Metabolism Clinical and Experimental 95 (2019) 84–92

Contents lists available at ScienceDirect

Metabolism Clinical and Experimental

journal homepage: www.metabolismjournal.com

FoxO1 localizes to mitochondria of adipose tissue and is affected by nutrient stress

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ARTICLE INFO

Article history:
Received 30 January 2019
Accepted 5 April 2019

Keywords:
ROS
PTPMT1
Nutrient restriction
Mitochondrial retrograde signaling

ABSTRACT

Objective: Mitochondria play pivotal roles in orchestrating signaling pathways in order to guarantee metabolic homeostasis under different stimuli. It has been demonstrated that the mito-nuclear communication is fundamental for facing physiological and/or stress-mediated cellular response through the activation of nuclear transcription factors. Here, we focused on the Forkhead box protein O1 (FoxO1) transcription factor that belongs to the FoxOs family proteins and is considered a "nutrients sensor" modulating the expression of nutrient-stress response genes.
Methods: In vitro and *in vivo* experimental systems, including 3T3-L1 white, X-9 beige and T37i brown adipocytes and different fat depots from C57BL/6 mice were used. The mitochondrial localization of FoxO1 was demonstrated by western blot analysis, confocal microscopy and chromatin immunoprecipitation assay, after sub-cellular compartment isolation. RT-qPCR analysis was used to evaluate the expression of antioxidant and mitochondrial genes after modulation of FoxO1 activity/localization. Treatment with diverse reactive oxygen species (ROS) species/sources were performed and assessed by cytofluorimetric analysis.
Results: We demonstrated that FoxO1 not exclusively localizes to cytosol and nucleus of adipocytes but also to mitochondria where it binds to mitochondrial DNA. We also proved that mitochondrial FoxO1 is phosphorylated upon normal feeding condition. Mitochondrial FoxO1 responds to starvation leaving mitochondrial compartment by ROS-mediated activation of the mitochondrial phosphatase PTPMT1. Indeed, FoxO1 de-phosphorylation and mito-to-nucleus shuttling was observed under starvation. Moreover, we provided evidence that ROS species/sources are able to differently modulate the mitochondrial localization of FoxO1.
Conclusion: The ability to localize at different cell compartments, including mitochondria, highlights a different layer of regulation of FoxO1 necessary for assuring a fast and efficient nutrient-stress response in white/beige adipose tissue. FoxO1 could be thus endorsed in the list of transcription factors involved in the mito-nuclear communication where ROS can act as upstream signals.

1. Introduction

High and chronic intake of nutrients exceeding energy expenditure results in adipose tissue (AT) enlargement, one of the most characterizing aspect of obesity. However, AT is not just an inert fat sink but represents a dynamic organ able to respond to diverse external stimuli. Such different responses mainly depend on two canonical types of adipose tissue: the white adipose tissue (WAT) that is designed for energy storage [1] and supply under high metabolic demand (*e.g.* during exercise or fasting); the brown adipose tissue (BAT) that is thermogenic and uses fatty acid oxidation for maintaining body temperature following cold exposure [2].

Upon specific circumstances (*e.g.* exposure to cold, caloric restriction), a third subtype of adipocytes called beige or brite (*brown-in-white*) can be found interspersed in WAT, especially in the subcutaneous fat, leading to the acquisition of an intermediate phenotype between WAT and BAT [3]. Nutrient restriction (NR) can modulate the metabolic activity of beige adipocytes through a tight mito-to-nuclear communication [4]. Regular mitochondrial activity is fundamental for energetic homeostasis. However, beyond ATP production, mitochondria produce key metabolites and generate biochemical signals for regulation of metabolic pathways as well as apoptotic cell death. On the contrary, impairment of mitochondrial functions results in mitochondrial disorders and metabolic diseases such as insulin resistance and type 2

Abbreviations: NR, nutrient restriction; CM, control medium; eWAT, epididymal white adipose tissue; iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; Tot, total; Nuc, nuclear; Mit, mitochondrial; *Ad lib*, *ad libitum*; DHE, Dihydroethidium; NAC, N-*Acetyl*Cysteine; AD, alexidine dihydrochloride; Rot, rotenone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; *t*-BH, tert-butyl; PTPMT1, Protein Tyrosine Phosphatase, mitochondrial 1; ROS, reactive oxygen species.

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https://doi.org/10.1016/j.metabol.2019.04.006
0026-0495/

diabetes [5–7]. Mitochondria contain their own DNA; however, the majority of mitochondrial proteome derives from nuclear-encoded genes through the activation of nuclear transcription factors. Consequently, mitochondrial biogenesis and homeostasis depend on signaling pathways that advice the nucleus of mitochondrial needs or dysfunctions. This is partly performed by cytosolic mediators, including reactive oxygen species (ROS), or even mitochondrial-derived peptides that regulate transcription factors important for mito-nuclear communication (*e.g.* FOXOs, NFE2L2, NF-kB) [8–10]. Among the transcription factors orchestrating AT remodelling and metabolic adaptations is the forkhead homeobox type protein O1 (FoxO1). Recent works have demonstrated that it is upregulated by NR in adipocytes improving lipid catabolism, antioxidant response [11,12] and white-to-brown conversion [4,13]. FoxO1 is considered a nutrient sensing factor and a crucial metabolic regulator. It performs the nutrient sensing function in AT by shuttling between nucleus and cytosol under specific dietary requirements. It has been reported that insulin has an inhibitory action on FoxO1 as it mainly promotes FoxO1 nuclear export and proteasomal degradation [14]. On the contrary, we demonstrated that ROS produced by NR promote FoxO1 nuclear entrapment for the induction of nuclear-encoded mitochondrial stress defensive proteins, such as uncoupling protein 1 (UCP1) and superoxide dismutase 2 (SOD2), leading to improved mitochondrial homeostasis [4].

In this report we provide evidence of mitochondrial localization of FoxO1 in white/beige adipocytes under feeding condition that was modulated by NR changing mitochondrial-encoded genes expression. Moreover, we highlighted the involvement of ROS and FoxO1 post-translational modification as determinants of its localization in different cellular compartments.

2. Materials and methods

2.1. Mice and treatments

We conducted all mouse experiments in accordance with accepted standard of humane animal care and with the approval by local (Institutional Animal Care and Use Committee, Tor Vergata University) and relevant national (Ministry of Health) committees. C57BL/6 adult male mice were purchased from Harlan Laboratories S.r.l. (Urbino, Italy). For *in vivo* experiments, twelve C57BL/6 were randomly divided into two age-matched (4 months old) groups: *ad libitum* fed (Ctrl) by standard diet and NR. NR was performed by 20 h fasting. In this period, each NR mouse had free access to water and was kept on a 12:12 h light:dark cycle at constant temperature of 21–23 °C. Mice were sacrificed by cervical dislocation, and tissue samples were collected as reported by Mann et al. [15] and immediately processed for total protein, mRNA extraction and mitochondria isolation.

2.2. Cell lines, differentiation, transfections and treatments

Murine 3T3-L1 murine pre-adipocytes, murine T37i cell line and murine X9 cells were obtained, grown and differentiated as previously reported [4]. For all cell lines, NR was carried out by using Dulbecco's Phosphatase Buffered Saline (DPBS) with calcium/magnesium supplemented with 1% pen/strep (Lonza) for 4 h. All experiments were performed in fully differentiated adipocytes (day 8).

Fully differentiated 3T3-L1-white were transfected with FoxO1 or PTPMT1 siRNAs (Santa Cruz Biotechnology) or scramble siRNAs (Santa Cruz Biotechnology) by using DeliverX Plus kit (Affymetrix). Adipocytes were subjected to NR 48 h after transfection.

Nutrient refill (re-feed) was performed by culturing starved differentiated adipocytes in complete culture medium (CM) (Dulbecco's Modified Eagle's Medium-high glucose: 4.5 g/l glucose) supplemented with 1% pen/strep, 2 mM L-Glutamine (Lonza) and 10% Fetal Bovine Serum (EuroClone) for 1 h. *N*-acetylcysteine (NAC) (Sigma-Aldrich) was dissolved in DPBS and added in culture medium at final concentration of 2 mM 1 h prior to NR and maintained throughout the experiment. Doses and treatment conditions of rotenone, carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), hydrogen peroxide (H_2O_2), tert-butyl hydroperoxide (*t*-BH) (all from Sigma-Aldrich) are reported in figure legends.

2.3. Gel electrophoresis and Western blotting

Cells and AT were lysed in RIPA buffer (50 mM Tris-HCL pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% NP-40) supplemented with protease inhibitors cocktail (VWR life sciences). Western blotting analysis was performed as previously described [16] by using the following antibodies against: FoxO1 (#2880) and Phospho-FoxO1 (Ser256) (#84192) (Cell Signaling Technology); LDH (sc-33781), Sp1 (sc-59), TOMM20 (sc-11415), p-Tyr (sc-508), p-Ser (sc-81514), SOD2 (sc-137254), H2B (sc-10808), vDAC (sc-8828), Aco2 (sc-130677) (Santa Cruz Biotechnology); UCP1 (ab10983) (Abcam); H3 (06-755), ubiquitin (MAB1510) (EMD Millipore). For electrophoresis of mitochondrial, cytosolic and nuclear preparations, the equivalent amount of protein was loaded. Carbonyls were determined by OxyBlot Protein Oxidation Detection Kit (EMD Millipore).

2.4. RT-qPCR analysis

Total RNA was extracted using TRItidy G™ reagent (PanReac Applichem). Equal amount of RNA was used for retrotranscription with PrimeScript RT Reagent Kit (Takara Bio, USA, Inc.). qPCR was performed in triplicates by using validated qPCR primers (BLAST), SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara) and Real Time PCR LightCycler II (Roche Diagnostic, Indianapolis, IN, USA). Primer sequences are shown in Supplemental Table 1. The relative mRNA levels were determined by using the $2^{-\Delta\Delta Ct}$ method and were normalized to β-actin.

2.5. Nuclear and mitochondrial fractionation

Nuclear fraction was obtained from cells and tissues lysed in Nucleus Buffer (NB) containing 0.1% Triton, 0.5 M Sucrose, 15 mM Tris-HCL pH 7.5, 60 mM KCl, 0.25 mM EDTA, 0.125 mM EGTA, 0.1 mM DTT and phosphatase and protease inhibitors. Nuclei were collected by centrifugation at 600 ×*g* for 10 min at 4 °C. Supernatant was discarded and pellet (nuclei) was resuspended in NB and centrifuged at 600 ×*g* for 10 min to remove nuclear debris. Mitochondria from differentiated adipocytes and WAT were obtained as described and modified respectively by Wieckowski et al. [17] and by Cardamone et al. [18]. Briefly, the cells were homogenized in a Teflon potter and centrifuged to separate cytoplasm from nuclei and unbroken cells (Fig. S1A). The supernatant (cytosol) was then centrifuged to collect mitochondria-enriched fraction (Fig. S1A). Finally, crude mitochondria were layered on top of different percentage of Percoll solution, and mitochondria were separated through a Percoll self-forming gradient according to the method of Wieckowsky et al. [17]. For mitochondrial subfractionation gradient-purified mitochondria were resuspended in hypotonic solution (10 mm KCl, 2 mm HEPES, pH 7.2, 1.5 μg/μl digitonin) on ice for 10 min with gentle agitation. One-third volume of hypertonic solution (1.8 mm sucrose, 2 mm ATP, 2 mm $MgSO_4$, 2 mm HEPES, pH 7.2) was then added and incubated for an additional 5 min. After centrifugation at 10000 ×*g* for 10 min, it is possible to collect the outer membrane and the intermembrane space in the supernatant and mitoplasts in the pellet (Supplemental Fig. 1A).

2.6. Chromatin immunoprecipitation assay

ChIP assay was performed as previously described [19]. Briefly, after crosslinking, nuclei and mitochondria extracted from 3T3-L1 adipocytes were fragmented by ultrasonication using 4 × 15 pulse (output 10%,

duty 30%). Samples were precleared with pre-adsorbed salmon sperm ProteinG/agarose beads (1 h, 4 °C) and then overnight immunoprecipitation using anti-FoxO1 (Cell Signaling Technology) and control IgG antibody was carried out. After de-crosslinking (1% SDS at 65 °C for 3 h), qPCR was used to quantify the promoter binding with 30 cycles total (95 °C, 1 s; 60 °C, 30 s; 72 °C, 60 s) with primers listed in Supplemental Table 2. Results are expressed as fold enrichment with respect to IgG control.

2.7. Confocal microscopy

Cells were seeded directly on glass coverslips, fixed with 4% paraformaldehyde and permeabilized by incubation with 0.2% Triton X-100. 3T3-L1 and X9 adipocytes were incubated with anti-FoxO1 (Cell Signaling Technology) and TOMM20 (Santa Cruz Biotechnology) antibodies. After incubation with the appropriate AlexaFluor-conjugated secondary antibody (Life Technologies), images were captured through an Olympus Fluoview 1000 Confocal Laser Scanning System (Applied Precision Inc., Issaquah, WA, USA) equipped with a CDD camera. Nuclei were stained with Hoechst 33342 (10 μg/ml). Co-localization plugin (ImageJ Software, Bethesda, MD, USA) was used for nuclear FoxO1 localization. FoxO1/TOMM20 co-localization was analysed on 3T3-L1 and X9 cells subjected to CM or to NR for 6 h.

2.8. Cytofluorimetric analysis of ROS

ROS were determined by incubating cells with the fluorescent probe MitoSOX RED (5 μM) or Dihydroethidium (DHE) (1 μM) for 30 min at 37 °C. Subsequently, cells were collected and used for cytofluorimetric analysis by FACScalibur instrument (Beckton and Dickinson, San Josè, CA, USA).

2.9. Statistical analysis

The results are presented as means ± S.D. For multiple comparisons, statistical evaluation was conducted by ANOVA, followed by the post-hoc Tukey comparison, by using GraphPad Prism 7 Software (GraphPad Software). The comparison of only two variables was performed by Student's *t*-Test. Differences were considered to be significant at $p < 0.05$.

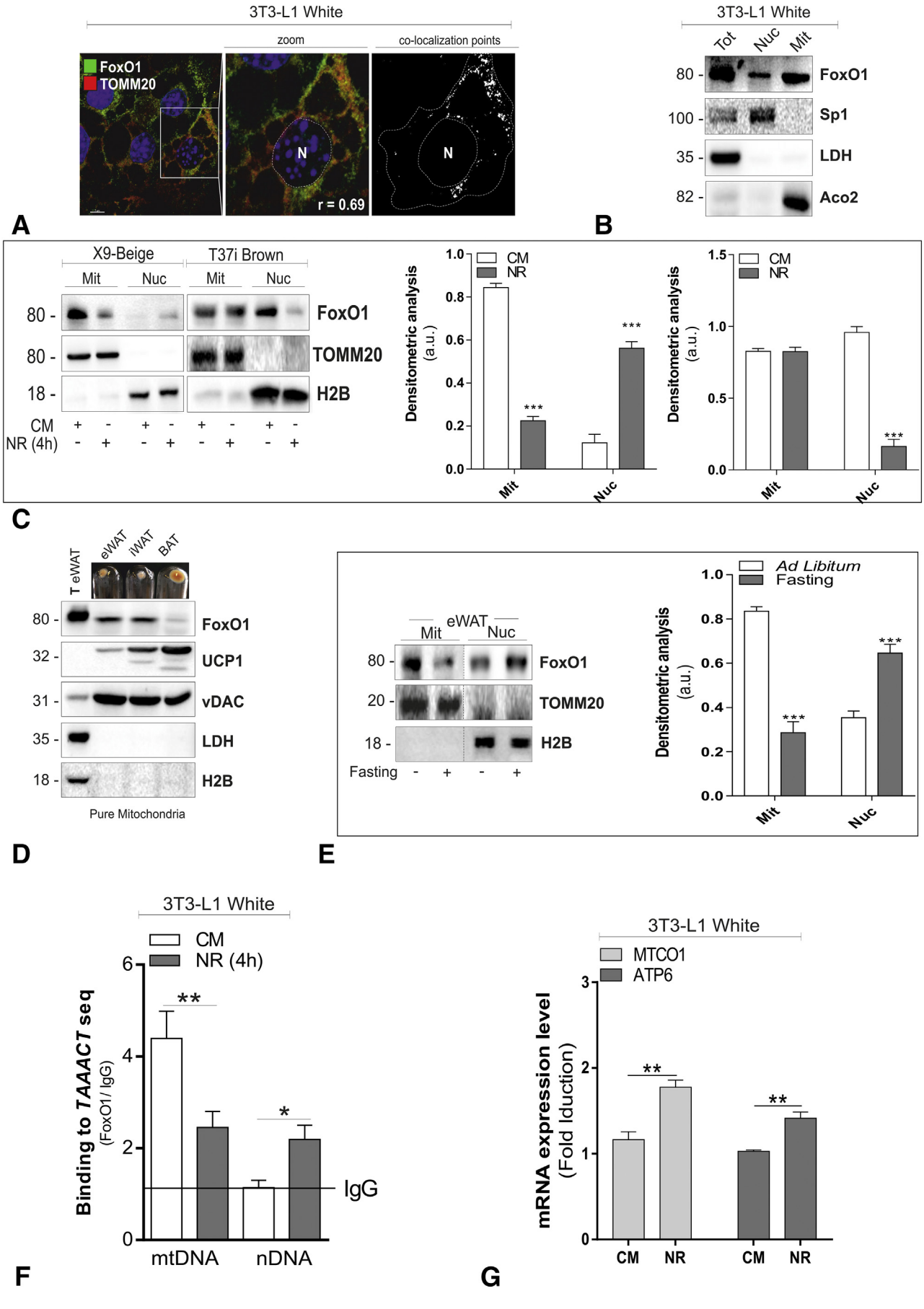
3. Results

3.1. FoxO1 has a mitochondrial localization in adipose tissues and cells

We have previously demonstrated that NR promotes cytosolic FoxO1 translocation into nuclear compartment in AT where it is responsible for increased transcription of genes related to lipid catabolism and stress adaptation [4,16]. Through confocal microscopy we noticed that under basal condition the staining of FoxO1 within the cytoplasm was heterogeneous with a punctate distribution that nicely coincided with that of mitochondrial lattice, as evidenced by TOMM20 counterstaining (Fig. 1A). Indeed, the calculation of the Pearson's correlation coefficient between FoxO1 and TOMM20 signals gave a significant value of $r = 0.69$ (Fig. 1A). This observation prompted us to more deeply characterize FoxO1 mitochondrial localization. Basically, we carried out Western blot analysis on purified mitochondria from 3T3-L1 adipocytes. Supplemental Fig. 1A shows the procedure adapted to obtain a highly pure mitochondrial fraction. Nuclear and cytosolic contaminations of purified mitochondria were excluded by performing Western blot analysis of Sp1 and LDH, respectively (Fig. 1B). The high content of Aco2 stated for efficient mitochondrial isolation (Fig. 1B). The use of antibodies against FoxO1 clearly evidenced FoxO1 presence at both mitochondrial and nuclear fractions of 3T3-L1 adipocytes under basal metabolic conditions (Fig. 1B). To verify that the reactive Western blot band actually corresponded to FoxO1 we made the immunoblotting after FoxO1 downregulation through RNA interference of 3T3-L1 cells. Supplemental Fig. 1B clearly demonstrated lack of the cross-reactive band of FoxO1 at both mitochondrial and nuclear fractions.

Fat cells display different phenotypes in relation to their role in energy and lipid homeostasis. Therefore, we also analysed the nuclear and mitochondrial localization of FoxO1 in X9-beige and T37i brown adipocytes. Fig. 1C shows the presence of FoxO1 in the purified mitochondria of both cell lines, indicating a role for its mitochondrial localization independently of adipocyte phenotype. Considering that NR induces the shuttling of cytosolic FoxO1 into the nucleus, we tested the outcome of this condition on FoxO1 mitochondrial localization. We treated X9-beige and T37i brown cells with DPBS for 4 h, as previously reported [16], in order to induce a NR condition. Fig. 1C shows that the nuclear content of FoxO1 is increased with a concomitant decrease in mitochondria of X9-beige cells. Moreover, confocal microscopy analysis showed a good co-localization with TOMM20 ($r = 0.64$) (Supplemental Fig. 1C) under fed condition that is lost after NR for 6 h ($r = 0.18$). A completely different trend was observed in T37i brown cells where nuclear content of FoxO1 decreases under NR with no change in mitochondrial fraction (Fig. 1C). Next, we evaluated the presence of FoxO1 in mitochondria *in vivo* purifying mitochondria from different mice fat depots. Fig. 1D reports Western blotting of purified mitochondria from eWAT (epididymal WAT), iWAT (inguinal WAT) and BAT of *ad libitum* fed mice. FoxO1 content was high in eWAT and iWAT with respect to BAT, confirming its localization at mitochondrial level also in *in vivo* fat depots and especially in white adipocytes. The figure shows a high purity of the mitochondrial fraction as demonstrated by the lack of LDH and H2B staining and high content of the mitochondrial porin vDAC. The different content of UCP1, which is mainly expressed in BAT, confirmed the AT phenotypes. Furthermore, the mitoplasts obtained through mitochondrial subfractionation highlighted the genuine localization of FoxO1 in mitochondrial matrix (Supplemental Fig. 1E). The different localization of mitochondrial FoxO1 observed in *in vitro* cell models in response to NR was also tested in *in vivo* analysing nuclear/mitochondrial shuttling of FoxO1 in eWAT of fasted mice. As reported in Fig. 1E, fasting causes a reduction of FoxO1 level in mitochondria and an enrichment in the nucleus. Supplemental Fig. 1D reports FoxO1 content in pure mitochondria from eWAT and BAT upon fasting, where it is clearly demonstrated that mitochondrial FoxO1 in BAT is unresponsive to NR. To envisage a role for FoxO1 in mitochondria, we verified the nutrient-dependent binding of FoxO1 to the D-loop region of mtDNA, where it is present the core recognition motif for FoxO proteins GTAAA(C/T)A

Fig. 1. Mitochondrial localization of FoxO1 *in vitro* and *in vivo*. (A) Mitochondrial localization of FoxO1 in 3T3-L1 white adipocytes determined by confocal microscopy after co-staining with FoxO1 (green) and TOMM20 (red) antibodies. Co-localization signals were estimated by calculating Pearson's coefficient ($r = 0.69$) in complete medium (CM). (B) Western blot analysis of FoxO1 in total, nuclear and mitochondrial extracts from 3T3-L1 white adipocytes. Sp1, LDH and Aco2 are purity control markers for nuclei, cytosol and mitochondria respectively. (C) Representative western blot analysis of FoxO1 in mitochondrial and nuclear extracts from X9-beige and T37i brown adipocytes (upper panel) in CM and after 4 h of nutrient restriction (NR). TOMM20 and H2B are purity control markers for mitochondria and nuclei respectively. The right panels report the densitometric analysis of mitochondrial and nuclear FoxO1 immunoreactive bands with respect to loading controls. Data are shown as means ± SD ($n = 4$). (D) Western blot analysis of FoxO1 and UCP1 in purified mitochondria from adipose tissue of mice fed *ad libitum*. T eWAT = Total lysate. eWAT = epididymal white adipose tissue. iWAT = inguinal white adipose tissue. BAT = brown adipose tissue. vDAC, LDH and H2B are purity control markers for mitochondria, cytosol and nuclei respectively. Immunoblots are from one experiment representative of four that gave similar results (E) Representative western blot analysis of FoxO1 in mitochondria and nuclei purified from eWAT of mice fed *ad libitum* or subjected to 20 h fasting (upper panel) ($n = 4$ mice per group). TOMM20 and H2B are purity control markers for mitochondria and nuclei respectively. The right panel reports the densitometric analysis of mitochondrial and nuclear FoxO1 immunoreactive bands with respect to loading controls. Data are shown as means ± SD ($n = 4$) (F) Binding activity of FoxO1 on mtDNA or nDNA determined by ChIP in 3T3-L1. Bar graphs are expressed as means ± SD ($n = 4$; $^{*}p < 0.05$; $^{**}p < 0.01$ *vs* CM) (G) MTCO1 and ATP6 mRNA expression levels assayed by RT-qPCR in 3T3-L1 white adipocytes in CM and 4 h of NR.


3T3-L1 White
zoom
co-localization points
FoxO1
TOMM20
N
r = 0.69
A
3T3-L1 White
Tot
Nuc
Mit
80 -
FoxO1
100 -
Sp1
35 -
LDH
82 -
Aco2
B
X9-Beige
T37i Brown
Mit
Nuc
80 -
FoxO1
80 -
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31 -
vDAC
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18 -
H2B
Pure Mitochondria
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FoxO1
20 -
TOMM20
18 -
H2B
Fasting
Ad Libitum
Fasting
Densitometric analysis
(a.u.)
E
3T3-L1 White
CM
NR (4h)
Binding to TAAACT seq
(FoxO1/ IgG)
**
*
IgG
mtDNA
nDNA
F
3T3-L1 White
MTCO1
ATP6
mRNA expression level
(Fold Induction)
**
CM
NR
G

[20]. By ChIP assay we observed a clear enrichment of FoxO1 at the D-Loop region under basal condition, while a diminished occupancy of FoxO1 on mtDNA was detected upon NR (Fig. 1F). Moreover, the increased binding of FoxO1 to a nuclear consensus sequence after NR was evidenced (Fig. 1F), in line with our previous results [12]. This scenario prompted us to investigate the effect of NR on the expression of mitochondrial encoded genes. Fig. 1G shows that the mRNA expression levels of MTCO1 (cytochrome C oxidase subunit I of respiratory complex IV) and ATP6 (ATP synthase Fo subunit 6) are significantly increased after 4 h of NR, suggesting an inhibitory effect of FoxO1 on mtDNA.

3.2. Mitochondrial FoxO1 is phosphorylated

It has been reported that phosphorylation of FoxO1 on key insulin/Akt target residues is mandatory for its nuclear exclusion and cytoplasmic retention [14]. We observed that under basal condition a portion of mitochondrial FoxO1 is phosphorylated on serine 256 (p-FoxO1) as it is for the nuclear isoform (Fig. 2A and B). NR induced a significant decrement of phosphorylated FoxO1 in cytosol, nuclei and mitochondria. Moreover, nutrient refilling tends to re-establish the levels of FoxO1 isoforms mainly in the cytosol (Fig. 2A and B).

The PTPMT1 (Protein Tyrosine Phosphatase, mitochondrial 1) is a family member of the dual-specific protein tyrosine phosphatase exclusively anchored to the matrix face of the inner mitochondrial membrane, where it is involved in bioenergetics regulation [21]. We first evaluated the levels of phosphoserine (p-Ser) and phosphotyrosine (p-Tyr) in pure mitochondria upon NR. Fig. 2C shows a significant decrement of phosphorylated proteins following NR and a concomitant increase in the mRNA levels of PTPMT1 in 3T3-L1 cells and in X9-beige cells (Fig. 2D). The latter result was also recapitulated in WAT depots of fasted mice (Fig. 2E). On the contrary, no change in the levels of the phosphatase was observed in T37i brown cells under NR and in BAT of fasted mice. This reinforces the idea that the mitochondrial FoxO1 shuttling is typical of white/beige fat cells, strictly involved in the response to NR, with respect to brown adipocytes which are deputed to adaptive thermogenesis [4].

3.3. FoxO1-mediated anti-stress response is affected by PTPMT1 activation

It is well known that FoxO1 is an upstream transcriptional regulator of metabolic genes counteracting NR and of mitochondria antioxidant response in white and beige adipocytes, inducing UCP1 and SOD2 [4]. From data so far obtained, we wondered whether the nutrient-dependent phosphorylation of mitochondrial FoxO1 may be also involved in such processes. To this aim, we selectively inhibited PTPMT1 with alexidine dihydrochloride (AD) [22] in 3T3-L1 and X9-beige adipocytes. Fig. 3A shows that under starvation FoxO1 was retained in the mitochondria after PTPMT1 inhibition and at the same time the induction of SOD2 and UCP1 was abrogated. The silencing of PTPMT1 by siRNA (Supplemental Fig. 2A) confirmed the effects on FoxO1 mitochondrial localization as well as SOD2 and UCP1 levels (Supplemental Fig. 2B).

Therefore, PTPMT1 phosphatase may have a role in NR-mediated mito-nuclear communication by releasing FoxO1 from mitochondria and allowing the adaptive response to NR. This hypothesis is in line with the effect of PTPMT1 silencing on MTCO1 gene expression that was not affected by NR under this condition (Supplemental Fig. 2C). It is also worth to notice that PTPMT1 inhibition *per se* resulted in mitochondria-encoded gene modulation that deserves further investigation (Supplemental Fig. 2C).

SOD2 and UCP1 are part of the antioxidant response orchestrated by FoxO1 under NR [4]. For this reason, we analysed the occurrence of oxidative unbalance by measuring mitochondrial ROS production and carbonyls content upon PTPMT1 inhibition. Fig. 3B shows that oxidative burst in mitochondria after NR was even exacerbated upon inhibition of the phosphatase. Consequently, we determined the effect of the antioxidant *N*-acetyl cysteine (NAC) on the expression levels of PTPMT1 in 3T3-L1 and X9-beige cells. Fig. 3C shows that NAC treatment completely abrogates the NR-mediated increase in PTPMT1 mRNA expression. To verify whether the effects of NAC on PTPMT1 activation impinge on FoxO1 phosphorylation and localization, mitochondria were isolated from 3T3-L1 cells subjected to NR with/without NAC. As shown in Fig. 3D, after NAC co-treatment FoxO1 is retained inside mitochondria as a phosphorylated isoform demonstrating that ROS deriving from NR are necessary for FoxO1 dephosphorylation and mitochondrial delocalization. The impact of NAC on FoxO1 subcellular localization after NR treatment was also confirmed in X9-beige cells (Supplemental Fig. 2D).

3.4. Mitochondrial localization of FoxO1 is modulated by ROS

As ROS production during NR was demonstrated to be required for FoxO1 mitochondrial delocalization upon NR, we investigated the effects of different ROS sources such as: mitochondrial (rotenone and CCCP) and external/cytoplasmic (H_2O_2 and *t*-BH) ones. 3T3-L1 adipocytes were treated with rotenone for different times and a significant ROS increase was demonstrated (Fig. 4A) and associated with an increment of ubiquitinated proteins (Supplemental Fig. 2E). Notably, the pure mitochondrial fractions obtained under rotenone treatment displayed an increase in FoxO1 content (Fig. 4B-left panel). This phenomenon was recapitulated by treatments with H_2O_2 and *t*-BH (Fig. 4B-right panel) indicating that an increased ROS flux is not sufficient to induce mitochondrial FoxO1 extrusion. On the contrary, CCCP led to reduced FoxO1 inside mitochondria (Fig. 4B-right panel) and to the activation of an integrated stress-response program similar to NR characterized by increased PTPMT1 and SOD2 expression (Fig. 4D–E). Likewise, the release of FoxO1 from mitochondria after NR or CCCP treatment was associated with upregulation of mitochondria-encoded genes MTCO1 and ATP6 (Fig. 4E–F). Finally, the fact that H_2O_2 treatment resulted in unaltered expression of PTPMT1, SOD2 (Fig. 4D–E) and mtDNA-encoded genes (Fig. 4E–F), further supports the importance of PTPMT1-mediated induction of FoxO1 shuttling for an efficient anti-stress response.

4. Discussion

FoxO1 has pivotal roles in mitochondrial metabolism in response to different nutritional conditions [4]. NR-induced mild redox imbalance promotes FoxO1 nuclear accumulation inducing enhanced mitochondrial oxidative capacity by transcription of lipolytic genes [16]. Moreover, FoxO1 has an active role in managing mitochondrial oxidative stress by increasing expression of UCP1 and SOD2 [4,13]. Herein, another unexpected role of FoxO1 in the control of mitochondrial function has been disclosed. Our results clearly showed a mitochondrial localization of this transcription factor in adipocytes under basal metabolic condition, both *in vivo* and *in vitro*. These data are in line with previous findings showing a mitochondrial localization of another member of FoxO family, FoxO3a [23,24]. Actually, it is well known that many proteins can enter mitochondria without any consensus sequence of mitochondrial localization, for example p53, STAT3 as well as FoxO3a [25]. However, it is nice to notice that the interrogation of the COMPARTMENTS database [26], which predicts protein localization by combining high-throughput microscopy-based screens and primary sequence analysis, provided a high confidence score for FoxO1 mitochondrial localization (Supplemental Fig. 2F).

Our data indicate that FoxO1 can regulate cellular responses to nutritional and oxidative stress, by redistributing itself among the different subcellular compartments and allowing a mitochondrial adaptive response specific for the type of adipose cell/tissue. In fact, even though T37i brown adipocytes show FoxO1 at mitochondrial level, they respond to NR without delocalization of mitochondrial FoxO1 but with decreased levels of the nuclear pool. A completely different trend was observed for 3T3-L1 white and X9-beige adipocytes, in which

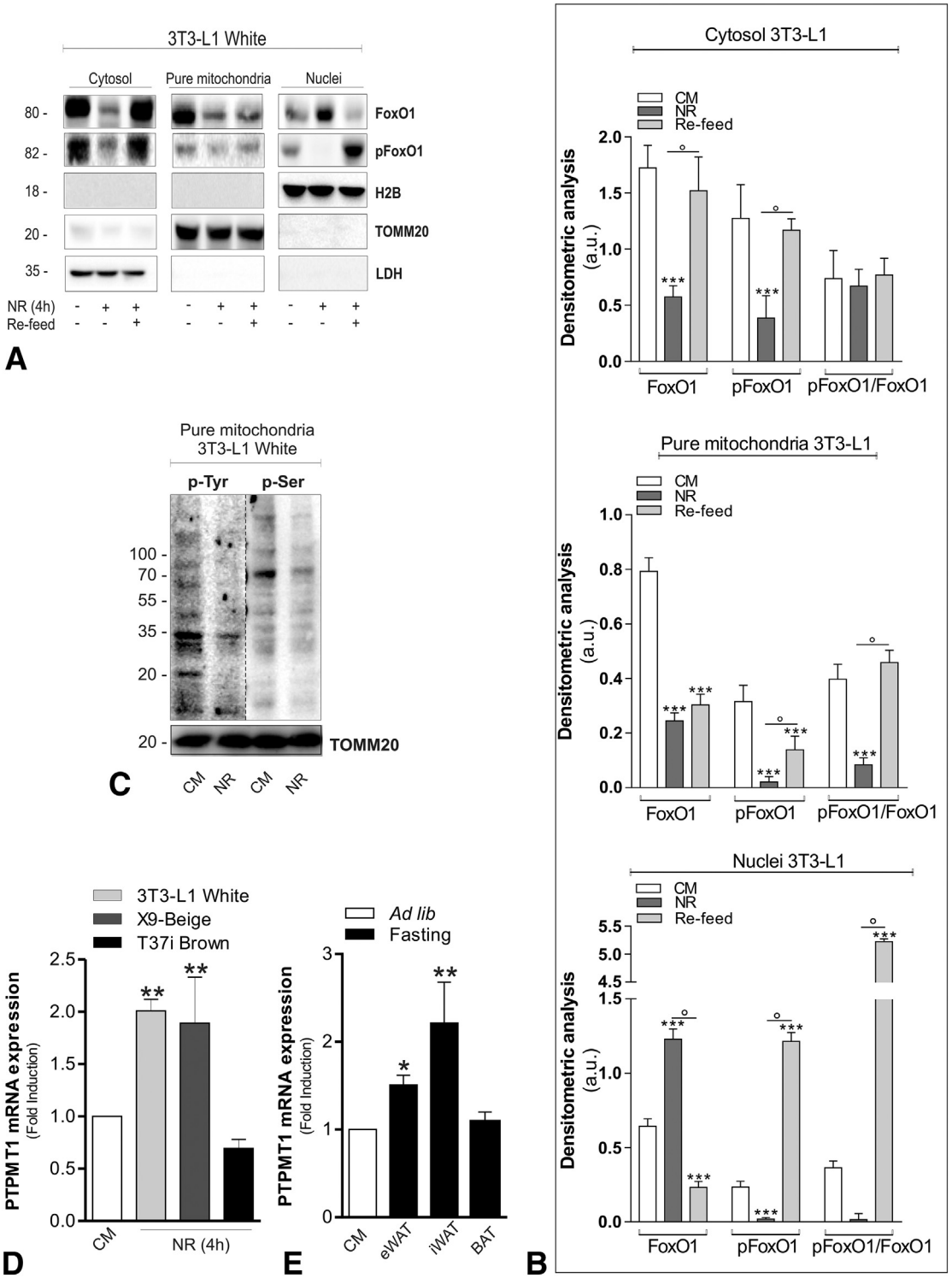

Fig. 2. Phosphorylated FoxO1 localizes to mitochondria and is affected by nutrients. (A) Western blot analysis of FoxO1 and phosphorylated FoxO1 at ser256 (p-FoxO1) in cytosol, mitochondria and nuclei isolated from 3T3-L1 white adipocytes subjected to 4 h of NR and 1 h nutrient refill with CM (Re-Feed). TOMM20, H2B and LDH are used as purity control markers or loading controls. Immunoblots are from one experiment representative of four that gave similar results (B) Densitometric analysis of immunoreactive bands. Data are shown as means ± SD (n = 4; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 *vs* CM; °p < 0.05). (C) Western blot analysis of p-Tyrosine (p-Tyr) and p-Serine (p-Ser) levels of purified mitochondria from 3T3-L1 white in CM and 4 h of NR. A parallel western blot was performed to probe TOMM20 as loading control. Immunoblots are from one experiment representative of two that gave similar results (D–E) PTPMT1 mRNA expression levels assayed by RT-qPCR *in vitro* (3T3-L1 white, X9-beige, T37i brown adipocytes in CM and 4 h NR) (D) and *in vivo* (eWAT, iWAT and BAT from C57BL/6 mice) (n = 4 mice per group) (E). Fasting = 20 h. *Ad lib* = *Ad libitum*. Bar graphs are expressed as means ± SD (n = 4; *p < 0.05 **, p < 0.01 *vs* CM or *Ad Libitum*).

mitochondrial FoxO1 significantly decreases upon NR, with a concomitant increase in the nucleus. The same fluctuations were revealed in AT of *ad libidum*-fed mice with respect to starved ones. Also in this case FoxO1 mitochondrial localization in BAT was unaffected upon fasting with respect to WAT, confirming the functional difference of such fat depots in response to nutrient stress [27]. Therefore, we can speculate that the delocalization of FoxO1 at mitochondrial level could be mostly related to the signaling processes connecting nutrient availability to

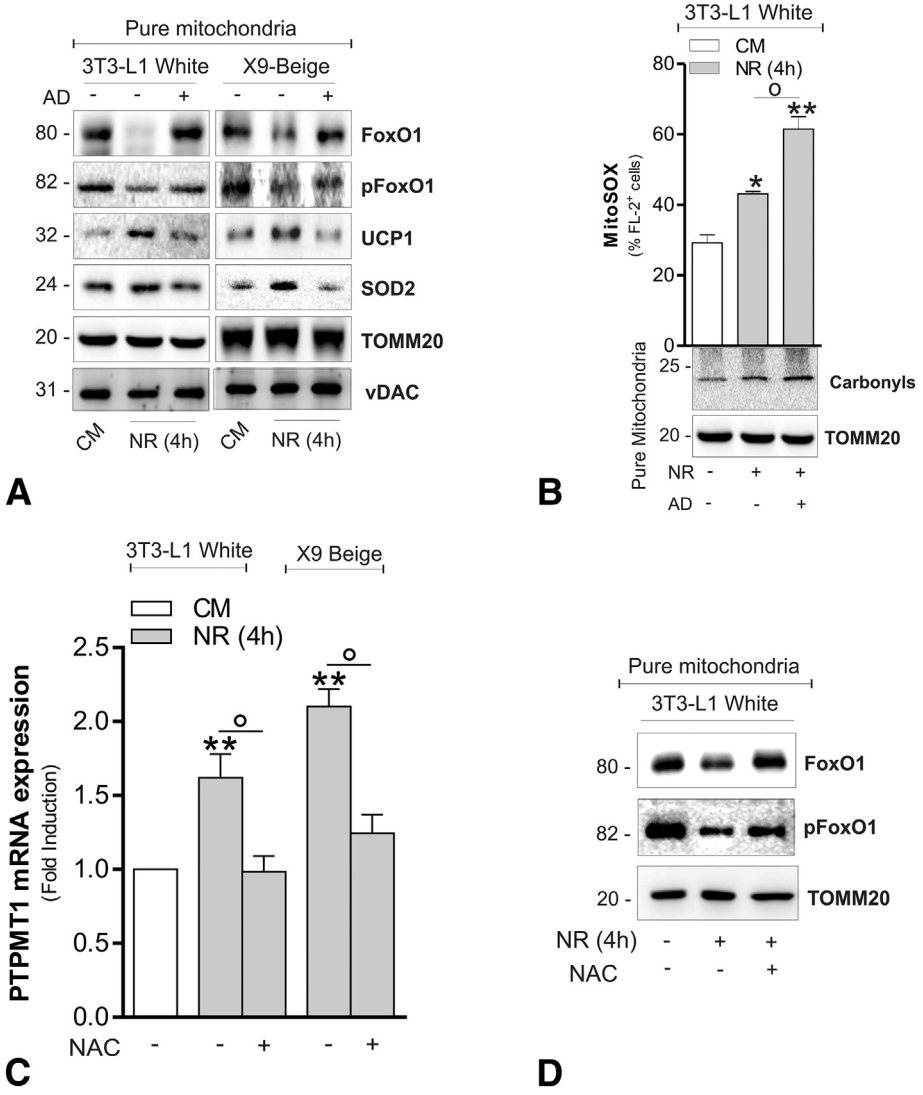

Fig. 3. PTPMT1 activation is involved in FoxO1 delocalization and associated anti-stress response. (A) Western blot analysis of FoxO1, phosphorylated FoxO1 at ser256 (p-FoxO1), UCP1 and SOD2 in mitochondrial extracts of 3T3-L1 white and X9-beige adipocytes treated with 5 μM of the PTPMT1 inhibitor alexidine dihydrochloride (AD). TOMM20 and VDAC are used as loading control. Immunoblots are from one experiment representative of three that gave similar results (B) Cytofluorimetric detection of mitochondrial ROS by MitoSOX probe in 3T3-L1 white adipocytes treated with NR (4 h) and AD (upper panel). Bottom panel, Western blot analysis of carbonyls of purified mitochondria. TOMM20 is used as loading control. Immunoblots are from one experiment representative of three that gave similar results. (**C**) PTPMT1 mRNA expression levels assayed by RT-qPCR in 3T3-L1 white and X9-beige adipocytes subjected to NR (4 h) and treated with 2 mM *N*-acetyl cysteine (NAC). (D) Western blot analysis of FoxO1 and phosphorylated FoxO1 at ser256 (p-FoxO1) in mitochondrial extracts of 3T3-L1 white treated with 2 mM NAC. TOMM20 is used as loading control. Immunoblots are from one experiment representative of three that gave similar results. Data are shown as means ± SD (n = 4). *p < 0.05; **p < 0.01 *vs* CM; °p < 0.05.

"nutrient-sensing" response. Even though the physiological role of FoxO1 at mitochondrial level requires further investigations, the fact that it is linked to mtDNA under basal metabolic condition and its release is associated with activation of mtDNA transcription after NR, indicates that FoxO1 can act as a transcription repressor in mitochondria. A dietary regimen such as NR would result in FoxO1 decrement at mitochondrial level and increase into the nucleus to modulate genes involved in nutritional and/or oxidative stress response. The restoration of the original distribution of FoxO1 into mitochondria in our re-feeding condition seems not efficient and it may require a different time of recovery or a more complicated reloading process. The latter hypothesis can be supported by the fact that FoxO1 seems to undergo post-translational modification before leaving mitochondria and this might impair its rapid return into the organelle under our re-feeding condition. In fact, we detected a percentage of FoxO1 phosphorylated at Ser256 at mitochondrial level and this modified form decreases under NR. Along with this, the mitochondrial phosphatase PTPMT1, an enzyme able to respond to nutrient and oxidative stress [21], is upregulated during NR treatment both in our *in vitro* and *in vivo* experimental systems, indicating an active involvement of such phosphatase in the NR mediated-stress condition. The impaired release of FoxO1 from mitochondria under starvation conditions when PTPMT1 activity is inhibited strongly indicates that FoxO1 de-phosphorylation is required for its migration outside mitochondria. Moreover, under our experimental condition, the increased levels of PTPMT1 were strictly related to ROS production. In fact, the use of the antioxidant NAC inhibits the elevation of PTPMT1 level and the NR-mediated FoxO1 shuttling. PTPMT1 inhibition increases ROS levels and oxidative damage dampening the release of FoxO1 from mitochondria and the activation of mitochondria-encoded genes as well as of UCP1 and SOD2. Therefore, these results demonstrate the great importance of PTPMT1/FoxO1 crosstalk under NR-increased ROS flux in order to orchestrate an efficient cell response.

Finally, a very interesting aspect of our results was the relation among different sources of ROS and FoxO1 mitochondrial localization. The treatment with the uncoupler CCCP mimicked the effects of NR in

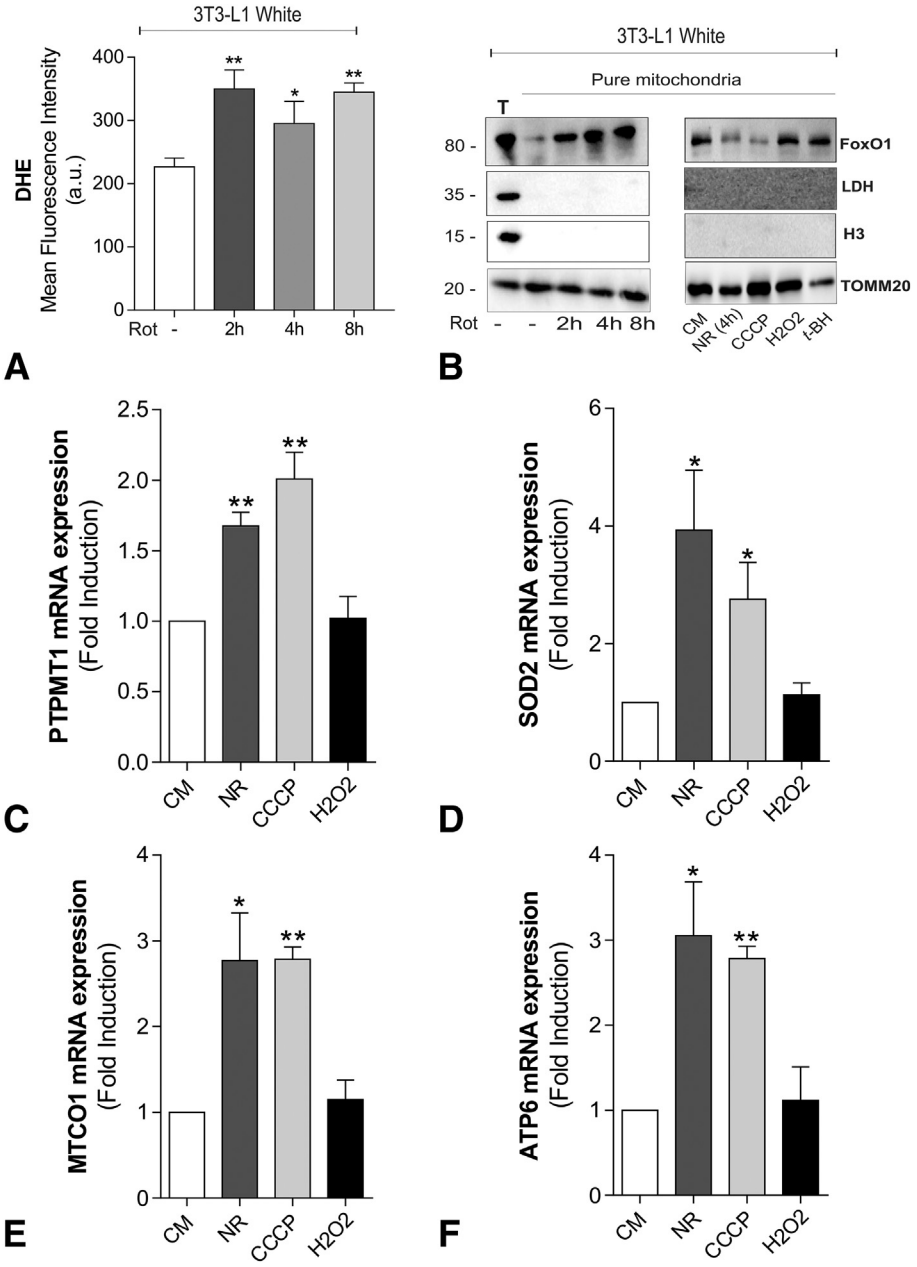

Fig. 4. Mitochondrial localization of FoxO1 is differently affected by alternative ROS sources. (A) Cytofluorimetric determination of ROS by DHE probe in 3T3-L1 white adipocytes treated with 1 μM of rotenone (Rot) for different time points. Data are shown as means ± SD (n = 6) *p < 0.05; **p < 0.01 *vs* untreated. (B) Western blot analysis of FoxO1 in purified mitochondria of 3T3-L1 white adipocytes treated with 1 μM of rotenone (left panel) or NR (4 h), 15 μM of CCCP (1 h), 500 μM of H_2O_2 (30 min) and 1 mM of *t*-BH (30 min). LDH and H3 are purity control markers. TOMM20 was used as loading control. Immunoblots are from one experiment representative of three that gave similar results. T = Total lysate. (C–D) PTPMT1 and SOD2 mRNA expression levels assayed by RT-qPCR in 3T3-L1 white treated with NR (4 h), CCCP (1 h) and H_2O_2 (30 min). (E–F) MTCO1 and ATP6 mRNA expression levels assayed by RT-qPCR in 3T3-L1 white adipocytes treated with NR (4 h), CCCP (1 h) and H_2O_2 (30 min). Data are shown as means ± SD (n = 3). *p < 0.05; **p < 0.01 *vs* CM.

terms of FoxO1 decrement in mitochondria, activation of PTPMT1, SOD2 and mtDNA-encoded genes. This is in line with the fact that starvation is also associated with mitochondrial depolarization [4]. On the contrary, as in the *ad libitum* feeding condition, the mitochondria sited ROS production by rotenone or the exogenous sources of ROS, such as H_2O_2 or *t*-BH, retain FoxO1 into the mitochondria, indicating that its subcellular distribution is not a mere consequence of ROS burst. Accordingly, H_2O_2 treatment condition has no effect on mitochondria-encoded gene, PTPMT1 induction and consequently on SOD2 transcriptional level, resembling the results obtained after the use of AD. All this evidence supports the hypothesis that de-phosphorylation of FoxO1 is a prerequisite for its migration from mitochondria, unlocking mitochondrial gene expression and the activation of antioxidant stress response at nuclear level.

Although further investigations will be necessary to deeply clarify the physiological role of FoxO1 in mitochondrial compartment, what emerges from this work is that ROS are important players in the commitment of FoxO1 localization and in the activation of cell response to nutrients. However, ROS need to be finely tuned (source, level, targets) in order to specifically turn on the transduction of the signaling processes governed by FoxO1.

Author contribution

D.L.B., L.I., K.A. and M.R.C. designed experiments; D.L.B. and L.I. performed the research in cooperation with F.C. and M.R.; K.A. and D.L.B.

revised the manuscript; M.R.C., L.I. and F.C. interpreted the data and wrote the manuscript.

Funding

This work was supported by the Italian Association for Cancer Research (AIRC, n. IG15403 M.R.C.), from MIUR/PRIN 2017 (n. 2017A5TXC3) and the European Foundation for the Study of Diabetes (D.L.B.)

Conflict of interest

All the authors declare that there are not any competing financial interests in relation to this work.

Acknowledgment

We thank people involved in animal housing and handling at the Stazione per la tecnologia animale (STA) of the University of Rome Tor Vergata.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.04.006.

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