

# Identification of TFG- and Autophagy-Regulated Proteins and Glycerophospholipids in B Cells

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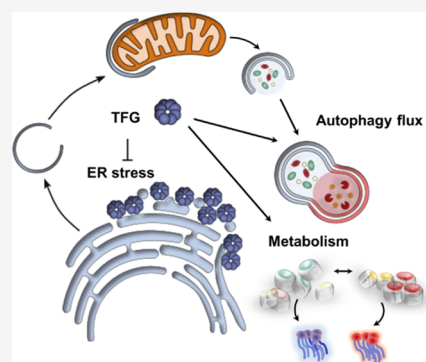
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**ABSTRACT:** Autophagy supervises the proteostasis and survival of B lymphocytic cells. *Trk-fused gene* (TFG) promotes autophagosome–lysosome flux in murine CH12 B cells, as well as their survival. Hence, quantitative proteomics of CH12tfgKO and WT B cells in combination with lysosomal inhibition should identify proteins that are prone to lysosomal degradation and contribute to autophagy and B cell survival. Lysosome inhibition via NH<sub>4</sub>Cl unexpectedly reduced a number of proteins but increased a large cluster of translational, ribosomal, and mitochondrial proteins, independent of TFG. Hence, we propose a role for lysosomes in ribophagy in B cells. TFG-regulated proteins include CD74, BCL10, or the immunoglobulin JCHAIN. Gene ontology (GO) analysis reveals that proteins regulated by TFG alone, or in concert with lysosomes, localize to mitochondria and membrane-bound organelles. Likewise, TFG regulates the abundance of metabolic enzymes, such as ALDOC and the fatty acid-activating enzyme ACOX9. To test consequently for a function of TFG in lipid metabolism, we performed shotgun lipidomics of glycerophospholipids. Total phosphatidylglycerol is more abundant in CH12tfgKO B cells. Several glycerophospholipid species with similar acyl side chains, such as 36:2 phosphatidylethanolamine and 36:2 phosphatidylinositol, show a dysequilibrium. We suggest a role for TFG in lipid homeostasis, mitochondrial functions, translation, and metabolism in B cells.

**KEYWORDS:** autophagy, autophagy flux, BCL10, lysosome, B cell, endoplasmic reticulum stress, ribosome, immunoglobulin, plasma cell, JCHAIN, metabolism, phosphatidylglycerol, TRK-fused gene



## INTRODUCTION

During their differentiation into antibody (Ab)-secreting plasma cells, activated B cells undergo a plethora of transcriptional, metabolic, and morphologic changes. These events support cell survival as well as synthesis, assembly, and secretion of Ab by plasma cells. Dimerization of IgA and pentamerization of IgM Ab require the immunoglobulin (Ig) J chain (IgJ; JCHAIN). Consequently, JCHAIN becomes strongly upregulated in plasma cells, albeit its mRNA is present in all B cell subsets. In line, JCHAIN abundance is controlled by protein degradation<sup>1</sup> but the underlying mechanism is not known.<sup>2</sup> The antigen-specific activation of B cells depends on the B cell receptor (BCR).<sup>3</sup> After T-cell-dependent B cell activation, B cells form germinal centers to undergo positive selection, differentiation into memory B cells and plasma cells, class switch recombination, and somatic hypermutation of their Ig genes by integrating signals from the BCR and CD40.<sup>4</sup> These processes are coordinated by transcription factors such as nuclear factor  $\kappa$ B (NFKB), B cell lymphoma (BCL) 6, inhibitor of DNA binding 3 (ID3), or JUNB.<sup>5</sup> Classical NFKB activation after BCR stimulation involves phosphorylation of NFKB inhibitor  $\alpha$  (IKBA) through inhibitor of IKBA kinase (IKK)  $\alpha$  and  $\beta$  activation via IKK $\gamma$ , leading to proteasomal degradation of IKBA

and B cell activation.<sup>6,7</sup> The proximal BCR signals are connected to the IKK complex and JUN N-terminal kinase (JNK) by CARD-containing MAGUK protein 1/Caspase recruitment domain-containing protein 11 (CARMA1/CARD11).<sup>8</sup> CARD11 forms a complex with BCL10 and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), the CBM (CARD11/BCL10/MALT1) complex, which leads to IKK/NFKB activation. Consequently, null mutations of CARD11 abrogate antigen receptor-induced activation of NFKB, JNK, and mammalian target or rapamycin (MTOR) C1,<sup>9</sup> leading to loss of lymphocyte activation, proliferation, and adaptive immunity. Conversely, somatic gain-of-function mutations of CBM components can cause diffuse large B cell lymphoma (DLBCL), T-cell leukemia, or lymphoproliferative disorders.<sup>9</sup> Thus, the CBM complex is essential for the growth

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control of lymphocytes. Phosphorylation and ubiquitination of BCL10 can positively and negatively regulate function and destruction of CBM complexes.<sup>10</sup> Moreover, in Bruton's tyrosine kinase-dependent DLBCL cell lines, the CBM complex is part of a supramolecular BCR-protein complex attached to lysosomes that fosters oncogenic, prosurvival NFKB, and mTOR signaling.<sup>11</sup> Lysosomes are an essential part of autophagy, the JNK and MTORC1 pathways regulate autophagy,<sup>12</sup> and BCL10 is a selective autophagy substrate in T cells.<sup>13</sup> Macroautophagy/autophagy is a process starting with autophagy receptors binding damaged organelles or ubiquitinated proteins that leads to their engulfment by a double membrane vesicle. This establishes an autophagosome, which fuses with lysosomes to form an autolysosome (reviewed in ref 14). Engulfed cargo and their receptors become degraded during this process, which can be blocked by prevention of lysosomal acidification, for instance using NH<sub>4</sub>Cl.<sup>15,16</sup> Autophagy involves a dynamic series of vesicular events managed by ATG (autophagy-related) genes. An important kinase controlling autophagy is ULK1 (homologous to ATG1 in yeast).<sup>16</sup> The autophagy vesicle matures by lipidation of an ATG protein (Atg8 in yeast, LC3 and GABARAP subfamilies in mammals), which is required for autophagy vesicle expansion and closure. Germinal center B cells apply noncanonical autophagy to ensure mitochondrial homeostasis, while plasma cells rely on atg5-dependent autophagy for sustainable antibody production and survival.<sup>17,18</sup> Autophagy also cooperates with the ubiquitin proteasome system (UPS) and ER-associated degradation (ERAD) in controlling survival of normal and malignant plasma cells.<sup>19</sup>

In a proteomic screen for interaction partners of CARD11, we identified a 50 kDa protein named TRK-fused gene (TFG) in the mouse B lymphoma line CH27.<sup>20</sup> TFG consists of 397 amino acids structured into an N-terminal PB1 protein–protein interaction domain,<sup>21</sup> a coiled-coil oligomerization domain, and a C-terminal proline-rich region. TFG interacts with IKK $\gamma$ ,<sup>22</sup> is involved in structural organization of the ER,<sup>23</sup> the ER–Golgi intermediate compartment (ERGIC),<sup>24</sup> and COPII vesicle transport. TFG is a regulator of the ubiquitin proteasome system (UPS) and modifies proteasomal targeting of ER proteins.<sup>25</sup> While fusions of the *tfg* gene with other genes can lead to oncogenic fusion proteins that drive lymphomas and leukemias,<sup>26</sup> *tfg* mutations can cause spastic paraplegia, optic atrophy, neuropathy, and dominant axonal Charcot–Marie–Tooth disease type 2.<sup>27</sup>

We found that *Tfg* mRNA is strongly expressed in primary plasma cells compared to nonactivated B cells and that TFG protein abundance is higher in *in vitro* differentiated plasmablasts than in resting B cells, suggesting that TFG is involved in plasma cell homeostasis. TFG supports the survival of mouse CH12 B lymphoma cells by preventing apoptosis, which could potentially be mediated by prosurvival NFKB signaling via CARD11/BCL10. In CH12 B cells, TFG is also required to maintain ER integrity as well as autophagy flux as evidenced by LC3 turnover assays, tandem fluorescent LC3 reporter, and electron microscopy.<sup>28</sup> The function of TFG in autophagy flux has been confirmed in other cell types.<sup>29</sup> Because TFG abundance maintains integrity of the ER and autophagy flux, regulates the UPS system and, thereby, ULK1 stability,<sup>30</sup> we reasoned that the protein composition of CH12*tfg*WT and KO B cells should differ. Identifying changes in the proteome of CH12*tfg*WT versus KO B cells in the presence or absence of NH<sub>4</sub>Cl should first reveal potential mechanisms regulating

TFG-dependent survival, ER integrity, and autophagy flux, which are crucial for activated B cells and plasma cells, and second also reveal proteins whose abundance is controlled by lysosomal acidification independently of TFG.

## MATERIALS AND METHODS

### B Cell Culture

CH12*tfg*WT and CH12*tfg*KO B cells<sup>28</sup> were cultured in R10 medium (RPMI1640 [Gibco Life Technologies, 31870-025], 10% fetal calf serum (FCS), 2 mM glutamate [Gibco Life Technologies, 25030-024], 1 mM sodium pyruvate [Gibco Life Technologies, 11360-039], 50 U/mL penicillin G, 50  $\mu$ g/mL streptomycin [Gibco Life Technologies, 15140-122], 50  $\mu$ M  $\beta$ -mercaptoethanol [Gibco Life Technologies, 31350-010]) at 37 °C, and 5% CO<sub>2</sub>. Primary mouse B cells were prepared from spleen and activated with a lipopolysaccharide (LPS) for 24 h as described.<sup>31</sup>

### Glycerophospholipid Analysis

Glycerophospholipids (PC, PE, PI, PS, PG, PA) in B cells were analyzed by nano-electrospray ionization tandem mass spectrometry (Nano-ESI-MS/MS) with direct infusion of the lipid extract (*Shotgun Lipidomics*): 14–45  $\times$  10<sup>6</sup> cells were homogenized in 300  $\mu$ L of Milli-Q water using the Precellys 24 Homogenizator (Peglab, Erlangen, Germany) at 6.500 rpm for 30 s. The protein content of the homogenate was routinely determined using bicinchoninic acid. To 100  $\mu$ L of the homogenate, 400  $\mu$ L of Milli-Q water, 1.875 mL of methanol/chloroform 2:1 (v/v), and internal standards (125 pmol PC 17:0/20:4, 132 pmol PE 17:0/20:4, 118 pmol PI 17:0/20:4, 131 pmol PS 17:0/20:4, 62 pmol PG 17:0/20:4, 75 pmol PA 17:0/20:4; Avanti Polar Lipids) were added. Lipid extraction and nano-ESI-MS/MS analysis were performed as previously described.<sup>32</sup> Endogenous glycerophospholipids were quantified by referring their peak areas to those of the internal standards. The calculated glycerophospholipid amounts were normalized to the protein content of the tissue homogenate. The data have been deposited at the Metabolomics Workbench<sup>33</sup> (Project ID PR001515, Study ID: ST002360, doi: 10.21228/M8VQ5S).

### Western Blot

Cells were washed in phosphate buffered saline (PBS) and lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 25 mM Tris/HCl pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenyl-methyl-sulfonyl-fluoride on ice for 15 min. The lysate was cleared by centrifugation at 10,000g at 4 °C for 15 min, and supernatants were prepared for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to standard procedures. After SDS-PAGE, proteins were transferred onto a nitrocellulose membrane, the membrane was washed with deionized water, and stained with Ponceau S. The membrane was blocked with 5% skim milk powder in TBST (150 mM NaCl, 25 mM Tris/HCl pH 7.5, 0.1 Tween-20), followed by incubation with primary antibody (diluted in TBST containing 3% bovine serum albumin (BSA), 0.1% NaN<sub>3</sub>) and four washes with TBST. The membrane was incubated with secondary horseradish peroxidase (HRP) conjugated antibody diluted in 5% skim milk powder in TBST. Anti-TFG antibody was described previously.<sup>28</sup> Other antibodies were mouse anti-ACTIN (Sigma-Aldrich, A1978), rabbit anti-ACTIN, ACOT9, ALDOC (Sigma-Aldrich, A2066, HPA035533, HPA003282), rabbit anti-MAP1LC3B/LC3 and BCL10 (Cell Signaling Technology, 2775S and 4237S), goat

antirabbit IgG-HRP (Jackson ImmunoResearch, 111-035-046), and goat antimouse IgG-HRP (Jackson ImmunoResearch, 115-035-008). After being washed, the membrane was incubated with enhanced chemiluminescence (ECL) before exposure to X-ray films. Quantification was performed by densitometry of scanned X-ray films with ImageJ 1.52p. Band intensities were measured, and the background of X-ray films was subtracted. The relative abundance of Actin bands in each lane was quantified, and other bands were normalized to relative Actin band intensity.

### Generation of Tryptic Peptides

Proteomic analysis was performed as described.<sup>34</sup> Briefly, we pooled in total  $1.5 \times 10^8$  cells from each 5 WT and 5 *tfgKO* subclones of CH12 cells. Clones were treated individually for each experimental condition with 1% phosphate buffered saline (PBS) or with 1% of 5 M  $\text{NH}_4\text{Cl}$  for 2 h in complete RPMI160 medium under normal culture conditions. The samples were washed with PBS and split into technical replicates of  $1 \times 10^7$  cells. The pelleted cells were shock-frozen using liquid nitrogen and stored at  $-80^\circ\text{C}$  until further processing. The cells were lysed in 30 mM Tris base with 7 M urea and 2 M thiourea, pH 8.5. The cells were sonicated on ice twice with an ultrasonic homogenizer for 15 s at 90% of the maximum power. Cell debris was removed by centrifugation for 20 min at 21,000g and  $4^\circ\text{C}$ . Samples containing 20  $\mu\text{g}$  of total protein were prepared and diluted with 50 mM ammonium bicarbonate to 2 M urea and incubated for 20 min with 1  $\mu\text{L}$  of benzamide at room temperature (RT). Cysteine residues were reduced with 5 mM tris(2-carboxyethyl)phosphine and alkylated with 50 mM chloroacetamide for 5 min at  $45^\circ\text{C}$ .<sup>35</sup> Each sample was then digested with Lys-C (protein-to-enzyme ratio 1:100, FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany) for 30 min at  $37^\circ\text{C}$ . Subsequently, trypsin (protein-to-enzyme ratio 1:50, Promega, Mannheim, Germany) was added for overnight digestion at  $37^\circ\text{C}$ . The reaction was stopped by acidification with trifluoroacetic acid (TFA) (1% [v/v] final concentration) and 10  $\mu\text{g}$  of the peptide sample was desalted using an in-house prepared C18 Stage tip cartridge using three layers of C18 material. The cartridges were washed with 1 mL of methanol followed by 1 mL of 70% acetonitrile (ACN) with 0.1% TFA. The column was conditioned twice with 0.4 mL of 0.1% TFA before the sample was applied. Peptides were washed with 0.4 0.1% TFA and eluted with two times 30  $\mu\text{L}$  of 70% ACN. The peptides were lyophilized using an  $\alpha$  1–2 LDplus system (Christ, Osterode am Harz, Germany).

### Proteomic Analysis

Reversed-phase high-performance liquid chromatography (HPLC) was performed on an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific, Dreieich, Germany) equipped with two Waters precolumns and a Waters nano *m/z* analytical column (75  $\mu\text{m} \times 250$  mm, 3  $\mu\text{m}$ , 100 Å, Thermo Fisher Scientific).<sup>34</sup> The HPLC was connected to a QExactive Plus instrument with the following parameter settings: spray voltage, 1.8 kV; capillary voltage, 200 V; automatic gain control (AGC),  $3 \times 10^6$  ions; max. ion time (IT), 60 ms. Full scans were acquired in the orbitrap with a mass range of *m/z* 375–1700 and a resolution (R) of 70,000 at *m/z* 200. A TOP12 method was used, and the parameters were as follows: normalized collision energy (NCE), 28%; dynamic exclusion (DE), 45 s; AGC, 5000; max. IT, 120 ms. A solvent system consisting of 0.1% FA (solvent A), 86% ACN, and 0.1% FA (solvent B) was used for

peptide separation. The RSLC was operated with a flow rate of 0.250  $\mu\text{L}/\text{min}$  for the analytical column.

Raw data were searched against Uniprot *Mus musculus*. The proteome set used MaxQuant version 1.5.4.0. The species was restricted to MaxQuant default settings unless stated otherwise. Database searches were conducted with trypsin and Lys-C (for quantitative proteome analysis) as proteolytic enzymes, a maximum number of three missed cleavages, and mass tolerance of 4.5 ppm for the precursor and 0.5 Da (CID data) or 20 ppm (HCD data) for fragment ions. Two missed cleavages were allowed. Carbamidomethylation of cysteine residues was set as fixed modification, and oxidation of methionine and N-terminal acetylation were applied as variable modifications. The minimum number of unique peptides was set to 1. A false discovery rate (FDR) of 1% was applied to both peptide and protein lists. For global proteome analysis, “label-free” and “iBAQ” quantification as well as “match between runs” were enabled with default settings. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>36</sup> partner repository with the data set identifier PXD039180 (<https://www.ebi.ac.uk/pride/archive/projects/PXD039180>).

### Bioinformatic Analysis

Functional analysis of proteomic data was performed using Microsoft R studio and the DEP package version 1.4.1 for single protein expression values for 3221 quantified proteins. In brief, raw expression data were adjusted to contain only protein ID, Uniprot code, protein name, gene name, and expression values of 20 samples. Raw data were imported into R studio and supplemented with meta data to assign raw data with sample information. Duplicates were excluded within the different ID columns by making the data set unique, and log2 transformation of the data set was reversed. Subsequently, data were written into a summarized experiment format and normalized. Using protein-wise linear models combined with empirical *t*-test-based Bayes statistics, *p*-value calculation for the comparison pairs WT/PBS + KO/PBS, KO/PBS + KO/ $\text{NH}_4\text{Cl}$ , WT/ $\text{NH}_4\text{Cl}$  + KO/ $\text{NH}_4\text{Cl}$ , and WT/PBS + WT/ $\text{NH}_4\text{Cl}$  was performed. Significant data were filtered using the cut off values of *p* < 0.05 and expression fold-change > 1.5. Exported data sets were used for the analysis of regulation of single protein expression. Volcano plots were generated using the DEP plot. The IDs of significantly regulated proteins were functionally classified using gene ontology (GO) enrichment analysis (<http://pantherdb.org/>)<sup>37</sup> using the current release (Version 18.0, released 2023-09-17). PANTHER overrepresentation test was performed with the version released on 2022-07-12 by probing the test set against the complete mouse genome (21997 genes) using Fisher's exact *t*-test and correction by the false discovery rate (FDR). Functional protein clusters were identified by STRING (*Mus musculus* proteins, default settings) (<https://string-db.org/>).<sup>38</sup>

### Flow Cytometry and Cell Sorting

$2 \times 10^6$  to  $4 \times 10^6$  cells were pelleted in FACS tubes (Micronic, Lelystad, Netherlands) at 300g for 5 min at  $4^\circ\text{C}$  and resuspended in 50  $\mu\text{L}$  of unlabeled anti-FCGR3/CD16-FCGR2B/CD32 Ab (Invitrogen, 14-0161-86), 10  $\mu\text{g}/\text{mL}$  in FACS buffer (PBS, 2% fetal calf serum [FCS, Gibco Life Technologies, 10270-106], 0.05% sodium azide [Carl Roth, K305.4]) for 15 min on ice. Cells were washed once with FACS buffer by centrifugation at 300g for 5 min at  $4^\circ\text{C}$ , resuspended in 50  $\mu\text{L}$  of FACS buffer containing the respective fluorochrome-

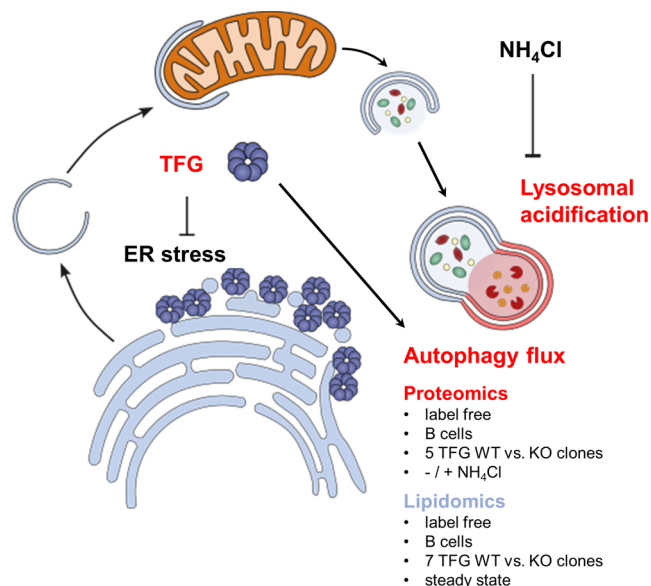


coupled Abs (anti TACI/CD267 PE, clone eBio8F10-3, eBioscience, anti CD138 PECy7, clone 281-2, Biolegend, anti CD19 BV510, clone 6DS, Biolegend, anti B220 BV421, clone RA3-6b2, Biolegend, anti CD98PE, #128207, Biolegend, anti CD74-FITC, clone In-1, Pharmingen, anti CD20-APC.Cy7, #302314, Biolegend), and incubated for 20 min on ice in the dark. For intracellular flow cytometric analysis, the fix and perm kit from An der Grub (distributed by DIANOVA, Hamburg, Germany) was used according to the manufacturer's instructions. Intracellular staining for JCHAIN was performed after surface staining, fixation, and permeabilization with rabbit anti JCHAIN antibody SP105 (Thermo, MAS-16419), followed by a secondary goat antirabbit antibody conjugated to Alexa 647 (Jackson). The cells were washed twice with FACS buffer by centrifugation at 300g for 5 min at 4 °C. Data were acquired using a Gallios flow cytometer (Beckman Coulter, Brea). Analyses were performed using Kaluza versions 1.3 and 2.1 (Beckman Coulter, Brea). For some experiments, the fluorescence intensity was normalized to the cell size as described previously.<sup>39</sup>

## RESULTS

### Identification of Proteins Controlled Quantitatively by TFG and NH<sub>4</sub>Cl

Autophagy flux requires the fusion of autophagosomes with lysosomes<sup>40</sup> and can be blocked by NH<sub>4</sub>Cl, which specifically prevents lysosomal function.<sup>41</sup> TFG is required for autophagy flux.<sup>28,29</sup> Therefore, proteins degraded by autophagy flux via TFG, or required for autophagy flux elicited by TFG, should be identifiable by comparing the proteome of CH12tfgWT and KO B cells upon inactivation of the lysosomal flux. Hence, we performed label-free quantitative proteomics<sup>34</sup> with previously established CH12tfgWT and KO B cell clones,<sup>28</sup> either treated with PBS or NH<sub>4</sub>Cl (Figure 1).



**Figure 1.** Biological background and experimental setup. To test whether the regulation of autophagy flux by TFG alters the protein composition of B cells, we devised an experimental setup that quantifies proteins in CH12tfgWT and KO B cells with intact or blocked autophagy flux. The second experimental approach was lipid analysis in CH12tfgWT and KO B cells in the steady state.

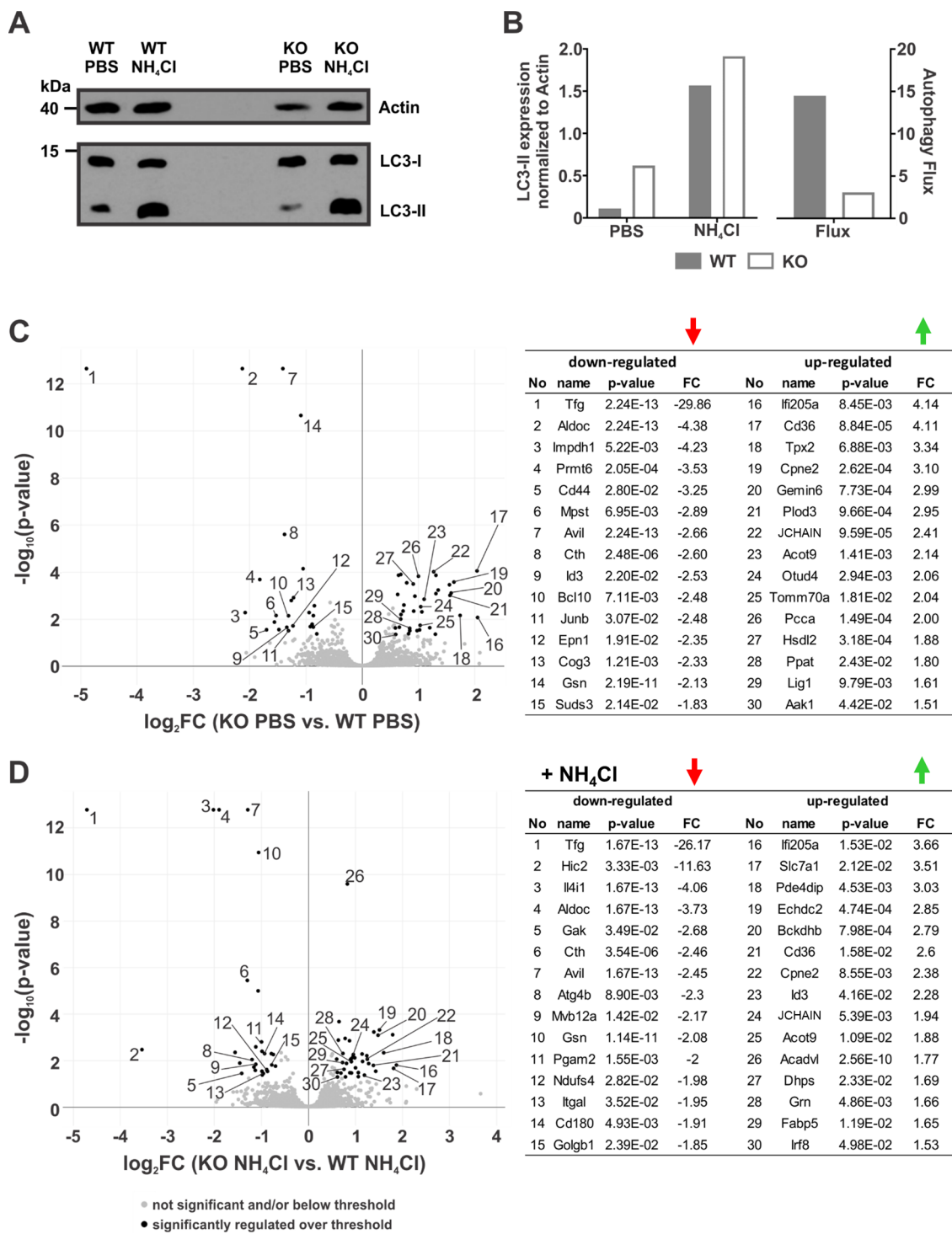
NH<sub>4</sub>Cl treatment of up to 4 h is a recommended reagent to study autophagy flux<sup>40</sup> but in order to avoid excessive cell death, we restricted the NH<sub>4</sub>Cl treatment to 2 h. Because TFG has functions independent of autophagy, we expected several groups of proteins: First, proteins are up- or downregulated by TFG without NH<sub>4</sub>Cl treatment (group 1). Second, proteins are regulated by TFG-mediated lysosomal targeting, i.e., proteins that are changed in CH12tfgKO B cells compared to CH12tfgWT B cells and simultaneously in NH<sub>4</sub>Cl-treated vs untreated CH12tfgWT B cells (group 2). Third, proteins are regulated by NH<sub>4</sub>Cl in both CH12tfgKO B cells and CH12tfgWT B cells, representing presumably the largest fraction (group 3). We first corroborated the reduction of autophagy flux in CH12tfgKO B cells by NH<sub>4</sub>Cl treatment and quantification of LC3-II abundance by Western Blot (Figure 2A,B). Cell lysates from the same experiment were subjected to a quantitative proteomic analysis. In total, we quantified over 3000 proteins in CH12tfgWT and KO B cells (Table S1), of which 52 proteins were significantly up- or downregulated by TFG only (Tables S2 and S3; group 1).

The 15 top significantly up- and downregulated proteins in CH12tfgKO B cells are shown in Figure 2C and are arranged by the extent of their regulation; the previously described knockout of TFG<sup>28</sup> was confirmed by a large negative fold-change of ~30 (Figure 2C). Please find all significantly regulated proteins in Tables S2 and S3. We also identified proteins that were either up- or downregulated in CH12tfgWT and KO B cells in the presence of NH<sub>4</sub>Cl (Figure 2D). Notably, as can be seen in Figure 2C,D, some proteins hardly changed their abundance upon NH<sub>4</sub>Cl treatment, such as Ifi205 or JCHAIN, while others did. For instance, ATG4B was downregulated upon inhibition of lysosomes by NH<sub>4</sub>Cl only in CH12tfgKO B cells (#8 in Figure 2D), confirming a linkage of TFG to autophagy.

Nineteen proteins were upregulated in CH12tfgKO B cells compared to WT B cells and at the same time in CH12tfgWT B cells treated with NH<sub>4</sub>Cl (Tables S2 and S3, group 2). These represent proteins that are targeted by TFG to lysosomal degradation (TPX2, GEMIN6, PLOD3, THYN1, MRRF, USF2, MRPS16, RRP9, CHTOP, PLRG1, OTUD4, and TOMM70A, PCCA, DNAJC11, MRPL30, PPAT, POLD3, ETFDH, LIG1, and ATAD3). Interestingly, four proteins were downregulated in CH12tfgKO B cells treated with NH<sub>4</sub>Cl (Table S3) (SU2DS3, PROSER1, SH3BP1, ID3). 123 proteins were differentially abundant in CH12tfgWT as well as CH12tfgKO cells treated with NH<sub>4</sub>Cl compared to PBS-treated cells. 81 of those proteins became more abundant with NH<sub>4</sub>Cl treatment, meaning that these proteins are delivered continuously to the lysosomal pathway in B cells independently of TFG (Table S3, group 3, up). Unexpectedly, we also identified 42 proteins that were decreased in CH12tfgWT cells treated with NH<sub>4</sub>Cl (Table S3, group 3, down). Figure 3 depicts a Venn diagram of the different groups.

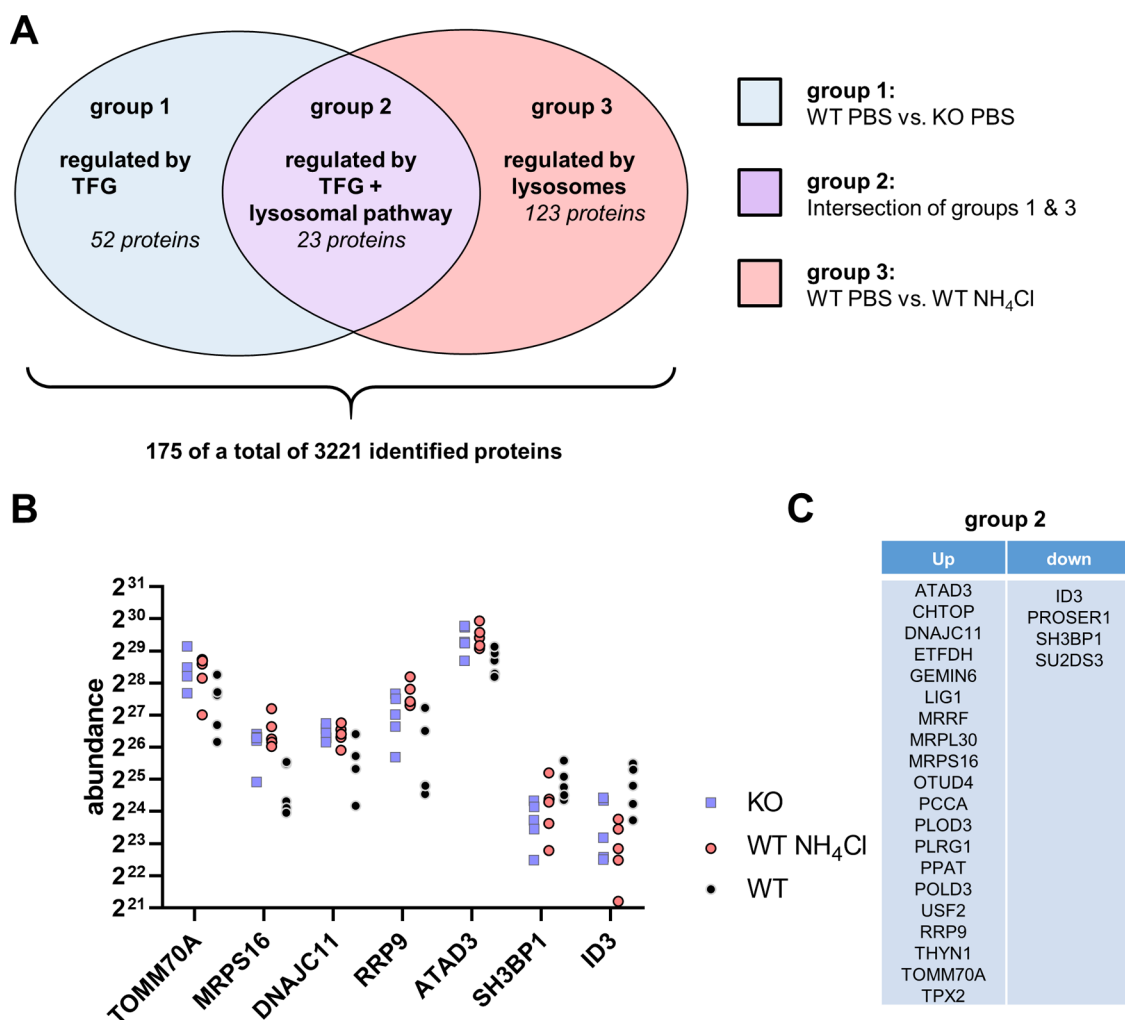
### Characterization of Biological Processes Controlled by TFG and Lysosomes

To reveal potential biological processes regulated by TFG and/or lysosomal flux, we determined PANTHER (<http://pantherdb.org/>) gene ontology (GO) processes and protein classes as well as functional clusters by STRING (<https://string-db.org/>). Proteins that were regulated specifically by TFG (Table S3 and Figure S3) comprise the protein classes protein modifying enzyme, scaffold/adaptor protein, membrane traffic protein, DNA metabolism protein, protein-binding activity



**Figure 2.** Regulation of protein abundance by TFG and  $\text{NH}_4\text{Cl}$  in CH12 B cells. (A) CH12tfgWT and KO B cells were left untreated (PBS controls) or treated with  $\text{NH}_4\text{Cl}$  for 2 h. Cell lysates of three clones were pooled, separated by 15% SDS-PAGE, and analyzed by Western Blotting with anti-ACTIN or LC3 antibodies. Molecular mass standards are shown on the left. (B) Autophagy flux is depicted as the ratio of Actin-normalized LC3-II of  $\text{NH}_4\text{Cl}$ -treated vs nontreated cells. (C) Total cell extracts of PBS-treated CH12tfgWT and KO B cells were digested with trypsin and analyzed by liquid chromatography-mass spectrometry (LC-MS)/MS (data are combined from two experiments. In the first experiment, three different CH12tfgWT and KO B cell clones were analyzed and in the second experiment, two additional independent CH12tfgWT and KO B cell clones). Volcano plots and tables show the 15 most upregulated or downregulated proteins in CH12tfgWT and KO B cells. (D) Total cell extracts of  $\text{NH}_4\text{Cl}$ -treated CH12tfgWT and KO B cells were digested with trypsin and analyzed by LC-MS/MS. Volcano plots and table show the 15 most up- or downregulated proteins in CH12tfgWT and KO B cells.

modulator, transmembrane signal receptor, defense/immunity protein, RNA metabolism protein, calcium-binding protein, cytoskeletal protein, gene-specific transcriptional regulator, translational protein, metabolite interconversion enzyme, chromatin/chromatin-binding or -regulatory protein (Figures S1 and S2). Notably, the largest group contained metabolite



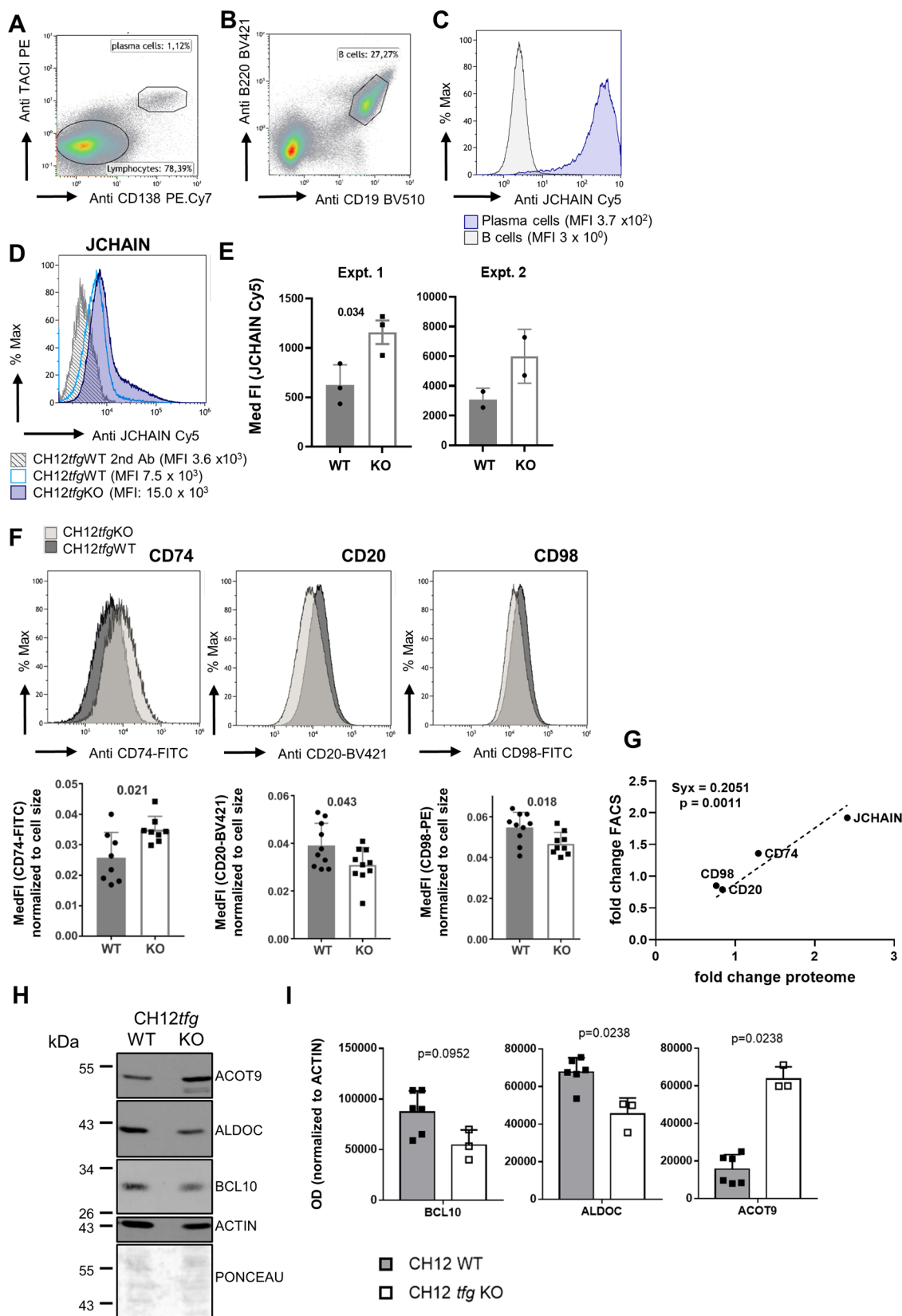
**Figure 3.** Identification of proteins regulated by TFG and NH<sub>4</sub>Cl. (A) Venn diagram of protein groups regulated by TFG (group 1), NH<sub>4</sub>Cl (group 3), and the intersection of TFG- and NH<sub>4</sub>Cl-regulated processes (group 2). (B) Representative raw quantification data of some proteins identified in group 2 (up- or downregulated in WT cells treated with NH<sub>4</sub>Cl and untreated KO cells; Table S1). Please note that some symbols overlap. (C) List of proteins in group 2.

interconversion enzymes (ALDOC, HSDL2, ACOT9, CTH, IMPDH1, and MPST1), suggesting that TFG controls metabolic pathways via enzyme abundance. The modulation of protein abundance via TFG could be direct or indirect, via regulation of transcription factors, such as JUNB or ID3 (Figure 2C). We found no statistical overrepresentation of the TFG-regulated proteins in any GO pathway.

The 23 proteins of group 2 are of special interest because they are targeted to lysosomal degradation via TFG (Figure 3). They may either represent effectors or targets of TFG-supported autophagy flux and belong to protein classes protein modifying enzyme (OTUD4, PLOD3), transporter (TOMM70A), chaperone (DNAJC11), DNA metabolism protein (LIG1, POLD3), cytoskeletal protein (TPX2), gene-specific transcriptional regulator (ID3, USF2), translational protein (MRPS16, MRPL30, MRRF), and metabolite interconversion enzyme (ETFDH, PPAT, PCCA) (Figure S3). STRING analysis revealed clusters of proteins involved in the mitochondrial network organization (DNAJC11, ATAD3A) and mitochondrial translation (MRPS16, MRPL30, and MRRF) (Figure S4). Remarkably, PANTHER (<http://pantherdb.org/>) overrepresentation analysis<sup>42</sup> showed that the proteins of group 2 are more abundant in mitochondrial cellular compartments when

compared to the total mouse genome (Figure S3). This suggests that TFG targets selected mitochondrial proteins or even mitochondria to lysosomes. We conclude that TFG intrinsically controls the abundance of proteins (depicted in Figure 2C; see also Table S3). In addition, there is an interplay between TFG and the lysosomal system. Notably, TFG and lysosomal inhibition seem to regulate mitochondrial physiology, in particular.

Group 3 UP is composed of proteins that become more abundant with NH<sub>4</sub>Cl treatment independent of TFG (Table S3). They belong mainly to the classes RNA metabolism (ZNF622, SRP14, DDX1, TRMT1, RCL1, DBR1, FBL, FAM98B, ILF3, TRMT1, POLR1E, DUS3L, and HDLBP), metabolite interconversion enzymes (ACLY, PFKM, ALDH18A1, ACP6, UQCR10, UQCRQ, GNPDA2, PGAM2, GPI, PRPSAP2, GMDS, NDUFV1, ELAC2, PGAM5, and BLVRA), protein modifying enzymes (PITRM1, SCYL1, MAPK3, ILK, and OGT) and, most robustly, a cluster of cytosolic and mitochondrial translational proteins (MRPL27, GTPBP1, MRPL3, RPL35A, RPS16, RPL34, WARS, RPS5, RPS11, FARSA, MRPL45, FARSB, MRPS2, EEFSEC, EIF2B4, RPS2, MRPL16, and RPS25) (Figures S5 and S6). The top ten hits in a PANTHER (<http://pantherdb.org/>) overrepresenta-



**Figure 4.** Confirmation of proteomic data by flow cytometry and Western Blotting. (A–C) Mouse bone marrow cells were stained with antibodies against CD19, B220, CD138, TACI, fixed, permeabilized, and stained with rabbit anti JCHAIN antibody followed by a secondary antirabbit antibody. (A) Plasma cell gate (TACI<sup>+</sup>, CD138<sup>+</sup>) and (B) B cell gate (CD19<sup>+</sup>, B220<sup>+</sup>). (C) JCHAIN expression in B cells or plasma cells: mean fluorescence intensity (MFI) is depicted below. (D) CH12tfgKO and WT cells were stained with antirabbit antibody alone or with rabbit anti JCHAIN antibody followed by a secondary antibody. MFIs are depicted below. (E) Quantification of JCHAIN abundance in CH12tfgKO and WT cells after background subtraction of secondary antibody staining. Two experiments (experiments) with five different clones per genotype in total are shown. Each dot represents one clone. Mean of median FI  $\pm$  standard deviation (SD). Statistical significance (experiment 1) was calculated by Student's *t*-test. (F)



Figure 4. continued

CH12tgfWT and KO B cells (7 clones each) were stained with antibodies against surface CD20 and CD98 or fixed, permeabilized, and stained with antibodies against CD74 and then analyzed by flow cytometry. The fluorescence intensity was normalized to the cell size and is depicted as the median intensity. Each dot represents one clone with mean  $\pm$  SD. Statistical significance was calculated by the Mann–Whitney *U*-test. (G) Fold-change of protein abundance of CH12tgfKO B cells was calculated relative to CH12tgfWT cells for flow cytometry and correlated to the relative changes as quantified by LC-MS/MS. (H) CH12tgfKO and WT B cells were lysed and subjected to Western Blotting with antibodies as indicated, and a part of the blot is shown. (I) Quantification of the Western Blot shown in panel (G). The background was subtracted and the optical density (OD) of the given protein was normalized to the OD of Actin. Statistical significance was calculated by Mann–Whitney *U*-test.

tion analysis<sup>42</sup> for cellular compartments were related to ribosomes (Table S3 and Figure S6). Hence, these data suggest that CH12 B cells undergo permanent lysosomal degradation of ribosomal proteins even in continuous nutrient-rich cultures.

Group 3 DOWN is composed of proteins that become less abundant with NH<sub>4</sub>Cl treatment independent of TFG (Figure S7). They belong mainly to the groups gene-specific transcriptional regulators (TRP53, ID3, LRRFIP2, RUNX3, BHLHE41), RNA metabolism (DNA2, TAF7, MRM1), metabolite interconversion enzymes (ECHDC2, ZADH2, ACADSB), and protein modifying enzymes (SCPEP1, UBE2B, TXNIP, UBE2S, USP48), in particular ubiquitin-processing enzymes (UBE2B, UBE2S, USP48). There was an overrepresentation in organelles and intracellular anatomic structures and a functional protein cluster centered around TRP53 (Figure S8). To conclude, proteins that are regulated by NH<sub>4</sub>Cl/lysosomes in B cells are mainly involved in gene transcription, RNA metabolism and metabolism, translation, and protein modification. Thus, the lysosomal function extends beyond clearance and antigen presentation.

#### Independent Verification of TFG-Controlled Proteins

To certify the proteomic data, we applied two independent methods, flow cytometry and Western Blot. We first focused on flow cytometry (Figure 4), commencing with JCHAIN.

JCHAIN is required for dimerization and mucosal transport of IgA and for pentamerization of IgM Ab.<sup>2</sup> JCHAIN protein quantity is much higher in plasma cells than in B cells, regulated on mRNA and protein level but the latter mechanism is unknown.<sup>1</sup> We first assessed whether JCHAIN abundance is higher on the protein level in plasma cells. To this end, we surface-stained splenic lymphocytes (primary mouse plasma cells, positive for CD138 and TACI (transmembrane activator and CAML interactor (TACI)/TNFRSF13B)<sup>43</sup> and B cells, identified by antibodies directed against a CD45 isoform, B220, and CD19), and then permeabilized the cells for intracellular staining of JCHAIN (Figure 4A–C). By flow cytometry, we then corroborated that there is more JCHAIN in plasma cells than in B cells and more JCHAIN in CH12tgfKO than in WT B cells (Figure 4D,E). Subsequently we focused on proteins whose abundance is only slightly regulated on the proteomic level (Tables S1 and S2) but known to play a role in B cells and are easily amenable to flow cytometric detection. CD20 suppresses plasma cell differentiation,<sup>44</sup> CD74 is the MHCII invariant chain,<sup>45</sup> and CD98 is an amino acid transporter and plasma cell marker.<sup>46</sup> Because we assessed rather small alterations, we ruled out potential effects of altered cell size by normalizing fluorescence to cell size.<sup>38</sup> Remarkably, CD20, CD74, CD98, and JCHAIN expression as assessed by flow cytometry (Figure 4D,F) showed the very same pattern of regulation as with unbiased proteomic analysis (Figure 4G). This experiment highlights the superior accuracy of the proteomic data. Additionally, we aimed at confirming metabolic interconversion enzymes ALDOC and ACOT9, as well as BCL10, an important

regulator of NF $\kappa$ B signaling and prosurvival factor in B cells. In this regard, the Western Blots of CH12tgfKO and WT B cells revealed similar trends as those uncovered by proteomics: ALDOC and BCL10 were both less abundant in CH12tgfKO B cells (1.6-fold each), while ACOT9 was 3.7-fold more abundant (Figure 4H,I).

#### Involvement of TFG in Lipid Homeostasis

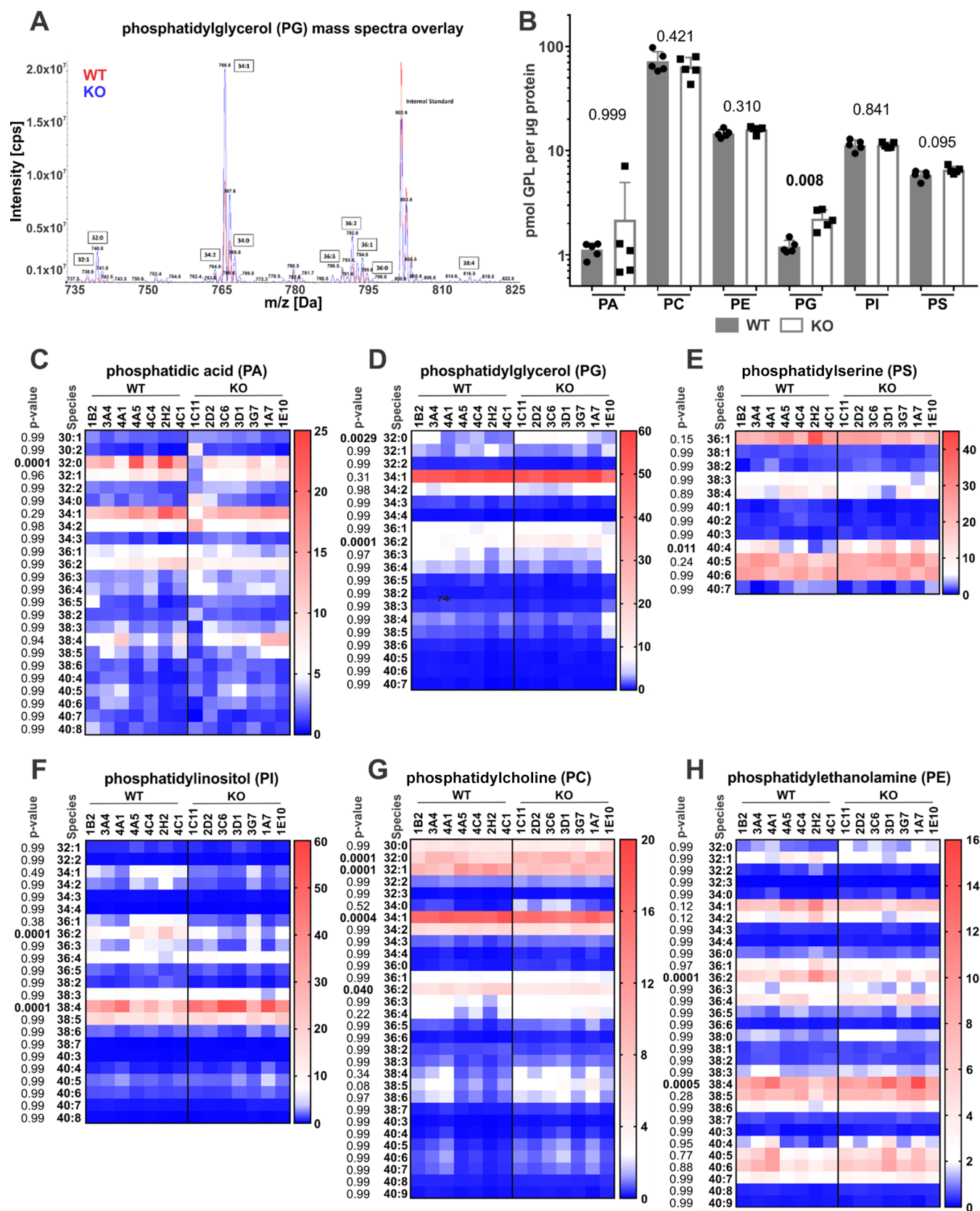
Autophagy is inherently a membrane- and lipid-dependent process. For instance, LC3 becomes lipidated by phosphatidylethanolamine (PE) during autophagy flux.<sup>40</sup> TFG has previously been described as the “lipogenic regulator” in sebocytes due to its upregulation by the insulin-like growth factor (IGF)-1, leading to increased triglycerides.<sup>47</sup> TFG is upregulated during plasmablast differentiation, and B cell differentiation into plasma cells does depend on de novo lipogenesis via glucose.<sup>48</sup> However, a clear mechanism and specific lipid classes regulated by TFG are unknown. The metabolite interconversion enzymes ACOT9 (Acyl CoA:thioesterase 9) and HSDL2 (hydroxysteroid dehydrogenase-like 2) are negatively regulated by TFG (Figure 2C). ACOT9 activates short-chain fatty acids (SCFA) for de novo lipogenesis, thereby, promoting triglycerides.<sup>49</sup> HSDL2 is localized in peroxisomes and is presumably involved in fatty acid metabolism.<sup>50</sup> These notions suggested that TFG could regulate lipid homeostasis by the regulation of enzymes involved in lipid metabolism. Therefore, we wondered whether TFG indeed contributes to lipid homeostasis. Because HSDL2 is not upregulated in CH12tgfKO cells treated with NH<sub>4</sub>Cl (Figure 2C,D), we chose to perform shotgun lipidomic analyses of glycerophospholipids (GPL)<sup>31,32</sup> from untreated CH12tgfWT and KO B cells (Table S4). Figure 5A depicts a representative neutral loss mass spectrum showing phosphatidylglycerol (PG) from CH12tgfWT and KO B cells.

Notably, among the examined GPL, only PG was elevated in total in CH12tgfKO compared to WT B cells, while there was no difference for total phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine (PS), or phosphoinositols (PI) (Figure 5B). Nevertheless, despite the total amounts of PA, PE, PI, PC and PS being similar, some particular FA species did reveal quantitative differences in CH12tgfKO B cells (Figure 5C–H). For instance, 32:0 PA was reduced in CH12tgfKO B cells, while 32:0 PG and 32:0 PC were elevated. 36:2 PE and 36:2 PI were lower but 36:2 PG was higher. Some specific longer and unsaturated lipids, such as 38:4 PE and PI, or 40:4 PS, were also elevated. Hence, TFG limits PG abundance and contributes to an equilibrium of selected GPL with similar acyl side chains.

#### DISCUSSION

To gain more insight into the mechanisms and consequences of autophagy in B cells, we characterized the proteome and a part of the lipidome of murine CH12tgfKO and WT B lymphoma cells





**Figure 5.** TFG limits the quantity of phosphatidylglycerol but stabilizes selected saturated glycerophospholipids in CH12 B cells. (A) Glycerophospholipids of CH12<sup>tg</sup>WT and KO B cells (experiment 1, two clones, experiment 2, five clones each) were analyzed via direct infusion MS/MS (Shotgun Lipidomics). A representative neutral loss mass spectrum of phosphatidylglycerol species from WT (red) and KO (blue) CH12 B cells (overlay) is shown, with small numbers indicating the masses of the  $[M+NH_4]^+$  precursor ions and large numbers indicating the total number of carbon atoms and the total number of double bonds within the two fatty acyl chains. (B) The absolute abundance (pmol/ $\mu\text{g}$  protein) of the glycerophospholipids (GPL) phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylcholine (PC),

Figure 5. continued

phosphatidylethanolamine (PE), and phosphatidylserine (PS) in CH12*tfg*WT and KO B cells is depicted (mean  $\pm$  SD, each dot represents one clone). Significance was calculated using the Mann–Whitney *U*-test. (C–H), The relative abundance (Mol %) of subspecies of phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) glycerophospholipids of CH12*tfg*WT and KO B cells is depicted as a heatmap. Each symbol represents one clone. *P*-value and lipid species on the left of each heatmap. The first number depicts the total number of carbon atoms, and the second number depicts the total number of double bonds within the two fatty acyl chains. Combined results from two experiments, *N* = 2, *n* = 7; statistics calculated using two-way analysis of variance (ANOVA).

in steady state. In addition, we also performed proteomics of CH12*tfg*KO and WT cells when the lysosomal activity was inhibited by NH<sub>4</sub>Cl. Although our approach cannot discriminate between bulk autophagy, selective autophagy, or the endosomal–lysosomal pathway,<sup>51</sup> it is clear that most of the cytosolic, nuclear, and organelle proteins degraded by lysosomes are routed there via autophagy.<sup>14</sup> In total, we identified 3221 proteins in CH12 B cells. The quantification of protein abundance in the absence or presence of NH<sub>4</sub>Cl or TFG identified clusters of proteins that were either regulated by NH<sub>4</sub>Cl treatment, TFG (i.e., genotype-driven), or both. Because we have used several different genetically independent CH12*tfg*KO B cell clones for our analyses,<sup>28</sup> clonal artifacts can be excluded. Yet, the proteome and lipidome changes may arise from long-term adaptation of the transformed B cells to *tfg* inactivation. A future task will be to determine their relation to primary B cells and other cell types.

We have identified substrates and processes, such as lipid homeostasis, downstream of TFG and lysosomal activity. Importantly, we also identified proteins regulated by lysosomes independently of TFG. Interestingly, many proteins that appear to be stabilized by NH<sub>4</sub>Cl (i.e., targeted to lysosomes) are proteins of the cytosolic and mitochondrial translation machinery. Our experiments were conducted under nutrient-rich conditions in a normal culture. Hence, CH12 B cells continuously shuttle ribosomal proteins to the lysosome, a process that is called ribophagy.<sup>52,53</sup> Because large amounts of ribosomes are assembled under nutrient-rich conditions, there may be the need to remove excess ribosomes in response to changing environmental conditions.<sup>53</sup> Homeostatic ribophagy in B cells could also target defective ribosomes as a quality control mechanism.<sup>52</sup> It is tempting to speculate that ribophagy aids the catabolic function of autophagy by limiting the protein translation in B cells. This is consistent with a positive regulation of autophagy by mTOR inhibition<sup>53–57</sup> and consistent with the requirement for canonical autophagy in plasma cells.<sup>18</sup> Specifically, activated *atg5*<sup>−/−</sup> B cells develop an enlarged ER, which initially promotes plasma cell differentiation via upregulation of the transcription factor BLIMP1, leading to more antibody synthesis. However, despite *atg5*<sup>−/−</sup> B cells expanding their ER and BLIMP1 expression, they cannot sustain this phenotype due to a depletion of ATP and cell death. Consequently, *atg5*<sup>−/−</sup> plasma cells are outcompeted, and sustained antibody responses are dampened.<sup>18</sup> Albeit antibody secretion is initially increased, it should be noted that ribosomal proteins were only sparsely regulated in 3-day LPS-activated *atg5*<sup>−/−</sup> B cells.<sup>18</sup> *Atg5*<sup>−/−</sup> B cells may already have adapted during their development and during the 3 days of activation. In contrast to primary LPS-activated B cells whose lifetime is 3–4 days, we analyzed transformed B cells cultivated over weeks for the ongoing experiments. Those cell lines may have adapted as well; therefore, there is a necessity to analyze primary *tfg*<sup>−/−</sup> B cells once available. Nevertheless, compensatory mechanisms, in particular metabolic adaptations, are physiological in the sense

that long-lived cells or clones, such as tumor cells, continuously adapt in response to established or new mutations. In previous experiments, we assessed adaption of CH12*tfg*KO B cells to ER stress by Western Blot and found a mildly elevated abundance of HSPA5/BiP<sup>28</sup> which, however, was not reflected in our proteomic data. Post-translational modifications of HSPA5<sup>58</sup> in CH12*tfg*KO cells may influence binding of HSPA5 to membranes or binding of anti HSPA5 antibodies.

To our surprise, a large fraction of proteins was not up- but downregulated by NH<sub>4</sub>Cl. Proteins stabilized by NH<sub>4</sub>Cl may secondarily suppress other proteins, such as TRP53, perhaps as part of a feedback survival mechanism linked to the nucleolus.<sup>59</sup> Alternatively, because lysosomal and UPS pathways are intertwined,<sup>60</sup> the proteasome may take over under conditions of lysosomal inhibition. In other systems, it has been shown previously that NH<sub>4</sub>Cl upregulates TRP53 by a ROS-mediated mechanism,<sup>61,62</sup> which may not take place here. However, NH<sub>4</sub>Cl can indeed cause cell death. Consequently, we decided to use a conservative 2 h treatment.<sup>40</sup> While this short treatment may underestimate actual lysosomal flux, it may in turn actually identify the most relevant pathways controlled by lysosomes.

Speaking of complementary mechanisms, proteins regulated by TFG, but not by NH<sub>4</sub>Cl, could well be linked to the function of TFG in the UPS system,<sup>25,30</sup> a possibility that warrants further investigation. Unexpectedly, ATG4B was downregulated in CH12*tfg*KO B cells treated with NH<sub>4</sub>Cl. ATG4B is a protease that immediately removes the C-terminal residues of LC3 to produce the LC3-I isoform.<sup>63</sup> During autophagy flux, the LC3-I isoform is converted to LC3-II by the addition of PE to the new C-terminus. However, inactive ATG4B blocks lipidation of LC3 paralogues, resulting in the inhibition of autophagy.<sup>63</sup> Thus, the regulation of ATG4B abundance by TFG and NH<sub>4</sub>Cl provides potential clues as to TFG-mediated autophagy flux, but deciphering a clear mechanism requires more work. Interestingly, LC3 lipidation works best with 36:2 PE (DOPE).<sup>64</sup> Strikingly, exactly this particular PE species was reduced in CH12*tfg*KO B cells, which exhibit reduced autophagy flux. Hence, a regulation of 36:2 PE by TFG may underlie autophagy flux in CH12 B cells.

An intriguing result was that some of the most quantitatively TFG-suppressed proteins are ACOT9, CD36, and HSDL2, all of which modify lipid synthesis and homeostasis.<sup>65</sup> It should be noted that those proteins are regulated by TFG independent of lysosomes by unclear mechanisms. A previous study has shown that the TFG protein was increased in a time- and dose-dependent manner by IGF-1 in sebocytes and that TFG overexpression increased triglycerides globally as assessed by thin layer chromatography.<sup>47</sup> ACOT9 promotes triglyceride biosynthesis.<sup>49</sup> Consequently, we reasoned that there should be altered lipids in CH12*tfg*KO B cells in the steady state and covered the GPL pool of CH12*tfg*KO B cells in depth by neutral loss mass spectrometry. The main finding was the limitation of PG abundance by TFG, i.e., there was more PG in CH12*tfg*KO B cells. PG is the biosynthetic precursor of Cardiolipin, a major

structural phospholipid in mitochondria.<sup>66</sup> It is tempting to speculate for future research that lipid metabolism in mitochondria may be regulated by TFG. The second important finding was that GPL with similar acyl side chains shows a disequilibrium in CH12tfgKO B cells. Specifically, 32:0 PA and 32:0 PE are reduced in CH12tfgKO B cells, while 32:0 PG and 32:0 PC are elevated; 36:2 PI is lower but 36:2 PG is higher. There may be an ongoing conversion of 32:0 PA to 32:0 PG via lipid phosphate phosphohydrolase-driven generation of 32:0 diacylglycerol in CH12tfgKO B cells.<sup>67</sup> Why PG is increased in CH12tfgKO cells remains elusive because enzymes involved in PG synthesis (CGI-58/ABHD5, PGS1, PTPMT1)<sup>68</sup> were not regulated in our hands. Therefore, the upregulation of PG could hint the regulation of mitophagy via mitochondrial fission in CH12tfgKO cells.<sup>68</sup> In support of this speculation, one surprising GO term characterizing TFG-regulated proteins was the “mitochondrial matrix”. Moreover, the finding that TOMM70A was elevated in CH12tfgKO B cells could point to impaired mitophagy.<sup>69</sup> Albeit we did not observe signs of defective mitochondria by electron microscopy in an earlier study,<sup>28</sup> in light of TOMM70A and PG regulation, this deserves further investigation.

With respect to B cells, TFG controlled the abundance of proteins involved in B cell activation (BCL10)<sup>7</sup> and the germinal center reaction (ID3).<sup>5</sup> Moreover, TFG may be a strong candidate for the regulation of JCHAIN in different plasma cell subsets, which is a yet unresolved but crucial issue for understanding IgA and IgM homeostasis as well as the regulation of anti-inflammatory antibody responses.<sup>2</sup> While BCL10 is selectively targeted by autophagy in T cells,<sup>13</sup> TFG stabilizes BCL10 in CH12 B cells. The stabilization of BCL10 abundance by TFG is congruent with our finding of TFG as part of a CARD11 complex in B cells and suggests that TFG is a positive regulator of the CBM pathway. This is potentially in line with its role as a positive regulator of NFκB activity<sup>22</sup> and its role in lymphomas and leukemias.<sup>26</sup>

In summary, the work presented here generated new insights and hypotheses on the function of lysosomes and TFG in B cells, like the regulation of ribosomes, mitochondrial function, or lipid homeostasis. Testing those hypotheses will aid in understanding the autophagy, lymphocyte activation and proliferation, and immunity.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.3c00713>.

Characterization of proteins and pathways regulated by TFG in a NH<sub>4</sub>Cl-independent manner by PANTHER pathway analysis (Figure S1); interactions of proteins regulated by TFG in a NH<sub>4</sub>Cl-independent manner (Figure S2); characterization of proteins and pathways controlled by TFG in a NH<sub>4</sub>Cl-dependent manner by PANTHER pathway analysis (Figure S3); interaction of proteins controlled by TFG in a NH<sub>4</sub>Cl-dependent manner and their connection to cellular components (Figure S4); characterization of proteins and pathways upregulated by NH<sub>4</sub>Cl in a TFG-independent manner by PANTHER pathway analysis (Figure S5); interaction of proteins upregulated by NH<sub>4</sub>Cl in a TFG-independent manner and their connection to cellular components (Figure S6); characterization of proteins and pathways

negatively controlled by NH<sub>4</sub>Cl in a TFG-independent manner via PANTHER pathway analysis (Figure S7); interaction of proteins downregulated by NH<sub>4</sub>Cl in a TFG-independent manner and their connection to cellular components (Figure S8); uncut Western Blots of Figure 2 (Figure S9); and uncut Western Blots of Figure 4 (Figure S10) (PDF)

List of all identified proteins in CH12 B cells (Table S1) (XLSX)

Differences in protein abundances in CH12tfgWT and KO B cells ± NH<sub>4</sub>Cl (Table S2) (XLSX)

Simplified depiction of regulated proteins and pathway classification (Table S3) (XLSX)

List of identified glycerophospholipids in CH12 B cells (Table S4) (XLSX)

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## Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

Ab, antibody; Ag, antigen; ASC, antibody-secreting cells; ATG, autophagy-related; BCR, B cell receptor; BCL, B cell lymphoma; CpG, nonmethylated CpG oligonucleotide; DLBCL, diffuse large B cell lymphoma; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FO, follicular; GO, gene ontology; HC, heavy chain; Ig, immunoglobulin; IKBA, NFKB inhibitor  $\alpha$ ; IKK, inhibitor of IKBA kinase; JNK, JUN N-terminal kinase; LC, light chain; MTOR, mammalian target or rapamycin; NFKB, nuclear factor of kappa light polypeptide gene enhancer in B cells; TACI, transmembrane activator and CAML interactor; TLR, toll-like receptor; UPR, unfolded protein response

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