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

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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

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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.