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DOR undergoes nucleo-cytoplasmic shuttling, which involves passage through the nucleolus

Caroline Mauvezin [a,b,c,*], Ana Sancho [a,b,c], Saska Ivanova [a,b,c], Manuel Palacin [a,b], Antonio Zorzano [a,b,c]

[a] Institute for Research in Biomedicine (IRB Barcelona), C/Baldiri Reixac 10, 08028 Barcelona, Spain
[b] Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain
[c] CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Spain

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ABSTRACT

DOR is a bi-functional protein that regulates transcription and enhances starvation-induced autophagy. While autophagy has been mostly described as a stress–response mechanism, cells also need autophagy to maintain homeostasis in basal conditions. However, the mechanisms regulating basal autophagy still remain unknown. Our results show that DOR acts in basal autophagy. Indeed, DOR already undergoes nucleo-cytoplasmic shuttling in basal conditions and, surprisingly, DOR exits continuously the nucleus and traverses the nucleolus. However, the nucleolus integrity is not essential for both DOR nucleo-cytoplasmic shuttling and DOR function on basal autophagy. Taken together, we propose that DOR exit from the nucleus is essential for basal autophagy stimulation even under nucleolus disruption.

1. Introduction

Adaptation to the extra-cellular environment is essential for cell viability. Indeed, cells respond to substrate scarcity by activating catabolic pathways, such as the ubiquitin proteasome system (UPS) and lysosomal-autophagy-related degradation machinery [1]. Macroautophagy (hereafter referred to as autophagy) is directly implicated in human health. Perturbations of this process are described in a wide range of diseases, including cancer, neurodegeneration and aging [2]. Autophagy was first described as a stress–response mechanism. During periods of starvation, the stimulation of autophagy is crucial to provide cells with the amino acids necessary to maintain metabolism and the ATP levels compatible with survival. Nutrient-sensitive autophagy is a highly regulated process, mainly by mTOR and AMPK in mammals [3]. However, autophagy also occurs constitutively in basal conditions in order to maintain homeostasis. Indeed, cells use basal autophagy as a cell repair system to degrade damaged organelles and long-lived proteins [4]. The mechanisms responsible for basal, and therefore mTOR-independent, autophagy activation are still unclear. Recent data revealed some of the potential mechanisms of activation or inhibition of autophagy and identified new molecules involved in the autophagic process at various levels [5]. For example, new components of the ATG8 conjugation, vesicle elongation, and autophagosome assembly sub-network were identified, such as TECPR1 protein, which interacts with the ATG12-ATG5-ATG16 complex and a related protein TECPR2 which associates with ATG8 orthologs. Autophagy occurs in the cytosol, however nuclear compartments, such as the nucleolus, are also implicated in stress–response mechanisms [6]. Recently, a new Atg8-interactor protein, which positively regulates stress-induced autophagy was identified, namely DOR (Diabetes- and Obesity-related protein, also called TP53inp2 (UniProt ID: Q8CFU8) [7,8]. DOR is also a transcriptional co-activator of thyroid hormone receptors in mammalian cells [9], and a co-activator of ecdisone receptors in Drosophila [10]. More recently, DOR bi-functionality was enlighten by the finding of two highly conserved regions playing a role in both transcription activity and in autophagy [11]. Thus, this protein presents two functions, acting either in the nucleus or in the cytosol. Here we proposed to investigate the impact of DOR intracellular distribution on basal autophagy.

2. Materials and methods

2.1. Cell lines and transfection

HeLa cells were maintained in DMEM (Invitrogen, 41966052) supplemented with 10% FBS (Invitrogen, 10270106), penicillin

Abbreviations: DOR, diabetes and obesity related protein; NES, nuclear export signal; NPM, nucleophosmin

* Corresponding author at: Institute for Research in Biomedicine (IRB Barcelona), C/Baldiri Reixac 10, 08028 Barcelona, Spain.
E-mail address: caroline.mauvezin@gmail.com (C. Mauvezin).

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(100 μU/ml) and streptomycin (100 μg/ml) (Invitrogen, 15140122) and 2% Hepes (Sigma, H3375). Amino acid starvation was induced by incubating the cells in Hank's Balanced Salt Solution (HBSS) (Invitrogen, 14025-050) with 10% dialyzed FBS, 2% Hepes and penicillin (100 μU/ml) and streptomycin (100 μg/ml). Cells were incubated in DMEM with 2 μM Rapamycin (Sigma, R8781) for 2 h or with 0.1 μg/ml Adriamycin (Sigma, D1515) for 16 or 18 h.

For transient transfection assays, cells were typically plated onto a 100-mm cell plate and transfected with 3 μg DNA using the PEI method. Cells were incubated for 18 h at 37 °C in 5% CO_2 and the medium was then removed and replaced by fresh DMEM. DOR-RFP construct was generated by In-Fusion™ Dry-down PCR (Clontech, 639606). NPM-GFP and NPMdL-GFP constructs were gifts from Dr. Weber. GFP-LC3 plasmid was kindly provided by Dr. Lavandero. DOR NES mutant (L36A, L37A, I38A and L40A) was produced by direct mutagenesis using QuickChange Site-Directed Mutagenesis Kit (Stratagene, 200518) as previously described [8].

2.2. Live imaging

HeLa cells were transiently transfected with either DOR-GFP or DOR-RFP alone or in combination with NPM-GFP or NPMdL-GFP. Wide-field images of live cells were captured using an Olympus 1X81 microscope equipped with Cell^R software. Cells were plated onto 35 mm glass bottom culture dishes (MatTek, P35G-0-10-C). Individual culture dishes with 2 ml of cell medium were stored in an imaging chamber with CO_2 and temperature control. The Cell^R imaging system was equipped with 60 × 1.1 NA water and 100× oil 1.30 NA objectives, MT-20 illumination unit (150 W xenon/mercury mix bulb), and an Hamamatsu Orca ER camera. GFP and RFP were excited using 470/40 nm and 545/30 nm band-pass filters, respectively. Emission was collected using 525/50 nm (GFP), and 617/73 nm (RFP) band-pass filters. Data were processed using ImageJ (NIH).

2.3. Immunofluorescence assays and confocal microscopy

After specific treatments, cells were fixed in ice-cold methanol or 4% paraformaldehyde and washed twice with PBS. Immunocytochemistry assays were performed using anti-DOR (1/150) generated in our laboratory [10], anti-PML nuclear bodies (Santa Cruz, sc-966) and anti-fibrillarin (Abcam, ab4566). Hoechst (1/2000) (Molecular Probes, H-3570) was used to label DNA. Cells were mounted in Fluoromount (Electron Microscopy Sciences, 17984-25). Immunofluorescence microscopy of cells and image analysis were performed as previously described [8].

The percentage of cells with GFP-LC3-positive punctate structures was obtained by counting 50 positive cells in each working condition of two independent experiments and results were expressed as the mean ± standard error.

3. Results

The transcriptional co-activator DOR has been previously characterized as a nuclear protein that partially co-localizes with PML (promyelocytic leukemia) nuclear bodies [9]. Moreover, DOR also acts in the cytosol by interacting with the autophagic machinery [8]. Here, we proposed to study DOR dynamics in transiently transfected HeLa cells in basal conditions performing DOR-RFP live imaging experiments. Surprisingly, DOR already left the nucleus in steady-state conditions and formed punctuated structures in the cytosol that were also highly dynamics (Fig. 1A and Video 1). DOR also moved rapidly inside the nucleus itself, suggesting that the intra-nuclear localization of this protein is variable and organized.

Interestingly, DOR also localized in compartments that were not stained by Histone2 (Fig. 1B and Video 2). Indeed, DOR-RFP localized in nucleoplasm but accumulated in large nuclear bodies when transiently transfected in He La cells (Fig. 1C (arrows) and Video 3). Additionally, we tracked DOR-positive particles that moved through these large nuclear sections (Fig. 1D and Video 4). Only a few nuclear compartments encompass a large portion of the nucleus and do not contain DNA or histone proteins, among these the nucleolus.

Consequently, we specifically studied by immunofluorescence assays the intra-nuclear localization of DOR and fibrillarin, a nucleolar protein of the dense fibrillar component (DFC) sub-region [12]. Interestingly, in basal conditions, DOR partially co-localized with fibrillarin (Fig. 2A and B).

To support the idea that a portion of DOR protein localizes at the nucleolus, we performed live imaging in HeLa cells transiently transfected with DOR-RFP and Nucleophosmin-GFP. Nucleophosmin (NPM) is a nucleolar protein, marker of the granular component (GC), which is essential for the export of ribosome subunits from the nucleolus to the cytoplasm. Our results showed that DOR and NPM co-localized at the nucleolus site in normal growth conditions (Fig. 2C and Video 5).

Next, we proposed to examine DOR behavior once the nucleolus compartment is disrupted. To this end, we induced nucleolar disruption in two different manners. First, we overexpressed in HeLa cells GFP-NPMdL (dominant negative form) together with DOR-RFP and we performed live imaging experiments. Over-expression in cells of NPMdL, which has its NES signal mutated, has been previously described to stay blocked in the nucleolus, to bind ribosomes and therefore to induce a blockade of ribosomes export from the nucleolus to the cytoplasm [13]. In the presence of NPMdL, DOR still undergoes a nucleo-cytoplasmic shuttle (Fig. 3A and Video 6), however DOR nucleolar localization is altered and over time DOR protein lost its ability to localize at the nucleolus (Fig. 3B). Then, HeLa cells were transiently transfected with DOR-GFP and treated with Adriamycin (0.1 μg/ml) for 18 h. Under these conditions, Adriamycin, a topoisomerase II inhibitor, induces cell cycle arrest leading to DNA damage but not cell death [14]. Live imaging assays showed that DOR-RFP access to the cytosol is not restricted upon DNA damage (Fig. 3C and Video 7). Indeed, we detected a large amount of DOR-positive structures in the cytosol under Adriamycin treatment (Fig. 3C, arrows). These data support the idea that nucleolar integrity is not essential for DOR nucleo-cytoplasmic shuttle in HeLa cells.

To better understand the physiological role of the nucleolus in the autophagic function of DOR protein, we quantitatively analyzed basal autophagy under nucleolus-disrupted conditions. To this end, HeLa cells were co-transfected with GFP-LC3 together with RFP or DOR-RFP and treated with Adriamycin for 16 h. Immunofluorescence assays were performed and GFP-LC3-positive spots were counted for each condition. Adriamycin has been shown to induce autophagy in cardiomyocytes [15]. Indeed, the authors showed that Adriamycin, a potent anti-tumor drug known to cause heart failure, markedly increased autophagic flux and that activation of autophagy mediates Adriamycin cardiotoxicity. In our model, Adriamycin treatment also caused an increase in the number of GFP-LC3-positive structures (Fig. 4) in control (RFP) HeLa cells (1.96 fold, $p < 0.0001$). DOR over-expression enhanced autophagosomes formation under basal conditions (Fig. 4) as we previously described [8]. In these cells, the addition of Adriamycin provoked a similar increase in the number of autophagic vacuoles (1.89 fold, $p < 0.0001$) in comparison with control RFP-transfected cells, suggesting that this increase is specifically due to Adriamycin treatment. In all, these data indicate that the disruption of the nucleolus induced by Adriamycin does not affect the function of DOR in basal autophagy.

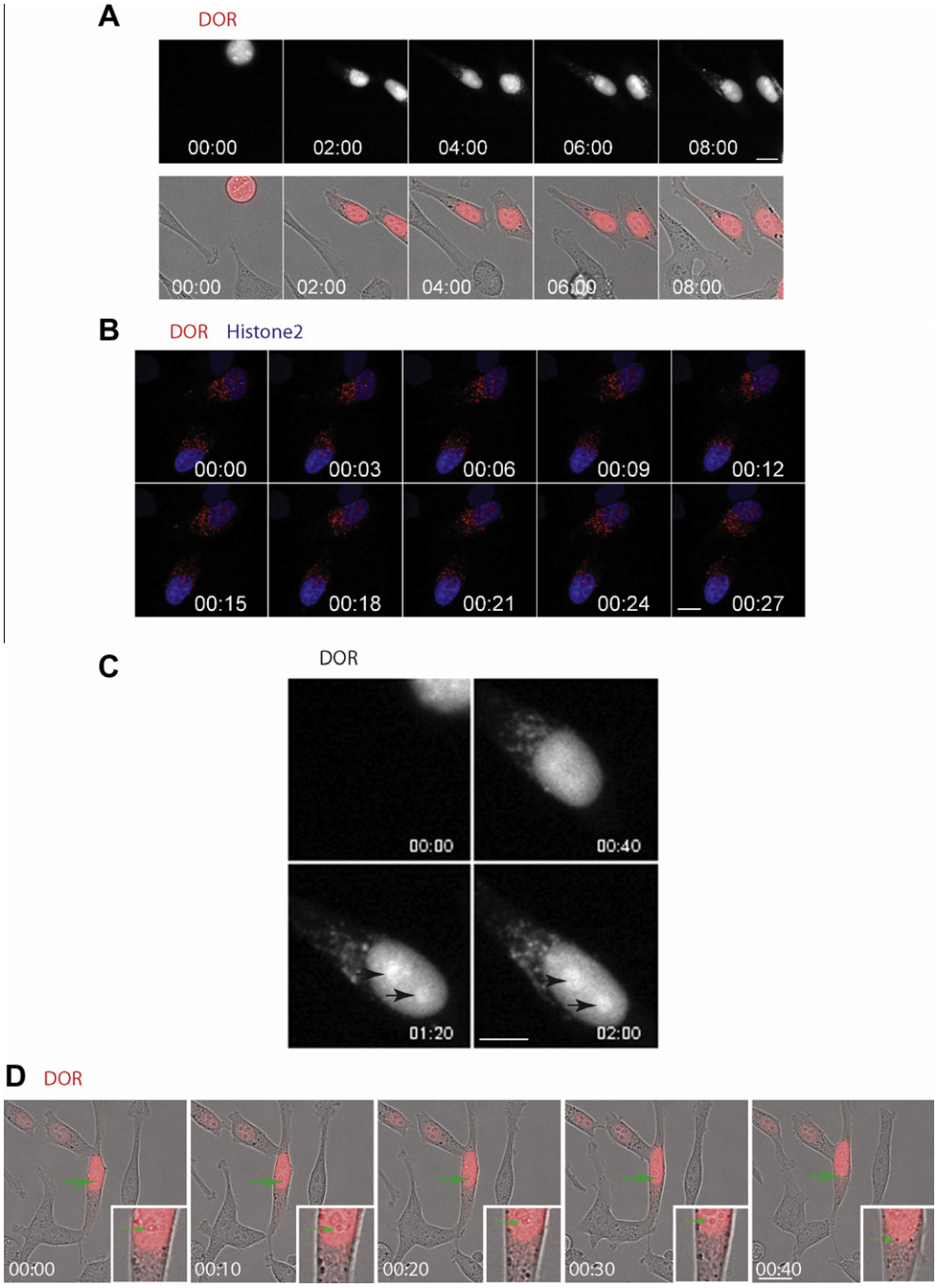

Fig. 1. (A) DOR is a dynamic protein in basal conditions. Sequence of images from live imaging experiment performed in HeLa cells transiently transfected with DOR-RFP. Transmission images were merged to obtain a clear picture of the DOR-RFP movement and the status and movement of the cell itself during the recording of the video. Scans were made every 5 min. Autofocus was displayed with transmission channel. Scale bar, 10 μm. (B) DOR accumulates in nuclear compartments, which do not contain DNA. Sequence of images from live imaging performed in HeLa cells transiently transfected with DOR-GFP and Histone2-mCherry. Scans were made every minute for 40 min. DOR-GFP was labeled in red and Histone2 in blue for better clarity. Scale bars, 10 μm. (C) DOR protein accumulates in large nuclear compartments. Zoom of live imaging experiment in which HeLa cells were transiently transfected with a plasmid encoding for DOR-RFP. Arrows indicate accumulation of DOR-RFP. Scale bar, 5 μm. (D) DOR passes through the nucleolus prior to its nuclear exit. Sequence of images of live imaging experiment that was performed in HeLa cells transiently transfected with DOR-RFP. We followed the status and movement of the cells with transmission images (in gray). DOR-RFP was labeled in red. Scans were made every 5 min for 40 min. Green arrows indicate DOR-positive particle. Scale bar, 10 μm.

Finally, we took advantage of the mutant form of DOR (hereafter referred to as NES mutant), which lacks the capacity to leave the nucleus and acts as a dominant negative form [8]. Immunofluorescence assays were performed in HeLa cells transiently transfected with the NES mutant and various nuclear compartments were monitored with specific markers. The mutant form was found adjacent to PML nuclear bodies in basal or autophagy-activation conditions (Fig. 5A). Interestingly, a large portion of NES mutant accumulated in the nucleolus as it maintained its capacity to co-localize with fibrillarin under basal state (Fig. 5B).

In all, our results showed that DOR is a dynamic protein that already undergoes nucleo-cytoplasmic shuttling in basal conditions and transiently localizes at the nucleolus.

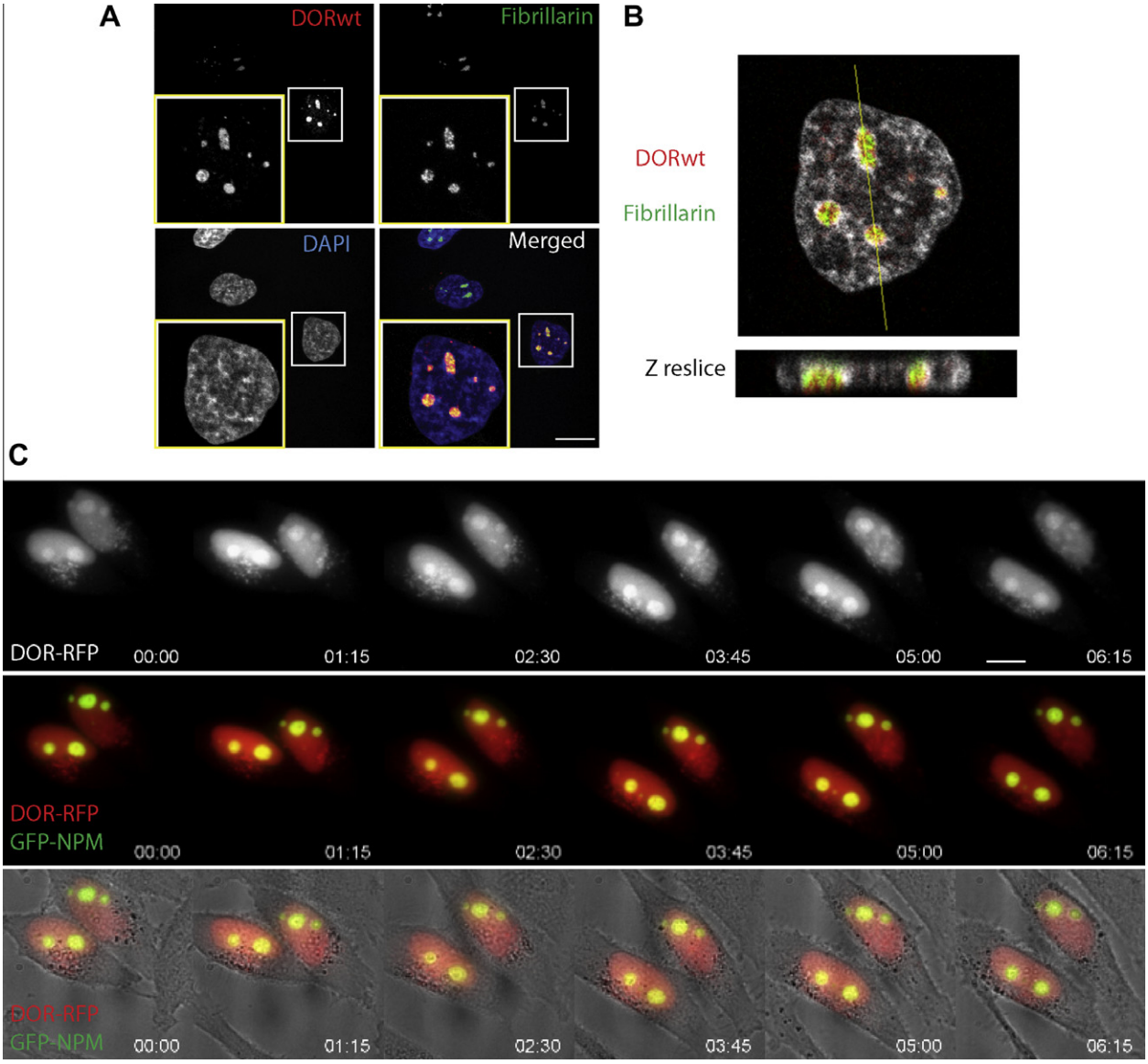

Fig. 2. (A) DOR co-localizes with fibrillarin a nucleolar protein. Confocal images of HeLa cells transiently transfected with DOR wild-type. DOR and fibrillarin were detected by immunofluorescence and respectively labeled in red and green. Nuclei were stained with DAPI and detected in blue. Scale bar, 10 μm. (B) The colocalization between DOR and fibrillarin is partial. Z-relice from ImageJ plugin. The nucleus is marked in gray; DOR wild-type is labeled in red and fibrillarin in green. (C) DOR co-localizes with another nucleolar protein Nucleophosmin (NPM). Sequence of images from live imaging experiment performed in HeLa cells transiently transfected with DOR-RFP and NPM-GFP. Transmission images were merged to obtain a clear picture of the DOR-RFP movement and the status and movement of the cell itself during the recording of the video. Scans were made every 5 min. Autofocus was displayed with transmission channel. Scale bar, 10 μm.

4. Discussion

A major finding of this study is the demonstration by live imaging that DOR continuously exits the nucleus. We previously found that during stress conditions linked to mTOR inhibition, the nucleus is depleted of DOR [8]. DOR translocation is a determining factor for the protein to up-regulate autophagosome formation as the NES mutant, which is unable to exit the nucleus, acts as a dominant-negative form [8]. Here, we discovered and characterized DOR exit from the nucleus under basal state. Autophagy operates continuously to maintain cellular homeostasis, even though it has been mainly characterized as a stress–response mechanism. Thus, the signaling pathways involved in basal autophagy activation and/or maintenance are still unknown. Previous analysis of DOR gain- and loss-of-function showed an impact on autophagy already in basal conditions [8]. So far, we believed that the effect of DOR on basal autophagy could be conferred by the bi-functional character of DOR protein and therefore a potential role of DOR on autophagy at the transcriptional level (data not shown). However, here our results pointed out that DOR actually can operate in basal autophagy as it continuously shuttles in and out of the nucleus. Interestingly we observed that, while shuttling, DOR passes through the nucleolus and a large portion of the protein co-localizes with two nucleolar proteins, respectively, Fibrillarin and NPM. However, cancellation of DOR NES motif traps an abundant part of the protein into the nucleolus and prevents its nucleo-cytoplasmic shuttle.

Interestingly, recent data indicate that various proteins such as p21, MDM2-p53 and hsp70 harbor a similar behavior and a nucleolar localization [14,16,17]. For example, MDM2 presents a NES signal that is required for p53 nuclear-cytoplasmic shuttling [16].

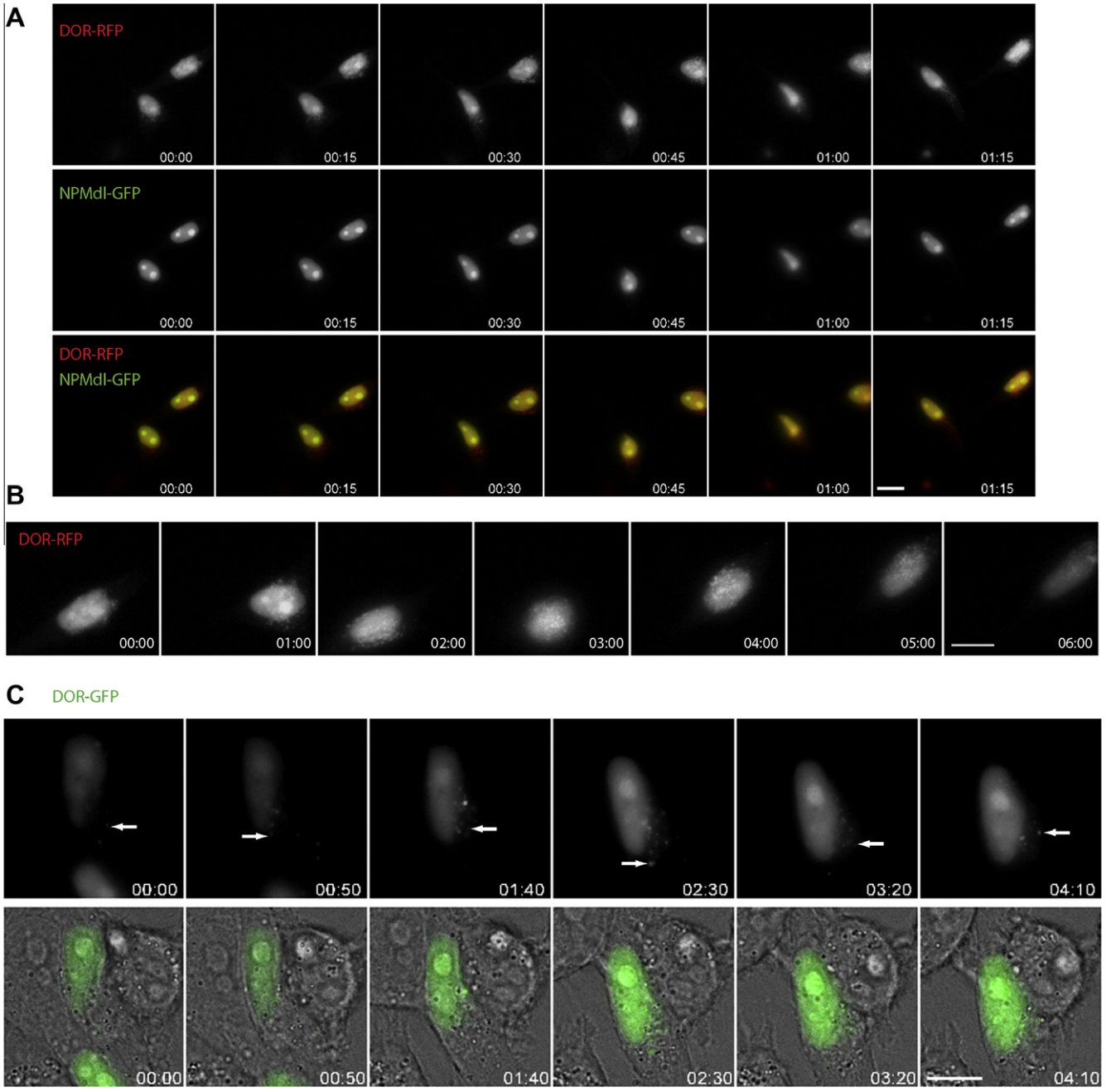

Fig. 3. (A) DOR nucleo-cytoplasmic shuttle is not impaired by nucleolar disruption. Sequence of images from live imaging experiment performed in HeLa cells transiently transfected with DOR-RFP and NPMdL-GFP. Scans were made every 5 min. Scale bar, 10 μm. (B) DOR nuclear redistribution under nucleolar disruption. Sequence of images from live imaging experiment performed as described in panel (A). Scans were made every 5 min. Scale bar, 10 μm. (C) Sequence of images from live imaging experiment performed in HeLa cells transiently transfected with DOR-GFP and treated with Adriamycin (0.1 μg/ml). Scans were made every 5 min. Scale bar, 10 μm.

Interestingly, p53 and MDM2 have recently been associated with PML nuclear bodies and the nucleolus [18]. DOR was also described as a PML-associated protein [9], and is here characterized to partially localize at the nucleolus. The nucleolus is not only responsible for the assembly of ribosomal RNA, but it acts as a sensor of cellular stress [6]. Interestingly, an autophagy-related protein, Raptor (an mTOR-Cl partner) was recently described to co-localize with the nucleolar fibrillarin [19]. Upon DNA damage, the nucleolus is disrupted, leading to a serial of compensatory intra-cellular responses. DOR nucleo-cytoplasmic shuttling is not prevented by nucleolar disruption upon Adriamycin-induced DNA damage or overexpression of NPM dominant-negative form as shown here. These data suggest that nucleolar integrity is not essential for DOR trafficking. Moreover, the autophagic activity of DOR protein is not altered upon DNA damage-induced nucleolus disruption, suggesting that the nucleolus is neither playing a role in the basal autophagy activation triggered by DOR protein. Interestingly, cytoplasmic heat shock protein hsp70 also moves in and out of the nucleus, however, cell stresses inhibit its nucleo-cytoplasmic shuttling [17]. Those authors showed that the inhibition of the shuttling causes a transient sequestration of the hsp70 chaperone in nuclei and more precisely into the nucleolus. This type of behavior resembles that of DOR observed in the present study. In our case, however, DOR shuttling is not abolished upon nucleolar-directed cellular stress and therefore passage through the nucleolus is not essential for DOR intracellular movement.

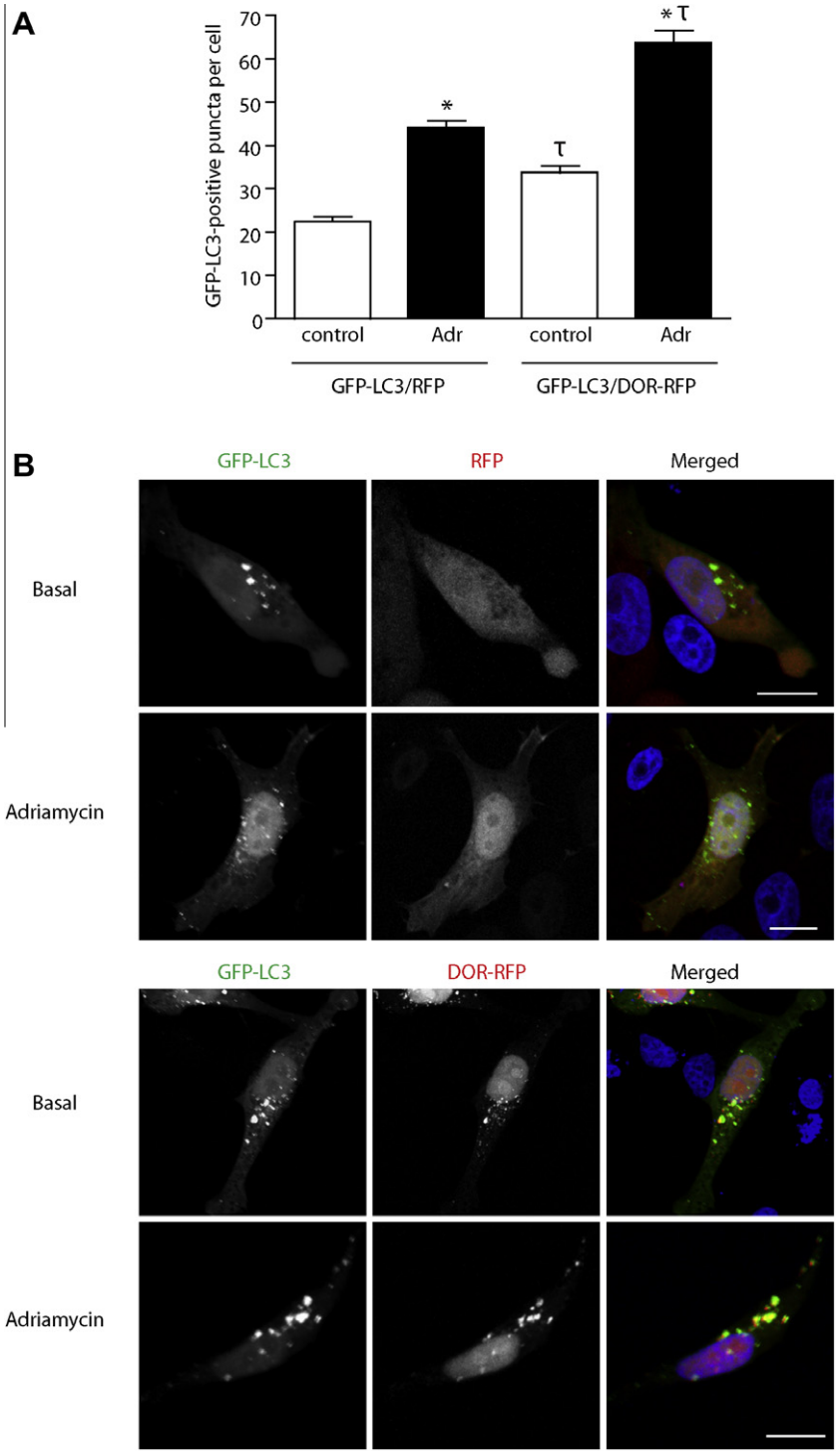

Fig. 4. Nucleolar disruption does not alter DOR function in basal autophagy. (A) Image analysis on the abundance of GFP-LC3 structures. GFP-LC3-positive spots were counted in 50 transfected HeLa cells. Data are mean ± SEM of two independent experiments. *, significant effects of Adriamycin, at $p < 0.0001$; τ, significant effects caused by DOR overexpression, at $p < 0.0001$. (B) Representative confocal images of HeLa cells co-transfected with GFP-LC3 together with RFP or DOR-RFP and subjected to basal growth conditions (basal) or incubated with Adriamycin for 16 h. Nuclei were stained with DAPI. Scale bars, 10 μm.

However, NES mutant remains trapped in the nucleolus meaning that the region encompassing the NES sequence at the N-terminal end of the protein is necessary for its nucleolar association and dissociation. Interestingly, this region is highly conserved through evolution and contains also the LIR (LC3 Interacting Region) domain [11]. Here we showed that the integrity of the nucleolus is essential for neither DOR-driven stimulation of basal autophagy nor DOR cytoplasmic localization; however, the function of this route still needs to be elucidated. Indeed, DOR may go to the nucleolus to probably exert an unknown function potentially related with ribosomal machinery. In all cases, further experimentations are needed to properly support any of these ideas.

In all, our results showed the following: (1) DOR is not only a stress-sensor protein but is a dynamic protein undergoing

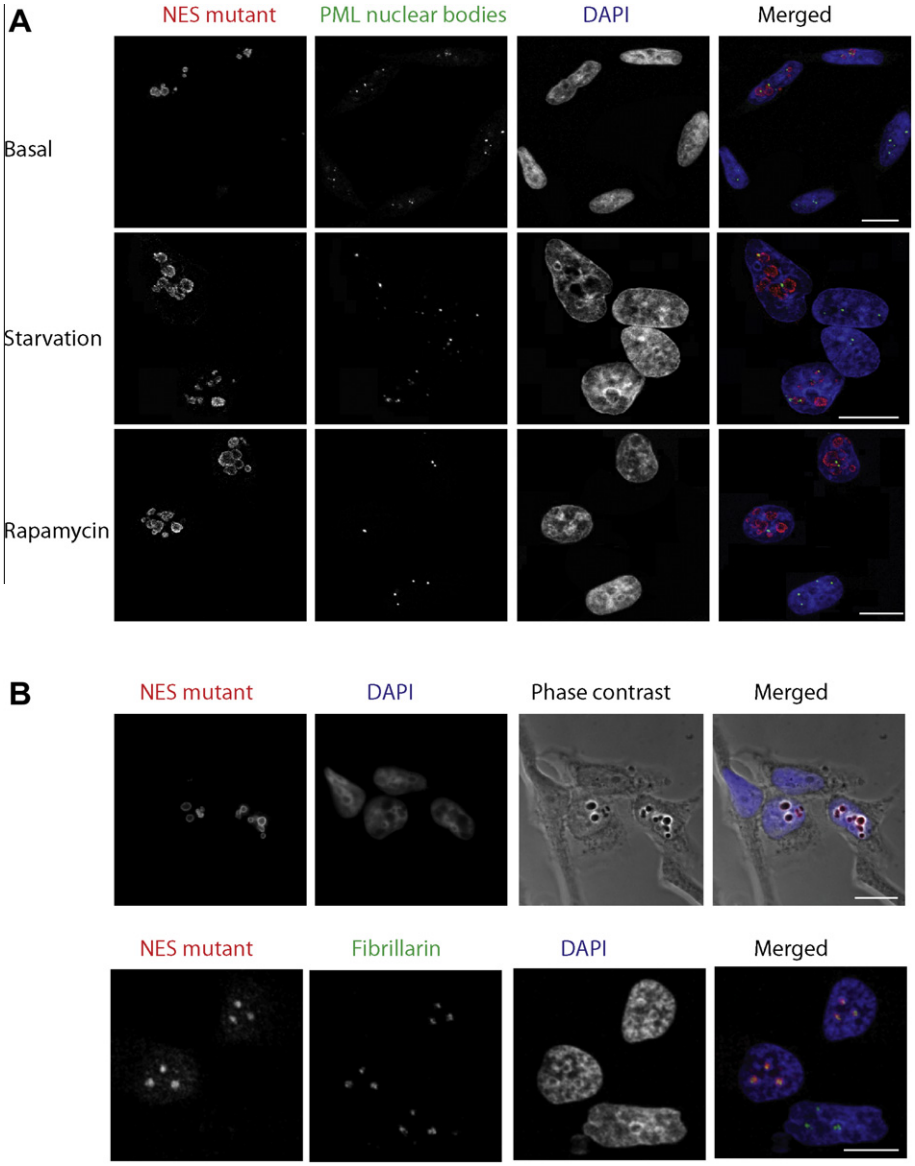

Fig. 5. NES mutant localizes in the nucleolus. (A). Cellular distribution of a mutant form of DOR (L36A/I37A/I38A/L40A), named NES mutant, in transiently transfected HeLa cells incubated with DMEM (basal), with HBSS (amino acid starvation) for 1 h, or with 2 μM rapamycin for 3 h. The intracellular localization of DOR (red) and PML nuclear bodies (green) was determined by indirect immunofluorescence. Nuclei were stained with DAPI and detected in blue. Scale bars, 10 μm. (B). The NES mutant accumulates in the nucleolus and co-localizes with fibrillarin. HeLa cells were transiently transfected with the DOR NES mutant and subjected to immunofluorescence. The NES mutant was detected in red, fibrillarin in green and nuclei in blue. Scale bars, 10 μm.

nucleo-cytoplasmic shuttle under normal growth conditions, (2) DOR transiently localizes at the nucleolus, (3) DOR translocation to the cytosol is not prevented upon nucleolar disruption induced by DNA damage or inhibition of ribosome nuclear export, (4) basal autophagic activity of DOR is not altered by Adriamycin-induced nucleolar disruption and (5) NES motif is essential for DOR nucleolar exit. To summarize, DOR is a scaffold protein that regulates both basal and stress-induced autophagy once being cytosolic.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.06.032.

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