Mitochondrial fragmentation in excitotoxicity requires ROCK activation

Alejandro Martorell-Riera, Marc Segarra-Mondejar, Manuel Reina, Ofelia M. Martínez-Estrada, Francesc X. Soriano.

Department of Cell Biology and CELLTEC-UB. Faculty of Biology, University of Barcelona, Av. Diagonal, 643, 08028 Barcelona, Spain.

Correspondence to: Francesc X. Soriano, Department of Cell Biology, Faculty of Biology, University of Barcelona, Av. Diagonal, 643, 08028 Barcelona, Spain Tel. +34 934029046, Fax +34934034607. f.x.soriano@ub.edu

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Abstract.

Mitochondria morphology constantly changes through fission and fusion processes that regulate mitochondrial function, and it therefore mitochondrial dynamics plays a prominent role in cellular homeostasis. Cell death progression is associated with mitochondrial fission. Fission is mediated by the mainly cytoplasmic Drp1, which is activated by different post-translational modifications and recruited to mitochondria to perform its function. Our research and other studies have shown that in the early moments of excitotoxic insult Drp1 must be nitrosylated to mediate mitochondrial fragmentation in neurons. Nonetheless, mitochondrial fission is a multistep process in which filamentous actin assembly/disassembly and myosin-mediated mitochondrial constriction play prominent roles. Here we establish that in addition to nitric oxide production, excitotoxicity-induced mitochondrial fragmentation also requires activation of the actomyosin regulator ROCK. Although ROCK1 has been shown to phosphorylate and activate Drp1, experiments using phosphor-mutant forms of Drp1 in primary cortical neurons indicate that in excitotoxic conditions, ROCK does not act directly on Drp1 to mediate fission, but may act on the actomyosin complex. Thus, these data indicate that a wider range of signaling pathways than those that target Drp1 are amenable to be inhibited to prevent mitochondrial fragmentation as therapeutic option.

Introduction

Mitochondria are dynamic organelles that continually fuse and divide. The balance between fusion and fission determines mitochondrial morphology, which has important roles in different aspects of cell health, including regulation of metabolism, protection against oxidative stress, calcium homeostasis and cell death [1-6]. The mitochondrial fusion/fission machinery is formed by four proteins of the family of large GTPases [7]. Three proteins mediate mitochondrial fusion: two mitofusins, Mfn1 and Mfn2, which mediate outer mitochondrial membrane (OMM) fusion; and Opa1 which mediates inner mitochondrial membrane (IMM) fusion. Drp1 is the only reported pro-fission GTPase and it mediates both OMM and IMM fission. Drp1 is largely cytosolic, but when activated by different post-translational signals, including phosphophorylation, nitrosylation and SUMOylation, it is recruited to mitochondria where it mediates fission [7].

Although many questions remain unanswered, more detail about the mitochondrial fission mechanism has been revealed in recent years. Mitochondrial division events have been reported to occur predominantly at sites of contact between ER and mitochondria. Evidence suggests that ER wrap around the mitochondria to mark sites of mitochondrial division [8]. Actin polymerizes in ER-mitochondria contact sites through ER-located formin protein INF2 [9]. Myosin II is recruited to actin filaments and its motor activity mediates contraction of the actin filaments to constrain mitochondria [10]. Pre-constricted mitochondria cause Drp1 to be recruited to mitochondria and to oligomerize into rings around the fission site [11, 12]. GTP hydrolysis causes constriction of the oligomeric ring, which leads to mitochondrial division [13, 14].

Although fission per se is not noxious, cell death is associated with mitochondrial fragmentation [6]. In neurons, overactivation of glutamate receptors leads tocell death in a

process called excitotoxicity. One of the hallmarks of excitotoxicity is mitochondrial fragmentation and dysfunction. We recently reported that mitochondrial fragmentation in excitotoxicity takes place in two phases: a fast, reversible phase mediated by Drp1 and a second delayed, irreversible mitochondrial fragmentation caused by the downregulation of profusion protein Mfn2 as a consequence of the degradation of the transcription factor MEF2, which regulates basal Mfn2 expression in neurons. A reduction in Mfn2 levels has a profound effect on mitochondrial function and facilitates Bax recruitment to mitochondrial a process that enhances delayed excitotoxic death [4].

In line with other reports, we found that the early, fast phase of mitochondrial fragmentation is induced by nitric oxide synthase (NOS)-mediated Drp1 recruitment to mitochondria and activation [4, 15]. Given the complexity of the mitochondrial fission process and the role of different factors, we investigated whether more than one signal could be regulating the early phase of mitochondrial fission in excitotoxicity. We found that Rho-associated coiled-coil containing protein kinase (ROCK) activation, a key regulator of actin cytoskeletal dynamics (for reviews see refs. [16, 17]), is necessary for mitochondrial fission.

Results and discussion

To test the possibility that, in addition to NOS activation, other signaling pathways may regulate the early mitochondrial fragmentation in excitotoxicity, we pretreated primary cultures of cortical neurons with the inhibitors of different known post-translational Drp1 modifiers before inducing excitotoxicity by bath application of NMDA. Calcineurin (Cn) is a calcium-dependent serine/threonine phosphatase that has been reported to induce mitochondrial fragmentation by dephosphorylating Drp1 at Ser-637 to promote its mitochondrial localization and activation [18, 19]. Surprisingly, we found that inhibition of Cn with CsA did not block NMDA mediated mitochondrial fragmentation and expression of a constitutively active CnA significantly reduced mitochondrial fragmentation in excitotoxicity (Fig. 1A). To corroborate these unexpected results, we used Ser-637 mutants of Drp1, non-phosphorylatable Drp1-S637A and phosphomimetic, Drp1-S637D, and in accordance with the lack of effect of the pharmacological and genetic modifiers of the Cn pathway, we found that these mutants did not prevent NMDA mediated mitochondrial fragmentation (Fig. 1B).

The fact that active CnA overexpression reduced excitotoxicity-mediated mitochondrial fragmentation suggests that phosphorylation events may promote mitochondrial fragmentation in excitotoxicity. ROCK1 is a downstream RhoA effector whose activation in podocytes and endothelial cells promotes mitochondrial fragmentation by phosphorylating Drp1 [20]. Given that RhoA is activated in as little as three minutes after activation of the NMDAR [21], we tested whether ROCK could be mediating the early NMDA-induced mitochondrial fragmentation. We found that two unrelated ROCK inhibitors, fasudil and Y-27632 [22], also reduced NMDA mediated mitochondrial fragmentation (Fig. 2A and B). Our research and other studies have shown that activation of NOS blocks excitotoxicity-induced mitochondrial fragmentation [4, 15].

Thus, we combined NOS inhibitor 7-nitroindazole and ROCK inhibitor Y-27632 to test whether these two pathways could act synergically, but there was no additional protection against mitochondrial fragmentation when the two inhibitors were combined (Fig. 2C and D), which would suggest that these inhibitors are acting at different steps of the same pathway.

Intriguingly, ROCK1 mediates mitochondrial fission by phosphorylating Drp1 at human Ser-637 (mouse isoform b Ser-600), but the use of the Ser-637 phospho-mutants of Drp1 indicates that phosphorylation of this residue is not necessary for excitotoxicity-dependent mitochondrial fragmentation (Fig. 1B). Nevertheless, fasudil and Y-27632 inhibit both ROCK isoforms, ROCK1 and ROCK2, and although ROCK1 is ubiquitously expressed in adult mice, it shows lower expression levels in the brain than other tissues whereas ROCK2 shows higher expression levels in the brain [22]. Thus, it cannot be ruled out that in excitotoxicity ROCK2 is preferentially activated and that ROCK2 cannot phosphorilate Drp1. But this requires further investigations.

Next, we tested other two Drp1 forms with described phosphorylatable residues mutated, Drp1-S579A (corresponding to human Drp1-Ser616; [23]) and Drp1-S693A [24] and no protection against excitotoxicity-mediated mitochondrial fragmentation was observed (not shown). To test the possibility that ROCK phosphorylated Drp1 in a new, undescribed residue, we immunoprecipitated with a phosphor-Ser antibody followed by western blot with anti Drp1, but no changes were detected after the NMDA treatment (not shown). Despite these negative results, we cannot rule out the possibility that there is a new phosphorylation site in Drp1 regulated by ROCK that was not detected by the method used. Taken as a whole, the evidence suggests that ROCK was not acting directly on Drp1.

Mitochondrial fission is a multistep process, and mounting evidence indicates that actin cytoskeleton dynamics have a key role in the mitochondrial fission process [9, 25-27]. The role of actin cytoskeleton dynamics in excitotoxicity-induced mitochondrial fragmentation was demonstrated by the diminished mitochondrial fission in neurons pre-treated with the actin-polymerization inhibitor cytochalasin D, but not by the tubulin-polymerization blocker nocodazol (Fig. 3). The ROCK family is best known for its well-characterized roles in regulating actin cytoskeleton dynamics [28]. By phosphorylating numerous downstream substrates, ROCK mediates actin filament stabilization and generation of actomyosin contractile force. Current models of mitochondrial fission suggest that Drp1 is recruited to pre-constricted sites by actin-myosin contraction in ER-mitochondria interaction sites [10]. ROCK proteins are key regulators of actin-myosin contraction. To promote contraction, ROCK can directly phosphorylate the myosin regulatory light chain (RLC) to activate it, but it acts primarily by phosphorylating and inhibiting PP1, which is responsible for the dephosphorylation and inhibition of myosin [28]. The phosphorylation of myosin II RLC by ROCK after NMDA receptor activation has been reported previously[29, 30]. In agreement with the indicated role of myosin activity in mitochondrial fragmentation, the use of myosin II inhibitor blebbistatin blocked NMDA-mediated mitochondrial fragmentation (Fig. 4A and B).

We then analyzed how the use of ROCK inhibitor Y-27632 and myosin inhibitor blebbistatin affected the recruitment of Drp1 to the mitochondria. In basal conditions mitochondria showed an elongated morphology with Drp1-GFP presenting a diffuse and weak signaling within the cytosol. Application of NMDA produced mitochondrial fragmentation and strong signal of Drp1-GFP that co-localized with mitochondria. The

use of ROCK or myosin inhibitors blocked the Drp1 translocation to mitochondria in excitotoxicity and consequently the mitochondrial fragmentation (Fig. 4 C and D).

Concluding remarks

Mitochondrial morphology has a profound effect on cell function and viability. Its control depends on the balance between fusion and fission processes. A number of reports have shown the complexity of mitochondrial fission. Drp1 is the only known active executer of mitochondrial fission, but to perform its function it requires to be modified by different post-translational modifications and the participation of accessory proteins [7]. In addition to Drp1 activation, as shown in a number of recent studies mitochondrial fission requires coordinated intervention of the ER and actin polymerization. Actin can organize into a variety of architectures to drive a range of cellular processes which depends of a number of accessory proteins [31]. The motor protein myosin II plays an important role regulating actin contraction. At the same time, myosin II activity is regulated by phosphorylation and dephosphorylation [28]. Here we have shown that signals that affect actomyosin dynamics must work in coordination with Drp1 modification to mediate mitochondrial fission, since the individual inhibition of each signaling pathway is sufficient to block mitochondrial fragmentation in excitotoxicity, and thus increase the level of complexity in the regulation of the fission process, but expand the therapeutic options aimed to block mitochondrial fragmentation as neuroprotective strategy [32].

Materials and methods

Cell culture and treatments

Cortical neurons from E21 Sprague Dawley rats were cultured as described previously [33]. Experiments were performed after a culturing period of 10–11 days during which cortical neurons develop a rich network of processes, express functional NMDA-type and AMPA/kainate-type glutamate receptors, and form synaptic contacts. Prior to stimulations and transfections, neurons were transferred from growth medium to a medium containing 10% MEM (Life technologies), 90% salt-glucose-glycine (SGG) medium (SGG: 114 mM NaCl, 0.219 % $NaHCO_3$, 5.292 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 1 mM glycine, 30 mM glucose, 0.5 mM sodium pyruvate, 0.1% phenol red; osmolarity 325 mosm/l).

Neurons were treated with 30 μM NMDA (Sigma). The following inhibitors were added 30 minutes before the NMDA treatment: cyclosporine A (1 μM; Merck Millipore), 7-nitorindazole (5 μM; Sigma), Y-27632 (10 μM; Sigma), fasudil (10 μM; Santa Cruz), nocodazole (10 μM; Merck Millipore), cytochalasin D (1 μM; Sigma) and blebbistatin (10 μM, Abcam).

Transfection and plasmids

Transfections were performed at DIV 8 with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer instructions. The experiments were performed 44 hours later.

The plasmids used were mtRFP [34], CnA was gift from Antonio Zorzano (IRB-Barcelona, Spain), GFP-Drp1 was a gift from AM van der Bliek (UCLA, USA), Drp1-S637A was a gift of Jennifer Lippincott-Schwartz (NIH, Bethesda, USA) and Drp1-S637D was gift from Zhigao Wang (UT Soutwestern, USA).

Imaging studies

For mitochondrial morphology analysis, neurons were transfected with mitochondrially targeted RFP. After the treatment, neurons were fixed and nuclei were stained with DAPI. The number of live neurons with tubular or fragmented mitochondria was counted.

Drp1 localization was determined by transfecting cortical neurons with GFP-Drp1 and mtRFP. Forty-eight hours after transfection neurons were treated when appropriate with 30 μM NMDA and fixed.

Statistical analysis

Statistical testing involved two-tailed student's T-tests. All data are presented as mean ± s.e.m. of at least three independent experiments (n).

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

Figure legends.

Figure 1. A phosphorylation event participates in NMDA-induced mitochondrial fragmentation. Mitochondrial morphology analysis of neurons transfected with mitochondria-targeted RFP (mtRFP) and treated with NMDA (30 μM) for 1 hour in the absence or presence of (A) calcineurin inhibitor CsA (1 μM) or overexpressing constitutively active calcineurin A (CnA); B) NMDA induced mitochondrial fragmentation is not affected by overexpression of the indicated Drp1 phosphomutants. *$p<0.05$ (n=3), .

Figure 2. ROCK activation is necessary for NMDA-induced mitochondrial fragmentation. Mitochondrial morphology analysis and representative images of neurons transfected with mtRFP and treated with NMDA (30 μM) for 1 hour in the absence or presence of (A, B) ROCK inhibitors fasudil (Fas; 10 μM) or Y-27632 (10 μM) and (C, D) a combination of Y-27632 and NOS inhibitor 7-nitroindazole (5 μM; in Arg-free medium). Scale bar= 10 μM. *$p<0.05$ (n=3)

Figure 3. Actin dynamics is necessary for NMDA-induced mitochondrial fragmentation. Mitochondrial morphology analysis of neurons transfected with mitochondria-targeted RFP and treated with NMDA (30 μM) for 1 hour in the absence or presence of cytochalasin D (Cyt. D;1 μM) or nocodazole (Noco; 10 μM). *$p<0.05$ (n=3-6)

Figure 4. ROCK activation modifies actomyosin dynamics to promote NMDA-induced Drp1 recruitment to mitochondria. A) Mitochondrial morphology analysis and representative image (B) of neurons treated with NMDA in the presence or absence of myosin inhibitor blebbistatin (Bleb; 10 μM). (n=4). C) Analysis of the localization of Drp1 after the indicated treatments and (D) representative images of neurons transfected

with plasmids encoding GFP-Drp1 and mtRFP. After 48 hours neurons were stimulated with NMDA (30 μM) for 1 hour in the absence or presence of the indicated inhibitors, and fixed and visualized under a confocal microscope. Yellow scale bar= 10 μM ; white scale bar= 5 μM *$p<0.05$ (n=4).

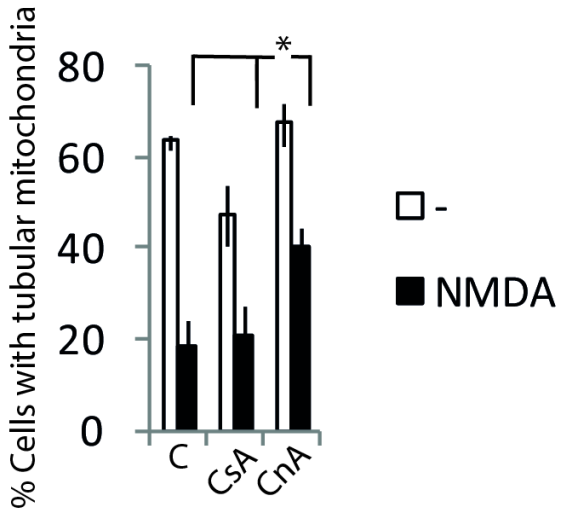
References.

1. Bach D, Pich S, Soriano FX, Vega N, Baumgartner B, Oriola J, Daugaard JR, Lloberas J, Camps M, Zierath JR, et al. Mitofusin-2 Determines Mitochondrial Network Architecture and Mitochondrial Metabolism. Journal of Biological Chemistry 2003; 278:17190-7.
2. Shutt T, Geoffrion M, Milne R, McBride HM. The intracellular redox state is a core determinant of mitochondrial fusion. 2012.
3. de Brito OM, Scorrano L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature 2008; 456:605-10.
4. Martorell-Riera A, Segarra-Mondejar M, Muñoz JP, Ginet V, Olloquequi J, Pérez-Clausell J, Palacín M, Reina M, Puyal J, Zorzano A, et al. Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and delayed neuronal death. The EMBO Journal 2014; 33:2388-407.
5. Youle RJ, van der Bliek AM. Mitochondrial Fission, Fusion, and Stress. Science 2012; 337:1062-5.
6. Westermann B. Mitochondrial fusion and fission in cell life and death. Nature reviews Molecular cell biology 2010; 11:872-84.
7. Liesa M, Palacín M, Zorzano A. Mitochondrial Dynamics in Mammalian Health and Disease. Physiological Reviews 2009; 89:799-845.
8. Friedman JR, Lackner LL, West M, DiBenedetto JR, Nunnari J, Voeltz GK. ER Tubules Mark Sites of Mitochondrial Division. Science 2011; 334:358-62.
9. Korobova F, Ramabhadran V, Higgs HN. An Actin-Dependent Step in Mitochondrial Fission Mediated by the ER-Associated Formin INF2. Science 2013; 339:464-7.
10. Korobova F, Gauvin Timothy J, Higgs Henry N. A Role for Myosin II in Mammalian Mitochondrial Fission. Current Biology 2014; 24:409-14.
11. Labrousse AM, Zappaterra MD, Rube DA, van der Bliek AM. C. elegans Dynamin-Related Protein DRP-1 Controls Severing of the Mitochondrial Outer Membrane. Molecular Cell 1999; 4:815-26.
12. Bleazard W, McCaffery JM, King EJ, Bale S, Mozdy A, Tieu Q, Nunnari J, Shaw JM. The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. Nat Cell Biol 1999; 1:298-304.
13. Smirnova E, Shurland D-L, Ryazantsev SN, van der Bliek AM. A Human Dynamin-related Protein Controls the Distribution of Mitochondria. The Journal of Cell Biology 1998; 143:351-8.
14. Ingerman E, Perkins EM, Marino M, Mears JA, McCaffery JM, Hinshaw JE, Nunnari J. Dnm1 forms spirals that are structurally tailored to fit mitochondria. The Journal of Cell Biology 2005; 170:1021-7.
15. Barsoum MJ, Yuan H, Gerencser AA, Liot G, Kushnareva Y, Graber S, Kovacs I, Lee WD, Waggoner J, Cui J, et al. Nitric oxide-induced mitochondrial fission is regulated by dynamin-related GTPases in neurons. Embo J 2006; 25:3900-11.
16. Schofield AV, Bernard O. Rho-associated coiled-coil kinase (ROCK) signaling and disease. Critical Reviews in Biochemistry and Molecular Biology 2013; 48:301-16.
17. Olson MF. Applications for ROCK kinase inhibition. Current Opinion in Cell Biology 2008; 20:242-8.
18. Cribbs JT, Strack S. Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. EMBO reports 2007; 8:939-44.

19. Cereghetti GM, Stangherlin A, de Brito OM, Chang CR, Blackstone C, Bernardi P, Scorrano L. Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. Proceedings of the National Academy of Sciences 2008; 105:15803-8.
20. Wang W, Wang Y, Long J, Wang J, Haudek Sandra B, Overbeek P, Chang Benny HJ, Schumacker Paul T, Danesh Farhad R. Mitochondrial Fission Triggered by Hyperglycemia Is Mediated by ROCK1 Activation in Podocytes and Endothelial Cells. Cell Metabolism; 15:186-200.
21. Semenova MM, Maki-Hokkonen AMJ, Cao J, Komarovski V, Forsberg KM, Koistinaho M, Coffey ET, Courtney MJ. Rho mediates calcium-dependent activation of p38[alpha] and subsequent excitotoxic cell death. Nat Neurosci 2007; 10:436-43.
22. Mueller BK, Mack H, Teusch N. Rho kinase, a promising drug target for neurological disorders. Nat Rev Drug Discov 2005; 4:387-98.
23. Losón OC, Song Z, Chen H, Chan DC. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Molecular Biology of the Cell 2013; 24:659-67.
24. Chou C-H, Lin C-C, Yang M-C, Wei C-C, Liao H-D, Lin R-C, Tu W-Y, Kao T-C, Hsu C-M, Cheng J-T, et al. GSK3beta-Mediated Drp1 Phosphorylation Induced Elongated Mitochondrial Morphology against Oxidative Stress. PloS one 2012; 7:e49112.
25. De Vos KJ, Allan VJ, Grierson AJ, Sheetz MP. Mitochondrial Function and Actin Regulate Dynamin-Related Protein 1-Dependent Mitochondrial Fission. Current Biology 2005; 15:678-83.
26. DuBoff B, Götz J, Feany Mel B. Tau Promotes Neurodegeneration via DRP1 Mislocalization In Vivo. Neuron; 75:618-32.
27. Li S, Xu S, Roelofs BA, Boyman L, Lederer WJ, Sesaki H, Karbowski M. Transient assembly of F-actin on the outer mitochondrial membrane contributes to mitochondrial fission. The Journal of Cell Biology 2015; 208:109-23.
28. Riento K, Ridley AJ. ROCKs: multifunctional kinases in cell behaviour. Nature reviews Molecular cell biology 2003; 4:446-56.
29. Rex CS, Gavin CF, Rubio MD, Kramar EA, Chen LY, Jia Y, Huganir RL, Muzyczka N, Gall CM, Miller CA, et al. Myosin IIb Regulates Actin Dynamics during Synaptic Plasticity and Memory Formation. Neuron; 67:603-17.
30. Hodges JL, Newell-Litwa K, Asmussen H, Vicente-Manzanares M, Horwitz AR. Myosin IIB Activity and Phosphorylation Status Determines Dendritic Spine and Post-Synaptic Density Morphology. PloS one 2011; 6:e24149.
31. Blanchoin L, Boujemaa-Paterski R, Sykes C, Plastino J. Actin Dynamics, Architecture, and Mechanics in Cell Motility. Physiological Reviews 2014; 94:235-63.
32. Perez-Pinzon MA, Stetler RA, Fiskum G. Novel mitochondrial targets for neuroprotection. J Cereb Blood Flow Metab 2012; 32:1362-76.
33. Soriano FX, Leveille F, Papadia S, Bell KF, Puddifoot C, Hardingham GE. Neuronal activity controls the antagonistic balance between peroxisome proliferator-activated receptor-gamma coactivator-1alpha and silencing mediator of retinoic acid and thyroid hormone receptors in regulating antioxidant defenses. Antioxid Redox Signal 2011; 14:1425-36.
34. Legros F, Lombès A, Frachon P, Rojo M. Mitochondrial Fusion in Human Cells Is Efficient, Requires the Inner Membrane Potential, and Is Mediated by Mitofusins. Molecular Biology of the Cell 2002; 13:4343-54.

Figure 1

A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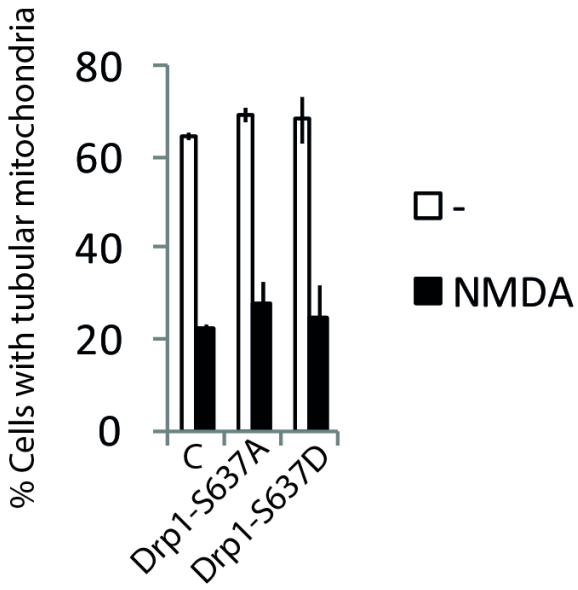
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Figure 2

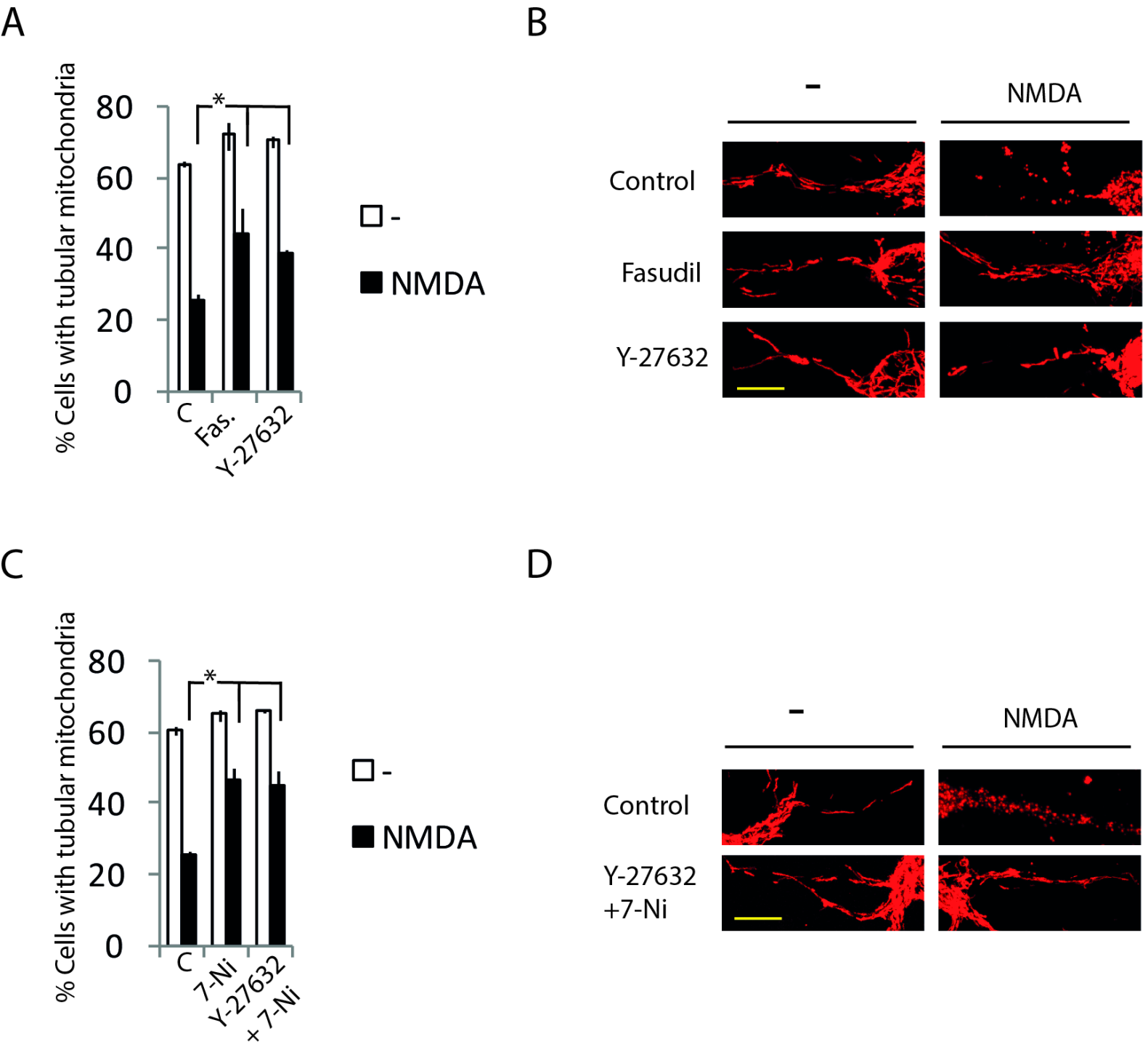
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Figure 3

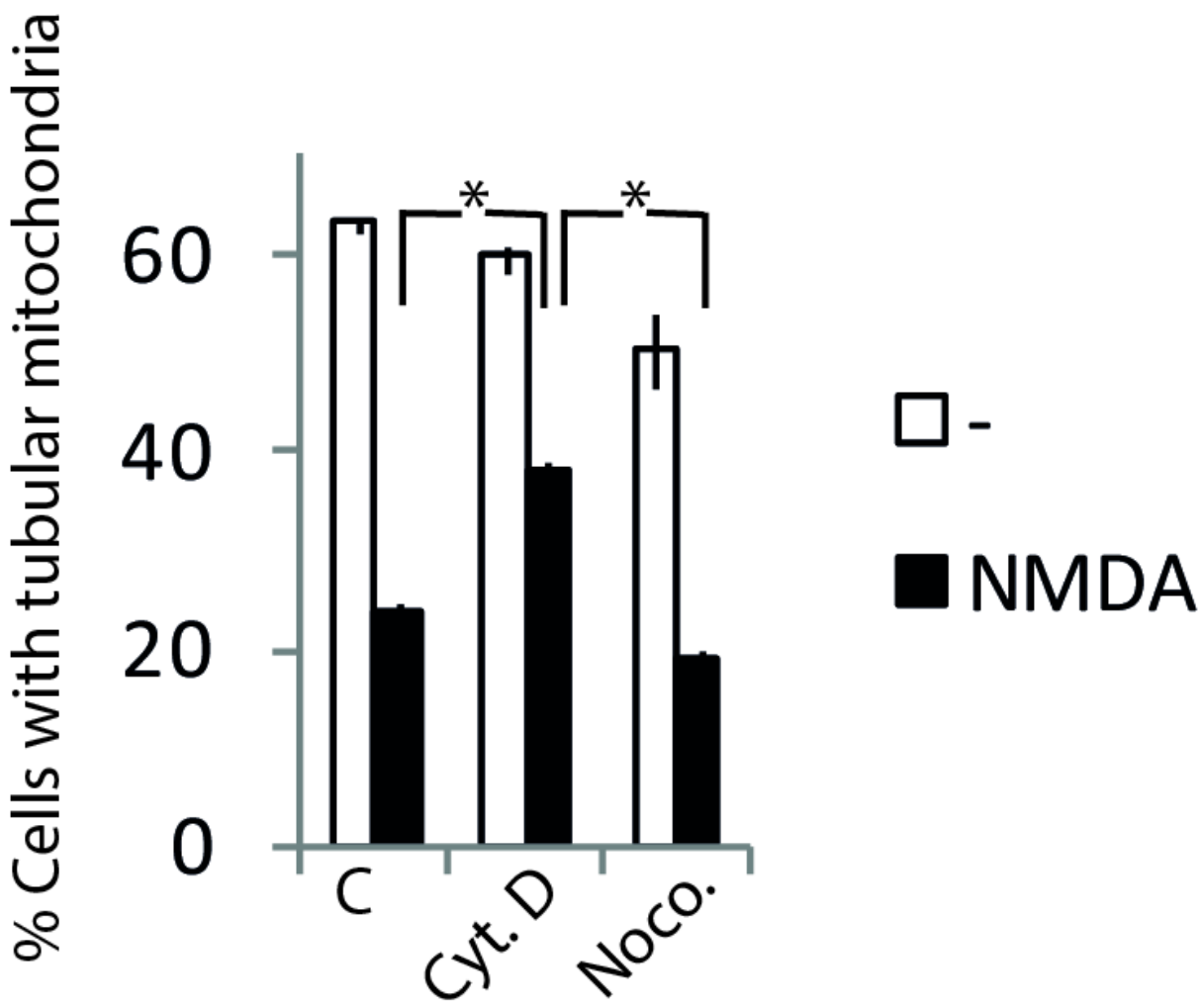

Figure 4

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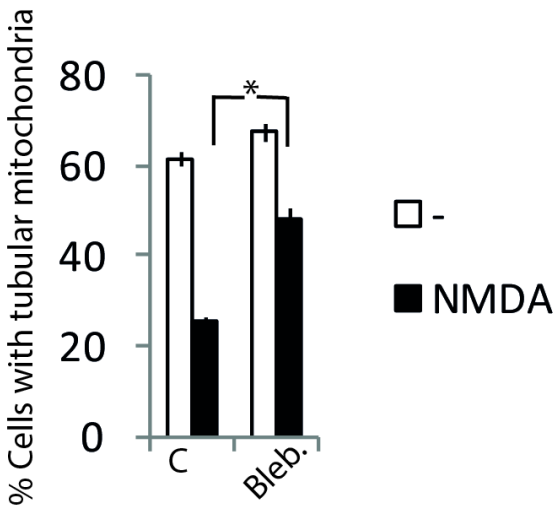

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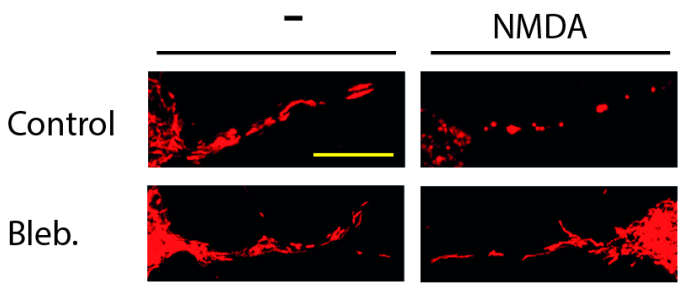

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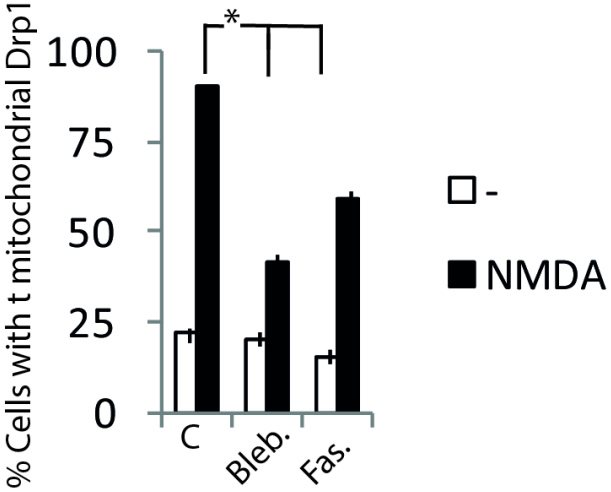

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