

HSPA9/Mortalin Mediates Axo-Protection by Modulating Mitochondrial Dynamics in Neurons

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HSPA9/MORTALIN MEDIATES AXO-PROTECTION BY MODULATING MITOCHONDRIAL DYNAMICS IN NEURONS

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ABSTRACT

Mortalin is a mitochondrial chaperone protein involved in quality control of proteins imported into the mitochondrial matrix, which was recently described as a sensor of neuronal stress. Mortalin is down-regulated in neurons of patients with neurodegenerative diseases and levels of Mortalin expression are correlated with neuronal fate in animal models of Alzheimer's disease or cerebral ischemia. To date, however, the links between Mortalin levels, its impact on mitochondrial function and morphology and, ultimately, the initiation of neurodegeneration, are still unclear. In the present study, we used lentiviral vectors to over- or under-express Mortalin in primary neuronal cultures. We first analyzed the early events of neurodegeneration in the axonal compartment, using oriented neuronal cultures grown in microfluidic-based devices. We observed that Mortalin down-regulation induced mitochondrial fragmentation and axonal damage, whereas its over-expression conferred protection against axonal degeneration mediated by oxidative stress. We next demonstrated that Mortalin levels modulated mitochondrial morphology by a direct action on DRP1 phosphorylation, thereby further illustrating the crucial implication of mitochondrial dynamics on neuronal fate in degenerative diseases.

INTRODUCTION

Neurodegeneration is a pathological phenomenon resulting from a complex interaction between environmental factors and genetic predispositions, occasioning the death of specific subpopulations of neurons. Nerve terminals are affected before neuronal cell bodies, which leads to neuronal disconnection and synapse impairment that may contribute to the early cognitive symptoms in patients (Vickers et al., 2009). However, the modalities of the retraction process of axons and its implication in the propagation of the degenerative signal, called *dying back*, remain elusive and seem to significantly differ from those involved in conventional apoptosis (Adalbert and Coleman, 2013; Raff et al., 2002). Indeed, axons and somas react very differently to stress, and axons have higher protective signaling capacities than neuronal cell bodies. Insights have been brought by the analysis of Wallerian degeneration (Babetto et al., 2010; Coleman and Freeman, 2010), which enlightened the central role of mitochondria and mitochondrial dynamics in the synaptic and axonal dysfunctions that mark the initiation of neurodegeneration (Cheng et al., 2010; Lassus et al., 2016; Magnifico et al., 2013). Moreover, we recently established a strong association between mitochondrial fragmentation, axonal vulnerability and retrograde spreading of degenerative processes (Lassus et al., 2016). Accordingly, we also demonstrated the neuroprotective ability of a viral protein, the X protein of Borna disease virus, which increases mitochondrial filamentation, thereby preserving mitochondrial function in the axonal compartment of stressed neurons and promoting neuronal survival in models of Parkinson's disease (PD) (Ferre et al., 2016; Szelechowski et al., 2014). Consequently, manipulating mitochondrial morphology and dysfunction in axons might have broad therapeutic implications, especially for neurodegenerative diseases (Court and Coleman, 2012; Schapira and Patel, 2014).

Mortalin (or Hspa9, Grp75, PBP74, mtHsp70) is a mitochondrial chaperone belonging to the heat shock 70 kDa protein family, which has been closely associated with mitochondrial

sensitivity to stress in degenerative neurons (Burbulla et al., 2010). Mortalin was originally described as an actor of protein translocation into the matrix (D'Silva et al., 2004; Schneider et al., 1996). Mortalin was next related to sensing of oxidative stress and activation of antioxidant pathways (Tai-Nagara et al., 2014), thereby conferring resistance against oxidative stress-induced apoptosis (Mylonis et al., 2017), as well as against *in vitro* neurotoxicity, including models of ischemia, synucleinopathy or amyloid β ($A\beta_{1-42}$) toxicity (Liu et al., 2015; Voloboueva et al., 2008; Xu et al., 2009). The neuroprotective properties of Mortalin were shown to rely on its ability to preserve the mitochondrial membrane potential and to protect from the accumulation of reactive oxygen species and the peroxidation of lipids (Qu et al., 2012; Qu et al., 2011). The protein amount of Mortalin is thought to be of critical importance for neuronal fate, since decreased levels of Mortalin were found in brains of PD (Cook et al., 2015; Jin et al., 2006) or Alzheimer's disease patients (Park et al., 2014). However, the link between Mortalin gene mutations and risk for neurodegenerative diseases is still unclear (Chung et al., 2017; De Mena et al., 2009; Osorio et al., 2007). Interestingly, the impact of Mortalin levels on neuronal fate seems to be closely related to mitochondrial function and morphology. Indeed, Mortalin down-regulation decreases mitochondrial respiration (Yang et al., 2011) and produces a drop of mitochondrial targeting to synapses (Zhu et al., 2013). It also worsens $A\beta$ -induced mitochondrial fragmentation and decreases ATP production (Park et al., 2014). In contrast, Mortalin overexpression was shown to be protective *in vitro* (Qu et al., 2012; Qu et al., 2011), leading to extended cellular lifespan, from nematodes to humans (Kaul et al., 2003; Yokoyama et al., 2002). However, the connections between Mortalin levels, mitochondrial physiology and neuronal fate during neurodegeneration are still missing, especially in the distal axonal compartment.

In this study, we sought to improve our knowledge on both the neuroprotective ability of Mortalin as well as on the intrinsic mechanisms of neuronal degeneration. We first observed

that mitochondrial stress induces down-regulation of Mortalin in primary cultures of cortical neurons. To assess the impact of the variation of Mortalin levels on axonal susceptibility to mitochondrial stress, we used oriented neuronal cultures grown in microfluidic-based devices, which allow a physical separation of axons from their cell bodies and we mimicked the first steps of neurodegeneration by inducing a distal oxidative stress (Lassus et al., 2016; Magnifico et al., 2013; Szelechowski et al., 2014). We used lentiviral vectors to modulate Mortalin levels and we analyzed the impact on mitochondrial morphology, dynamics and sensitivity to distal oxidative stress.

RESULTS

Mortalin expression levels correlate with neuronal fate under oxidative stress conditions.

In PD, Mortalin expression levels are strongly correlated to neuronal degeneration (Burbulla et al., 2010; Cook et al., 2015; Jin et al., 2006; Liu et al., 2015; Pennington et al., 2010). Considering the importance of defects in mitochondrial respiratory complex I activity, both in the human disease and in animal models of PD (Schapira, 1992; Schapira et al., 1990; Schapira et al., 1989), we first examined expression levels of Mortalin in rat primary cortical neurons treated with rotenone, a lipophilic cell-permeable toxin of complex I that induces ROS overproduction and oxidative stress, leading to PD symptoms (Betarbet et al., 2000). Interestingly, we observed that treating neurons with 10 nM rotenone for 4 h was sufficient to decrease Mortalin expression by roughly 50 % (Figure 1A, Sup Figure 1). To further establish the pathophysiological link between Mortalin expression levels and neuronal death induced by oxidative stress, we modulated Mortalin amounts in primary cortical neurons and we analyzed neuronal outcome after inducing oxidative stress. This was achieved by transducing neurons with lentiviral vectors expressing either rat Mortalin (LV-Mortalin), or an shRNA directed against rat Mortalin transcript (LV-shMortalin) to over- or under-express Mortalin, respectively

(Figure 1B, Sup Figure 2). Since a 50 % decrease of Mortalin expression was observed in stressed neurons, we used titers of LV preparations sufficient to roughly double or halve Mortalin protein amount when compared to non-transduced neurons. A lentiviral vector expressing a control shRNA (scrambled sequence of the shA, LV-shScr) was used as a control of Mortalin silencing. Transduced neurons were grown for 11 days *in vitro* (DIV) and then treated or not with rotenone (10 nM) for 24 hours. Neurons were then fixed and neuronal health was appraised by analyzing nuclear pyknosis (Figure 2). Interestingly, in basal conditions, neurons under-expressing Mortalin were more pyknotic than control ones and displayed only 63 % of healthy neurons, as compared to 85 % for control neurons and 80% in neurons overexpressing Mortalin. After rotenone treatment, cultures displayed a 25 % drop in healthy neurons, with the notable exception of neurons overexpressing Mortalin, which were protected from rotenone insult. Importantly, this protection was also evidenced when measuring mitochondrial membrane potential, as Mortalin overexpression protected from rotenone-induced mitochondrial failure (Sup Figure 3). Hence, Mortalin expression levels are directly correlated with both neuronal health and the ability to resist to oxidative stress.

Mortalin expression levels drive axonal sensitivity to mitochondrial stress.

Mitochondrial dysfunction and oxidative stress occur early and distally in the pathological process of neurodegeneration. We previously showed that mitochondrial physiology strongly gates axonal vulnerability towards direct axonal insults and that high doses of rotenone are needed to trigger direct axonal degeneration (Lassus et al 2016). Thus, we addressed the influence of Mortalin expression on axonal integrity and sensitivity to oxidative stress, using rat primary cortical neurons grown in microfluidic devices and transduced with LV-Mort, LV-shMortalin or LV-shScr. Mature and transduced neurons were then subjected (or not) to a strong treatment with 1 μ M rotenone in low glucose medium, applied in the axonal chamber for 16 hours. Indeed, we previously showed that somas and axons behave differently when treated

with rotenone: this treatment applied in the axonal chamber induces axonal fragmentation through mitochondrial dysfunction, without any overt sign of apoptosis in the somatic chamber (Kilinc et al., 2011; Magnifico et al., 2013; Szelechowski et al., 2014). In contrast, the same treatment applied in the somatic chamber induces massive apoptosis of the whole culture and the axons appear completely fragmented (index of fragmentation reaching about 0.5). After 16 hours of axonal treatment, neurons were fixed and stained for DAPI and β III-tubulin, to evaluate, respectively, nuclear and axonal integrity (Figure 3A). Consistent with our previous findings, axons of non-transduced neurons presented severe fragmentation after rotenone treatment (mean fragmentation index of 0.31 as compared to 0.06 for untreated, Figure 3B). Interestingly, neurons under-expressing Mortalin exhibited strong baseline levels of axonal fragmentation (0.36), which was further enhanced after rotenone treatment (0.52), reaching levels observed when rotenone was added directly in the somatic chamber. In sharp contrast, neurons over-expressing Mortalin were almost completely protected from rotenone insult (Figure 3), since they presented a very slight and non-significant increase of fragmentation when treated with rotenone (0.09 *versus* 0.07 without rotenone). Hence, Mortalin expression levels in neurons have a direct impact on the axonal sensitivity to mitochondrial oxidative stress.

Mitochondria morphology and dynamics are modulated by Mortalin expression levels in neurons.

Previously, Park *et al.* documented that Mortalin downregulation exacerbates mitochondrial fragmentation induced by A β (1–42) (Park et al., 2014). We also showed that mitochondrial fragmentation was correlated with axonal vulnerability and the ability to spread “death signals” (Lassus et al., 2016). We therefore assessed the impact of modulating Mortalin expression levels on mitochondrial morphology, as well as on rotenone-induced mitochondrial fission in axons. To this aim, we performed immunofluorescence assays with Tom20, to reveal the

mitochondrial network of primary cortical neurons transduced with LV-Mortalin or LV-shMortalin (Figure 4A) and measured the lengths of mitochondria within neurites (Figure 4B). In untreated neurons, mitochondrial length was unevenly distributed in three categories, from short ($< 2 \mu\text{m}$, 60 % of total network), intermediate (2-6 μm , 35 %) and long ($> 6 \mu\text{m}$, 5 %) mitochondria. Interestingly, Mortalin down-regulation enhanced the proportion of short mitochondria (70 %) to the detriment of intermediate and long mitochondria (respectively, 29% and 1%). Conversely, over-expression of Mortalin had the opposite effect, with a decrease of shorter and an increase of longer mitochondria (38%, 39% and 23% for short, intermediate and long mitochondria, respectively). This increased filamentation of the mitochondrial network in neurons over-expressing Mortalin was kept under oxidative stress conditions (Sup Figure 4), suggesting a crucial role of Mortalin on mitochondrial morphology in degenerative neurons. Mitochondria morphology is controlled by the activity of different GTPases. Among them, Drp1 (Dynamin Related Protein 1) drives mitochondrial fission (Pitts et al., 1999). We thus wondered whether Mortalin control of mitochondrial elongation could result from changes in the molecular processes regulating mitochondrial Drp1 activity. In particular, the fission activity of Drp1 depends on its phosphorylation status, phosphorylation on serine 616 being associated with enhanced mitochondrial fission (Taguchi et al., 2007). Interestingly, we observed that the S616 phosphorylated form of Drp1 was strongly reduced in neurons over-expressing Mortalin (-35 % compared to control). Conversely, Drp1 S616 phosphorylation was enhanced (+ 25 %) in neurons expressing reduced Mortalin levels (Figure 5A and B).

DISCUSSION

Although it has been documented that Mortalin levels are reduced in the degenerative brain of PD patients (Cook et al., 2015), this observation has not been linked to date with any genetic risk with degenerative diseases (Chung et al., 2017; De Mena et al., 2009). Thus, the

interplay between this mitochondrial chaperone and the neuronal degeneration process remains largely unknown. In the present work, we show that Mortalin expression levels in neurons are directly correlated with the control of mitochondrial stress, through the modulation of axonal vulnerability. These observations corroborate the link between the oxidative stress management mediated by Mortalin, already described in non-neuronal cells (Qu et al., 2011), as well as its down-regulation in patients' brains.

Mechanistically, we demonstrate that Mortalin over-expression is axoprotective and that this property correlates with its ability to increase mitochondrial length, by interfering with the activation of the mitochondrial fission protein Drp1. This observation is in line with the recent correlation between Mortalin up-regulation and inhibition of Fis1, a Drp1 mitochondrial receptor, upon antioxidant treatment of hepatocytes (Liu et al., 2017). Our data also further document the fundamental importance of mitochondrial dynamics in axons at the early stages of neurodegeneration (Annweiler et al., 2014; Kim et al., 2015; Lassus et al., 2016; Magnifico et al., 2013; Szelechowski et al., 2014). It should, however, be reminded that mitochondrial dynamics is also required for essential processes regulating cellular physiology, particularly in neurons, such as mitochondrial transport or quality control through mitophagy, as well as synaptic activity. More specifically, fission is thought to be an adaptive response to axonal injury and reduced mitochondria fission may impair mitochondrial targeting to the nerve terminals, thereby causing degeneration of specific neuronal populations (Berthet et al., 2014; Kiryu-Seo et al., 2016). Finally, although it is established that Mortalin over-expression is protective, its expression is not physiologically boosted in degenerative neurons. Hence, the modalities by which Mortalin-induced over-filamentation drives protection against axonal insults might underlie complex molecular processes that remain to be determined.

In degenerative neurons, mitochondrial functional and morphological impairments are associated with increased levels of ROS, inducing an oxidative stress situation that affects

nucleic acid stability, protein homeostasis and, ultimately, the functionality of ion channels and cellular defenses (Mancuso et al., 2010; Zhao et al., 2017). These events might be of critical importance in the progress of neurodegenerative pathologies. Mortalin was identified as one of the most oxidation-sensitive cellular proteins (Choi et al., 2004). Under conditions of oxidative stress, Mortalin is able to activate cellular antioxidant processes, mainly through sequestration of DJ-1 within mitochondria (Tai-Nagara et al., 2014). Moreover, Mortalin is found in multiple subcellular localizations such as the endoplasmic reticulum (ER), cytosol, cytoplasmic vesicles and mitochondria (Ran et al., 2000), where it interferes with different cellular pathways through binding to a wide range of specific protein partners (for review (Flachbartova and Kovacech, 2013). The majority of cellular Mortalin is, however, located within the mitochondrial matrix (Burbulla et al., 2010).

Finally, Mortalin - rather known as Grp75 for this function - also plays a crucial role by tethering mitochondria and ER, by connecting proteins from the ER membrane (IP3R) and the mitochondrial outer membrane (VDAC) at contact membrane locations called MAMs (mitochondria-associated membranes). MAMs structure and function are altered in several neurodegenerative pathologies such as PD, concomitantly with Mortalin down-regulation (Rodriguez-Arribas et al., 2016). Interestingly, MAMs functions and mitochondrial dynamics are closely related. In particular, Drp1-dependent mitochondrial fission occurs preferentially in the proximity of ER-mitochondria contacts (Friedman et al., 2011; Murley et al., 2013), while Ca^{2+} released from the MAMs activates Drp1, thereby causing mitochondria fission (Jeyaraju et al., 2009). Hence, our observations regarding the impact of Mortalin on Drp1 activation may further enlighten the decisive crosstalk between ER and mitochondria for proper management of stress, or - in case of prolonged or strong stress conditions - the initiation of neurodegeneration. To date, however, the involvement of MAMs in the axonal compartment and their precise role during the early stages of degeneration remains unclear. Further

examination of the interplay between mitochondrial dynamics and ER-mitochondria crosstalk in the axonal compartment may provide new insights for a better understanding of the degenerative process and, hopefully, for the development of efficient therapeutics against neurodegeneration.

METHODS

Primary cultures of cortical neurons

Pregnant Sprague-Dawley female rats were purchased from Janvier Labs. Prior to sacrifice, housing and care of rats was performed in a registered facility (agreement number: E3155508, renewed on Oct 24, 2019), in accordance with the European Union Council Directive 86/609/EEC. Our primary neuronal protocol received approval from the local ministry-approved committee on the ethics of animal experiments (CEEA-122; permit number: PI-U1043-DD-10). Primary cortical neurons were isolated from embryos at gestational day 17 by the papain dissociation method, as described previously (Ferre et al., 2016; Szelechowski et al., 2014). Before seeding, supports were coated with 500 µg/ml Poly-L-ornithine (Sigma), followed by 5 µg/ml laminin (Roche). Neurons were maintained in Neurobasal medium (Gibco) supplemented with 100 µg/ml penicillin/streptomycin, 2 mM glutamine, 2 % B-27 supplement (Gibco). Depending on the experiment, neurons were seeded in 24-wells plates, either directly (fluorometric analyses) or on 12 mm diameter glass coverslips for immunofluorescence (100,000 cells/well), in 60 mm plastic culture dishes for biochemistry ($1.5 \cdot 10^6$ cells/dish) or in microfluidic-based culture devices.

Microfluidic chips were prepared as described before (Ferre et al., 2016; Kilinc et al., 2011; Magnifico et al., 2013; Szelechowski et al., 2014), by molding polydimethylsiloxane (Sylgard 184, Dow Corning) into resin SU-8 silicon masters having a complementary positive pattern of the cell culture compartments and microchannels. Polymer prints and glass coverslips were

treated for 1 min with an air plasma generator, then both elements were bonded together and coated with Poly-L-ornithine and laminin. Primary neuronal cell suspensions were seeded in the somatic chambers by introducing 3 μl of a 5×10^7 cells/ml suspension in the upper reservoir, before filling both reservoirs with supplemented neuronal culture medium. A 10 μl differential medium volume was maintained between the somatic and axonal chambers, to ensure a permanent hydrostatic flux. Neurons were transduced with lentivectors (LV) 3 days after plating.

Construction and Production of LV

To overexpress Mortalin, the coding sequence of rat Mortalin was obtained by extracting mRNAs from neurons, followed by specific reverse transcription and PCR amplification. The PCR product was sequenced and inserted into the pTrip vector (kind gift from P. Charneau, Pasteur Institute, Paris) using *Bam*HI and *Xho*I restriction sites, downstream of the constitutive cytomegalovirus enhancer/chicken β -actin (CAG) promoter. To downregulate Mortalin expression, LV plasmids driving the expression of 29-mer short hairpin RNAs (shRNA) specific for Mortalin, or a scramble control sequence, together with GFP were purchased from Origene (CAT# TL704140). All LVs were produced and purified as described previously (Ferre et al., 2016; Szelechowski et al., 2014). Briefly, 10 T150 flasks were plated with 1.2×10^7 HEK-293T cells each, to reach 80 % confluence the following day. Then, HEK-293T cells were transfected with the packaging plasmids psPAX2 and pMD2.G (both obtained from Addgene), together with LV genomic plasmid (respectively, 14.6 μg , 7.9 μg , and 22.5 μg of endotoxin-free prepared plasmids mixed with 100 μL of GeneJuice (Merck) for each T150). Media containing lentiviral particles were harvested in serum-free OptiMEM (Gibco) 48 h later, cleared by low-speed centrifugation (150 g, 5 min at 4 $^{\circ}\text{C}$), and filtered through 45- μm capped syringe filters. LVs were lastly purified by ultracentrifugation through a 20 % sucrose cushion (80,000 g, 2 h, 4 $^{\circ}\text{C}$; SW32Ti rotor; Beckman Coulter) and lentiviral pellets were resuspended

in ice-cold PBS, pooled, and aliquoted in 10 μ L samples stored at -80°C . LV concentrations were systematically evaluated after transduction of HEK-293T cells with serial dilutions, by quantification of Mortalin levels by Western blot. LV-shRNA titers were also determined by counting GFP foci 72 h after transduction. We then used the LV suspension amounts necessary to either double or decrease by 50 % the expression levels of Mortalin, relative to endogenous levels in non-transduced neurons.

Mitochondria isolation for Western blotting

We performed sub-cellular fractionation to obtain mitochondria-enriched fractions. To this aim, 2.10^7 rat primary cortical neurons were pelleted, rinsed twice in PBS and resuspended in homogenization buffer (HB: 10 mM Tris-HCl, pH7.5, 2 mM MgCl₂, 10 mM KCl, 250 mM Sucrose, Protease Inhibitor cocktail, 0.5 mM DTT). Neurons were then frozen at -80°C and thawed, transferred to a 2 ml Potter homogenizer, triturated for 20 times and further centrifuged at 1,000 g for 10 minutes. The supernatant was then centrifuged at 12,000 g for 15 minutes, afterwards the pellet, containing mitochondria, was resuspended in HB + 1 % Triton X-100 for protein quantification by Western Blot.

Western blotting

For protein quantification, we proceeded as described before (Ferré et al., 2016). Indeed, protein concentrations were determined with a standard Bradford assay and samples were boiled for 10 min in Laemmli buffer. A total of 15 μ g of samples were loaded on 4–15 % Tris-glycine gels (Bio-Rad), run 1 h at 100 V, transferred onto nitrocellulose membranes and blocked for 1 h in Odyssey blocking buffer diluted 1:1 in Tris buffer saline (TBS). After blocking, membranes were incubated with primary antibodies diluted in blocking buffer, overnight at 4°C [rabbit polyclonal anti-Mortalin (Origene Technologies, 1/5,000); mouse monoclonal anti- β -actin, (Sigma-Aldrich, 1/100,000); mouse monoclonal anti-Hsp60 antibody (Santa Cruz Biotechnology, 1/1000)]. Membranes were then washed extensively in Tween-20 containing

TBS (TBST) and incubated for 1h at RT with secondary antibodies: goat anti-rabbit Alexa 680 and goat anti-mouse Alexa 770 (Biotium, 1/15,000) both diluted in Odyssey blocking buffer (1:1 v/v TBS). Laser scanning and analyses of the blots were performed using the Odyssey Infrared Imaging System (Li-Cor).

Immunofluorescence and imaging

Neurons were fixed with PBS containing 4 % paraformaldehyde preheated to 37 °C, for 20 min at room temperature before being stained as described earlier (Ferré et al., 2016). To this aim, cells were then permeabilized with PBS containing 0.25 % Triton X-100 for 5 min, rinsed twice with PBS, and blocked for at least 1 h with PBS containing 2.5 % normal goat serum and 3 % BSA. The cells were subsequently incubated for 2 h at 37 °C with primary antibodies diluted in blocking solution [anti- β III-tubulin (Sigma-Aldrich, 1:500); anti-translocase of outer mitochondrial membrane 20 (Tom20, Santa Cruz Biotechnology, 1:500), anti-cyclin B1 (Thermo Fisher Scientific clone V152, 1:200), anti-phospho-DRP1^{S616} (Cell Signaling Technologies, 1:1,000)], rinsed twice for 10 min each in PBS containing 0.1 % Triton X-100 and once in PBS, and then incubated 45 min at room temperature with Alexa-conjugated fluorescent secondary antibodies (Sigma-Aldrich, 1:500 dilution in PBS). After several washes in PBS, the cells were mounted in Vectashield medium containing DAPI (Vector Laboratories). All microscopy analyses and measurements were performed on an inverted SP8 confocal microscope (Leica Microsystems) fitted with x20 (microfluidics) or x63 (mitochondria and DRP1) objectives. For pyknosis analysis, the DAPI staining was considered in β III-tubulin⁺ cells. For each culture, a minimum of 100 nuclei were observed and assessed as healthy or pyknotic according to their morphology. For phospho-DRP1^{S616} quantifications, whole-cell fluorescence intensity was measured in β III-tubulin⁺ cells, using the integrative fluorescence intensity tool of the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Induction of oxidative stress

Neuronal cultures at 12 days *in vitro* (DIV12) were subjected to oxidative stress by adding different concentrations of the respiratory chain complex I inhibitor rotenone (Sigma-Aldrich), diluted in DMEM containing 1 g/L glucose (Gibco), supplemented with 2 mM glutamine, 1% penicillin/streptomycin, 2% B-27, 1% N2 supplements (Gibco). Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma, 100 μ M) was used as a positive control of mitochondrial respiration failure.

Analysis of axonal fragmentation of primary neurons in microfluidic-based culture devices

To assess the specific impact of oxidative stress on axons, we evaluated axonal fragmentation as previously described (Ferre et al., 2016; Szelechowski et al., 2014). Briefly, rotenone (1 μ M) diluted in low-glucose medium was applied in the somatic or axonal chambers (for, respectively, somatic or axonal stress) of neuronal cultures (DIV12), for 16 hours. Neurons were then fixed and stained for β III-Tubulin. The minor fraction of cultures displaying more than 25 % of pyknotic nuclei was discarded. The numbers of Tubulin spots were counted in 4 randomly-chosen fields in each axonal chamber using Imaris software (Bitplane), while total β III-Tubulin staining was assessed using the ImageJ software for each picture, thereby defining a fragmentation index. Staining with DAPI was systematically performed in the somatic chambers to check for nuclear integrity and ensure that the hydrostatic flux had been maintained to prevent leakage throughout axonal rotenone treatment.

Statistical analyses

Results are presented as means \pm SD. Data were analyzed by Mann-Whitney or 1-way/2-way ANOVA using *U*, Sidak's or Kruskal Wallis and Dunn's multiple comparisons as non-parametric *post hoc* tests with Prism software (GraphPad).

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AUTHOR CONTRIBUTIONS

Conceived and supervised the experiments: MS, DGD; Performed and analyzed experiments: CF, AT, AB, PB, MS, DGD; Designed and prepared microfluidic culture chambers: JMP, MCM; Wrote the paper: MS, DGD. All authors reviewed the manuscript and approved its final version.

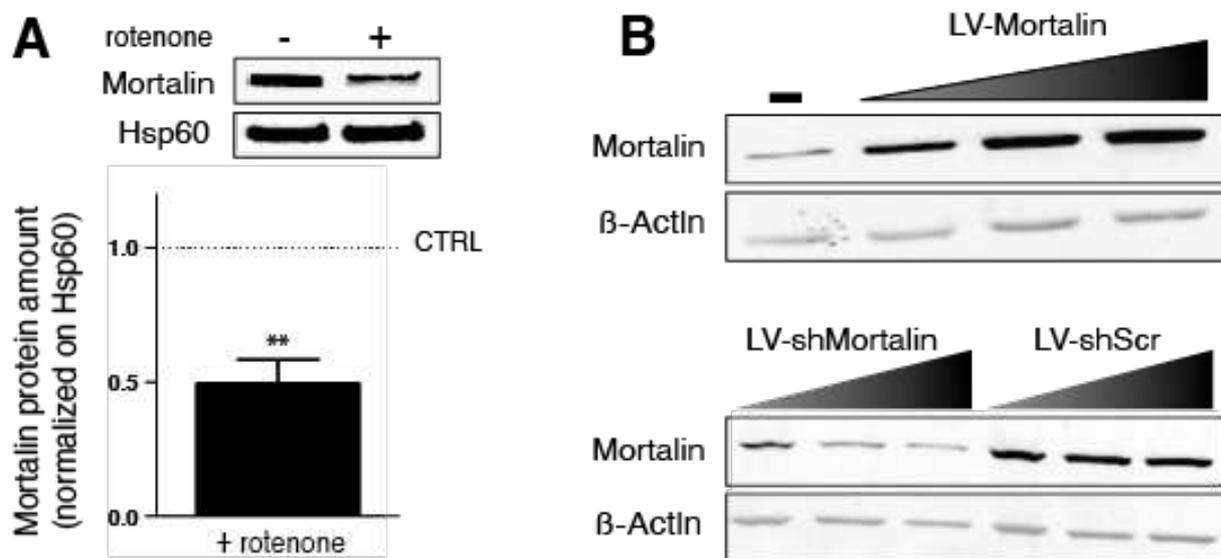


Figure 1: Mortalin is down-regulated in neurons upon oxidative stress.

(A) Rat embryonic primary cortical neurons (DIV 12) were treated or not with 10 nM rotenone (+ rotenone) for 4 hours and protein extracts were prepared from mitochondria-enriched fractions. Mortalin amounts were assessed by Western blot, using the mitochondrial chaperone Hsp60 or β -Actin as loading controls. The graph represents the normalized ratio relative to untreated neurons and is expressed as the mean \pm SD of 4 independent experiments. (B) Expression levels of Mortalin were modulated by transduction with lentiviral vectors (LV). Overexpression was achieved with a LV bearing an expression cassette for the rat Mortalin gene. Mortalin down-regulation was driven by cell transduction with a lentiviral vector expressing a specific shRNA directed against Mortalin (shMortalin), or a control scramble sequence (shScr, Origene TR704140, clone A). Different multiplicities of transduction (1, 2, 4) were used to test the percentage of over- or under-expression.

WB are presented as cropped parts of full blots displayed as supplemental information.

DIV: days *in vitro*. ** $p < 0.01$, by Mann-Whitney non parametric t-test.

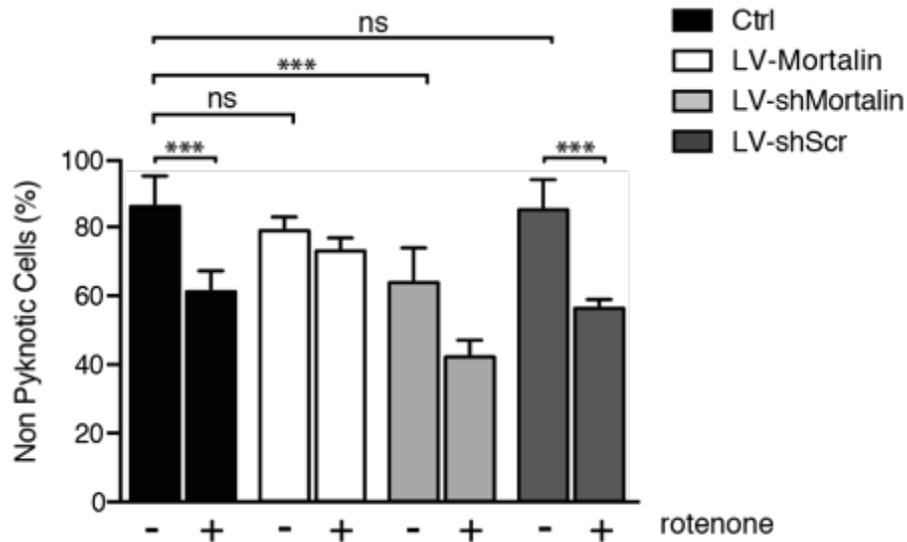


Figure 2: Mortalin levels control neuronal survival against oxidative stress.

Rat embryonic primary cortical neurons were transduced on DIV 3 with lentiviral vectors (LV) to induce over- (+100 %, LV-Mortalin) or under- (-50 %, LV-shMortalin) expression of Mortalin. A lentiviral vector expressing a shRNA with a scramble sequence was used as a control (LV-shScr). On DIV 11, neurons were treated or not with 10 nM rotenone for 24 hours and then fixed on DIV 12. Percentages of cells showing pyknosis (revealed by DAPI staining) are presented as means \pm SD of 4 independent experiments.

**** $p < 10^{-4}$, by 1-way ANOVA with Sidak's multiple comparison *post-hoc* test.

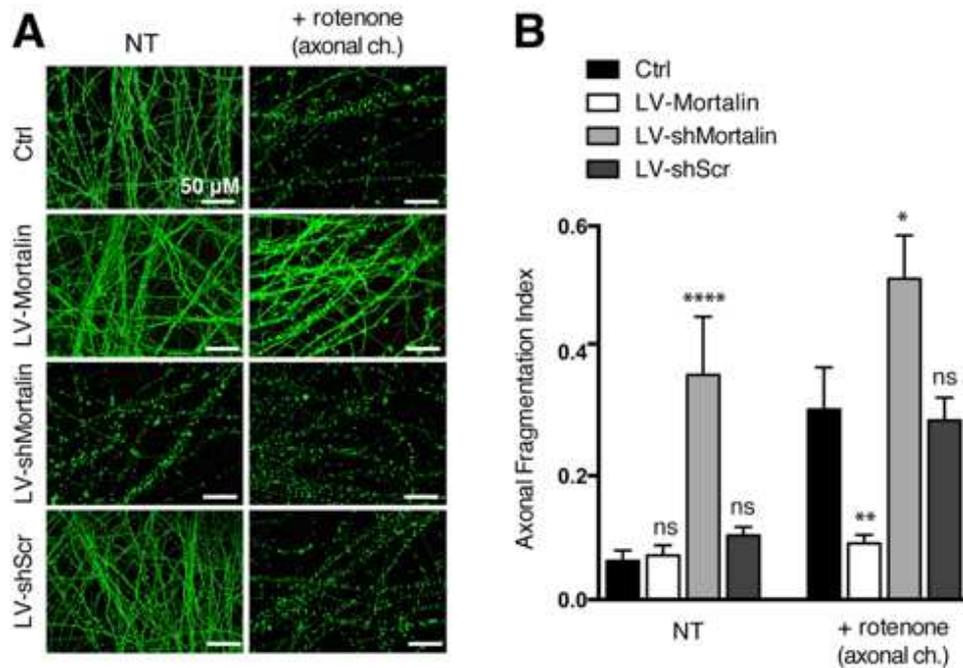


Figure 3: Mortalin expression controls axonal fate against a distal oxidative stress insult.

Rat embryonic primary cortical neurons were grown in microfluidic devices for 12 DIV. Neurons were transduced on DIV 3 with lentiviral vectors (LV) to induce over- (LV-Mortalin) or under- (LV-shMortalin) expression of Mortalin. A lentiviral vector expressing a shRNA with scramble nucleotide sequence was used as a control (LV-shScr). On DIV 11, neurons were treated or not with 1 μ M rotenone applied in the axonal chamber (+ rotenone) for 24 hours and fixed on DIV 12. (A) Pictures of β III-tubulin staining were randomly taken within axonal chambers (4 by condition) and the axonal fragmentation indexes were measured for each picture as the number of axonal dots per unit of tubulin staining. (B) Results are presented as means \pm SD of 4 to 10 independent microfluidic devices, from 4 independent neuronal cultures.

* $p < 0.05$, ** $p < 0.01$, **** $p < 10^{-4}$, by 1-way ANOVA with Sidak's multiple comparison *post-hoc* test.

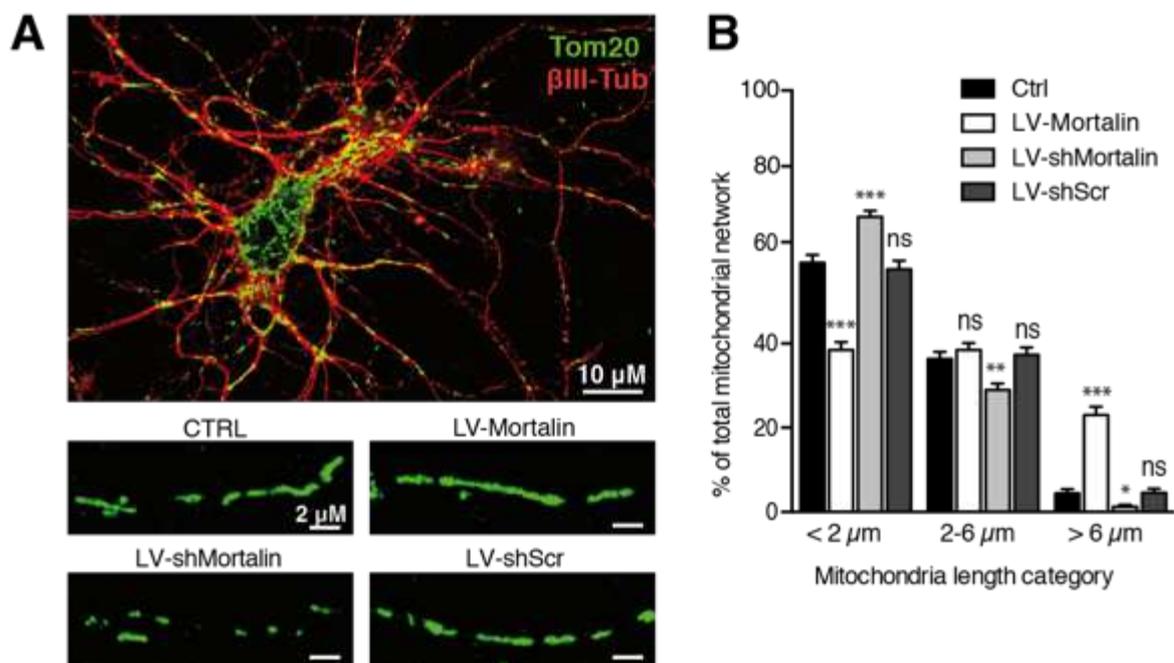


Figure 4: Mortalin modulates mitochondrial morphology in neuronal extensions.

Rat embryonic primary cortical neurons were transduced on DIV 3 with lentiviral vectors (LV) to induce over- (LV-Mortalin) or under- (-LV-shMortalin) expression of Mortalin. A lentiviral vector expressing a shRNA with a scramble sequence was used as a control (LV-shScr). (A) Neurons were fixed on DIV 12 and both neuronal and mitochondrial networks were revealed with, respectively, Tom20 and β III-tubulin (β III-Tub) staining. Pictures were randomly taken and mitochondrial lengths were measured in all neuronal extensions. (B) For each neuron, the sum of the lengths of all measured mitochondria was calculated (mitochondrial network), within which the relative proportions of short (<2 μ m), medium (2-6 μ m) and long (>6 μ m) mitochondria were considered. The graph represents means \pm SD of 18 to 40 neuronal mitochondrial networks, from 4 independent neuronal preparations.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by 2-way ANOVA, Kruskal Wallis test and Dunn's multiple comparisons as *post-hoc* tests.

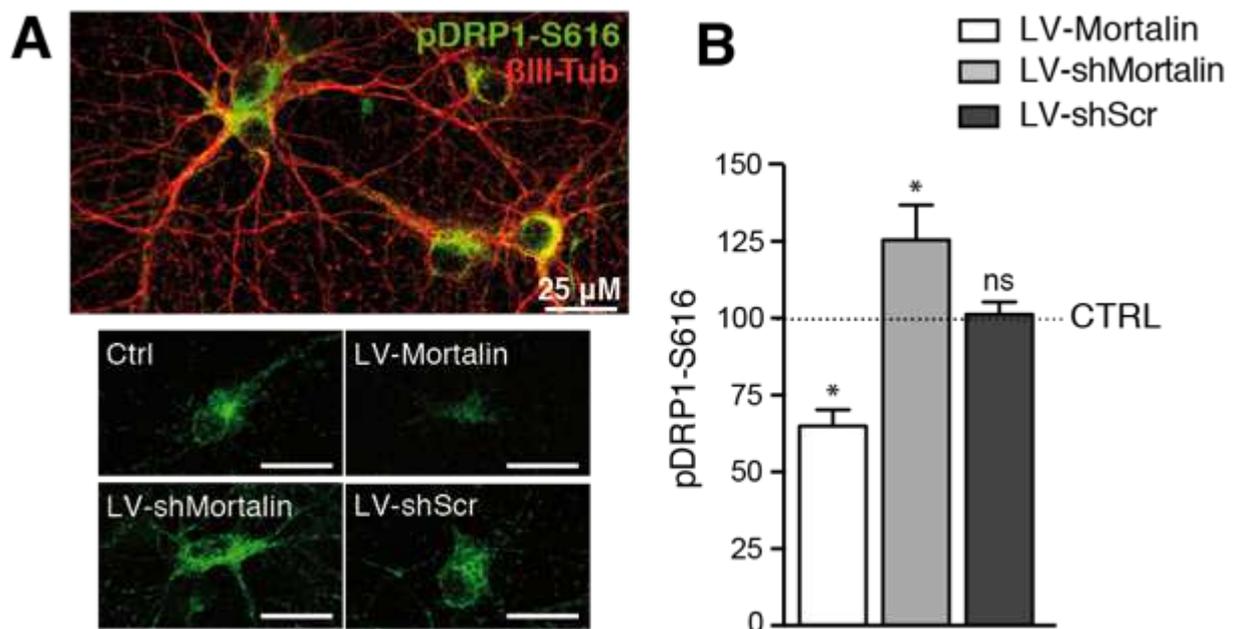


Figure 5: Mortalin impacts mitochondrial morphology by modulating Drp1-S⁶¹⁶ phosphorylation.

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** p<0.01, by Mann-Whitney non parametric test.

Figures

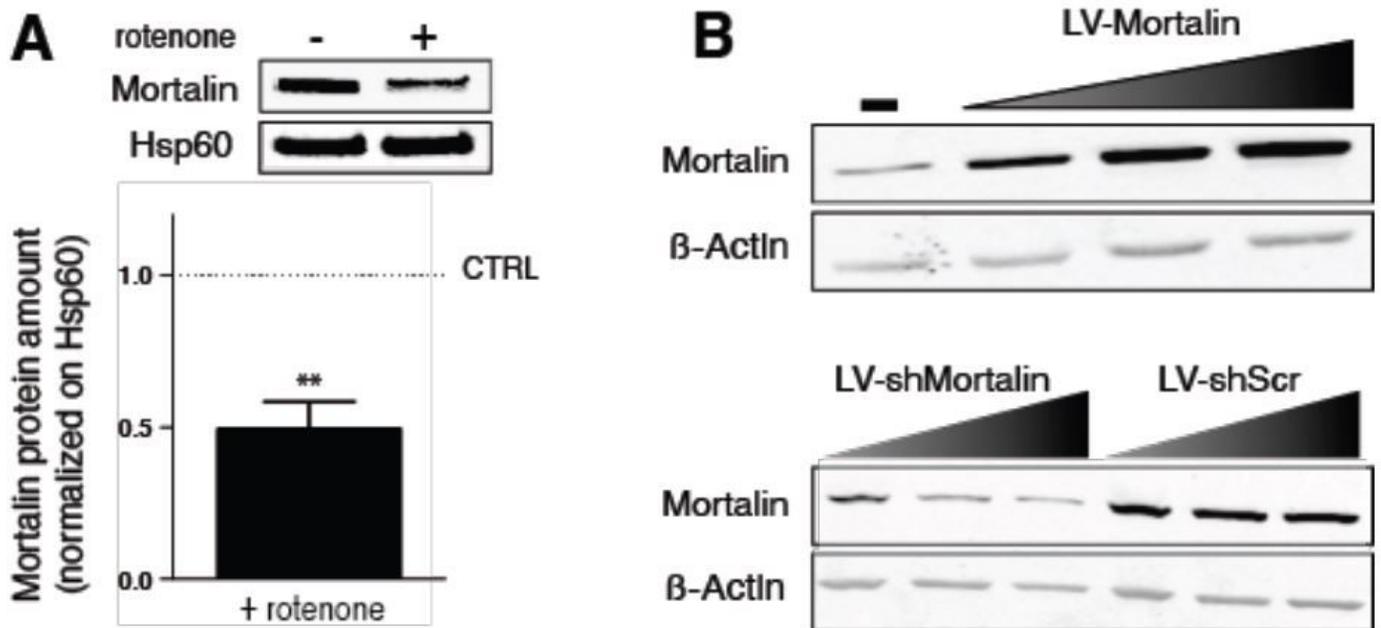


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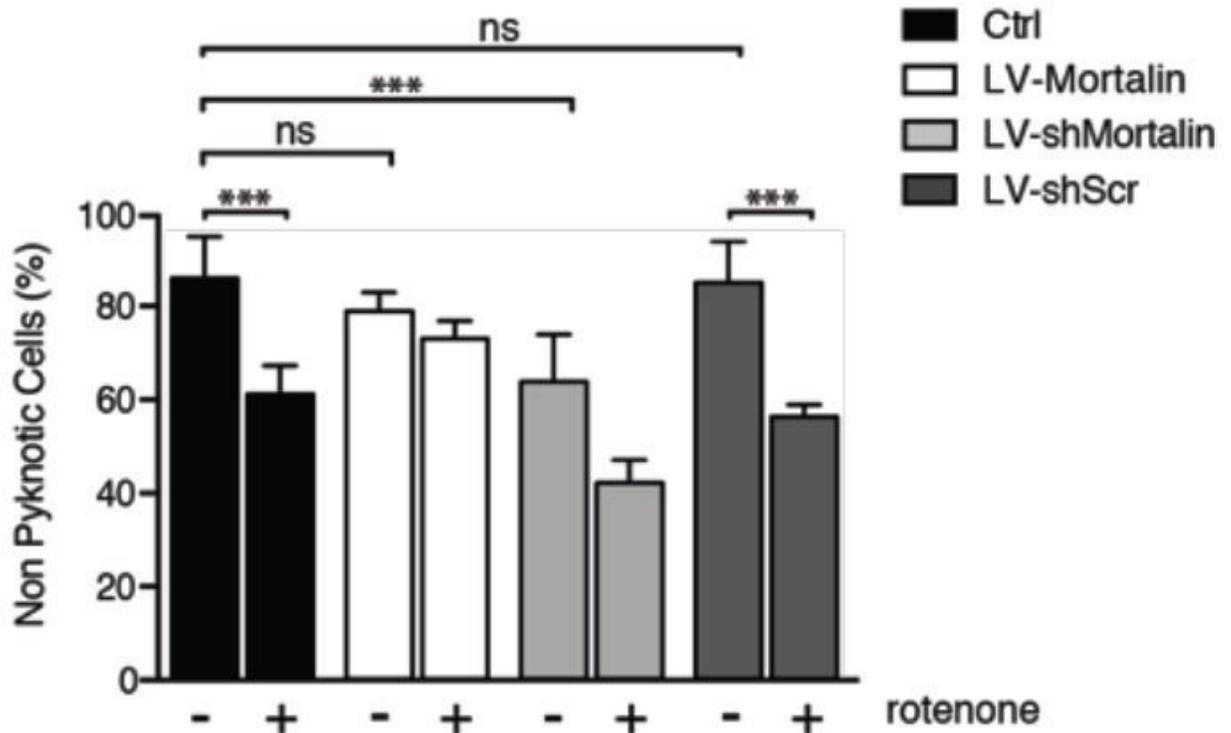


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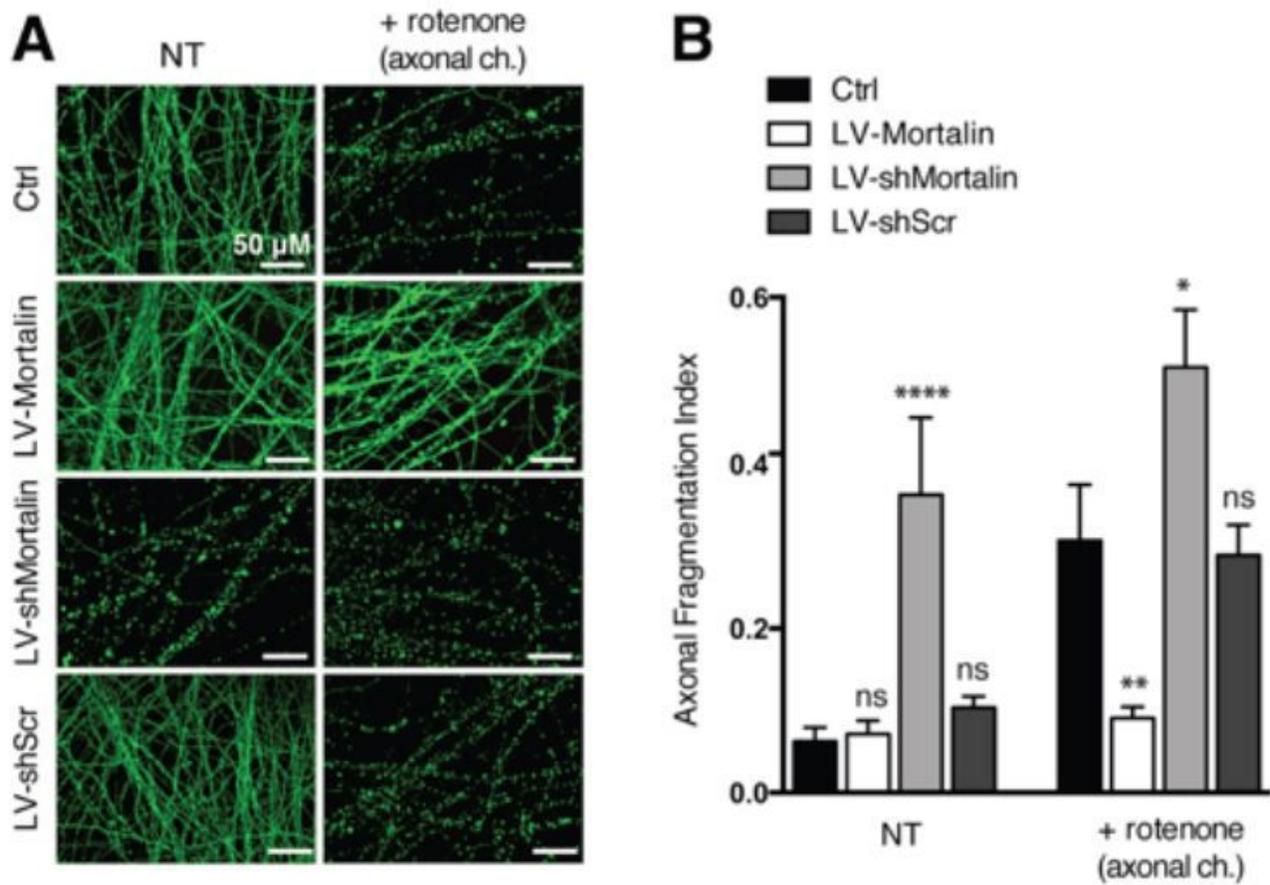


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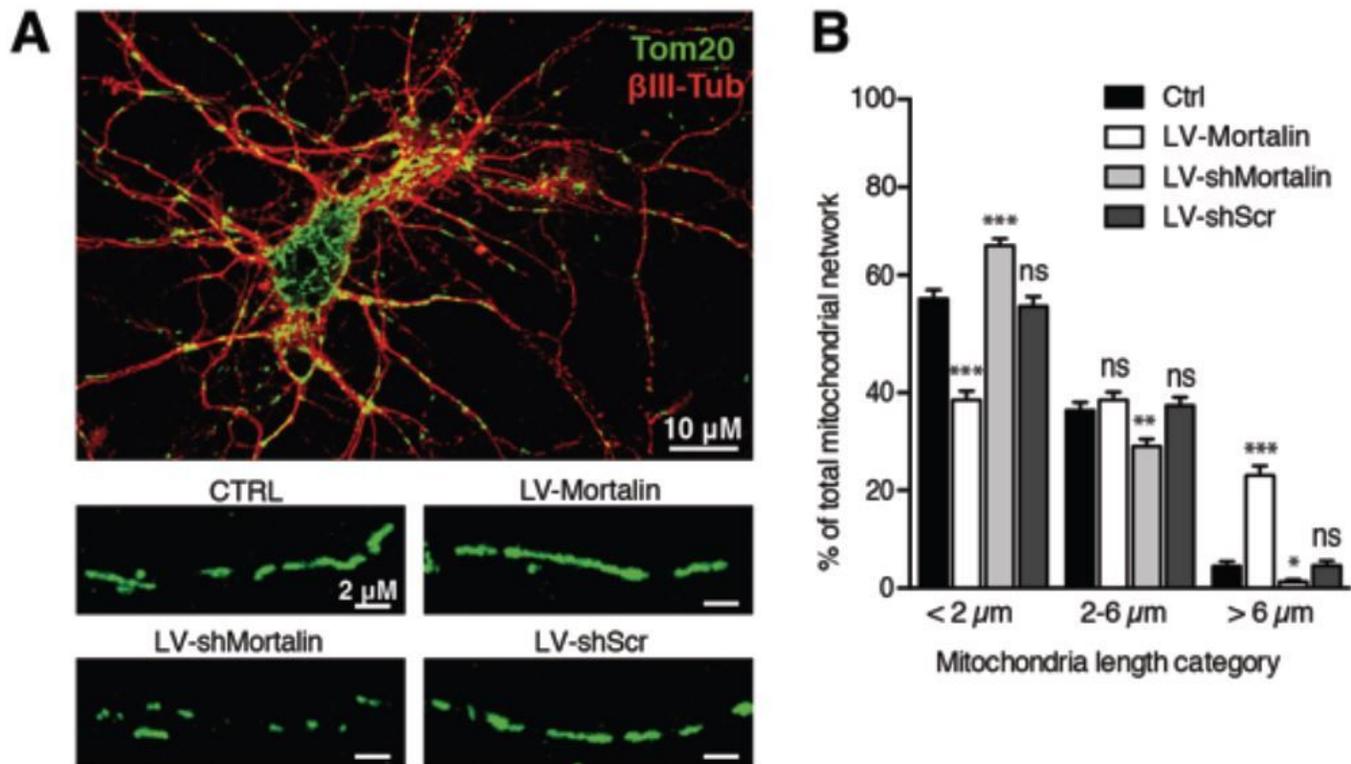


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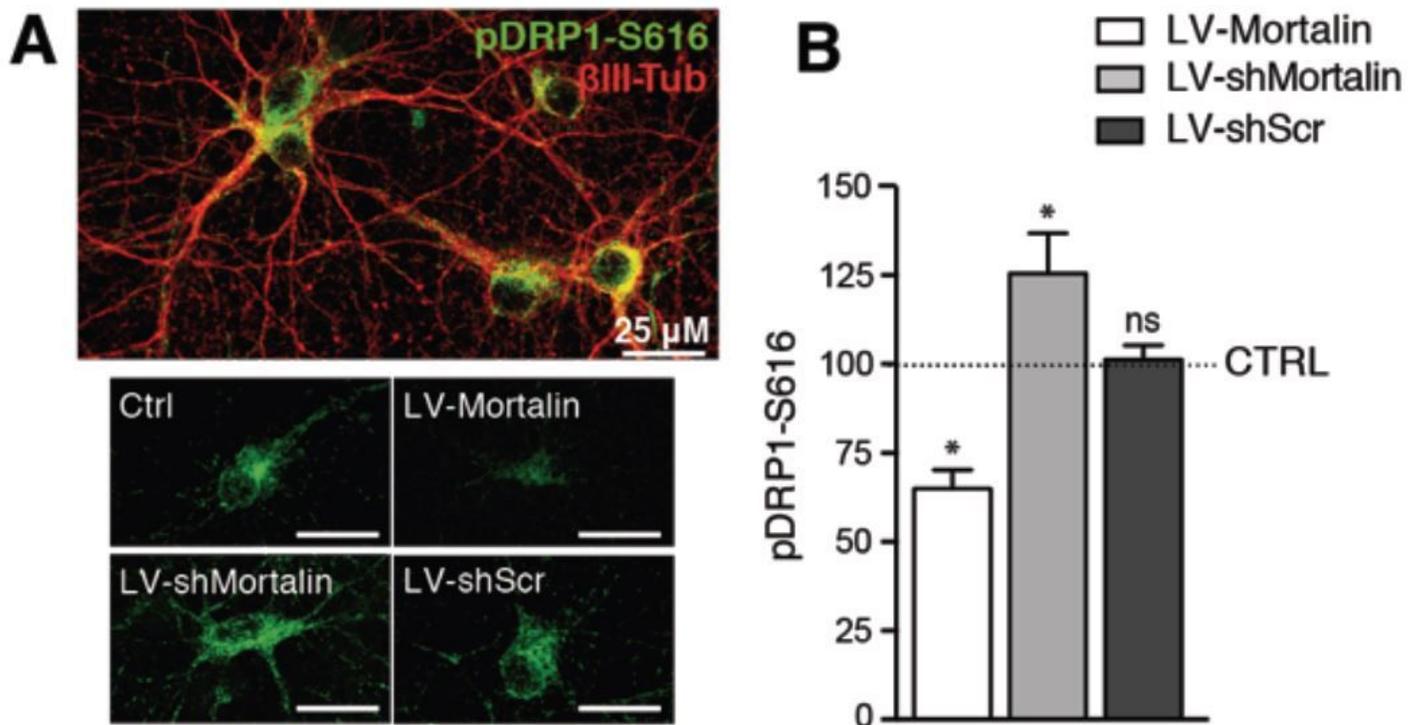


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