ATG16L1 autophagy pathway regulates BAX protein levels and programmed cell death

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Previously we reported that adipocyte SNAP23 (synaptosome-associated protein of 23 kDa) deficiency blocks the activation of macroautophagy, leading to an increased abundance of BAX, a pro-death Bcl-2 family member, and activation and adipocyte cell death both in vitro and in vivo. Here, we found that knockdown of SNAP23 inhibited the association of the autophagosome regulators ATG16L1 and ATG9 compartments by nutrient depletion and reduced the formation of ATG16L1 membrane puncta. ATG16L1 knockdown inhibited autophagy flux and increased BAX protein levels by suppressing BAX degradation. The elevation in BAX protein had no effect on BAX activation or cell death in the nutrient-replete state. However, following nutrient depletion, BAX was activated with a concomitant induction of cell death. Co-immunoprecipitation analyses demonstrated that SNAP23 and ATG16L1 proteins form a stable complex independent of nutrient condition, whereas in the nutrient-depleted state, BAX binds to SNAP23 to form a ternary complex independent of nutrient condition, whereas in the nutrient-depleted state, BAX binds to SNAP23 to form a ternary BAX–SNAP23–ATG16L1 protein complex. Taken together, these data support a model in which SNAP23 plays a crucial function as a scaffold for ATG16L1 necessary for the suppression of BAX activation and induction of the intrinsic cell death program.

SNARE proteins comprise a large family of proteins (~60 members in mammalian cells) that primarily function in the fusion of membrane compartments with each other (2–7). The specificity of SNARE protein formation of membrane fusion pores depends upon the complimentary interactions between appropriate cognate SNARE coiled-coil domains, SNARE protein subcellular compartmentalization, and interaction with various accessory factors (5, 8–19). Recently, SNARE proteins have been implicated in various aspects of the macroautophagy process (1, 20–26). In Saccharomyces cerevisiae the SNARE proteins Sso2p, Tlg2p, Sec9p, Sec22p, and Ykt6p have all been shown to be involved in the trafficking and organization of ATG9 during autophagosome biogenesis (20, 27, 28). In mammalian systems, VAMP3 is required for the maturation of ATG16L1 vesicles that fuse with ATG9 vesicles necessary for autophagosome formation (29, 30).

Previously, we reported that SNAP23 deficiency results in an ATG9-dependent, but ATG7-independent, induced intrinsic cell adipocyte cell death caused by the inhibition of a noncanonical autophagy pathway required for the lysosomal degradation of the pro-apoptotic protein BAX (1). In vivo, adipocyte-specific SNAP23 knockout results in the development of generalize lipodystrophy and subsequent insulin resistance and liver steatosis.

Because ATG9 is an important component in the initial stages of phagophore expansion (31–34) and interacts with ATG16L1-containing compartments (34, 35), ATG16L1 directly interacts with the ATG5–ATG12 complex that functions in the conjugation of phosphatidylethanolamine of LC3-I to generate LC3-II (36). ATG16L1 also contains a coiled-coil domain that is required for starvation-induced autophagy (35, 37), and coiled-coil domains mediate high-affinity SNARE proteins interactions (3, 4). In this study, we examined the potential interactions between SNAP23, ATG9, and ATG16L1 in the macroautophagy-dependent degradation of BAX and regulation of apoptotic cell death.

Results

SNAP23 regulates ATG16L1 and ATG9 subcellular localization

To characterize the potential interaction and morphological changes associated with SNAP23 deficiency, we transfected NIH3T3 fibroblast SNAP23 knockdown (SNAP23 shRNA) and control (NM shRNA) cells with CFP-ATG16L1 and RFP-ATG9 (under nutrient-depleted conditions to active autophagy (Fig. 1A)). Quantification of these confocal images demonstrated that the SNAP23 deficiency results in a decrease in colocalization of ATG16L1 with ATG9 (Fig. 1B), a reduction in the number of ATG16L1-positive compartments (Fig. 1C), and a decrease in the size of ATG16L1 compartments (Fig. 1D). Essentially identical results were obtained from independent NIH3T3 cell clones (Fig. S1A). We also confirmed that both clones using two distinct SNAP23 shRNAs resulted in efficient knockdown of SNAP23 (Fig. S1, B and C). In addition, the number and size of ATG16L1 vesicle compartments in nutrient-depleted 3T3L1 adipocytes was substantially reduced in SNAP23 shRNA compared with NM shRNA cells (Fig. 2, A–C). As controls, in nutrient-replete 3T3L1 adipocytes there was essentially no distinct ATG16L1 vesicle compartments in either NM shRNA or SNAP23 shRNA adipocytes with primarily diffuse cytoplasmic and perinuclear ATG16L1 labeling (Fig. 2A). Similarly, independent clones of NM shRNA and SNAP23 shRNA 3T3L1 adipocytes also resulted in a reduction in the

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number and size of ATG16L1 vesicle compartments in nutrient-depleted state (Fig. S2).

**ATG16L1 deficiency increases BAX protein levels, BAX activation, and cell death**

We previously reported that both SNAP23 and ATG9 deficiency resulted in an increase in BAX protein levels caused by an inhibition of autophagy-mediated lysosomal degradation (1). To examine whether ATG16L1 deficiency also affected autophagy and BAX protein levels, BAX immunoblots in ATG16L1 shRNA knockdown cells demonstrated a significant increase in steady-state BAX protein levels (Fig. 3, A–C). Treatment of the control NM shRNA cells with the protein synthesis inhibitor cycloheximide demonstrated a time-dependent decline of BAX protein levels (Fig. 3, D–F). In contrast, BAX levels were relatively unchanged in the ATG16L1 shRNA knockdown cells, consistent with a stabilization of BAX protein levels. Consistent with several previous studies, ATG16L1 deficiency suppressed autophagy as determined by the inhibition of LC3-II flux and inhibition of p62 degradation (Fig. 3, G–J).

We also confirmed these data from independent clones of NM shRNA and ATG16L1 shRNA NIH3T3 clones as shown in Fig. S3.
shRNA knockdown cells, nutrient depletion resulted in a marked increase in 6A7 immunofluorescence (Fig. 4A, panel 4) with over 40% of the cells positive for BAX activation (Fig. 4B). As shown in Fig. 4A (panels 5–8), there were no significant change in the levels or distribution of the mitochondrial inner membrane marker ATP5a in the NM shRNA cells in either the nutrient-replete or -depleted state. Similarly, there were no significant differences in the ATG16L1 shRNA cells in the nutrient-replete state. In contrast, nutrient depletion of the ATG16L1 shRNA cells resulted in a strong co-localization of activated BAX with the mitochondrial marker (Fig. 4A, panels 4, 8, and 12).

Consistent with a BAX-dependent cell death program, in the nutrient-replete state, there was no discernable cell death as detected by propidium iodine labeling in either control NM shRNA or ATG16L1 shRNA cells (Fig. 5A, panels 1 and 2). Nutrient depletion of the NM shRNA cells had a very small induction of cell death (Fig. 5, A, panel 3, and B). Because BAX was substantially activated only in the ATG16L1 knockdown cells under nutrient-depleted conditions, there was a concomitant increase in cell death (Fig. 5, A, panel 4, and B). The induction of cell death by nutrient depletion of ATG16L1-deficient cells was also confirmed by propidium iodide labeling in independent clones of NM shRNA and ATG16L1 shRNA (Fig. S4).

**SNAP23 associates with ATG16L1 and forms a ternary complex with BAX in the nutrient-depleted state**

Having demonstrated a functional interaction between SNAP23, ATG16L1, and BAX, we next investigated whether these proteins physically interact. Cell extracts from nutrient-replete and nutrient-depleted cells immunoblotted for ATG16L1 and SNAP23 demonstrated equal amounts of total protein (Fig. 6A). SNAP23 immunoprecipitation demonstrated the co-immunoprecipitation of ATG16L1 that was unaffected by the nutrient status, indicating that SNAP23 and ATG16L1 exist in a stable complex. Immunoprecipitation of BAX resulted in the co-immunoprecipitation of ATG16L1 in the nutrient-depleted state but not in the nutrient-replete state (Fig. 6B). Similarly, BAX immunoprecipitation also resulted in the co-immunoprecipitation of ATG16L1 in the nutrient-depleted state with substantially lower levels in the nutrient-replete state (Fig. 6C). These data indicate that SNAP23 and ATG16L1 are in a binary complex but form a ternary complex with BAX during nutrient depletion. To assess whether ATG16L1 or SNAP23 mediates the formation of the ternary complex, we examined BAX immunoprecipitates in NM shRNA, SNAP23 shRNA, and ATG16L1 shRNA knockdown cells in the nutrient-depleted state when BAX is normally in the ternary complex. As shown in Fig. 6D and quantified in Fig. 6 (E and F), BAX immunoprecipitation from control NM shRNA cells resulted in the co-immunoprecipitation of both ATG16L1 and SNAP23. In the SNAP23 shRNA knockdown cells, BAX was unable to co-immunoprecipitate either SNAP23 or ATG16L1. However, in the ATG16L1 shRNA knockdown cells, BAX immunoprecipitation also resulted in the immunoprecipitation of SNAP23. These data are consistent with SNAP23 binding to both BAX and ATG16L1 and functioning as the bridge/scaffold linking BAX and ATG16L1 into a ternary complex.
Discussion

We previously reported that adipocyte-specific SNAP23 knockout mice within in the first several months of age develop generalized lipodystrophy caused by an elevation of the BAX protein, BAX activation, and the BAX-dependent adipocyte cell death program that occurs during periods of nutrient deprivation in vivo and in vitro (1). The increase in BAX protein levels by SNAP23 deficiency was due to the inhibition of autophagy flux and prevention of lysosome-mediated BAX degradation that was phenocopied by ATG9 deficiency.

Several studies have shown that ATG9 is localized to the trans-Golgi network, late, early, and recycling endosomes and following amino acid starvation redistributes from the juxtanuclear trans-Golgi network and/or recycling endosomes to peripheral endosomes positive for LC3 (31, 37, 40). ATG16L1 also plays a critical role in the initiation of autophagy by localizing the ATG12–ATG5 complex to the phagophore assembly site, where the ATG12–ATG5 complex functions to covalently attach phosphatidylethanolamine to LC3-I and generate LC3-II necessary for the expansion and completion of the limiting autophagosome membrane (35, 41). Because ATG9 functionally intersects with that of ATG16L1 and ATG16L1 contains a coiled-coil domain, a hallmark of SNARE interactions, we speculated that SNAP23 and ATG16L1 may interact in the control of autophagy. Consistent with this hypothesis, SNAP23 deficiency reduced the amount of co-localized ATG16L1 with ATG9, the number of ATG16L1 puncta compartments, and the average size of these vesicular structures. ATG16L1 was also co-immunoprecipitated with SNAP23.
independent of the cellular nutrient state, indicating a constitutive binding interaction.

Previously, we found that both SNAP23 and ATG9 deficiency resulted in an inhibition of autophagy and stabilization of the BAX protein by preventing lysosomal degradation of BAX (1). ATG16L1 deficiency also inhibited autophagy and increased BAX protein levels by suppression of BAX degradation. More importantly, BAX co-immunoprecipitated with SNAP23, but only in the nutrient-depleted state, and was found to form a ternary complex between ATG16L1–SNAP23–BAX with SNAP23 functioning as a scaffold to link ATG16L1 and BAX together. Interestingly, the interaction of SNAP23 and Bax was enhanced by ATG16L1 deficiency. Although we do not know the molecular basis for this observation, one possibility is that the reduction of ATG16L1 prevents the trafficking of Bax from SMAP23 to autophagic vesicles, thereby increasing the steady-state association of Bax with SNAP23. In any case, our data provide a model in which SNAP23 plays two important roles in the control of autophagy (Fig. 7). First, SNAP23 functions to direct the ATG16L1–ATG12–ATG5 complex to the site of ATG9 where phagophore assembly and expansion occur. Thus, in the absence of SNAP23, this assembly of the phagophore is impaired with reduction in LC3-II formation and reduction in the size of the phagophore membranes.

Figure 4. ATG16L1 deficiency induces BAX activation. A, NM shRNA and ATG16L1 shRNA NIH3T3 cells were maintained under NR or ND conditions for 1 h. The cells were fixed and subjected to immunofluorescence microscopy using the BAX activation–specific mAb 6A7 (green, panels 1–4), the mitochondria–specific antibody ATP5a (red, panels 5–8), and merged images with DAPI (blue, panels 9–12). These are representative images from four independent determinations. Scale bars, 25 μm. B, the percentage of 6A7 staining positive cells in NM shRNA and ATG16L1 shRNA NIH3T3 cells cultured under NR or ND conditions for 1 h were quantified from ~300 cells in four independent experiments. All data represent the means ± standard deviation. ****, P ≤ 0.001 by unpaired two-tailed Student’s t test.
Second, SNAP23 serves to associate the pro-apoptotic cell death protein BAX to the expanding phagophore for its inclusion in the limiting membrane and subsequent trafficking to and degradation by the lysosome. In this manner, inhibition of autophagy by either SNAP23 or ATG16L1 deficiency suppresses BAX degradation, resulting in an increase in steady-state BAX protein levels.

Interestingly, although BAX is a pro-apoptotic cell death mediator despite an approximate 2-fold increase in BAX protein levels in normal nutrient-replete conditions, BAX remains inactive as detected by the conformational specific BAX activation antibody and by the absence of any significant cell death. However, nutrient depletion markedly activated BAX and induced cell death in the ATG16L1-deficient cells with little effect on control cells. This is similar to our previous findings in SNAP23 and ATG9 knockdown cells (1). One possibility for enhanced activation of BAX with only a relatively small change in BAX protein level is that the inhibition of autophagy also generates another signal that increases BAX activation. Alternatively, it is known that BAX activation occurs as a result of homologous and/or heterologous BAX oligomerization (42, 43). Thus, it is also possible that the small increase in BAX protein results in enhanced oligomerization resulting in autoactivation. Further studies are needed to distinguish between these possibilities. In summary, our data demonstrate that SNAP23 associates with ATG16L1, and in turn SNAP23 interacts with BAX to form a ternary complex under nutrient-depleted conditions. Disruption of these interactions results in a stabilization of the BAX protein and sensitizes cells to programmed cell death.

**Experimental procedures**

**Cell culture**

Murine 3T3L1 preadipocyte cell culture and adipocyte differentiation were prepared as previously described (38). Mouse fibroblast NIH3T3 cells (ATCC, catalog no. CRL-1658™) or 3T3L1 (ATCC, catalog no. CL-173™) adipocytes were cultured in nutrient-replete (NR) medium containing Dulbecco’s modified Eagle’s medium (11965092, Gibco) supplemented with 10% fetal bovine serum and 1× penicillin–streptomycin. To induce nutrient-depleted (ND) conditions, cells were placed in RPMI medium without amino acids, glucose, or glutamine (catalog no. R9010-01, US Biological Life Sciences) for 1 h for BAX activation and 6 h for propidium iodide staining as previously described (1). The cell lines were maintained in a 5% CO2 incubator at 37°C.

**Lentiviral shRNA knockdown and cDNA plasmids**

MISSION lentiviral shRNA bacterial glycerol stocks for ATG16L1 (TRCN0000339310 and TRCN0000351019), SNAP23 (TRCN0000325516 and TRCN0000305934), and NM (non-mammalian, SHC202V) control plasmids were obtained from Millipore–Sigma. cDNA pMRX-IP/SECFP-hATG16A1 (CFP-ATG16L1, catalog no. 58994, Addgene) and pMXs-puro-RFP-ATG9A (RFP-ATG9, catalog no. 60609, Addgene) were bought from Addgene. The cDNA plasmids and lentiviral shRNA plasmids were purified using the HiSpeed plasmid maxi kit (Qiagen, catalog no. 12662). The lentiviral shRNA plasmids were transfected into HEK293T cells along with lentiviral packaging mix (Millipore–Sigma, catalog no. SHP001) to produce lentivirus packed with shRNA according to the manufacturer’s instructions. 3T3-L1 preadipocytes were selected with puromycin (2.5 µg/ml) after lentivirus infection and then subjected to standard adipocyte differentiation. NIH3T3 cells were infected with the lentivirus and also selected with puromycin (2.5 µg/ml) for 3 days.

**Autophagy flux**

The cells were placed in RPMI medium without amino acids (catalog no. R8999, US Biological Life Sciences) for 4 h, with or without lysosome inhibitors (20 mM NH4Cl, 100 µM leupeptin). The cell lysates were then prepared as previously described an immunoblotted for LC3 (2775, Cell Signaling Technology) and p62 (PM045, MBL) (1). Net LC3-II and p62 flux was calculated as the difference in levels of LC3-II or p62 protein normalized to loading-control ACTIN protein levels, with and without lysosomotropic agent treatment for 2 h.
Quantification of intracellular membrane puncta

NIH3T3 NM shRNA and NIH3T3 SNAP23 shRNA cells were co-transfected with cDNAs encoding CFP-ATG16L1 and RFP-ATG9. The cells were then maintained in NR medium. 24 h later, the cells were placed in ND medium for 1 h. NM shRNA and SNAP23 shRNA expressing differentiated 3T3L1 adipocytes were also placed in ND medium for 1 h. The puncta per cell were then visualized by a fluorescence laser scanning confocal microscope and a Leica SP5 and counted. Co-localization of CFP-ATG16L1 and RFP-ATG9 under nutrient-depleted conditions was determined by Pearson’s coefficient. The average size of RFP-ATG16L1 vesicles/cell (AU) were analyzed by Imaris software.

Cell death determination

Plasma membrane permeability was determined by incubating cells with 250 ng/ml propidium iodide (PI) and 5 μg/ml Hoechst 33342 for 10 min and then counting PI-positive cells as dead cells under a microscope.

Immunoblot analysis

Cultured cells were washed with cold PBS and scraped and homogenized using Ceria stabilized zirconium oxide beads (MidSci) in radioimmune precipitation assay lysis buffer (catalog no. sc-24948, Santa Cruz Biotechnology) containing a

Figure 6. BAX–SNAP23–ATG16L1 form a ternary complex in the nutrient-depleted state. NIH3T3 cells were cultured in NR or ND medium for 1 h and cell lysates generated using a buffer that does not activate BAX as described under “Experimental procedures.” A, the lysates were directly immunoblotted or immunoprecipitated (IP) with SNAP23 antibody and then blotted with the ATG16L1 or the SNAP23 antibody. B, same culture condition as in A, were immunoprecipitated with the BAX antibody and immunoblotted with the SNAP23 or BAX antibody. C, the same cell lysates were immunoprecipitated with the BAX antibody and immunoblotted for ATG16L1 and BAX. D, the NM shRNA, SNAP23 shRNA, and ATG16L1 shRNA NIH3T3 cells were cultured in nutrient-depleted medium for 1 h, and cell lysate was generated, immunoprecipitated with the BAX antibody, and immunoblotted for ATG16L1, SNAP23, and BAX. These are representative immunoblots independently performed three times. E and F, the ratio of ATG16L1 (E) and SNAP23 (F) protein co-precipitated with BAX from four independent experiments. All data represent the means ± standard deviation. *, P ≤ 0.05; ****, P ≤ 0.001 by unpaired two-tailed Student’s t test.
SNAP23/ATG16L1 interaction regulates BAX-dependent cell death

Figure 7. Proposed model predicting the interaction of ATG16L1, SNAP23, and BAX required for BAX degradation. SNAP23 consistently interacts with ATG16L1. Following nutrient depletion, BAX interacts with SNAP23 to form a ternary ATG16L1–SNAP23–BAX complex. This allows BAX to associate with ATG16L1 and ATG9 membrane compartments that form an autophagosome. The BAX-associated autophagosome can then fuse with the lysosome to mediate BAX degradation.

protease and phosphatase inhibitor mixture (catalog no. 78442, Thermo Fisher Scientific). The cell homogenates were centrifuged for 15 min at 21,000 g at 4 °C, and the supernatants were collected for protein assay. Protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane using the iBlot Blotting System (Thermo Fisher Scientific). The immunoblot membrane was blocked with SuperBlock (TBS) blocking buffer (catalog no. 37535, Life Technologies, Thermo Fisher Scientific) and incubated with a primary antibody in the blocking buffer. The immunoblot membranes were washed in TBS with Tween-20 (TBST) and incubated with horseradish peroxidase–conjugated secondary antibody in TBST. The membrane was then washed with TBST and visualized through ECL (Pierce, Thermo Fisher Scientific). Image software was used to compare relative protein levels.

Detection of BAX activation by 6A7 immunofluorescence

NIH3T3 cells were grown on coverslips and treated for 1 h with NR or ND medium, as described above. The cells were washed once with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min and permeabilized with PBS containing 0.1% Triton X-100 for 10 min. The cells were blocked with PBS containing 3% bovine serum albumin fraction V (Roche) for 1 h and then incubated overnight with a 1:100 dilution of the activation–specific BAX antibody 6A7 (556467, BD Biosciences, 1:100 dilution) and a 1:100 dilution of the inner mitochondrial protein ATP5a (Abcam) at 4 °C. After washing with PBS, the slides were incubated with Alexa Fluor secondary antibodies (T13343, Thermo Fisher Scientific) in blocking solution at room temperature for 1 h, mounted with VECTASHIELD HardSet mounting medium, and stained with DAPI (Vector Laboratories). The fluorescent signals were visualized with an Axio Observer Z1 microscope (Carl Zeiss), and the signal intensity was quantitated using ImageJ software to integrate the 6A7 fluorescence intensity per cell.

Immunoprecipitation

NIH3T3 NM shRNA, SNAP23 shRNA, or ATG16L1 shRNA cells (90% confluence) were homogenized in 500 µl of IBC buffer (10 mM Tris, 1 mM EGTA, 200 mM sucrose) plus 0.5% CHAPS and protease/phosphatase inhibitors on ice and then centrifuged for 10 min. The 550 µg of supernatant protein in 330 µl were added to 6.5 µl of the BAX (catalog no. 2772, Cell Signaling Technology), 2 µl of ATG16L1 (catalog no. NB110-60928, Novus Biologicals), or 2 µl of SNAP23 (catalog no. ab3340, Abcam) antibodies and then incubated for 2 h at 4 °C. For each immunoprecipitation, 50 µl of Dynabeads were washed once with IBC buffer; CHAPS was then added, and the samples were mixed by rotation for 1 h at 4 °C. The beads were centrifuged, and the pellets were washed twice with IBC buffer containing CHAPS. Following washing, the pellets were resuspended in 75 µl of IBC buffer with SDS containing Laemmli sample buffer and denatured for 5 min at 95 °C. The samples were run in 12% NuPAGE Bis-Tris gel then and subjected to immunoblot analysis as described above, using IgG as control.

Statistical analyses

Group data are presented as the means ± S.E. Statistical significance was calculated using a paired or unpaired two-tailed Student’s t test. A p value of less than 0.05 was considered statistically significant.

Data availability

Figs. 1–7 are included in the article. Figs. S1–S4 are included in the supporting information.

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Abbreviations—The abbreviations used are: shRNA, short hairpin RNA; NR, nutrient-replete; AU, arbitrary unit(s); PI, propidium iodide; DAPI, 4',6-diamino-2-phenylindole.

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