPA-GFP-based mitochondrial fusion assay acquiring kinetic data of multiple cells in parallel using confocal microscopy. J Vis Exp, 2012(65): p. e3991.
32. O'Rourke, B., From bioblasts to mitochondria: ever expanding roles of mitochondria in cell physiology. Front Physiol, 2010. **1**: p. 7.
33. Osellame, L.D., T.S. Blacker, and M.R. Duchen, Cellular and molecular mechanisms of mitochondrial function. Best Pract Res Clin Endocrinol Metab, 2012. **26**(6): p. 711-23.
34. Desagher, S. and J.C. Martinou, Mitochondria as the central control point of apoptosis. Trends Cell Biol, 2000. **10**(9): p. 369-77.
35. Frey, T.G. and C.A. Mannella, The internal structure of mitochondria. Trends Biochem Sci, 2000. **25**(7): p. 319-24.
36. Russell, O. and D. Turnbull, Mitochondrial DNA disease-molecular insights and potential routes to a cure. Exp Cell Res, 2014. **325**(1): p. 38-43.
37. Malik, A.N. and A. Czajka, Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction? Mitochondrion, 2013. **13**(5): p. 481-92.
38. Rochaix, J.D., Regulation of photosynthetic electron transport. Biochim Biophys Acta, 2011. **1807**(3): p. 375-83.
39. Ghezzi, D. and M. Zeviani, Assembly factors of human mitochondrial respiratory chain complexes: physiology and pathophysiology. Adv Exp Med Biol, 2012. **748**: p. 65-106.
40. Hirst, J., Mitochondrial complex I. Annu Rev Biochem, 2013. **82**: p. 551-75.
41. Bleier, L. and S. Drose, Superoxide generation by complex III: from mechanistic rationales to functional consequences. Biochim Biophys Acta, 2013. **1827**(11-12): p. 1320-31.
42. Nakamoto, R.K., C.J. Ketchum, and M.K. al-Shawi, Rotational coupling in the F0F1 ATP synthase. Annu Rev Biophys Biomol Struct, 1999. **28**: p. 205-34.
43. Nakamoto, R.K., J.A. Baylis Scanlon, and M.K. Al-Shawi, The rotary mechanism of the ATP synthase. Arch Biochem Biophys, 2008. **476**(1): p. 43-50.
44. Capaldi, R.A. and R. Aggeler, Mechanism of the F(1)F(0)-type ATP synthase, a biological rotary motor. Trends Biochem Sci, 2002. **27**(3): p. 154-60.

45. Koopman, W.J., et al., OXPHOS mutations and neurodegeneration. EMBO J, 2013. **32**(1): p. 9-29.
46. Papa, S., et al., The oxidative phosphorylation system in mammalian mitochondria. Adv Exp Med Biol, 2012. **942**: p. 3-37.
47. Zorov, D.B., M. Juhaszova, and S.J. Sollott, Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol Rev, 2014. **94**(3): p. 909-50.
48. Turrens, J.F., Mitochondrial formation of reactive oxygen species. J Physiol, 2003. **552**(Pt 2): p. 335-44.
49. Packer, M.A., C.M. Porteous, and M.P. Murphy, Superoxide production by mitochondria in the presence of nitric oxide forms peroxynitrite. Biochem Mol Biol Int, 1996. **40**(3): p. 527-34.
50. Grivennikova, V.G. and A.D. Vinogradov, Mitochondrial production of reactive oxygen species. Biochemistry (Mosc), 2013. **78**(13): p. 1490-511.
51. Jensen, P.K., Antimycin-insensitive oxidation of succinate and reduced nicotinamide-adenine dinucleotide in electron-transport particles. I. pH dependency and hydrogen peroxide formation. Biochim Biophys Acta, 1966. **122**(2): p. 157-66.
52. Smeitink, J., L. van den Heuvel, and S. DiMauro, The genetics and pathology of oxidative phosphorylation. Nat Rev Genet, 2001. **2**(5): p. 342-52.
53. Maker, H.S., et al., Coupling of dopamine oxidation (monoamine oxidase activity) to glutathione oxidation via the generation of hydrogen peroxide in rat brain homogenates. J Neurochem, 1981. **36**(2): p. 589-93.
54. Zoccarato, F., P. Toscano, and A. Alexandre, Dopamine-derived dopaminochrome promotes H(2)O(2) release at mitochondrial complex I: stimulation by rotenone, control by Ca(2+), and relevance to Parkinson disease. J Biol Chem, 2005. **280**(16): p. 15587-94.
55. Raha, S. and B.H. Robinson, Mitochondria, oxygen free radicals, and apoptosis. Am J Med Genet, 2001. **106**(1): p. 62-70.
56. Hansford, R.G., B.A. Hogue, and V. Mildaziene, Dependence of H2O2 formation by rat heart mitochondria on substrate availability and donor age. J Bioenerg Biomembr, 1997. **29**(1): p. 89-95.
57. Lemarie, A., et al., Specific disintegration of complex II succinate:ubiquinone oxidoreductase links pH changes to oxidative stress for apoptosis induction. Cell Death Differ, 2011. **18**(2): p. 338-49.
58. Dong, L.F., et al., Mitochondrial targeting of vitamin E succinate enhances its pro-apoptotic and anti-cancer activity via mitochondrial complex II. J Biol Chem, 2011. **286**(5): p. 3717-28.
59. Brouillet, E., et al., Replicating Huntington's disease phenotype in experimental animals. Prog Neurobiol, 1999. **59**(5): p. 427-68.
60. Liot, G., et al., Complex II inhibition by 3-NP causes mitochondrial fragmentation and neuronal cell death via an NMDA- and ROS-dependent pathway. Cell Death Differ, 2009. **16**(6): p. 899-909.
61. Trumpower, B.L., The protonmotive Q cycle. Energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc1 complex. J Biol Chem, 1990. **265**(20): p. 11409-12.
62. Starkov, A.A. and G. Fiskum, Myxothiazol induces H(2)O(2) production from mitochondrial respiratory chain. Biochem Biophys Res Commun, 2001. **281**(3): p. 645-50.
63. Rieske, J.S., et al., On the antimycin-sensitive cleavage of complex 3 of the mitochondrial respiratory chain. J Biol Chem, 1967. **242**(21): p. 4854-66.

64. Huang, L.S., et al., Binding of the respiratory chain inhibitor antimycin to the mitochondrial bc1 complex: a new crystal structure reveals an altered intramolecular hydrogen-bonding pattern. J Mol Biol, 2005. **351**(3): p. 573-97.
65. Musatov, A. and N.C. Robinson, Susceptibility of mitochondrial electron-transport complexes to oxidative damage. Focus on cytochrome c oxidase. Free Radic Res, 2012. **46**(11): p. 1313-26.
66. Kadenbach, B., R. Ramzan, and S. Vogt, Degenerative diseases, oxidative stress and cytochrome c oxidase function. Trends Mol Med, 2009. **15**(4): p. 139-47.
67. Prabu, S.K., et al., Protein kinase A-mediated phosphorylation modulates cytochrome c oxidase function and augments hypoxia and myocardial ischemia-related injury. J Biol Chem, 2006. **281**(4): p. 2061-70.
68. Kulikov, A.V., et al., Cytochrome c: the Achilles' heel in apoptosis. Cell Mol Life Sci, 2012. **69**(11): p. 1787-97.
69. Sheridan, C. and S.J. Martin, Mitochondrial fission/fusion dynamics and apoptosis. Mitochondrion, 2010. **10**(6): p. 640-8.
70. Lenaz, G., Mitochondria and reactive oxygen species. Which role in physiology and pathology? Adv Exp Med Biol, 2012. **942**: p. 93-136.
71. Schaffer, S.W. and S. Suleiman, Mitochondria: The Dynamic Organelle. 2010: Springer.
72. Chan, D.C., Fusion and fission: interlinked processes critical for mitochondrial health. Annu Rev Genet, 2012. **46**: p. 265-87.
73. Benard, G. and M. Karbowski, Mitochondrial fusion and division: Regulation and role in cell viability. Semin Cell Dev Biol, 2009. **20**(3): p. 365-74.
74. Santel, A. and M.T. Fuller, Control of mitochondrial morphology by a human mitofusin. J Cell Sci, 2001. **114**(Pt 5): p. 867-74.
75. Griffin, E.E., S.A. Detmer, and D.C. Chan, Molecular mechanism of mitochondrial membrane fusion. Biochim Biophys Acta, 2006. **1763**(5-6): p. 482-9.
76. Song, Z., et al., Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. Mol Biol Cell, 2009. **20**(15): p. 3525-32.
77. de Brito, O.M. and L. Scorrano, Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature, 2008. **456**(7222): p. 605-10.
78. Merkwirth, C. and T. Langer, Mitofusin 2 builds a bridge between ER and mitochondria. Cell, 2008. **135**(7): p. 1165-7.
79. Schaaf, C.P., et al., Early-onset severe neuromuscular phenotype associated with compound heterozygosity for OPA1 mutations. Mol Genet Metab, 2011. **103**(4): p. 383-7.
80. Yu-Wai-Man, P., et al., Pattern of retinal ganglion cell loss in dominant optic atrophy due to OPA1 mutations. Eye (Lond), 2011. **25**(5): p. 596-602.
81. Spinazzi, M., et al., A novel deletion in the GTPase domain of OPA1 causes defects in mitochondrial morphology and distribution, but not in function. Hum Mol Genet, 2008. **17**(21): p. 3291-302.
82. Delettre, C., et al., OPA1 (Kjer type) dominant optic atrophy: a novel mitochondrial disease. Mol Genet Metab, 2002. **75**(2): p. 97-107.
83. Olichon, A., et al., Effects of OPA1 mutations on mitochondrial morphology and apoptosis: relevance to ADOA pathogenesis. J Cell Physiol, 2007. **211**(2): p. 423-30.
84. Zanna, C., et al., OPA1 mutations associated with dominant optic atrophy impair oxidative phosphorylation and mitochondrial fusion. Brain, 2008. **131**(Pt 2): p. 352-67.
85. Chen, H., J.M. McCaffery, and D.C. Chan, Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell, 2007. **130**(3): p. 548-62.

86. Hom, J. and S.S. Sheu, Morphological dynamics of mitochondria--a special emphasis on cardiac muscle cells. J Mol Cell Cardiol, 2009. **46**(6): p. 811-20.
87. Chang, C.R. and C. Blackstone, Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1. Ann N Y Acad Sci, 2010. **1201**: p. 34-9.
88. Palmer, C.S., et al., Adaptor proteins MiD49 and MiD51 can act independently of Mff and Fis1 in Drp1 recruitment and are specific for mitochondrial fission. J Biol Chem, 2013. **288**(38): p. 27584-93.
89. Loson, O.C., et al., Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Mol Biol Cell, 2013. **24**(5): p. 659-67.
90. Otera, H., et al., Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J Cell Biol, 2010. **191**(6): p. 1141-58.
91. Sesaki, H., et al., In vivo functions of Drp1: lessons learned from yeast genetics and mouse knockouts. Biochim Biophys Acta, 2014. **1842**(8): p. 1179-85.
92. Nakamura, T., et al., S-nitrosylation of Drp1 links excessive mitochondrial fission to neuronal injury in neurodegeneration. Mitochondrion, 2010. **10**(5): p. 573-8.
93. Figueroa-Romero, C., et al., SUMOylation of the mitochondrial fission protein Drp1 occurs at multiple nonconsensus sites within the B domain and is linked to its activity cycle. FASEB J, 2009. **23**(11): p. 3917-27.
94. Wang, H., et al., Parkin ubiquitinates Drp1 for proteasome-dependent degradation: implication of dysregulated mitochondrial dynamics in Parkinson disease. J Biol Chem, 2011. **286**(13): p. 11649-58.
95. Buhlman, L., et al., Functional interplay between Parkin and Drp1 in mitochondrial fission and clearance. Biochim Biophys Acta, 2014. **1843**(9): p. 2012-26.
96. Chen, L., et al., OPA1 mutation and late-onset cardiomyopathy: mitochondrial dysfunction and mtDNA instability. J Am Heart Assoc, 2012. **1**(5): p. e003012.
97. Bess, A.S., et al., Mitochondrial dynamics and autophagy aid in removal of persistent mitochondrial DNA damage in Caenorhabditis elegans. Nucleic Acids Res, 2012. **40**(16): p. 7916-31.
98. Meyer, J.N. and A.S. Bess, Involvement of autophagy and mitochondrial dynamics in determining the fate and effects of irreparable mitochondrial DNA damage. Autophagy, 2012. **8**(12): p. 1822-23.
99. Meeusen, S., et al., Mitochondrial inner-membrane fusion and crista maintenance requires the dynamin-related GTPase Mgm1. Cell, 2006. **127**(2): p. 383-95.
100. Frezza, C., et al., OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell, 2006. **126**(1): p. 177-89.
101. Cipolat, S., et al., OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc Natl Acad Sci U S A, 2004. **101**(45): p. 15927-32.
102. Campello, S., F. Strappazzon, and F. Cecconi, Mitochondrial dismissal in mammals, from protein degradation to mitophagy. Biochim Biophys Acta, 2014. **1837**(4): p. 451-60.
103. Bhatia-Kissova, I. and N. Camougrand, Mitophagy: a process that adapts to the cell physiology. Int J Biochem Cell Biol, 2013. **45**(1): p. 30-3.
104. Shutt, T., et al., The intracellular redox state is a core determinant of mitochondrial fusion. EMBO Rep, 2012. **13**(10): p. 909-15.
105. Shutt, T.E. and H.M. McBride, Staying cool in difficult times: mitochondrial dynamics, quality control and the stress response. Biochim Biophys Acta, 2013. **1833**(2): p. 417-24.

106. Ryan, M.T. and D. Stojanovski, Mitofusins 'bridge' the gap between oxidative stress and mitochondrial hyperfusion. EMBO Rep, 2012. **13**(10): p. 870-1.
107. Steiger-Barraissoul, S. and A. Rami, Serum deprivation induced autophagy and predominantly an AIF-dependent apoptosis in hippocampal HT22 neurons. Apoptosis, 2009. **14**(11): p. 1274-88.
108. Rambold, A.S., B. Kostelecky, and J. Lippincott-Schwartz, Fuse or die: Shaping mitochondrial fate during starvation. Commun Integr Biol, 2011. **4**(6): p. 752-4.
109. Gomes, L.C., G. Di Benedetto, and L. Scorrano, During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. Nat Cell Biol, 2011. **13**(5): p. 589-98.
110. Rambold, A.S., B. Kostelecky, and J. Lippincott-Schwartz, Together we are stronger: fusion protects mitochondria from autophagosomal degradation. Autophagy, 2011. **7**(12): p. 1568-9.
111. Kluck, R.M., et al., The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science, 1997. **275**(5303): p. 1132-6.
112. Wu, S., et al., Bax is essential for Drp1-mediated mitochondrial fission but not for mitochondrial outer membrane permeabilization caused by photodynamic therapy. J Cell Physiol, 2011. **226**(2): p. 530-41.
113. Brooks, C., et al., Fragmented mitochondria are sensitized to Bax insertion and activation during apoptosis. Am J Physiol Cell Physiol, 2011. **300**(3): p. C447-55.
114. Wasiak, S., R. Zunino, and H.M. McBride, Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death. J Cell Biol, 2007. **177**(3): p. 439-50.
115. Grohm, J., et al., Inhibition of Drp1 provides neuroprotection in vitro and in vivo. Cell Death Differ, 2012. **19**(9): p. 1446-58.
116. Wang, J., et al., Mdivi-1 prevents apoptosis induced by ischemia-reperfusion injury in primary hippocampal cells via inhibition of reactive oxygen species-activated mitochondrial pathway. J Stroke Cerebrovasc Dis, 2014. **23**(6): p. 1491-9.
117. Nakatsukasa, K., T. Kamura, and J.L. Brodsky, Recent technical developments in the study of ER-associated degradation. Curr Opin Cell Biol, 2014. **29C**: p. 82-91.
118. Livnat-Levanon, N. and M.H. Glickman, Ubiquitin-proteasome system and mitochondria - reciprocity. Biochim Biophys Acta, 2011. **1809**(2): p. 80-7.
119. Berndsen, C.E. and C. Wolberger, New insights into ubiquitin E3 ligase mechanism. Nat Struct Mol Biol, 2014. **21**(4): p. 301-7.
120. Chhangani, D., A.P. Joshi, and A. Mishra, E3 ubiquitin ligases in protein quality control mechanism. Mol Neurobiol, 2012. **45**(3): p. 571-85.
121. Scheffner, M. and S. Kumar, Mammalian HECT ubiquitin-protein ligases: biological and pathophysiological aspects. Biochim Biophys Acta, 2014. **1843**(1): p. 61-74.
122. Metzger, M.B., et al., RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. Biochim Biophys Acta, 2014. **1843**(1): p. 47-60.
123. Lok, G.T., et al., Differential regulation of RNF8-mediated Lys48- and Lys63-based poly-ubiquitylation. Nucleic Acids Res, 2012. **40**(1): p. 196-205.
124. Neutzner, A., R.J. Youle, and M. Karbowski, Outer mitochondrial membrane protein degradation by the proteasome. Novartis Found Symp, 2007. **287**: p. 4-14; discussion 14-20.
125. Tang, F., et al., RNF185, a novel mitochondrial ubiquitin E3 ligase, regulates autophagy through interaction with BNIP1. PLoS One, 2011. **6**(9): p. e24367.
126. Benard, G., et al., IBRDC2, an IBR-type E3 ubiquitin ligase, is a regulatory factor for Bax and apoptosis activation. EMBO J, 2010. **29**(8): p. 1458-71.
127. Park, Y.Y., et al., Loss of MARCH5 mitochondrial E3 ubiquitin ligase induces cellular senescence through dynamin-related protein 1 and mitofusin 1. J Cell Sci, 2010. **123**(Pt 4): p. 619-26.

128. Winklhofer, K.F., Parkin and mitochondrial quality control: toward assembling the puzzle. Trends Cell Biol, 2014. **24**(6): p. 332-41.
129. Yonashiro, R., et al., A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. EMBO J, 2006. **25**(15): p. 3618-26.
130. Park, Y.Y. and H. Cho, Mitofusin 1 is degraded at G2/M phase through ubiquitylation by MARCH5. Cell Div, 2012. **7**(1): p. 25.
131. Sugiura, A., et al., MITOL regulates endoplasmic reticulum-mitochondria contacts via Mitofusin2. Mol Cell, 2013. **51**(1): p. 20-34.
132. Wolf, D.H. and A. Stolz, The Cdc48 machine in endoplasmic reticulum associated protein degradation. Biochim Biophys Acta, 2012. **1823**(1): p. 117-24.
133. Jentsch, S. and S. Rumpf, Cdc48 (p97): a "molecular gearbox" in the ubiquitin pathway? Trends Biochem Sci, 2007. **32**(1): p. 6-11.
134. Vaz, B., S. Halder, and K. Ramadan, Role of p97/VCP (Cdc48) in genome stability. Front Genet, 2013. **4**: p. 60.
135. Puumalainen, M.R., et al., Chromatin retention of DNA damage sensors DDB2 and XPC through loss of p97 segregase causes genotoxicity. Nat Commun, 2014. **5**: p. 3695.
136. Bug, M. and H. Meyer, Expanding into new markets--VCP/p97 in endocytosis and autophagy. J Struct Biol, 2012. **179**(2): p. 78-82.
137. Wang, Q., C. Song, and C.C. Li, Molecular perspectives on p97-VCP: progress in understanding its structure and diverse biological functions. J Struct Biol, 2004. **146**(1-2): p. 44-57.
138. Chou, T.F., et al., Specific Inhibition of p97/VCP ATPase and Kinetic Analysis Demonstrate Interaction between D1 and D2 ATPase Domains. J Mol Biol, 2014. **426**(15): p. 2886-99.
139. Ballar, P., A. Pabuccuoglu, and F.A. Kose, Different p97/VCP complexes function in retrotranslocation step of mammalian ER-associated degradation (ERAD). Int J Biochem Cell Biol, 2011. **43**(4): p. 613-21.
140. Kloppsteck, P., et al., Regulation of p97 in the ubiquitin-proteasome system by the UBX protein-family. Biochim Biophys Acta, 2012. **1823**(1): p. 125-9.
141. Liu, S., et al., Structural and mechanistic insights into the arginine/lysine-rich peptide motifs that interact with P97/VCP. Biochim Biophys Acta, 2013. **1834**(12): p. 2672-8.
142. Yeung, H.O., et al., Insights into adaptor binding to the AAA protein p97. Biochem Soc Trans, 2008. **36**(Pt 1): p. 62-7.
143. Ye, Y., H.H. Meyer, and T.A. Rapoport, Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. J Cell Biol, 2003. **162**(1): p. 71-84.
144. Ye, Y., H.H. Meyer, and T.A. Rapoport, The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature, 2001. **414**(6864): p. 652-6.
145. Xu, S., et al., The AAA-ATPase p97 is essential for outer mitochondrial membrane protein turnover. Mol Biol Cell, 2011. **22**(3): p. 291-300.
146. Esaki, M. and T. Ogura, Cdc48p/p97-mediated regulation of mitochondrial morphology is Vms1p-independent. J Struct Biol, 2012. **179**(2): p. 112-20.
147. Tresse, E., et al., VCP/p97 is essential for maturation of ubiquitin-containing autophagosomes and this function is impaired by mutations that cause IBMPFD. Autophagy, 2010. **6**(2): p. 217-27.
148. Erzurumlu, Y., et al., A unique IBMPFD-related P97/VCP mutation with differential binding pattern and subcellular localization. Int J Biochem Cell Biol, 2013. **45**(4): p. 773-82.
149. Weihl, C.C., et al., Inclusion body myopathy-associated mutations in p97/VCP impair endoplasmic reticulum-associated degradation. Hum Mol Genet, 2006. **15**(2): p. 189-99.

150. Drose, S. and U. Brandt, Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. Adv Exp Med Biol, 2012. **748**: p. 145-69.
151. Fernandez-Checa, J.C., et al., Oxidative stress and altered mitochondrial function in neurodegenerative diseases: lessons from mouse models. CNS Neurol Disord Drug Targets, 2010. **9**(4): p. 439-54.
152. Wang, C.H., et al., Oxidative stress response elicited by mitochondrial dysfunction: implication in the pathophysiology of aging. Exp Biol Med (Maywood), 2013. **238**(5): p. 450-60.
153. Wang, X., et al., Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. Biochim Biophys Acta, 2014. **1842**(8): p. 1240-7.
154. Ayala-Pena, S., Role of oxidative DNA damage in mitochondrial dysfunction and Huntington's disease pathogenesis. Free Radic Biol Med, 2013. **62**: p. 102-10.
155. Lehmann, S. and L.M. Martins, Insights into mitochondrial quality control pathways and Parkinson's disease. J Mol Med (Berl), 2013. **91**(6): p. 665-71.
156. de Castro, I.P., L.M. Martins, and R. Tufi, Mitochondrial quality control and neurological disease: an emerging connection. Expert Rev Mol Med, 2010. **12**: p. e12.
157. Miriyala, S., A.K. Holley, and D.K. St Clair, Mitochondrial superoxide dismutase--signals of distinction. Anticancer Agents Med Chem, 2011. **11**(2): p. 181-90.
158. Kazak, L., A. Reyes, and I.J. Holt, Minimizing the damage: repair pathways keep mitochondrial DNA intact. Nat Rev Mol Cell Biol, 2012. **13**(10): p. 659-71.
159. Horvath, S.E. and G. Daum, Lipids of mitochondria. Prog Lipid Res, 2013. **52**(4): p. 590-614.
160. Langer, T. and W. Neupert, Heat shock proteins hsp60 and hsp70: their roles in folding, assembly and membrane translocation of proteins. Curr Top Microbiol Immunol, 1991. **167**: p. 3-30.
161. Van Dyck, L. and T. Langer, ATP-dependent proteases controlling mitochondrial function in the yeast Saccharomyces cerevisiae. Cell Mol Life Sci, 1999. **56**(9-10): p. 825-42.
162. Langer, T. and W. Neupert, Regulated protein degradation in mitochondria. Experientia, 1996. **52**(12): p. 1069-76.
163. Matsushima, Y. and L.S. Kaguni, Matrix proteases in mitochondrial DNA function. Biochim Biophys Acta, 2012. **1819**(9-10): p. 1080-7.
164. Sauer, R.T. and T.A. Baker, AAA+ proteases: ATP-fueled machines of protein destruction. Annu Rev Biochem, 2011. **80**: p. 587-612.
165. Alavi, M.V. and N. Fuhrmann, Dominant optic atrophy, OPA1, and mitochondrial quality control: understanding mitochondrial network dynamics. Mol Neurodegener, 2013. **8**: p. 32.
166. Twig, G., B. Hyde, and O.S. Shirihai, Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. Biochim Biophys Acta, 2008. **1777**(9): p. 1092-7.
167. Ding, W.X. and X.M. Yin, Mitophagy: mechanisms, pathophysiological roles, and analysis. Biol Chem, 2012. **393**(7): p. 547-64.
168. Novak, I., et al., Nix is a selective autophagy receptor for mitochondrial clearance. EMBO Rep, 2010. **11**(1): p. 45-51.
169. Springer, W. and P.J. Kahle, Regulation of PINK1-Parkin-mediated mitophagy. Autophagy, 2011. **7**(3): p. 266-78.
170. Corti, O. and A. Brice, Mitochondrial quality control turns out to be the principal suspect in parkin and PINK1-related autosomal recessive Parkinson's disease. Curr Opin Neurobiol, 2013. **23**(1): p. 100-8.
171. Koh, H. and J. Chung, PINK1 as a molecular checkpoint in the maintenance of mitochondrial function and integrity. Mol Cells, 2012. **34**(1): p. 7-13.

172. Koh, H. and J. Chung, PINK1 and Parkin to control mitochondria remodeling. Anat Cell Biol, 2010. **43**(3): p. 179-84.
173. Deng, H., et al., The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila. Proc Natl Acad Sci U S A, 2008. **105**(38): p. 14503-8.
174. Meissner, C., et al., The mitochondrial intramembrane protease PARL cleaves human Pink1 to regulate Pink1 trafficking. J Neurochem, 2011. **117**(5): p. 856-67.
175. Narendra, D.P., et al., PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol, 2010. **8**(1): p. e1000298.
176. Jin, S.M., et al., Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. J Cell Biol, 2010. **191**(5): p. 933-42.
177. Jin, S.M. and R.J. Youle, PINK1- and Parkin-mediated mitophagy at a glance. J Cell Sci, 2012. **125**(Pt 4): p. 795-9.
178. Chen, Y. and G.W. Dorn, 2nd, PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science, 2013. **340**(6131): p. 471-5.
179. Poole, A.C., et al., The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. PLoS One, 2010. **5**(4): p. e10054.
180. Lim, K.L., et al., Mitochondrial dynamics and Parkinson's disease: focus on parkin. Antioxid Redox Signal, 2012. **16**(9): p. 935-49.
181. Scarffe, L.A., et al., Parkin and PINK1: much more than mitophagy. Trends Neurosci, 2014. **37**(6): p. 315-24.
182. Cai, Q., et al., Spatial parkin translocation and degradation of damaged mitochondria via mitophagy in live cortical neurons. Curr Biol, 2012. **22**(6): p. 545-52.
183. Palomo, G.M. and G. Manfredi, Exploring new pathways of neurodegeneration in ALS: The role of mitochondria quality control. Brain Res, 2014.
184. Karbowski, M., et al., Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. J Cell Biol, 2002. **159**(6): p. 931-8.
185. Baker, M.J., T. Tatsuta, and T. Langer, Quality control of mitochondrial proteostasis. Cold Spring Harb Perspect Biol, 2011. **3**(7).
186. Ramanan, V.K. and A.J. Saykin, Pathways to neurodegeneration: mechanistic insights from GWAS in Alzheimer's disease, Parkinson's disease, and related disorders. Am J Neurodegener Dis, 2013. **2**(3): p. 145-75.
187. Price, D.L., S.S. Sisodia, and D.R. Borchelt, Genetic neurodegenerative diseases: the human illness and transgenic models. Science, 1998. **282**(5391): p. 1079-83.
188. Harvey, B.K., et al., Transgenic animal models of neurodegeneration based on human genetic studies. J Neural Transm, 2011. **118**(1): p. 27-45.
189. Dexter, D.T. and P. Jenner, Parkinson disease: from pathology to molecular disease mechanisms. Free Radic Biol Med, 2013. **62**: p. 132-44.
190. Neve, R.L., D.L. McPhie, and Y. Chen, Alzheimer's disease: a dysfunction of the amyloid precursor protein(1). Brain Res, 2000. **886**(1-2): p. 54-66.
191. Siuda, J., S. Fujioka, and Z.K. Wszolek, Parkinsonian syndrome in familial frontotemporal dementia. Parkinsonism Relat Disord, 2014.
192. Pickles, S. and C. Vande Velde, Misfolded SOD1 and ALS: zeroing in on mitochondria. Amyotroph Lateral Scler, 2012. **13**(4): p. 333-40.
193. Metcalf, D.J., et al., Autophagy and misfolded proteins in neurodegeneration. Exp Neurol, 2012. **238**(1): p. 22-8.

194. Ali, Y.O., B.M. Kitay, and R.G. Zhai, Dealing with misfolded proteins: examining the neuroprotective role of molecular chaperones in neurodegeneration. Molecules, 2010. **15**(10): p. 6859-87.
195. Spillantini, M.G. and M. Goedert, Tau pathology and neurodegeneration. Lancet Neurol, 2013. **12**(6): p. 609-22.
196. Bodles, A.M. and G.B. Irvine, Alpha-synuclein aggregation. Protein Pept Lett, 2004. **11**(3): p. 271-9.
197. Halliday, M. and G.R. Mallucci, Targeting the unfolded protein response in neurodegeneration: A new approach to therapy. Neuropharmacology, 2014. **76 Pt A**: p. 169-74.
198. Simpson, I.A., A. Carruthers, and S.J. Vannucci, Supply and demand in cerebral energy metabolism: the role of nutrient transporters. J Cereb Blood Flow Metab, 2007. **27**(11): p. 1766-91.
199. Reeve, A.K., K.J. Krishnan, and D. Turnbull, Mitochondrial DNA mutations in disease, aging, and neurodegeneration. Ann N Y Acad Sci, 2008. **1147**: p. 21-9.
200. Chern, C.J. and E. Beutler, Biochemical and electrophoretic studies of erythrocyte pyridoxine kinase in white and black Americans. Am J Hum Genet, 1976. **28**(1): p. 9-17.
201. Federico, A., et al., Mitochondria, oxidative stress and neurodegeneration. J Neurol Sci, 2012. **322**(1-2): p. 254-62.
202. Di Carlo, M., et al., Are oxidative stress and mitochondrial dysfunction the key players in the neurodegenerative diseases? Free Radic Res, 2012. **46**(11): p. 1327-38.
203. Gandhi, S. and A.Y. Abramov, Mechanism of oxidative stress in neurodegeneration. Oxid Med Cell Longev, 2012. **2012**: p. 428010.
204. Cartoni, R. and J.C. Martinou, Role of mitofusin 2 mutations in the physiopathology of Charcot-Marie-Tooth disease type 2A. Exp Neurol, 2009. **218**(2): p. 268-73.
205. Yonashiro, R., et al., Mitochondrial ubiquitin ligase MITOL blocks S-nitrosylated MAP1B-light chain 1-mediated mitochondrial dysfunction and neuronal cell death. Proc Natl Acad Sci U S A, 2012. **109**(7): p. 2382-7.
206. Koppen, M., et al., Variable and tissue-specific subunit composition of mitochondrial m-AAA protease complexes linked to hereditary spastic paraplegia. Mol Cell Biol, 2007. **27**(2): p. 758-67.
207. Atorino, L., et al., Loss of m-AAA protease in mitochondria causes complex I deficiency and increased sensitivity to oxidative stress in hereditary spastic paraplegia. J Cell Biol, 2003. **163**(4): p. 777-87.
208. Krishnamoorthy, R.R., et al., A forensic path to RGC-5 cell line identification: lessons learned. Invest Ophthalmol Vis Sci, 2013. **54**(8): p. 5712-9.
209. Van Bergen, N.J., et al., Recharacterization of the RGC-5 retinal ganglion cell line. Invest Ophthalmol Vis Sci, 2009. **50**(9): p. 4267-72.
210. Wood, J.P., et al., A comparison of differentiation protocols for RGC-5 cells. Invest Ophthalmol Vis Sci, 2010. **51**(7): p. 3774-83.
211. Kazmi, S.M. and R.K. Mishra, Opioid receptors in human neuroblastoma SH-SY5Y cells: evidence for distinct morphine (mu) and enkephalin (delta) binding sites. Biochem Biophys Res Commun, 1986. **137**(2): p. 813-20.
212. Lukas, R.J., S.A. Norman, and L. Lucero, Characterization of Nicotinic Acetylcholine Receptors Expressed by Cells of the SH-SY5Y Human Neuroblastoma Clonal Line. Mol Cell Neurosci, 1993. **4**(1): p. 1-12.

213. Nair, V.D., H.B. Niznik, and R.K. Mishra, NMDA and dopamine D2L receptor interaction in human neuroblastoma SH-SY5Y cells involves tyrosine kinase and phosphatase. Neuroreport, 1996. **7**(18): p. 2937-40.
214. Xie, H.R., L.S. Hu, and G.Y. Li, SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. Chin Med J (Engl), 2010. **123**(8): p. 1086-92.
215. Lummen, P., Complex I inhibitors as insecticides and acaricides. Biochim Biophys Acta, 1998. **1364**(2): p. 287-96.
216. Jenner, P., Parkinson's disease, pesticides and mitochondrial dysfunction. Trends Neurosci, 2001. **24**(5): p. 245-7.
217. Wright, A.K., M. Garcia-Munoz, and G.W. Arbuthnott, Slowly progressive dopamine cell loss--a model on which to test neuroprotective strategies for Parkinson's disease? Rev Neurosci, 2009. **20**(2): p. 85-94.
218. Marr, R.A. and D.M. Hafez, Amyloid-beta and Alzheimer's disease: the role of neprilysin-2 in amyloid-beta clearance. Front Aging Neurosci, 2014. **6**: p. 187.
219. Abbasowa, L. and N.H. Heegaard, A systematic review of amyloid-beta peptides as putative mediators of the association between affective disorders and Alzheimers disease. J Affect Disord, 2014. **168C**: p. 167-183.
220. Kummer, M.P. and M.T. Heneka, Truncated and modified amyloid-beta species. Alzheimers Res Ther, 2014. **6**(3): p. 28.
221. Hong, L., H.C. Huang, and Z.F. Jiang, Relationship between amyloid-beta and the ubiquitin-proteasome system in Alzheimer's disease. Neurol Res, 2014. **36**(3): p. 276-82.
222. Pinho, C.M., P.F. Teixeira, and E. Glaser, Mitochondrial import and degradation of amyloid-beta peptide. Biochim Biophys Acta, 2014. **1837**(7): p. 1069-74.
223. Kaminsky, Y.G., et al., Subcellular and metabolic examination of amyloid-beta peptides in Alzheimer disease pathogenesis: evidence for Abeta(25-35). Exp Neurol, 2010. **221**(1): p. 26-37.
224. Forloni, G., et al., Apoptosis mediated neurotoxicity induced by chronic application of beta amyloid fragment 25-35. Neuroreport, 1993. **4**(5): p. 523-6.
225. Cafe, C., et al., Oxidative stress after acute and chronic application of beta-amyloid fragment 25-35 in cortical cultures. Neurosci Lett, 1996. **203**(1): p. 61-5.
226. Vehvilainen, P., J. Koistinaho, and G. Gundars, Mechanisms of mutant SOD1 induced mitochondrial toxicity in amyotrophic lateral sclerosis. Front Cell Neurosci, 2014. **8**: p. 126.
227. Matos, C.A., S. de Macedo-Ribeiro, and A.L. Carvalho, Polyglutamine diseases: the special case of ataxin-3 and Machado-Joseph disease. Prog Neurobiol, 2011. **95**(1): p. 26-48.
228. Naon, D. and L. Scorrano, At the right distance: ER-mitochondria juxtaposition in cell life and death. Biochim Biophys Acta, 2014. **1843**(10): p. 2184-94.
229. Vij, N., AAA ATPase p97/VCP: cellular functions, disease and therapeutic potential. J Cell Mol Med, 2008. **12**(6A): p. 2511-8.
230. Haubenberger, D., et al., Inclusion body myopathy and Paget disease is linked to a novel mutation in the VCP gene. Neurology, 2005. **65**(8): p. 1304-5.
231. Kimonis, V.E., et al., Clinical studies in familial VCP myopathy associated with Paget disease of bone and frontotemporal dementia. Am J Med Genet A, 2008. **146A**(6): p. 745-57.
232. Bartolome, F., et al., Pathogenic VCP mutations induce mitochondrial uncoupling and reduced ATP levels. Neuron, 2013. **78**(1): p. 57-64.
233. Kimura, Y., et al., Different dynamic movements of wild-type and pathogenic VCPs and their cofactors to damaged mitochondria in a Parkin-mediated mitochondrial quality control system. Genes Cells, 2013. **18**(12): p. 1131-43.

234. Kim, N.C., et al., VCP is essential for mitochondrial quality control by PINK1/Parkin and this function is impaired by VCP mutations. Neuron, 2013. **78**(1): p. 65-80.
235. Karbowski, M. and R.J. Youle, Regulating mitochondrial outer membrane proteins by ubiquitination and proteasomal degradation. Curr Opin Cell Biol, 2011. **23**(4): p. 476-82.
236. Hemion, C., J. Flammer, and A. Neutzner, Quality control of oxidatively damaged mitochondrial proteins is mediated by p97 and the proteasome. Free Radic Biol Med, 2014. **75**: p. 121-8.
237. Benischke, A.S., et al., Proteasome-mediated quality control of S-nitrosylated mitochondrial proteins. Mitochondrion, 2014. **17**: p. 182-6.

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Conferences and Seminars

1. **Lei Fang**, Albert Neutzner, MARCH5 inactivation support mitochondrial function during neurodegenerative stress. 2014, Swiss Eye Research Week, Biel, Switzerland, Oral Presentation.
2. Jia Li, **Lei Fang**, Peter Meyer, Hanspeter E. Killer, Josef Flammer, and Albert Neutzner, Meningothelial cells are involved in the host defense against bacterial infection. 2013, Congress of the European Society of Ophthalmology, Copenhagen, Denmark, Poster Presentation.

List of publications

1. **Lei Fang**, Albert Neutzner, Josef Flammer, Maneli Mozaffarieh, The effect of Ginkgo Biloba and nifedipine on DNA breaks in circulating leukocytes of glaucoma patients. (2015 Manuscript submitted)
2. **Lei Fang**, Charles Hemion, Claudia Bippes, Josef Flammer, Albert Neutzner, Mitochondrial function in neuronal cells depends on p97/VCP/Cdc48-mediated quality control. Frontiers in Cellular Neuroscience, 2015, 9:16.
3. **Lei Fang**, Albert Neutzner, Maneli Mozaffarieh, Systemic oxidative stress and the Flammer-Syndrome: a focus on comet assay methodology. Journal of Visualized Experiment, (2015, Manuscript accepted)
4. **Lei Fang**, Michael Bärtschi, Maneli Mozaffarieh, The effect of Flammer-Syndrome on retinal venous pressure. BMC Ophthalmology, 2014, 14:121
5. Jia Li, **Lei Fang**, Peter Meyer, Hanspeter E. Killer, Josef Flammer, Albert Neutzner, Anti-inflammatory response following uptake of apoptotic bodies by meningothelial cells. Journal of Neuroinflammation, 2014, 11:35.
6. **Lei Fang**, Jia Li, Josef Flammer and Albert Neutzner, MARCH5 inactivation supports mitochondrial function during neurodegenerative stress. Frontiers in Cellular Neuroscience, 2013, 7:176.
7. Jia Li, **Lei Fang**, Hanspeter E. Killer, Josef Flammer, Peter Meyer and Albert Neutzner, Meningothelial cells as part of the central nervous system host defence. Biology of the Cell, 2013, 105:1-12.
8. **Lei Fang**, Charles Hemion, David Goldblum, Peter Meyer, Selim Orgul, Stephan Frank, Josef Flammer, Albert Neutzner, Inactivation of MARCH5 prevents mitochondrial fragmentation and interferes with cell death in a neuronal cell model. PLoS ONE, 7(12): 2012.
9. Anna Egger, Marijana Samardzija, Vithiyanjali Sothilingam, Naoyuki Tanimoto, Christina Lange, Silvia Salatino, **Lei Fang,** Marina Garcia-Garrido, Susanne Beck, Michal J. Okoniewski, Albert Neutzner, Mathias W. Seeliger, Christian Grimm, Christoph Handschin, PGC-1α determines light damage susceptibility of the murine retina, PloS ONE, 7(2): 2012.
10. Zhiyong Liu, **Lei Fang,** Phosphodiesterase-5 inhibitor and rat lung ischemia-reperfusion injury, Asian Cardiovascular and Thoracic Annals, 2012;20:42-47.
11. **Lei Fang,** Zeinab Barekati, Bei Zhang, Zhiyong Liu, Xiaoyan Zhong, Targeted therapy in breast cancer: what's new? Swiss Medical Weekly, 2011; 141:w13231.