BAG3 regulates formation of the SNARE complex and insulin secretion

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Insulin release in response to glucose stimulation requires exocytosis of insulin-containing granules. Glucose stimulation of beta cells leads to focal adhesion kinase (FAK) phosphorylation, which acts on the Rho family proteins (Rho, Rac and Cdc42) that direct F-actin remodeling. This process requires docking and fusion of secretory vesicles to the release sites at the plasma membrane and is a complex mechanism that is mediated by SNAREs. This transiently disrupts the F-actin barrier and allows the redistribution of the insulin-containing granules to more peripheral regions of the beta cell, hence facilitating insulin secretion. In this manuscript, we show for the first time that BAG3 plays an important role in this process. We show that BAG3 downregulation results in increased insulin secretion in response to glucose stimulation and in disruption of the F-actin network. Moreover, we show that BAG3 binds to SNAP-25 and syntaxin-1, two components of the t-SNARE complex preventing the interaction between SNAP-25 and syntaxin-1. Upon glucose stimulation BAG3 is phosphorylated by FAK and dissociates from SNAP-25 allowing the formation of the SNARE complex, destabilization of the F-actin network and insulin release.

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Bcl2-associated athanogene 3 (BAG3) protein is a member of BAG family of co-chaperones that interacts with the ATPase domain of the heat shock protein (Hsp) 70 through a specific domain known as BAG domain. In addition to this structural domain, BAG3 also contains a WW domain that is a protein interaction module that binds to the proline-rich motif, XPPXY1,2 and a proline-rich domain (PXXP) that modulates the interaction with SH3 domain-containing protein. These domains have been identified in a variety of signal transduction proteins that interact with plasma membrane receptor complexes or with components of the submembranous cytoskeleton suggesting that BAG3 might be a chaperone or a regulatory protein for proteins involved in cell migration and/or adhesion. Two BAG3 forms have been described so far: one is the full-length product of the bag3 gene having an apparent mass of 74 kDa; the other one is a shorter form found in association to neural synaptosomes. The full-length protein is normally localized in the cytoplasm and is mainly concentrated in the rough endoplasmic reticulum. Upon cell exposure to stressors, a slightly different molecular weight band can be observed and the protein runs as a doublet in a persistent secretion rate. The first phase is due to the readily releasable pool of dense-core granules in pancreatic beta cells, is released in two phases by exocytosis following glucose stimulation. The first phase results in a transient peak of secretion lasting only a few minutes, whereas the second phase maintains a lower but persistent secretion rate. The first phase is due to the triggering of the ATP-sensitive K(+) (K(ATP)) channel-dependent pathway that increases [Ca(2+)](i) and has been thought to discharge the granules from a readily releasable pool. The second phase of insulin secretion requires transport of the reserve granules pool to the plasma membrane and involves actin cytoskeleton remodeling. Insulin granule exocytosis requires docking and fusion of secretory vesicles to the release
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sites at the plasma membrane. This is mediated by a core machinery of membrane-associated soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which can be classified into two subfamilies: vesicle SNAREs (v-SNAREs) found on the vesicles and target SNAREs (t-SNAREs) found on the target membranes. These data suggest that in adhesion assembly, signaling and migration via its PXXP domain, BAG3 may negatively regulate adhesion, focal complexes at cell membrane. Earlier studies have demonstrated that isolated insulin-containing granules co-sediment with filamentous actin (F-actin), which is demonstrated that isolated insulin-containing granules to more peripheral regions of the β-cell, hence facilitating insulin secretion. It has been shown that BAG3 is involved in actin folding. It was also reported that BAG3 may negatively regulate adhesion, focal adhesion assembly, signaling and migration via its PXXP domain. These data suggest that in β-cells BAG3 could play a role in insulin release regulating intracellular trafficking or the exocytosis mechanism. Indeed using a mouse insulinoma β-TC-6 cell model here we show that BAG3 silencing affects F-actin polymerization state resulting in increased insulin secretion. Moreover, we show that BAG3 physically interacts with components of the SNARE complexes: SNAP-25/syntaxin-1, and is phosphorylated by FAK upon glucose stimulation. Our data suggest a model by which the activation of FAK after glucose entry, increases BAG3 tyrosine phosphorylation with subsequent loss of the BAG3/SNAP-25 interaction, thus allowing the formation of the t-SNARE complex and exocytosis of insulin vesicles.

Results

**BAG3 is highly expressed in human islet of Langerhans and co-localizes with insulin.** While studying expression of BAG3 in PDACs we noticed that pancreatic islets in normal pancreatic tissue surrounding the tumor strongly stained for BAG3, we therefore decided to further investigate the role of this protein in endocrine pancreatic cells. Indeed we confirmed this finding in additional pancreas sections and as shown in Figure 1a, BAG3 shows high expression in pancreatic islets, while the exocrine portion of the pancreas is as expected negative. We then chose to use an established mouse insulinoma cell line, β-TC-6, to further investigate the role played by BAG3 in endocrine pancreatic cells. As shown in the western blot in Figure 1b this cell line does express BAG3 that is localized in the cytoplasm of these cells (Figure 1c). Interestingly, BAG3 appears to co-localize with insulin-containing granules as shown by co-immunostaining (Figure 1d) and by subcellular fractionation (Figure 1e) that shows the presence of BAG3 in the insulin granule fraction.

**BAG3 knockdown decreases insulin cell content and increases its secretion.** We then tested the possibility that BAG3 influenced insulin levels and secretion. As shown in Figure 2a silencing BAG3 in β-TC-6 cells results in a significant reduction of their insulin content. Moreover, evaluation of insulin secretion in the supernatant by ELISA, following stimulation with 25-mM glucose, shows that this is strongly affected by BAG3 silencing. Under these experimental conditions there are two secretion peeks, one after 15 min and the other after 60 min (Figure 2b). BAG3 silencing results in increase of both early and late secretion. Similar results were obtained using a different siRNA to silence BAG3 excluding off-target effects (Supplementary Figure 1A).

**BAG3 knockdown impedes F-actin polymerization.** It has recently been shown that localized F-actin remodeling is a requisite for the normal biphasic pattern of nutrient-stimulated insulin secretion. F-actin constitutes a barrier that prevents docking and fusion of insulin granules to the plasma membrane allowing insulin exocytosis, hence preventing F-actin polymerization, markedly potentiates glucose-stimulated insulin secretion in a β-cell line. We therefore investigated the possibility that BAG3 affected insulin secretion by altering F-actin remodeling. To this end, we performed confocal experiments on β-TC-6 transfected with bag3 siRNA, using TRITC-conjugated phalloidin to visualize F-actin. As shown in Figure 2c and Supplementary Figure 1B, the actin cytoskeleton appears to be intact in the control cells, and after 15 min of glucose stimulation a redistribution of actin fibers becomes visible. This is consistent with the evidences that glucose induces F-actin depolymerization. Cells treated with NT siRNA revealed a similar pattern. Conversely, cells treated with bag3 siRNA display a clear disappearance of phalloidin staining, both in the unstimulated cells as well as in the cells stimulated with glucose, suggesting a failure in F-actin polymerization in BAG3-deficient β-cells.

**BAG3 interacts with t-SNARE complex: SNAP-25/syntaxin-1.** Cortical F-actin remodeling is known to couple granule mobilization to the SNARE exocytosis machinery. The formation of the t-SNARE complex by the interaction of SNAP-25 with syntaxin-1 at the plasma membrane is essential for the pairing with the vesicle v-SNARE complex. It is therefore possible that BAG3 affects F-actin remodeling by interacting with components of the SNARE complex. To test this hypothesis, we performed co-immunoprecipitation experiments in basal conditions and after 5, 15 and 30 min of glucose stimulation. β-TC-6 cell extracts were immunoprecipitated using anti-SNAP-25 (Figure 3a) and anti-syntaxin-1 (Figure 3b) antibodies and revealed using a polyclonal anti-BAG3 antibody. Interestingly, while BAG3 is pulled down with the antibody against SNAP-25 at basal levels and even more after 5 min of glucose stimulation, this interaction is lost at longer time points (Figure 3a). On the contrary BAG3 appears to interact with syntaxin-1 with the same intensity throughout the stimulation (Figure 3b).
We then investigated the possibility that BAG3 affected the interaction between syntaxin-1 and SNAP-25 and thus the fusion to the plasma membrane and exocytosis of insulin vesicles. As shown in Figure 3c treatment with glucose 25 mM for 15 min results in increased interaction between these two proteins, however, in the absence of BAG3 this interaction is far more pronounced following glucose stimulation. These data strongly suggest that BAG3 is an important regulator of the syntaxin-1/ SNAP-25 complex and prevents its formation in basal conditions, while under glucose stimulation BAG3 does not bind SNAP-25 allowing it to bind syntaxin-1 thus promoting exocytosis.

**FAK controls BAG3 tyrosine phosphorylation.** An essential part of the signaling leading to insulin secretion is FAK
autophosphorylation following glucose stimulation that leads to phosphorylation of other substrate proteins and the formation of the mulitprotic SNARE.\textsuperscript{28,49} We therefore checked whether FAK could interact with BAG3 and phosphorylate it. As shown in Figure 4a upon glucose stimulation there is an increase in BAG3 in the fraction containing phosphorylated proteins. As shown in Figure 4b BAG3 can be immunoprecipitated with FAK both in basal conditions and upon stimulation with glucose. Moreover, BAG3 immunoprecipitation in basal conditions and after 15 min of glucose stimulation followed by western blotting using a monoclonal anti tyrosine antibody (Figure 4c) shows that BAG3 is phosphorylated in tyrosine and that tyrosine phosphorylation increases upon glucose stimulation. Most importantly treatment with 1\textmu M Y15 a FAK specific inhibitor completely abrogates BAG3 phosphorylation, thus showing that BAG3 is FAK dependent and probably BAG3 is directly phosphorylated by FAK. As previously reported,\textsuperscript{28} under these experimental conditions, Y15 reduces insulin secretion (Figure 4d). Furthermore Y15 also reduces the interaction between SNAP-25 and syntaxin-1 (Figure 4e), and therefore SNARE complex formation. Finally, as shown in Figure 4f inhibiting BAG3 phosphorylation with the FAK inhibitor Y15 results in persistence of the interaction between BAG3 and SNAP-25 upon glucose stimulation.

Discussion

Insulin secretion by exocytosis of its storage granules is a complex mechanism that involves numerous proteins and is regulated by multiple pathways to finely control circulating
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Figure 3 BAG3 associates with SNAP-25 and syntaxin-1 and regulates the formation of the SNARE complex. β-TC-6 cells were stimulated with 25-mM glucose for the indicated time. Clarified whole-cell detergent extracts were prepared and immunoprecipitated with (a) anti-SNAP-25 or (b) anti-syntaxin-1 antibodies. Immune precipitates were analyzed by western blot with anti-SNAP-25, syntaxin-1, BAG3, Hsc-70 and GAPDH antibodies. Control immunoprecipitations were performed in parallel using mouse IgG. These results are representative of three sets of lysates prepared from independent experiments. (c) β-TC-6 cells at 80% confluence were transfected with a bag3-specific small interfering (si) RNA or a non-target (si) RNA (NT siRNA); 48 h after transfection, cells were stimulated with 25-mM glucose for 15 min. Clarified whole-cell detergent extracts were prepared and immunoprecipitated with anti-syntaxin-1. Immune precipitates were subjected to western blot with anti-SNAP-25 and GAPDH antibodies. Control immunoprecipitations were performed in a similar manner, using mouse IgG. These results are representative of two independent experiments.

Materials and Methods

Cell culture. Cells of the established murine β-TC-6 cell line were purchased from Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna (IZSLER, Lugo, Ra, Italy). Cells were grown in DMEM (LONZA Group Ltd, Basel, Switzerland) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

β-TC-6 cells at 80% confluence were transfected with a bag3-specific small interfering (si) RNA or a non-target (si) RNA (NT siRNA); 48 h after transfection, cells were stimulated with 25-mM glucose for 15 min. Clarified whole-cell detergent extracts were prepared and immunoprecipitated with anti-syntaxin-1. Immune precipitates were subjected to western blot with anti-SNAP-25 and GAPDH antibodies. Control immunoprecipitations were performed in a similar manner, using mouse IgG. These results are representative of two independent experiments.
Switzerland) culture medium containing 15% FBS; 25, 12.5 or 2.8 mM glucose; and 1% streptomycin/penicillin at 37°C in a 5% CO2 atmosphere. The medium was changed every 48 h, and cells were passaged once weekly using standard trypsin-EDTA concentrations.

**Tissue samples.** We analyzed tissue samples from tissue microarray TMA PA2082 (US Biomax, Inc., Rockville, MD, USA) that contained normal pancreas tissue samples from 11 donors (6 men and 5 women; mean ± S.D. age: 44.6 ± 19.3 years).

**Figure 4** BAG3 tyrosine phosphorylation by FAK disrupts the interaction between BAG3 and SNAP-25. (a) β-TC-6 cells were stimulated with 25-mM glucose for 15 min. Phosphorylated proteins were separated from unphosphorylated proteins using affinity chromatography. Then 20 µg of each sample was analyzed by western blot with anti-BAG3 antibody. Anti-p-ERK1/2 antibody was used as a control of the phosphorylated protein fraction and anti-GAPDH antibody as a control of the unphosphorylated fraction. These results are representative of two independent experiments. (b) β-TC-6 cells were stimulated with 25-mM glucose from 5–30 min. Clarified whole-cell detergent extracts were immunoprecipitated with an anti-FAK antibody. Immune precipitates were analyzed by western blot using anti-BAG3, FAK and GAPDH antibodies. Control immunoprecipitations were performed similarly using mouse IgG. These results are representative of three independent experiments. (c) β-TC-6 cells were stimulated with 25-mM glucose for 15 min with or without the FAK inhibitor Y15. Clarified whole-cell detergent extracts were immunoprecipitated with an anti-BAG3 antibody. Immune precipitates were analyzed by western blot using anti-phosphotyrosine and anti-ERK2 antibodies. Control immunoprecipitations were performed in parallel using mouse IgGs. These results are representative of three independent experiments. (d) Insulin secretory response of β-TC-6 cells treated with FAK inhibitor Y15. Insulin levels were evaluated by ELISA on β-TC-6 supernatants collected 15, 30 and 60 min after glucose stimulation. Data are mean ± S.E.M. (n = 2). *P < 0.05. (e) β-TC-6 cells were stimulated with 25-mM glucose for 15 and 30 min with the FAK inhibitor Y15. Clarified whole-cell detergent extracts were immunoprecipitated with an anti-SNAP-25 antibody. Immune precipitates were analyzed by western blot with anti-syntaxin-1 and Hsc-70 antibodies. Control immunoprecipitations were performed in parallel using mouse IgGs. These results are representative of two independent experiments.
Figure 5 Schematic illustration of the proposed role of BAG3 in insulin granule exocytosis. In basal conditions F-actin barrier (presumably stabilized by BAG3) prevents insulin granule mobilization to the plasma membrane. FAK autophosphorylation, upon glucose stimulation, leads to activation of the Rho family proteins (Rac-1 and Cdc42) that direct F-actin remodeling and to phosphorylation of BAG3. Phosphorylated BAG3 no longer binds SNAP-25 allowing its interaction with syntaxin-1 and the formation of the t-SNARE complex, thus promoting insulin secretion.

Antibodies and reagents. Antibodies recognizing ERK2 (sc-154), GAPDH (sc-32233), insulin (sc-9166), FAK (sc-271195) and Hsc-70 (sc-7298) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); SNAP-25 (mouse monoclonal) and syntaxin-1 (rabbit polyclonal) from Synaptic Systems (Göttingen, Germany); SNAP-25 (C-term; rabbit polyclonal) and syntaxin-1 (mouse monoclonal) from Antibodies-online (Aschen, Germany); p-tyrosine (# 9411), p-ERK1/2 (Thr202/Thr 404; # 9101) from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-BAG3 antibody TOS-2 (rabbit polyclonal) and AC-1 (mouse monoclonal) were purchased from Antibodies-online. FAK inhibitor 1,2,4,5-benzenetetraamine tetrahydrochlo ride (Y15) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Phalloidin conjugates TRITC (P 1951) was purchased from Santa Cruz Biotechnology, Inc. Briefly, 45 µM of anti-BAG3 mouse monoclonal antibody AC-1 for 2 h at room temperature, coverslips were washed three times with PBS 1x. After incubation with a 1:500 dilution of goat anti-rabbit IgG DyLight 594-conjugated antibodies (Jackson ImmunoResearch, Baltimore, PA, USA) and a 1:500 dilution of goat anti-mouse IgG DyLight 488-conjugated antibodies (Jackson ImmunoResearch) at room temperature for 45 min, coverslips were again washed for three times in PBS. F-actin was visualized using a dilution 1:1000 of TRITC-conjugated phalloidin, 45 min at room temperature. The coverslips were washed once with PBS 1x and once in distilled water and then mounted on a slide with interspaces containing 70% glycerol. Samples were analyzed using a confocal laser scanning microscope (Zeiss LSM confocal microscope, Oberkochen, Germany). Images were acquired in sequential scan mode by using the same acquisition parameters (laser intensities, gain photomultipliers, pinhole aperture and objective x63 or x150) when comparing experimental and control material. For production of figures, brightness and contrast gain were adjusted by taking care to leave a light cellular fluorescence background for visual appreciation of the lowest fluorescence intensity features and to help comparison among the different experimental groups. Final figures were assembled using Adobe Photoshop 7 and Adobe Illustrator 10 (San Jose, CA, USA).

Glucose-stimulated insulin secretion. β-TC-6 cells were plated in six-well plates at a density of 2.5 x 10^5 in DMEM with 2.8-mM glucose. At the beginning of the third day of subculture, cells were incubated once for 30 min at 37°C in Krebs-Ringer bicarbonate buffer (118.5-mmol/l NaCl; 2.54-mmol/l CaCl_2; 24.0-HO; 1.19-mmol/l KH_2PO_4; 4.74-mmol/l KCl; 25-mmol/l NaHCO_3; 1.19-mmol/l MgSO_4-7H_2O; 10-mmol/l HEPES (LONZA Group Ltd) and 0.1% bovine serum albumin (BSA), pH 7.4). Following incubation with a 1:100 dilution of anti-insulin polyclonal antibody, 3 µg/ml of anti-BAG3 mouse monoclonal antibody AC-1 for 2 h at room temperature, coverslips were washed three times with PBS 1x. After incubation with a 1:500 dilution of goat anti-mouse IgG DyLight 594-conjugated antibodies (Jackson ImmunoResearch, Baltimore, PA, USA) and a 1:500 dilution of goat anti-rabbit IgG DyLight 488-conjugated antibodies (Jackson ImmunoResearch) at room temperature for 45 min, coverslips were again washed for three times in PBS. F-actin was visualized using a dilution 1:1000 of TRITC-conjugated phalloidin, 45 min at room temperature. The coverslips were washed once with PBS 1x and once in distilled water and then mounted on a slide with interspaces containing 70% (v/v) glycerol. Samples were analyzed using a confocal laser scanning microscope (Zeiss LSM confocal microscope, Oberkochen, Germany). Images were acquired in sequential scan mode by using the same acquisition parameters (laser intensities, gain photomultipliers, pinhole aperture and objective x63 or x150) when comparing experimental and control material. For production of figures, brightness and contrast gain were adjusted by taking care to leave a light cellular fluorescence background for visual appreciation of the lowest fluorescence intensity features and to help comparison among the different experimental groups. Final figures were assembled using Adobe Photoshop 7 and Adobe Illustrator 10 (San Jose, CA, USA).

F-actin was visualized using a dilution 1:1000 of TRITC-conjugated phalloidin, 45 min at room temperature. Nuclei was visualized with a dilution 1:500 of DAPI, 10 min at room temperature. The coverslips were washed once with PBS 1x and once in distilled water and then mounted on a slide with interspaces containing 70% (v/v) glycerol. Samples were analyzed using a confocal laser scanning microscope (Zeiss LSM confocal microscope, Oberkochen, Germany). Images were acquired in sequential scan mode by using the same acquisition parameters (laser intensities, gain photomultipliers, pinhole aperture and objective x63 or x150) when comparing experimental and control material. For production of figures, brightness and contrast gain were adjusted by taking care to leave a light cellular fluorescence background for visual appreciation of the lowest fluorescence intensity features and to help comparison among the different experimental groups. Final figures were assembled using Adobe Photoshop 7 and Adobe Illustrator 10 (San Jose, CA, USA).

Co-immunoprecipitation. BAG3, SNAP-25, syntaxin-1 and FAK proteins were immunoprecipitated from β-TC-6 lysate using IP matrix mouse (sc-45042) purchased from Santa Cruz Biotechnology, Inc. Briefly, 45 µl of matrix were incubated with 3 µg of anti-BAG3 (Ac-1), anti-SNAP-25, anti-syntaxin-1 or anti-FAK antibodies for 1 h at 4°C in continuous shaking. Then the matrix was washed with

Confocal microscopy. Cells were cultured on coverslips in six-well plates to 60-70% confluence. At third day of culture, coverslips were washed in PBS 1x and fixed in 3.7% formaldehyde in PBS 1x for 30 min at room temperature, and then incubated for 5 min with PBS 1x 0.1-M glycine. After washing, coverslips were permeabilized with 0.1% Triton X-100 for 5 min, washed again and incubated with blocking solution (5% normal goat serum in PBS 1x) for 1 h at room temperature. Following incubation with a 1:100 dilution of anti-insulin polyclonal antibody, 3 µg/ml of anti-BAG3 mouse monoclonal antibody AC-1 for 2 h at room temperature, coverslips were washed three times with PBS 1x. After incubation with a 1:500 dilution of goat anti-mouse IgG DyLight 594-conjugated antibodies (Jackson ImmunoResearch, Baltimore, PA, USA) and a 1:500 dilution of goat anti-rabbit IgG DyLight 488-conjugated antibodies (Jackson ImmunoResearch) at room temperature for 45 min, coverslips were again washed for three times in PBS. F-actin was visualized using a dilution 1:1000 of TRITC-conjugated phalloidin, 45 min at room temperature. The coverslips were washed once with PBS 1x and once in distilled water and then mounted on a slide with interspaces containing 70% (v/v) glycerol. Samples were analyzed using a confocal laser scanning microscope (Zeiss LSM confocal microscope, Oberkochen, Germany). Images were acquired in sequential scan mode by using the same acquisition parameters (laser intensities, gain photomultipliers, pinhole aperture and objective x63 or x150) when comparing experimental and control material. For production of figures, brightness and contrast gain were adjusted by taking care to leave a light cellular fluorescence background for visual appreciation of the lowest fluorescence intensity features and to help comparison among the different experimental groups. Final figures were assembled using Adobe Photoshop 7 and Adobe Illustrator 10 (San Jose, CA, USA).
Peroxidase complex (Novocastra; Leica Microsystems, Milan, Italy) and developed after washing thoroughly with PBS, sections were incubated using a biotinylated room temperature with the monoclonal antibody AC-1 at a concentration of 3 g/ml. Serum in 0.1% PBS or BSA. To detect BAG3, samples were incubated for 1 h at 4°C. Subsequently, 300 μl of PBS + 0.1% Tween twice and centrifuged each time at 13,000 r.p.m. for 1 min at 4°C. Finally, the matrix was resuspended in 45 μl of Laemmli buffer 2x and stored at −20°C. The samples were then processed for western blot.

**Immunohistochemistry.** The immunohistochemistry protocol included deparaffinization in xylene, rehydration via decreasing concentrations of alcohol down to pure water, non-enzymatic antigen retrieval in citrate buffer (pH 6.0) for 30 min at 95°C, and endogenous peroxidase quenching with H2O2 in methanol for 20 min. After rinsing with PBS, the samples were blocked using 5% normal horse serum in 0.1% PBS or BSA. To detect BAG3, samples were incubated for 1 h at room temperature with the monoclonal antibody AC-1 at a concentration of 3 μg/ml. After washing thoroughly with PBS, sections were incubated using a biotinylated secondary anti-mouse IgG for 20 min, then rinsed, incubated using avidin-biotin-peroxidase complex (Novocastra; Leica Microsystems, Milan, Italy) and developed using diaminobenzidine (Sigma-Aldrich). Finally, the sections were counterstained using hematoxylin, dehydrated in alcohol, cleared in xylene and mounted using Permount (Fisher Scientific Inc., Milan, Italy).

**Subcellular fractionation.** Subcellular fractions of β/TC-6 cells were isolated as previously described. All procedures were performed on ice. Approximately, 4.5 × 106 cells grown in 15-cm dishes were washed two times with cold PBS and scraped in 1 ml homogenization buffer (20 mM Tris-HCl, pH 7.4; 0.5 mM EDTA; 0.5 mM EGTA; 250 mM sucrose; and 1 mM dithiothreitol) containing the following protease inhibitors: leupeptin (10 g/ml), aprotinin (4 g/ml), pepstatin (2 g/ml), and 0.5 mM trithiocyanate (Sigma-Aldrich). As previously described, all procedures were performed on ice. Approximately, 15 min and then at 25,000 × g of protein extracts obtained from 900 × g of protein extracts obtained from 900 × g of protein extracts obtained from

**Conflict of Interest**

BIOUNIVERSA s.r.l., which produces anti-BAG3 antibodies, provided them free of charges for this study. MF, AR, VDL and MCT are shareholders of the company BIOUNIVERSA that provided some of the used antibodies. The remaining authors declare no conflict of interest.

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