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TFEB activation promotes the recruitment of lysosomal glycohydrolases β-hexosaminidase and β-galactosidase to the plasma membrane

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ABSTRACT

Lysosomes are membrane-enclosed organelles containing acid hydrolases. They mediate a variety of physiological processes, such as cellular clearance, lipid homeostasis, energy metabolism and pathogen defence. Lysosomes can secrete their content through a process called lysosome exocytosis in which lysosomes fuse with the plasma membrane realising their content into the extracellular milieu. Lysosomal exocytosis is not only responsible for the secretion of lysosomal enzymes, but it also has a crucial role in the plasma membrane repair. Recently, it has been demonstrated that lysosome response to the physiologic signals is regulated by the transcription factor EB (TFEB). In particular, lysosomal secretion is transcriptionally regulated by TFEB which induces both the docking and fusion of lysosomes with the plasma membrane. In this work we demonstrated that TFEB nuclear translocation is accompanied by an increase of mature glycohydrolases β-hexosaminidase and β-galactosidase on cell surface. This evidence contributes to elucidate an unknown TFEB biological function leading the lysosomal glycohydrolases on plasma membrane.

1.Introduction

Lysosomes are involved in the degradation of a wide variety of structurally diverse substances into their basic building blocks, such as proteins, glycosaminoglycans, glycogen, nucleic acids, oligosaccharides and sphingolipids [1]. The coordinated expression of lysosomal genes is essential not only for the degradation and recycling of metabolic intermediates but also for other specific cellular processes in which lysosomes are involved, such as cell-surface receptor regulation and antigen presentation [2–4].

Mutations that cause lysosomal enzymes deficiency are at the basis of a group of more than 50 genetic disorders, called Lysosomal Storage Disorders (LSDs), characterised by the accumulation of autophagic and endosomal substrates. Most of LSDs are associated with abnormal brain development and mental retardation. In addition, they are characterised by intracellular deposition and protein aggregation, events also found in age-related neurodegenerative disorders, such as Alzheimer's and Parkinson's Diseases [5–8]. The clinical phenotypes observed in LSDs are not a simple consequence of the accumulation of a specific substrate in lysosomes, but they are rather associated with an impairment of endosomal–lysosomal trafficking and autophagy [9,10].

Currently, many reports indicate the association of glycohydrolases with the plasma membrane [11]. In particular, neuraminidase 3,β-hexosaminidase, β-galactosidase and β-glucosidase, working on the oligosaccharide chain of glycosphingolipids, are associated with the external leaflet of the plasma membrane, where they display both *cis* and *trans* activity, being capable to act as well on surface components of the neighbouring cells [12–16]. It has been also demonstrated that recruitment on plasma membrane of functionally related glycohydrolases increases in cells overexpressing the α-subunit of β-hexosaminidase [17]. Moreover, the association of fully processed β-hexosaminidase and β-galactosidase with plasma membrane lipid microdomains has been demonstrated [18].

β-hexosaminidase (Hex, EC 3.2.1.52) and β-galactosidase (Gal, EC 3.2.1.23) are both involved in the stepwise degradation of GM1 to GM3 ganglioside. Hex is an acidic glycohydrolase that cleaves terminal β-linked N-acetylglucosamine or N-acetylgalactosamine residues from oligosaccharides, glycolipids, glycoproteins and glycosaminoglycans [19], while Gal catalyses the

Abbreviations: flot-2, flotillin-2; Gal, β-galactosidase; GM1, Galβ1,3GalNAcβ1,4-(NeuAcα2,3)-Galβ1,4Glc-ceramide; GM2, 3GalNAcβ1,4-(NeuAcα2,3)-Galβ1,4Glc-ceramide; GM3, NeuAcα2,3Galβ1,4Glc-ceramide; GSLs, glycosphingolipids; Hex, β-hexosaminidase; MUGal, 4-methylumbelliferyl-β-D-galactopyranoside; MUG, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide; MUGS, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide-6-sulphate; TFEB, transcription factor EB.

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hydrolysis of terminal N-linked galactosyl moiety from oligosaccharides and glycosides [20]. Genetic deficiency of Hex and Gal leads to the occurrence of GM2 and GM1 gangliosidosis, respectively, two severe neurodegenerative LSDs [19]. Moreover, alterations of these enzymes and their membrane-associated forms have been observed in pregnancy [21], apoptosis [22], neurodegenerative disorders [8,23], and cancer [24].

Recently, it has been demonstrated that the adaptive response of lysosomes to physiologic changes is related to the activity of TFEB [25]. TFEB is a master gene that induces autophagy, lysosome biogenesis and up-regulation of lysosomal genes expression [26,27]. Moreover, it has been reported that TFEB induces the clearance of storage material in several LSD cell models by promoting lysosomal exocytosis [28], which is in turn responsible for the secretion of lysosomal content in extracellular environment and plasma membrane repair [29].

In this work, we overexpressed TFEB in HEK-293 cells and analysed cell surface-associated Hex and Gal enzymes to explore whether TFEB also modulates the levels of plasma membrane-associated glycohydrolases.

2. Materials and methods

2.1. Cell culturing

HEK-293 cells (ATCC, Manassas, VA, USA), were cultured in DMEM supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml Streptomycin in a humidified incubator under 5% CO_2 at 37 °C. Starvation was performed in HBSS medium (Sigma–Aldrich), with Ca and Mg, supplemented with 10 mM HEPES. The viability of the cells was estimated by examining their ability to exclude trypan blue.

2.2. TFEB cloning and cells transfection

Total RNA was extracted from HEK-293 cells with PureLink™ Total RNA Purification System (Invitrogen). cDNA was obtained by reverse transcription of total RNA with random hexamer primers and SuperScript™ II Reverse Transcriptase according to the manufacturer's procedure (Invitrogen). Finally, full-length human TFEB cDNA was obtained by PCR performed with Phusion® Hot Start II (Finzymes) using the following primers: i) forward 5′-ATGGTACCC CACCATGGCGTCACGCAT-3′; reverse 5′-ATGAATTC-TCACAGCACATCGCCCTCC-3′; ii) forward 5′-ATGAATTCCCACCATG-GCGTCACGCA-3′; reverse 5′-ATGGTACCGTCAGCA CATCGCCCTCC TC-3′. Full-length human TFEB was cloned (i) into the pcDNA™6/*myc*-His A vector (Invitrogen) to produce TFEB without tag; (ii) into the pEGFP-N1 vector in frame with EGFP cDNA. TFEB–EGFP cDNA was successively subcloned in pcDNA™6/*myc*-His A vector to obtain TFEB–EGFP. Cells were seeded in six-well plates at 70% confluence before transfection. Transfection was performed by using jetPEI™ (Polyplus transfection) according to the manufacturer's protocols. Transfectants for TFEB and TFEB–EGFP (TFEB cells and TFEB–EGFP cells) and with the empty vector (CTRL cells) were selected with 8 μg/ml Blasticidin (Sigma–Aldrich).

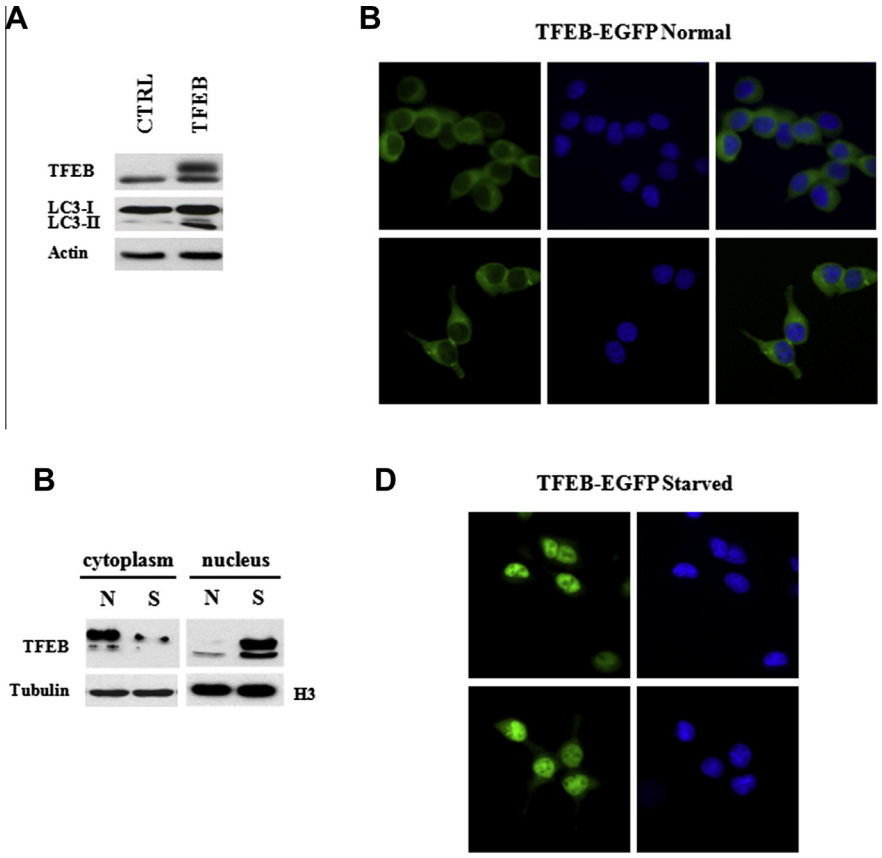

Fig. 1. Cells starvation promotes TFEB nuclear translocation. (A) Immunoblot analysis showing TFEB, LC3 and actin levels in extracts (30 μg of protein) from CTRL and TFEB cells. Representative immunoblotting of three independent experiments is reported. (B) Immunoblot analysis showing TFEB levels in cytosolic (30 μg of proteins) and nuclear (5 μg of proteins) extracts from TFEB cells, both in normal (N) and starved (S) conditions. Tubulin and H3 were used as cytosolic and nuclear markers, respectively. Representative immunoblotting of three independent experiments is reported. (C, D) Fluorescent microscopy images of TFEB–EGFP cells, both in normal and starved conditions. Magnification, 60×.

2.3. RNA extraction and real-time Q-PCR

cDNA from CTRL and TFEB cells, obtained as above, was used as template for the estimation of Hex β-subunit (HEXB), Hex α-subunit (HEXA) and Gal (GLB1) genes expression by quantitative PCR (Q-PCR) in a Stratagene Mx3000P Q-PCR machine (Agilent Technologies). β-Actin gene (ACTB) was amplified as endogenous control. Reactions and data analysis were obtained as reported in [18].

2.4. Preparation of cytosolic, nuclear and enriched plasma membrane extracts

CTRL and TFEB cells at 50% of confluence were washed twice in PBS and lysed with 50 mM Tris–HCl, 0.5% (v/v) Triton X-100 (TX-100), 137.5 mM NaCl, 10% (v/v) glycerol, 5 mM EDTA, pH 7.2 containing fresh protease and phosphatase inhibitor cocktail (Sigma–Aldrich). After 15 min on ice, nuclei were pelleted by centrifugation at 10,000*g* for 5 min at 4 °C, and the supernatants, corresponding to the cytosolic extracts, were recovered. Nuclei were washed twice with lysis buffer, centrifuged as above and lysed for 30 min on ice using 50 mM Tris–HCl, 150 mM NaCl, 1% (v/v) TX-100, 0.5% (w/v) deoxycholic sodium, 0.1% (w/v) SDS, pH 8.0 containing both fresh protease and phosphatase inhibitors. Debris were pelleted by centrifugation at 16,000*g* for 10 min at 4 °C, and the supernatants, corresponding to the nuclear extracts, were recovered.

Plasma membrane proteins were separated from soluble proteins using cell surface protein biotinylation and purification on avidin affinity column, as reported in [15]. Eluted cell surface proteins were precipitated with 10% TCA/acetone and the resulting pellets subjected to immunoblotting analysis.

Protein concentration was determined by the method of Bradford [30], using bovine serum albumin as standard.

2.5. Isolation of lipid microdomains

Lipid microdomains from CTRL and TFEB cells were isolated by discontinuous sucrose-density gradient centrifugation as previously described [18]. Eleven fractions of equal volume (450 μl) were collected from the top to the bottom of the gradient. In order to concentrate lipid microdomain vesicles, flot-2 positive fraction 3 was mixed with four volumes of TNE containing 1% (v/v) TX-100 in polyallomer centrifuge tube. Samples were ultracentrifuged at 60,000 rpm at 4 °C for 2 h using a TLA-100.3 rotor and an Optima Max ultracentrifuge. The pellets were resuspended in loading buffer for Western Blot analysis.

Cholesterol was depleted by incubating the cells in serum-free medium for 30 min at 37 °C in the presence of 10 mM Methyl-cyclodextrin (MbCD) (Sigma–Aldrich) prior to cell lysis.

2.6. Immunoblot and dot blot analysis

Proteins were separated by 10% SDS–PAGE under reducing conditions (Mini-Protean III, Biorad). Successively, proteins were transferred to PVDF membrane (Mini Trans-Blot Cell, Biorad) and reacted for 1 h with primary antibodies: goat polyclonal anti-TFEB (Abcam), rabbit polyclonal anti-H3 (Millipore), mouse monoclonal anti-β-actin (Sigma–Aldrich), mouse monoclonal anti-flot-2 (BD Biosciences), rabbit polyclonal anti-LAMP-1 (Millipore), mouse monoclonal anti-LC3 (Abcam), goat antiserum specific for Hex α-subunit, raised to a mixture of synthetic peptides belonging to the sequence of the human mature enzyme [18] and mouse anti-Gal (Sigma–Aldrich). After being washed using TBS containing 0.1% (v/v) Tween 20, the blots were incubated with secondary antibodies anti-goat IgG (Sigma–Aldrich), anti-rabbit IgG (GE Healthcare) or anti-mouse IgG (GE Healthcare), HRP-conjugated (Sigma–Aldrich), and were developed by ECL detection system (GE Healthcare).

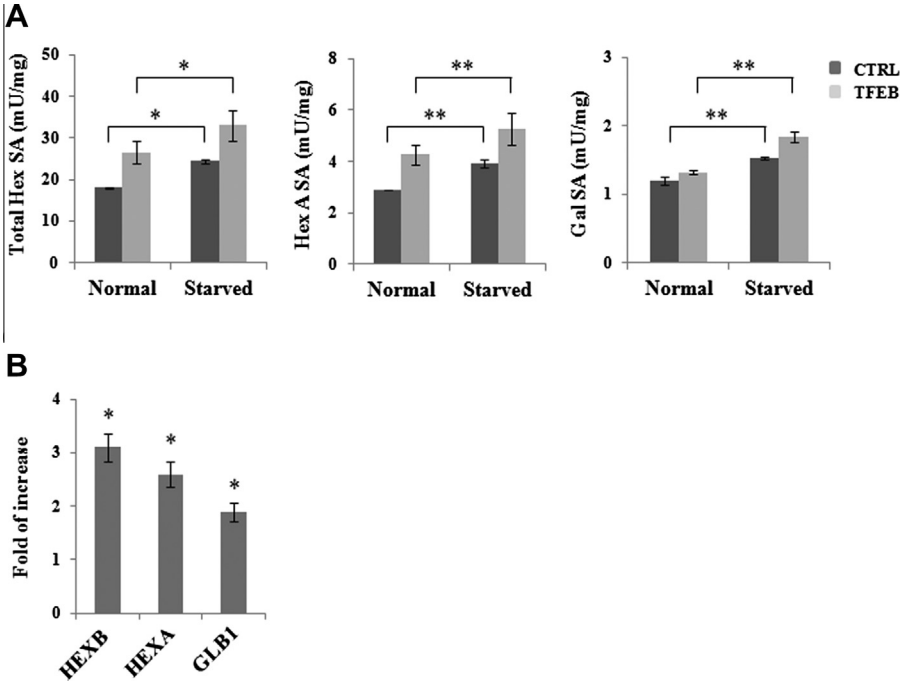

Fig. 2. TFEB modulates the expression of Hex and Gal. (A) Total Hex, Hex A and Gal specific activity (SA, mU/mg) in extracts from CTRL and TFEB cells, both in normal and starved conditions. Values are the mean ± SD of three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.05$ (starved *vs* normal cells) according to unpaired two tailed Student's *t* test. (B) Gene expression analysis by real-time Q-PCR. Reactions were performed in triplicate, using SYBR green binding to detect amplification. ACTB gene was used as endogenous standard. The fold expression of HEXB, HEXA and GLB1 genes in TFEB cells with respect to CTRL cells are reported. The values are expressed as Relative Quantity (RQ). The mean ± SD of three independent experiments is reported. $^{*}P < 0.01$ (TFEB *vs* CTRL cells) according to unpaired two tailed Student's *t* test.

Fractions from discontinuous sucrose-density gradient were spotted on nitrocellulose membrane (Biorad) for dot blot analysis and GM1 was revealed by incubation with cholera toxin B subunit biotin-conjugated (Sigma–Aldrich) for 1 h. The membrane was successively incubated with streptavidin HRP-conjugated (Pierce) and developed by ECL detection system.

2.7. Determination of glycohydrolase activities

Total Hex, Hex A and Gal lysosomal activities were determined as reported in [18]. Plasma membrane-associated Hex and Gal activities were determined in living cells plated in 12-wells microplates. The artificial substrates MUG, MUGS and MUGal were solubilized in DMEM–F12 medium, without phenol red, at the final concentration of 1 mM, 1 mM and 0.5 mM, respectively. At different incubation times, aliquots of the medium were fluorometrically analysed adding 5 volumes of 0.4 M glycine/NaOH buffer, pH 10.4.

One enzymatic unit (U) is the amount of enzyme that hydrolyses 1 mmol of substrate/min at 37 °C. Enzymatic activity was expressed as enzymatic unit $x10^{-3}$ (mU). Specific activity was expressed as mU/mg of protein.

2.8. Fluorescent microscopy analysis

TFEB–EGFP cells were plated onto glass coverslips and grown for 24 h. Starvation was performed as above. Cells were washed three times with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde/PBS for 30 min at RT. Slides were rinsed three times with PBS and mounted on glass coverslip using Vectashield with DAPI (Vector Laboratories). Fluorescence microscopy analysis was carried out using a Nikon TE2000 microscope through a 60x oil immersion objective. Image processing was performed using Adobe Photoshop CS software (Adobe Systems Incorporated).

3. Results

3.1. TFEB regulates expression and activity of Hex and Gal glycohydrolases

HEK-293 were stable transfected to overexpress TFEB or TFEB–EGFP. As expected, TFEB overexpression increased autophagosome formation, as attested by immunoblot analysis showing increased level of LC3II (Fig. 1 panel a). Moreover, as showed in Fig. 1 panel b, nutrient withdrawal induced TFEB nuclear translocation, whereas in normal conditions the transcription factor is mainly localised in the cytoplasm [27]. This result was also confirmed by Immunofluorescence microscopy which established the TFEB–EGFP nuclear translocation in starved cells (Fig. 1 panel c and d).

Control and TFEB cells, both in normal and starved conditions, were assayed for enzymatic activity of Hex using the two substrates MUG, which is hydrolysed by both α- and β-subunits forming Hex isoenzymes (Total Hex), and MUGS, which is hydrolysed only by the α-subunit-containing isoform (Hex A). Gal enzymatic activity was assayed using MUGal substrate. As reported in Fig. 2 panel a, Hex and Gal specific activities were increased in TFEB cells, both in normal and starved conditions, with respect to the control cells. The increase of glycohydrolase activities in starved cells is related to the TFEB activation which promotes its nuclear translocation [25].

The increase of Hex and Gal specific activities was due to transcriptional regulation, in fact, real-time Q-PCR experiments showed that the expression of both HEXB (5q13.3) and HEXA (15q24.1), the two evolutionarily related genes encoding for β and α subunits of Hex, and GLB1 gene (3p22.3) encoding for Gal, were all up-regulated in TFEB cells (Fig. 2 panel b).

3.2. Hex and Gal activity increases in the external leaflet of TFEB cells

TFEB overexpression results in an increased exposure of lysosomal membrane protein LAMP-1 on the cell surface, as shown in Fig. S1 panel a, where a significant enrichment in LAMP-1 was detectable in membrane fraction of TFEB cells with respect to control cells, indicating the occurrence of fusion event between lysosomes and plasma membrane. Enhancement of lysosomal exocytosis in TFEB cells with respect to the control cells was also

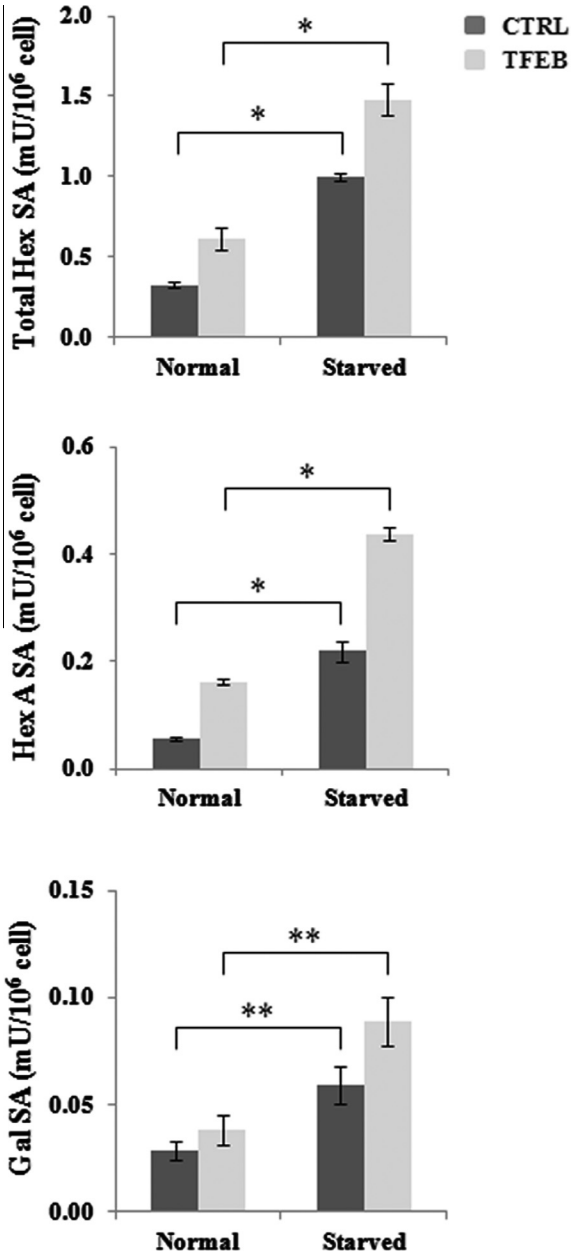

Fig. 3. TFEB overexpression promotes Hex and Gal translocation to the cell surface. Activity of plasma membrane Total Hex, Hex A and Gal was determined incubating living CTRL and TFEB cells in an isotonic medium containing the enzyme substrates. Data are expressed as specific activity (SA, mU/10^6 cells). Values are the mean ± SD of three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.05$ (starved *vs* normal cells) according to unpaired two tailed Student's *t* test.

confirmed by the increase of Hex and Gal enzymatic activity into the culture medium, as reported in Fig. S1, panel b.

In this context, to evaluate if there was also an enrichment of the cell surface associated Hex and Gal, TFEB and control cells were subjected to the *in vivo* enzyme activity assay [31]. This method is based on the observation that the fluorogenic substrates commonly used for the *in vitro* assay of glycohydrolases activity are not taken up by living cells and are hydrolysed by the enzyme associated to the external leaflet of the plasma membrane [17]. As shown in Fig. 3, Hex and Gal cell surface activities increased in TFEB cells, both in normal and starved conditions, with respect to control cells. This experiment clearly demonstrates that TFEB overexpression and/or activation induce a significant recruitment of Hex and Gal activities on the plasma membrane.

3.3. Hex and Gal are recruited in lipid microdomains of TFEB cells

To test if the glycohydrolases were homogeneously distributed on the cell surface or localised to specialised areas of the plasma membrane, we performed Hex and Gal assays after lipid microdomains purification. Cell lipid microdomains were obtained by flotation method as reported in 'Materials and methods'. Cells were treated with TNE containing 1% TX-100 and low-density floating material was separated from the unfloating material by using discontinuous sucrose-density gradient centrifugation. Our recently work [18] demonstrated that use of TX-100 as detergent provided a stringent method to exclude non-lipid microdomain proteins contamination. Fractions of the gradient were analysed for the presence of the specific lipid microdomain markers GM1 and

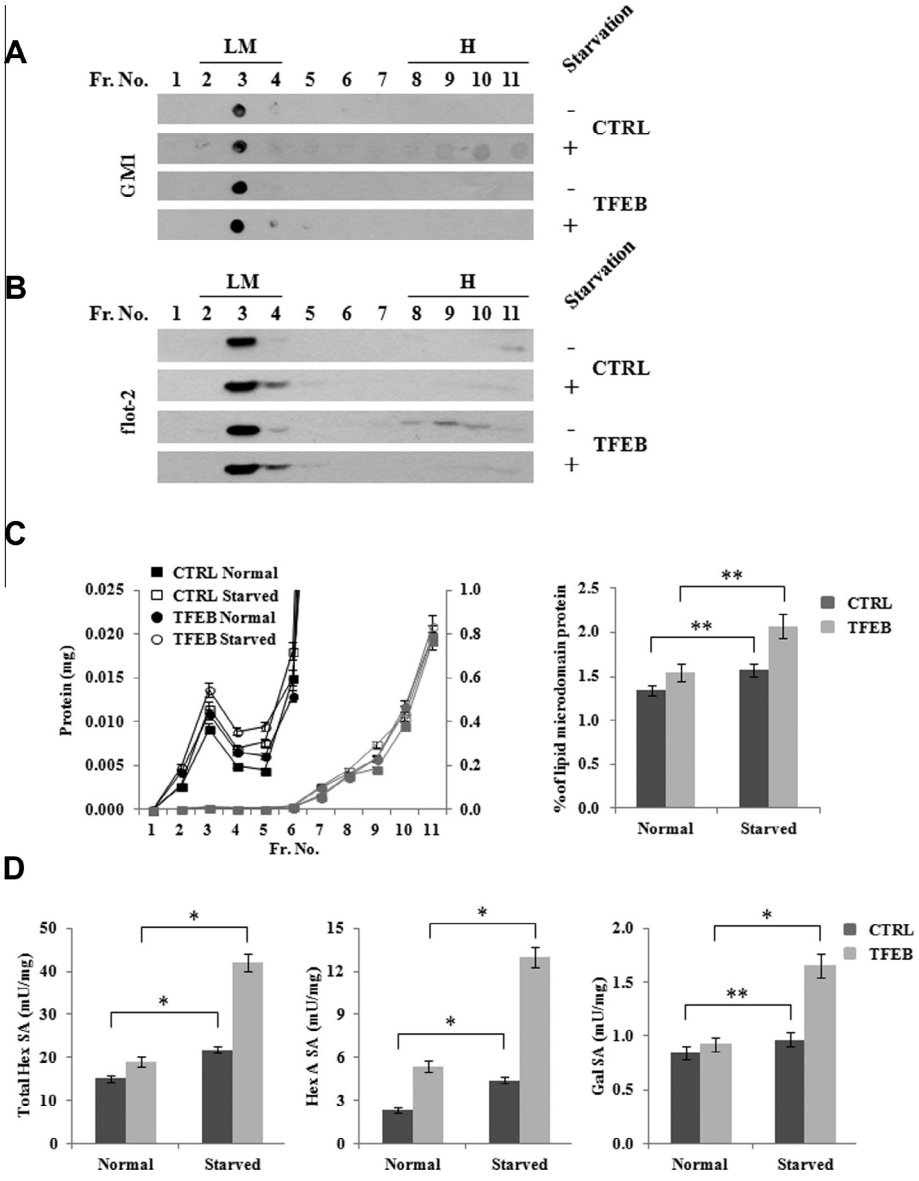

Fig. 4. Lipid microdomain Hex and Gal enzymatic activities increase consequently to the TFEB activation. CTRL and TFEB cells, both in normal and starved conditions, were resuspended in TNE buffer containing 1% TX-100. Cell extracts (2 mg of total proteins) were fractionated by discontinuous sucrose-density gradient. Eleven fractions were collected from the top of the gradient. (A) Aliquots of each fraction (1.5 μl) were spotted in a nitrocellulose membrane and the presence of GM1 was revealed by biotinylated CT-B. Representative Dot blot of three independent experiments is reported. (B) Aliquots of each fraction (30 μl) were subjected to immunoblotting for flot-2. Representative immunoblotting of three independent experiments is reported. (C) Protein distribution in the gradient is reported as mg of proteins recovered in each fraction (*gray line;* scale on the right). Enlarged data *(black line;* scale on the left) are also shown. Bar graphs reported the protein percentage recovered in lipid microdomain (LM) fractions with respect to the total proteins. Values are the mean ± SD of three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.05$ (starved *vs* normal cells) according to unpaired two tailed Student's *t* test. (D) Activity of Total Hex, Hex A and Gal in LM fractions from CTRL and TFEB cells, both in normal and starved conditions. Values are the mean ± SD of three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.05$ (starved *vs* normal cells) according to unpaired two tailed Student's *t* test. LM: Lipid microdomains; H: High density fractions.

flotillin-2 (flot-2). As shown in the Fig. 4 panel a and b, GM1 and flot-2 were highly enriched in the light-density fraction 3, corresponding to the 5–35% sucrose interface. Moreover, the protein content of this fraction corresponded to approximately 1.5–2.0% of the total proteins (Fig. 4 panel c). Interestingly, the overexpression and activation of TFEB resulted in an increase of lipid microdomain recovered proteins with respect to the control cells. Flot-2 positive fractions 2, 3 and 4 (LM fractions) were assayed for Hex and Gal enzyme activities. As reported in Fig. 4 panel d, the Hex and Gal specific activity showed an increase in TFEB cells, both in normal and starved conditions, with respect to control cells. In order to exclude contamination of the lipid microdomains with soluble lysosomal enzymes due to the experimental procedure, cells were treated with MbCD [32]. Cholesterol depletion induced by MbCD produced the disappearance of lipid microdomain-associated Hex and Gal enzymatic activity (data not shown).

To perform immunoblotting analysis of lipid microdomain-associated Hex and Gal, flot-2 positive fraction 3 from normal and starved TFEB cells was subjected to high-speed centrifugation. Results reported in Fig. S2 highlighted bands at 54 and 64 kDa for Hex α-subunit and Gal, respectively, corresponding to the fully processed forms of the proteins [19,20].

4. Discussion

Plasma membrane glycohydrolases may play important roles in both physiological and pathological conditions. For instance, catabolic events due to the activity of plasma membrane glycohydrolases have been described as the responsible for some of the changes in GSL patterns associated with neuronal development [33]. Moreover, plasma membrane glycohydrolases can contribute to the formation of pro-apoptotic ceramide from GSL [14].

Cell surface Hex and Gal are not randomly distributed on the cell membranes, but they are localised as mature forms in lipid microdomains [11,18], suggesting the translocation of glycohydrolases from lysosome to the cell surface [15,18,34]. In fact, after *de novo* synthesis, Hex and Gal, like other lysosomal proteins, are delivered from TGN to lysosomes, where they undergo to the final proteolytic cleavage [19]. Therefore, the presence of mature Hex and Gal on the cell surface implicates their lysosomal origin.

As recently pointed out by Ballabio's group, many evidences indicate that TFEB regulates multiple aspects of lysosomal dynamics [26], including the propensity of lysosomes to fuse with the plasma membrane [28], suggesting that the range of biological functions of TFEB still needs to be fully elucidated [25]. In this work, we demonstrated that TFEB nuclear translocation is accompanied by a significant increase of Hex and Gal activities on cell surface due to the activation of lysosomal exocytosis. Glycohydrolases Hex and Gal are both involved in the stepwise degradation of GM1 to GM3 ganglioside. It is notable that monosialogangliosides are instrumental in the formation of plasma membrane lipid microdomains [35] and participate to the modulation of signalling pathways by interacting with membrane proteins. Therefore, the associations of Hex and Gal on the cell surface close their natural substrates and the modulation of their level by TFEB strongly suggest a role of these enzymes in the *in situ* remodelling of plasma membrane, in order to quickly respond to cell physiological changes. For instance, an enrichment of Hex and Gal activities on plasma membrane of Gaucher disease fibroblasts has been reported and an increase of these enzymes trafficking toward the plasma membrane to respond to the enzymatic deficit has been suggested [36].

In conclusion, we demonstrated that the up-regulation of the lysosomal compartment due to the TFEB nuclear translocation is mirrored by Hex and Gal recruitment to the plasma membrane, where they are possibly involved in glycosphingolipids oligosaccharide modification processes [37]. The balance between glycosylation and deglycosylation of glycan structures directly at the cell surface may be functional: (i) to define the curvature of the membrane; (ii) to regulates transduction signaling pathway and, in general, (iii) for rapid cellular adaptation to environmental cues.

Results reported in this paper clearly indicate that lysosomal exocytosis induced by TFEB nuclear translocation is required not only for plasma membrane repair and lysosomal content secretion, but also for the recruitment of glycohydrolases on the cell surface.

Acknowledgments

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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.bbrc.2013.09.060.

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