Lysine trimethylation regulates 78-kDa glucose-regulated protein proteostasis during endoplasmic reticulum stress

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The up-regulation of chaperones such as the 78-kDa glucose-regulated protein (GRP78, also referred to as BiP or HSPA5) is part of the adaptive cellular response to endoplasmic reticulum (ER) stress. GRP78 is widely used as a marker of the unfolded protein response, associated with sustained ER stress. Here we report the discovery of a proteostatic mechanism involving GRP78 trimethylation in the context of ER stress. Using mass spectrometry–based proteomics, we identified two GRP78 fractions, one homeostatic and one induced by ER stress. ER stress leads to de novo biosynthesis of non-trimethylated GRP78, whereas homeostatic, METTL21A-dependent lysine 585–trimethylated GRP78 is reduced. This proteostatic mechanism, dependent on the posttranslational modification of GRP78, allows cells to differentially regulate specific protein abundance during cellular stress.

The endoplasmic reticulum (ER) is critical for protein biosynthesis and folding (1, 2). Disturbed ER homeostasis is characterized by accumulation of mis- and/or unfolded proteins and referred to as ER stress (ERS). To counteract the detrimental effects of ERS, cells have evolved a highly conserved unfolded protein response (UPR). The UPR is primarily an adaptive response that attenuates protein translation, induces ER-associated protein degradation, and increases ER chaperone expression (1, 2). When prolonged ERS persists, cells ultimately initiate cell death programs (3–5).

The ER-resident chaperone protein GRP78 plays a critical role in sensing ERS and triggering the UPR (1, 2). In particular, accumulating mis- and/or unfolded proteins place an increasing demand on GRP78 that results in its dissociation from the three transmembrane receptors PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1), leading to their activation and triggering the UPR (1, 2, 6, 7). The UPR induces an up-regulation of the chaperone GRP78 to enhance the folding capacity of the ER.

Although numerous studies have used the increase in GRP78 protein abundance, assayed by Western blotting, as a marker of ERS (8), little is known about GRP78 proteostasis and the role of posttranslational modifications in this process. We were intrigued by initial proteomics experiments in mouse podocytes undergoing ERS that did not reveal the expected up-regulation of GRP78 as detected by mass spectrometry. Subsequent work in podocytes, a mouse pancreatic beta islet cell line (MIN6), and HEK cells led to the discovery that, in podocytes and MIN6 cells, ERS triggers the de novo synthesis of non-trimethylated GRP78 and simultaneous degradation of existing, lysine-trimethylated GRP78. This posttranslational modification requires the activity of N-lysine methyltransferase 21A (METTL21), and the critical residue for trimethylation is lysine 585 of GRP78.

Results

Identification of a distinct, ERS-inducible GRP78

Podocytes are the only postmitotic cell population of the kidney, and therefore their adaptations to ERS may be particularly important for their survival as well as kidney health (9, 10). To better understand the molecular pathways involved in podocyte ERS, we analyzed podocytes in the presence or absence of the ERS inducer thapsigargin by MS-based proteomics, using isobaric tandem mass tag (TMT10, see “Experimental Procedures”) labeling for quantification, to characterize the ERS-associated podocyte proteome (Fig. 1A). Although GADD34, ATF4, and CHOP were up-regulated in the setting of ERS, as expected (9, 10), GRP78 was not significantly up-regulated (Fig. 1A). This result was contradictory to prior studies based on Western blot analyses for GRP78 protein abundance under the same conditions (9, 10).

We first confirmed the induction of GRP78 after thapsigargin-induced ERS at the mRNA (Fig. 1B) and protein levels (Fig. 1C). We next asked how MS proteomics experiments led to differences in GRP78 protein abundance detection compared with Western blot analyses. To identify the underlying
reason for the observed differences, we conducted a series of Western blot experiments using three different anti-GRP78 antibodies directed against different epitopes (Fig. 2A). In contrast to the anti-GRP78ERS antibody (3177, Cell Signaling Technology) that we (9, 10) and others (6) have routinely applied as a marker of ERS, anti-GRP78 antibodies directed against two other regions, on the N terminus (ab32618, Abcam) or the C terminus (MABC675, EMD Millipore), aligned with the proteomics data, showing no change in total GRP78 (GFP78TOT) after treatment with thapsigargin (Fig. 2B) as well as the ER stressor tunicamycin (an N-glycosylation inhibitor) (supplemental Fig. 1, A and B). The specificity of these antibodies was confirmed in GRP78-silenced podocytes (Fig. 2B). Anti-GRP78TOT (C-terminal) and anti-GRP78ERS antibodies were subsequently used to assess these two distinct GRP78 proteotypes in other cell types. Similar to podocytes, thapsigargin treatment did not alter GRP78TOT levels in MIN6 cells (Fig. 2C). In contrast, we noted increased levels of GRP78TOT as well as strong up-regulation of GRP78ERS in HEK cells (Fig. 2C).

Taken together, these results suggested that GRP78 protein abundance is regulated and fine-tuned and hinted at putative mechanisms for tight modulation of GRP78 proteostasis in specialized, differentiated cells such as MIN6 pancreatic beta cells and kidney podocytes.

Given that the anti-GRP78ERS antibody was produced by a synthetic peptide corresponding to the region surrounding G584, we hypothesized that a modification in its vicinity could differentiate this distinct GRP78ERS fraction. This prompted us to take a closer look at the individual -fold changes of the 58 unique peptides corresponding to GRP78 identified by proteomics. Although 57 of the peptides showed no change in abundance upon treatment with thapsigargin, a single peptide corresponding to residues Leu587-Lys597 (LSSEDKETMEK) was found to be consistently up-regulated in two independent proteomics experiments with two biological replicates each (Fig. 3A and supplemental Table 1).

Three lines of evidence led us to the hypothesis that a post-translational modification may affect anti-GRP78ERS antibody binding: a region of GRP78 consisting of 10 amino acids was exclusively up-regulated, as detected by MS proteomics (Fig. 3B); an antibody against the same region specifically detected GRP78ERS in three different cell types (Fig. 2C); and the two distinct GRP78 fractions are differentially regulated at the protein level in the setting of ERS (Fig. 2, B and C). We became interested in a GRP78 lysine residue conserved throughout evolution from yeast to human (Fig. 3B), corresponding to Lys586 in mouse and Lys585 in human GRP78 (Fig. 3A), and shown previously to undergo methylation (11–13). We thus looked more deeply into the proteomics data, allowing for lysine methylation in the database search. This analysis revealed two unique peptides (Leu583-Lys597 and Leu583-Lys592) that contained the trimethylated Lys586 (represented in blue, Fig. 3, A–C) and were down-regulated under conditions of ERS (Fig. 3, A and B). The identified single up-regulated peptide, Leu587-Lys597 (Fig. 3, A and C, and supplemental Table 1) was formed by tryptic cleavage after unmodified Lys586. Trypsin cannot cleave C-terminal...
to trimethylated Lys^{586}, which is why this modified residue was internal in both peptides observed to contain this site (Fig. 3, A and C). Cleavage was not observed after lysine 592 because it is bounded on the N- and C-terminal side with acidic amino acids that are known to hinder cleavage by trypsin. Taken together, these experiments suggest that two proteotypes of GRP78 are present, one that is trimethylated at Lys^{586} and one that is not, and that this difference could be responsible for the protein fraction detected by the C-terminal GRP78\textsuperscript{TOT} antibody but not the anti-GRP78\textsuperscript{ERS} antibody.

**Simultaneous de novo synthesis of GRP78\textsuperscript{ERS} and degradation of baseline GRP78**

To understand the proteostasis of GRP78, we asked whether ERS converts baseline GRP78 abundance into GRP78\textsuperscript{ERS} or whether the effect of ERS is restricted to a de novo synthesized GRP78\textsuperscript{ERS} fraction, as also suggested by the strong induction of GRP78 mRNA (Fig. 1B). Podocytes were treated with thapsigargin in the presence or absence of the translation inhibitor cycloheximide. Cycloheximide blocked the thapsigargin-mediated GRP78\textsuperscript{ERS} induction, whereas GRP78\textsuperscript{TOT} levels decreased, indicating that baseline, pre-ERS GRP78 may be degraded during ERS (Fig. 4A). In support of this, adding hydroxychloroquine, an inhibitor of lysosomal protein degradation, resulted in the preservation of GRP78\textsuperscript{TOT} abundance. The restored GRP78\textsuperscript{TOT} did not correlate with an increase in GRP78\textsuperscript{ERS}, confirming that these are two distinct GRP78 fractions (Fig. 4A). The proteasome inhibitor lactacystin did not block the degradation of baseline GRP78 (supplemental Fig. 1C). Together, these data show that GRP78\textsuperscript{ERS} is de novo synthesized under ERS, whereas baseline GRP78 is likely degraded in the lysosome.

The proteomics experiment suggested that homeostatic, pre-ERS GRP78 is trimethylated on Lys^{586}. We confirmed this using a lysine trimethylation-specific antibody (K-3Me) that detected a 78-kDa protein corresponding in molecular size and abundance to GRP78\textsuperscript{TOT} (Fig. 4A). Although GRP78 is a chaperone that binds unfolded proteins during ERS, it has also been reported that it can form pools of inactive oligomers ready to be activated as needed (14). We therefore wondered whether extended heat denaturing could further dissociate GRP78-containing protein complexes to increase the fraction of linearized, monomeric GRP78 detectable by Western blotting. To this end, we heat-denatured protein lysates for 4–24 h (Fig. 4B) and noted that, indeed, this correlated with a progressively increased abundance of linearized, monomeric GRP78 (Fig. 4B) (14). In this experiment, GRP78 abundance in control, non-ERS cells correlated with the protein abundance recognized by the K-3Me antibody at 78 kDa, which was absent in ERS-induced podocytes (Fig. 4B), in keeping with the notion that only homeostatic, pre-ERS GRP78 is trimethylated. To further investigate whether the observed lysine trimethylation protein band at 78 kDa corresponds to baseline, pre-ERS GRP78, we looked for the 78-kDa lysine trimethylation protein band in GRP78-depleted cells. As expected, the 78-kDa lysine trimethylation protein band was diminished after GRP78 silencing (Fig. 4C). Together, these results confirmed the MS data that lysine trimethylation is responsible for the differences between these two distinct GRP78 fractions.

Based on previous studies, a candidate enzyme for trimethylation of Lys^{586} in mouse GRP78 (or Lys^{585} in human GRP78) is METTL21A (11, 12), which has been annotated as a cytosolic enzyme but also localizes to the ER (Fig. 4E), likely because of its C-terminal ER-targeting peptide REDL (mouse) or KEDL (human). We asked whether depleting podocytes of METTL21A would lead to a reduction in GRP78 trimethylation and whether this would affect GRP78 proteostasis. In METTL21A-silenced podocytes, GRP78\textsuperscript{TOT} levels remained constant, but GRP78\textsuperscript{ERS} became detectable in the absence of thapsigargin/ERS, as assessed by several UPR markers (Chop (Fig. 4C) and Chop, Xbp1s, and Gadd34 (Fig. 4D)). In addition,
the amount of detectable GRP78ERS correlated with the Mettl21a knockdown efficiency (Fig. 4D). These findings supported Lys585 (Lys586) trimethylation of GRP78 by METTL21A.

Last, to further evaluate Lys585 as the residue responsible for trimethylation and as a marker to distinguish baseline GRP78 from GRPERS, we generated the trimethylation-resistant point mutant myc-tagged human GRP78(K585R) and expressed it in HEK cells. Overexpression itself induced an ERS response, as indicated by the presence of endogenous GRP78ERS. In immunoprecipitation experiments using anti-myc, anti-GRPERS detected wild-type, myc-tagged GRP78 but not the point mutant myc-GRP78(K585R) (Fig. 4F). This experiment thus confirmed that Lys586 is essential for anti-GRP78ERS binding, and it is therefore a critical residue to distinguish non-methylated GRP78ERS from baseline, trimethylated GRP78 (Fig. 4F).

**Discussion**

This study revealed lysine trimethylation of GRP78 as a distinguishing characteristic of its baseline, pre-ERS fraction compared with a trimethylation-independent, inducible, ERS-associated fraction. We were led to this discovery by MS proteomics.
Lys$^{585}$ trimethylation and GRP78 proteostasis

**A**

|          | DMSO | Thapsi |
|----------|------|--------|
| Time [h] | 3    | 6      | 9      |
| 78 kDa   |      |        |        |
| GRP78$^{ERS}$ |      |        |        |
| GRP78$^{TOT}$ |      |        |        |
| K-3Me    |      |        |        |
| CHOP     |      |        |        |
| LC3B     |      |        |        |
| GAPDH    | 3    | 6      | 9      |

**B**

|          | DMSO | Thapsi |
|----------|------|--------|
| Heat-denatured | 10min | 4h | 8h | 24h |
| 78 kDa   |      |        |        |
| GRP78$^{ERS}$ |      |        |        |
| GRP78$^{TOT}$ |      |        |        |
| K-3Me    |      |        |        |
| CHOP     |      |        |        |
| GAPDH    | 3    | 6      | 9      |

**C**

|          | scrambled | GRP78 | Mettl21a |
|----------|-----------|-------|----------|
| sh1      |  -        | +     | -        |
| sh1      |  -        | +     | -        |
| sh2      |  -        | -     | +        |
| sh3      |  -        | -     | +        |
| sh4      |  -        | -     | +        |

**D**

|          | Mettl21a | Grp78 | Chop | Xbp1s | Gadd34 |
|----------|----------|-------|------|-------|--------|
|          | 0.25     | 1     | 1    | 1     | 1      |
|          | 1        | 2     | 2    | 2     | 2      |
|          | 2        | 4     | 4    | 4     | 4      |
|          | 4        | 8     | 8    | 8     | 8      |
|          | 8        | 16    | 16   | 16    | 16     |

**E**

|          | METTL21A | PDI |
|----------|----------|-----|
|          |          |     |

**F**

|          | IP: MYC |
|----------|---------|
|          | 79 kDa  |
|          | 78 kDa  |
|          | 79 kDa  |
|          | 27 kDa  |

**G**

Homeostatic ER

ER stress

CH

3Me K585

GRP78

HCOQ

Lysosome

ER stress

18882 J. Biol. Chem. (2017) 292(46) 18878–18885
experiments on samples from cells under ERS, which revealed a posttranslationally modified fraction of GRP78.

An intriguing discovery in this study is that, in highly differentiated cells such as podocytes, ERS induces de novo biosynthesis of GRP78, whereas baseline METTL21A-dependent trimethylated GRP78 undergoes degradation (Fig. 4G). Of interest, ERS-mediated lysosomal degradation was also observed for other ER-resident chaperones (supplemental Fig. 1D), which is in agreement with a recent study (15). These findings suggest a substantial turnover of ER-resident proteins during chronic ERS. We speculate that this previously unrecognized mechanism may be a particularly important adaptive response in postmitotic cells whose defenses against cellular stressors must be heightened to secure survival (16). Although future work is needed to better understand cell-specific versus conserved mechanisms of GRP78 and other ER chaperone proteostasis, our results show that pancreatic beta cells (MIN6) and kidney podocytes exhibit elaborate and efficient regulation of GRP78 turnover as part of their adaptive response to ERS.

The absence of METTL21A-dependent trimethylation at Lys585 on GRP78 is intriguing, because this lysine is conserved among ER chaperone/heat shock proteins, and it has been shown to affect heat shock protein A8 (HSPA8) function (11, 12). Moreover, mouse Lys586 or human Lys585 is located in the “lid” domain of GRP78, which is open in the ATP-bound state (low affinity for substrates) and closes upon ATP hydrolysis (stable substrate binding) (11, 17). Therefore, it is intriguing to speculate that the lack of posttranslational modification may alter the conformation of GRP78 in a way that may be beneficial during ER stress. Future studies will address whether the trimethylation of Lys585/Lys586 affects GRP78 substrate binding, localization, or susceptibility to lysosomal degradation. In conclusion, our study reveals a previously unrecognized complexity in GRP78 proteostasis under conditions of cellular stress, with implications for cellular response to stress across a wide range of cell types.

**Experimental procedures**

**Cell culture**

Mouse podocytes were cultured as described before (18, 19) in RPMI 1640 medium (21875, Invitrogen) supplemented with 10% FBS (10270, Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin (15140, Invitrogen) and interferon-γ (Cell Sciences) on type I collagen (BD Biosciences) during the proliferation process, whereas induction of differentiation was achieved by a thermoshift to 37 °C without interferon-γ in 6-well plates (mRNA isolation), 10-cm dishes (protein isolation for Western blotting), and 15-cm dishes (proteomics). Experiments were performed on day 11 of differentiation. HEK293 cells and MIN6 cells were cultured in DMEM (41965, Invitrogen) supplemented with 10% FBS and penicillin/streptomycin.

**shRNAs, vectors, and lentiviruses**

shRNAs (in PLKO.1 puro vectors) directed against GRP78 (sh1, 5'-CCGTACATCAATTGTATATT-3'; sh2, 5'-GGA-GACTGCTAGGCGGTATTT-3'; sh3, 5'-GGGGACATTGAAGCTTAAA-3') and Mettl21a (sh1, 5'-CGCATATGAGG-GGATAGA-3'; sh2, 5'-TACTTGGAGCTGTGATTGA-3'; sh3, 5'-GACCCTGAGGCGTATTT-3'; sh4, 5'-ACGGATCGAGAAGTAGATTACA-3') were obtained from Sigma. A 21-nt scrambled sequence lacking any homology to mammalian genes (5'-CAACAAGATGAAAGACCAACCA-3', Sigma) served as a control.

**Proteomics**

Total protein was isolated from podocytes treated with 2.5 μM thapsigargin or vehicle (DMSO) for 16 h. Protein lysates, two biological replicates per condition, were prepared in 8 M urea, 75 mM NaCl, 1 mM EDTA in 50 mM Tris HCl (pH 8), 2 μg/ml aprotinin (A6103, Sigma), 10 μg/ml leupeptin (11017101001, Roche), and 1 mM PMSF (78830, Sigma), and deep coverage quantitative mass spectrometric analysis was performed as reported previously (24–26) following TM10 labeling and offline HPLC fractionation. All MS data were interpreted using the Spectrum Mill software package v5.0 prerelease (Agilent Technologies, Santa Clara, CA). Similar MS/MS spectra acquired on the same precursor m/z within ±60 s were merged. MS/MS spectra were excluded from searching when they failed the quality filter by not having a sequence tag length > 0 or did not have a precursor MH+ in the range of 750–6000. MS/MS spectra were searched against the UniProt Database.

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**Figure 4. The proteostasis of GRP78 under ERS is lysine trimethylation-dependent.** A, induction of GRP78ERS and degradation of trimethylated/homeostatic GRP78. Shown is a Western blot analysis of the dynamics of the two GRP78 fractions during ERS. Podocytes were treated with thapsigargin (Thapsin) in the presence or absence of cycloheximide (CH, 10 μM) and/or hydroxylchloroquine (HCQ, 150 μM) for the indicated times. CHOP served as an ERS marker, the accumulation of light chain 3B (LC3B) as a marker for the inhibition of autophagy/lysosomal degradation, and GAPDH as a loading control, respectively. B, protein lysates were heat-denatured as indicated and analyzed by Western blotting for GRP78ERS, GRP78TOT, lysine trimethylation, and CHOP. C, Western blot analysis of GRP78ERS, GRP78TOT, lysine trimethylation, and CHOP in GRP78- and METTL21A-depleted podocytes. GAPDH served as a loading control. D, mRNA levels of Mettl21a and the ERS markers GRP78, Chop, Xbp1s, and Gadd34 in Mettl21a-silenced and scrambled control podocytes were analyzed by qPCR. E, immunocytochemistry of METTL21A and the ER marker protein disulfide isomerase (PDI) in podocytes. Representative images are shown. F, co-immunoprecipitation (IP) experiments in HEK cells expressing Myc-tagged GRP78K585R versus wild-type GRP78. G, proposed model for the regulation of GRP78 proteostasis by Mettl21a-mediated trimethylation and lysosomal degradation. Under homeostatic conditions, the bulk of GRP78 is trimethylated at Lys585/Lys586. In podocytes experiencing ERS, the protein abundance of non-methylated GRP78ERS is strongly induced, whereas trimethylated GRP78 undergoes lysosomal degradation. As a consequence, overall protein abundance of GRP78 (GRP78TOT) remains largely constant.
Lys^{585} trimethylation and GRP78 proteostasis

mouse protein database. All spectra were allowed ± 20 ppm mass tolerance for precursor and product ions, 30% minimum matched peak intensity, and “trypsin allow P” enzyme specificity with up to four missed cleavages. For the proteome search, carbamidomethylation at cysteine was searched as a fixed modification, and TMT10 labeling was required at lysine, but peptide N termini were allowed to be either labeled or unlabeled. Allowed variable modifications for whole proteome searches were acetylation of protein N termini and oxidized methionine. Allowed variable modifications for the trimethylation search were trimethylation of lysine and oxidation of methionine with a precursor MH+ shift range of -200 to 100 Da. Identities interpreted for individual spectra were automatically designated as confidently assigned using the Spectrum Mill autovalidation module to use target-decoy–based false discovery rate estimates to apply score threshold criteria. Peptide level TMT ratios were calculated as the median of all PSM level ratios contributing to a protein subgroup.

Western blotting

Western blotting was done according to standard protocols (9, 10). Antibodies against GRP78ERS (3177, Cell Signaling Technology), GRP78 (ab32618, Abcam), GRP78TOT (MABC675, EMD Millipore), Myc tag (2272, Cell Signaling Technology), ubiquitin (3933, Cell Signaling Technology), ATF4 (11815, Cell Signaling Technology), CHOP (sc-7351, Santa Cruz Biotechnology), GADD34 (sc-825, Santa Cruz Biotechnology), trimethyl lysine (14680, Cell Signaling Technology), METTL21A (FAM119A, ab84523, Abcam), and GAPDH (EMD Millipore) were applied at dilutions of 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:500, 1:500, 1:300, and 1:10,000, respectively. The immunoblots were detected by enhanced chemiluminescence (34078, Thermo Fisher Scientific) in a G:Box (GeneSys software, Syngene). Representative experiments of at least three repeats are shown.

Quantitative real-time RT-PCR

Total RNA was extracted (74104, Qiagen), and cDNA was synthesized using random hexamers (N8080127, Thermo Fisher Scientific) and Maxima H Minus reverse transcriptase (EP0752, Thermo Fisher Scientific) according to the protocols of the manufacturer. Real-time qPCR was performed in a total volume of 20 μl using 2× GoTaq® qPCR Master Mix (Promega) and 0.75 μM particular primers. Gapdh was used as internal control. The experiments were carried out in a 7500 Fast Real Time PCR System (Applied Biosystems) with an initial denaturation at 95 °C for 2 min and 40 cycles of amplification (95 °C for 3 s and 60 °C for 30 s). The following primer sequences (5’-3’) were used: Gapdh (F-CTGCACCACCAACTGTCCAG, R-GGCATGTTGCTGATGAG), GRP78 (F-CACGAGATGCGGACATTGA, R-AGGGCCTCCACCTCAGA), Chop (F-CAACAGAGGTCCACGCA, R-GGCACCTCCACTCTCCAGA), Gadd34 (F-AGAGAAGACCAAGGGACGG, R-AACACCTGAGCCGCCCTCTG), Bips1 (F-TGCTGATCCACGACCGT, R-CTGACGACTGCTGGGAGG), and Mettl21a (F1-CCAGAAATTCCATAA-GCCTCTCG, R1-TTCCGATCGTATGACCA).

Statistics

A representative experiment of at least three repeats is shown. Individual experiments were performed in triplicates, and data are expressed as means and standard deviations. Significance of differences was calculated with analysis of variance and Bonferroni post hoc tests using the Prism 6 program. The significance level was set to p < 0.05.

Immunocytochemistry

Podocytes grown on CellCarrier-96 Ultra microplates (PerkinElmer Life Sciences) were fixed for 10 min in PBS containing 4% paraformaldehyde (Electron Microscopy Sciences) and permeabilized for 15 min in 0.5% Triton X-100 (Sigma, in PBS). Blocking solution (11096176001, Roche) was applied for 1 h. Antibodies specific for METTL21A (21079-1-AP, Protein-Tech) and protein disulfide isomerase (ADI-SPA-891-F, Enzo Life Sciences) were incubated for 1 h in blocking solution at a concentration of 1:200. Alexa 488-labeled secondary antibodies against mouse (A11029, Life Technologies) and Alexa 647-labeled secondary antibodies against rabbit (A21246, Life Technologies) IgG were applied at 1:500 together with Hoechst 33342 (H3570, Life Technologies) 1:2000 in blocking solution for 1 h. Cells were imaged by confocal microscopy (Operetta CLS, PerkinElmer Life Sciences) using a ×63 water objective.

Author contributions—J. S., N. W., and O. A. designed and performed the experiments. M. D. L. and M. O. performed experiments. O. A. performed mass spectrometric analyses in collaboration with N. D. U. and S. A. C. J. S., N. W., M. O., and O. A. analyzed the data. J. S., N. S. U., S. A. C., and A. G. wrote the paper. A. G. supervised the project. All authors read and agreed with the content of the manuscript.

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