Non-canonical ubiquitination of the cholesterol-regulated degron of squalene monoxygenase

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Running title: Serine ubiquitination and cholesterol regulation

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ABSTRACT

Squalene monoxygenase (SM) is a rate-limiting enzyme in cholesterol synthesis. The region comprising the first 100 amino acids, termed SM N100, represents the shortest cholesterol-responsive degron and enables SM to sense excess cholesterol in the endoplasmic reticulum (ER) membrane. Cholesterol accelerates the ubiquitination of SM by membrane-associated ring-CH-type finger 6 (MARCH6), a key E3 ubiquitin ligase involved in ER-associated degradation. However, the ubiquitination site required for cholesterol regulation of SM N100 is unknown. Here, we used SM N100 fused to GFP as a model degron to recapitulate cholesterol-mediated SM degradation and show that neither SM lysine residues nor the N-terminus impart instability. Instead, we discovered four serines (Ser-59, Ser-61, Ser-83, and Ser-87) that are critical for cholesterol-accelerated degradation, with MS analysis confirming Ser-83 as a ubiquitination site. Notably, these two clusters of closely spaced serine residues are located in disordered domains flanking a 12-amino acid-long amphipathic helix (residues Gln-62–Leu-73) that together confer cholesterol responsiveness. In summary, our findings reveal the degron architecture of SM N100, introducing the role of non-canonical ubiquitination sites and deepening our molecular understanding of how SM is degraded in response to cholesterol.

INTRODUCTION

Cholesterol synthesis is an important metabolic pathway implicated in many diseases. Dysregulation of cholesterol synthesis is a feature of numerous cancers (1, 2) and neurological disorders (3–5). Statins are used to lower cholesterol levels by inhibiting 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR), the first rate-limiting enzyme in this pathway (6, 7). The enzymes in cholesterol biosynthesis are regulated transcriptionally by the master transcription factor SREBP-2 (8). Cells also utilize post-translational mechanisms to regulate de novo cholesterol biosynthesis, with sterols accelerating the degradation of at least three enzymes (9–12).

Squalene monoxygenase (SM) is the second rate-limiting enzyme in cholesterol synthesis (10). Earlier research investigated SM as a pharmacological target for reducing cholesterol levels (13, 14). More recently, SQLE, the gene encoding SM, has gained attention as an oncogene. Its altered gene expression is associated with perturbed cholesterol homeostasis and tumour progression, prompting investigation of the therapeutic potential of SM in certain cancers (15–18). Given its biological significance in cholesterol homeostasis and disease, there is an increasing need to understand the regulatory mechanisms controlling SM. It is known that SM is degraded by the ubiquitin-proteasome system in response to excess cholesterol, and that the first 100 amino acids of SM (termed SM N100) is necessary for this to occur (10). When SM N100 is fused to GFP (SM N100-GFP), it confers the ability of normally stable GFP to be degraded by cholesterol, making...
this the shortest known transferrable cholesterol-regulated degron. SM N100 is anchored to the endoplasmic reticulum (ER) membrane via a re-entrant loop, which along with a 12-amino acid-long amphipathic helix (residues Gln-62–Leu-73) is needed to sense changes in cholesterol content of the ER (19–21).

The cholesterol-accelerated degradation of SM is part of a protein quality control process known as endoplasmic reticulum associated-degradation (ERAD) (22). Efforts to dissect ERAD mechanisms often use model substrates such as the mammalian ERAD substrate, the T-cell receptor α-chain (TCRα) (23–25). However, the regulation of many ERAD substrates is perplexing given the diverse requirements of each substrate within the ERAD landscape (26). To add to the complexity, lysine ubiquitination is not always the main signal for degradation, with non-canonical ubiquitination on alternative residues being reported (27–31). This has been exemplified in a number of ERAD substrates (24, 32–34).

SM remains degraded by the ubiquitin-proteasome system under excess cholesterol conditions even when all lysine residues in the SM N100 degron are mutated (10). The only reported SM N100 ubiquitination site is Lys-90 (35–41). As lysine residues do not mediate degradation of SM, it seems likely that alternative non-lysine sites are ubiquitinated, such as the α-NH₂ group at the N-terminus (30, 31). We generated a construct where mCherry was introduced to sterically hinder the N-terminus of SM N100 (Fig. 1, right), thus impairing potential N-terminal ubiquitination. Introducing this bulky tag resulted in slight blunting (24%) of cholesterol regulation of SM N100 but no change in protein expression when comparing untreated conditions (Fig. 1, D and E). We therefore concluded that the internal lysine residues and the N-terminus are not major ubiquitination sites necessary for cholesterol to accelerate degradation of SM N100.

RESULTS

The N-terminus and lysine residues are dispensable for SM N100 cholesterol-accelerated degradation – We have previously shown that SM N100 is degraded via the ubiquitin-proteasome system when excess cholesterol is present, even when all five lysine residues (Lys-15, Lys-16, Lys-82, Lys-90, Lys-100) have been substituted with arginine (10). Here, we confirm our findings with this lysine-less construct (Fig. 1A, left) but importantly include quantification (Fig. 1, B and C).

Since lysine sites are not crucial for cholesterol-accelerated degradation of SM N100, we hypothesized that SM N100 undergoes non-canonical ubiquitination. Non-canonical residues that can be ubiquitinated include serine, threonine, cysteine and the free α-NH₂ group at the N-terminus (30, 31). We generated a construct where mCherry was introduced to sterically hinder the N-terminus of SM N100 (Fig. 1A, right), thus impairing potential N-terminal ubiquitination. Introducing this bulky tag resulted in slight blunting (24%) of cholesterol regulation of SM N100 but no change in protein expression when comparing untreated conditions (Fig. 1, D and E). We therefore concluded that the internal lysine residues and the N-terminus are not major ubiquitination sites necessary for cholesterol to accelerate degradation of SM N100.

SM N100 cholesterol-accelerated degradation requires serine residues located in the second half – Aside from the N-terminus, the only other known non-canonical ubiquitination sites are serine, threonine and cysteine residues (Fig. 2A). We hypothesized that it is likely that SM N100 is ubiquitinated at more than one non-canonical site, as observed in other ERAD substrates (24, 32, 34). To test this, we mutated clusters of serines, threonines or cysteines to alanine. We first generated five main cluster mutants (T3A/T9A/T11A, S43A/C46A, S59A/S61A, S67A/S71A and S83A/S87A) (Fig. 2A). Residues Thr-22 and Cys-31 were not included in the mutations given they are embedded in the ER membrane (20) and hence likely to be inaccessible for ubiquitination. SM N100 mutants of the initial five clusters (Fig. 2A, T3A/T9A/T11A, S43A/C46A, S59A/S61A, S67A/S71A and S83A/S87A, clusters 1 to 5)
showed no change in cholesterol regulation (Fig. 2, B and C). We next combined mutations of multiple clusters and observed eight mutants of SM N100 showed loss of cholesterol regulation to varying degrees (Fig. 2, B and C, clusters 7 to 9 and 11 to 15). Five mutants also displayed increased protein expression (Fig. 2, B and C, 7 and 12 to 15).

Next, we pooled the data to deduce the contribution of these five clusters to cholesterol regulation. Overall, losing residues Ser-59 and Ser-61 from any construct resulted in the greatest loss of cholesterol regulation (Fig. 2D, minus S59/S61). Ser-67, Ser-71, Ser-83 and Ser-87 also contributed to cholesterol regulation although to a lesser extent than that of Ser-59 and Ser-61 (Fig. 2D). Here, our observations reveal serine residues in the second half of SM N100 are necessary for the cholesterol-accelerated degradation of SM N100.

**MARCH6 degradation of SM N100 is impeded by loss of serine residues** – MARCH6 is the E3 ubiquitin ligase that degrades SM and SM N100-GFP, indicating that MARCH6 must ubiquitinate the SM N100 degron (22). We next determined if the SM N100 serine mutants are still regulated by MARCH6. If the identified serine residues required for cholesterol-accelerated degradation of SM N100 (Fig. 2) are indeed ubiquitination sites, MARCH6 may target these serine residues.

We initially investigated three main constructs. We compared a mutant containing serine, threonine and cysteine point mutations in the first half of SM N100 (Fig. 3A, cluster 6) with a mutant having serine to alanine point mutations in the second half (Fig. 3A, cluster 12). We also included an SM N100 mutant with all eleven potential non-canonical ubiquitination sites replaced with alanine (Fig. 3A, cluster 15). This allowed us to discriminate whether functional non-canonical ubiquitination sites existed in the first half, second half or in the entire SM N100 degron.

To this end, we co-transfected SM N100 mutants with siRNA targeting MARCH6. When MARCH6 is knocked down, this increases SM N100 WT protein levels, indicating rescue. When all potential non-canonical ubiquitination sites were mutated to alanine within the first half of SM N100, MARCH6 knockdown still increased SM N100 protein levels, indicated by the unchanged MARCH6 response (Fig. 3A, cluster 6). Serine to alanine replacement in the second half of SM N100 resulted in a minimal increase in protein levels when MARCH6 was knocked down, as demonstrated by reduction of the MARCH6 response (Fig. 3A, cluster 12). When all serine, threonine and cysteine residues were mutated to alanine, MARCH6 response was also reduced (Fig. 3A, cluster 15).

The serine residues in the second half disrupts MARCH6 regulation (Fig. 3A), suggesting that the ubiquitination sites are more likely to be in the second half of SM N100, rather than the first half. We have shown there are other SM N100 mutants lacking cholesterol regulation and hypothesized these mutants may also exhibit perturbed MARCH6 regulation. We tested three such mutants which are L42A, F35A/S37A/L65A/I69A and the amphipatic helix deletion (∆Q62–L73) (21). When MARCH6 is knocked down, these mutants showed a blunted response (Fig. 3B). These findings suggest that these mutated sites (Fig. 3, A and B) impede MARCH6 degradation of SM N100 and may have functional roles in regulating distinct steps of degradation, including ubiquitination.

**Distinct serine requirement of full-length SM and SM N100** – To further narrow down which serine residues in the second half of SM N100 are critical for cholesterol-accelerated degradation, we utilized SM N80 (encoding the first 80 amino acids of SM) fused to GFP, which remains responsive to cholesterol (21) (Fig. 4A). SM N80 excludes residues 81 to 100 of SM N100, therefore missing Ser-83, Ser-87 and Lys-90. Lys-90 is a reported ubiquitination site (35–41) that is not needed for the cholesterol-accelerated degradation of SM or SM N100 (10, 21). Using this construct, we have a minimal protein which is still degraded in response to excess cholesterol, further ruling out a role for the Lys-90 ubiquitination site.

Here, we investigated how mutating the remaining available serine residues, Ser-59, Ser-61, Ser-67 and Ser-71 to alanine would affect cholesterol regulation (Fig. 4A). Introducing S67A/S71A (cluster 4) mutations alone did not affect SM N80 cholesterol regulation (Fig. 4, B...
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and C). In contrast, SM N80 S59A/S61A (cluster 3) displayed blunted cholesterol regulation and SM N80 S59A/S61A/S83A/S71A (cluster 9) showed a similar loss of cholesterol regulation, suggesting S59A/S61A are the key residues (Fig. 4, B and C). Unlike the SM N80 S59A/S61A mutant (cluster 3), when Ser-59/Ser-61/Ser-83/Ser-87 (cluster 11) were mutated to alanine in SM N100, the SM N100 S59A/S61A/S83A/S87A mutant showed less blunting of cholesterol regulation and an increase in protein expression (Fig. 2, B and C, cluster number 11). Collectively, our data from SM N100 and SM N80 narrow down our search for potential ubiquitination sites to four serine residues, Ser-59, Ser-61, Ser-83 and Ser-87.

We next asked whether the effects from the stabilizing mutations identified from SM N100 and SM N80 could be transferred to full-length SM. We introduced mutations S59A/S61A/S83A/S87A into full-length SM, but the protein expression and cholesterol regulation remained unchanged (data not shown). However, replacing all the threonine, cysteine and serine residues to alanine (Fig. 4A) in the first 100 amino acids of full-length SM (except Thr-22 and Cys-31 which are buried in the ER membrane) significantly blunted cholesterol regulation without any change in protein expression (Fig. 4, D and E). In conclusion, full-length SM requires more than the four critical serine residues identified in SM N100 for cholesterol-accelerated degradation. Seven additional residues in the N-terminal domain (Thr-3, Thr-9, Thr-11, Ser-43, Cys-46, Ser-67, Ser-71) may also be needed.

Evidence of serine ubiquitination from destabilizing effects of Ube2J2 and mass spectrometry – In yeast, the E3 ubiquitin ligase Doa10 cooperates with two E2 ubiquitin-conjugating enzymes, Ubc6 and Ubc7, to ubiquitinate ERAD substrates (43–45). MARCH6 is the human ortholog of yeast Doa10 (46), while Ube2J2 and Ube2G2 are the human orthologs of yeast Ubc6 and Ubc7, respectively (47, 48). During this study, two independent groups showed that Ube2J2 is needed to degrade endogenous full-length SM (49, 50). Given that both human Ube2J2 and yeast Ubc6 have been shown to attach ubiquitin on serine residues (42, 51), we hypothesized Ube2J2 regulates SM by targeting the SM N100 degron through serine ubiquitination.

We co-transfected Ube2J2 constructs with SM N100-GFP-V5 and assessed how these impact SM N100 (Fig. 5A). Upon overexpression of the catalytically inactive Ube2J2 C94S mutant (52), SM N100 levels increased. We also performed immunoprecipitation of cells stably overexpressing SM N100 after cholesterol treatment and analyzed the products using mass spectrometry. We identified a tryptic peptide where Ser-83 was modified with a di-glycine remnant, confirming our hypothesis of ester linked ubiquitin to serine residues (Fig. 5B). Taken together, we demonstrate that serine ubiquitination occurs on SM N100, with Ube2J2 being the likely E2 ubiquitin-conjugating enzyme mediating this process.

Human SM N100 serine residues confer instability to chicken SM N100 – We previously investigated the conservation of SM N100 in other species including chicken, zebrafish and lamprey (21). We reported chicken SM N100 is not cholesterol regulated and showed high protein expression (21). We were interested to see if the serine residues in human SM N100 were conserved in chicken SM N100, particularly those influencing protein levels and cholesterol regulation (Figs. 2 and 4). Focusing on the second half of SM N100, the human residues Ser-59, Ser-61, Ser-83 and Ser-87 are Gln-58, Pro-60, Pro-82 and Ser-87 in the corresponding chicken SM N100 sequence (Fig. 6A). Since Ser-59, Ser-61, Ser-83 and Ser-87 are required for cholesterol regulation in human SM N100 (Fig. 4B), we questioned if chicken SM N100 was not regulated due to the lack of three of these four critical serine residues.

A recurring theme in degrons is that the degradation properties should be transferable (53). We therefore mutated chicken SM N100 residues Gln-58, Pro-60 and Pro-82 to serine residues. Interestingly, there was a stepwise reduction of protein levels in the serine mutants of chicken SM N100 (Fig. 6, B and C). However, these new serine sites were not sufficient to enable cholesterol regulation of chicken SM N100 to the same extent as that of human SM N100 WT. Therefore, our findings show the serine residues confer instability to chicken SM N100, which
normally exhibits a 3 to 4-fold increase in protein levels relative to human SM N100.

Given residues Gln+62–Leu+73 form an amphipathic helix essential for cholesterol regulation in our human SM N100 model (21), we reasoned the lack of cholesterol regulation in chicken SM N100 may be due to differences in this region. A closer examination revealed the hydrophobicity and hydrophobic moment of this region does not differ greatly between the two species (Fig. 6D). If chicken SM N100 also forms an amphipathic helix in this region, we postulate that the lack of cholesterol regulation in chicken SM N100 may be a result of sequence differences when comparing the helices between two species.

Of note, the serine clusters Ser+59, Ser+61, Ser+83 and Ser+87 are situated near the amphipathic helix. Disordered regions are considered important structural elements in degrons (54–57). We next examined SM N100 using 13 disordered predictors and presented the data as the number of times a residue was predicted to be present in a disordered region. The second half of SM N100 was more frequently identified as being disordered than the first half. Our residues of interest, Ser+59, Ser+61, Ser+83 and Ser+87, were also found to be disordered, based on 5 to 9 different predictors depending on the serine (Fig. 6E). Taken together, we have identified four regulatory serine residues Ser+59, Ser+61, Ser+83 and Ser+87, which are part of a disordered region, with Ser+83 being a confirmed ubiquitination site.

DISCUSSION
In this study, we sought to understand the ubiquitination events occurring during the cholesterol-accelerated degradation of SM. Our earlier work provided considerable insights (20, 21) into how cholesterol regulates conformational changes in SM N100 leading to proteasomal degradation, but the essential ubiquitination residues have eluded us until now.

The most striking discovery in this study is that the cholesterol-accelerated degradation of SM N100 depends on non-lysine residues, specifically serines. We identified four serines in the second half of SM N100 (Ser+59, Ser+61, Ser+83 and Ser+87) that are important for cholesterol regulation. These residues are situated in disordered regions on either side of the amphipathic helix (Gln-62–Leu-73) of SM N100 (Fig. 6E). Since disordered regions are implicated in proteasomal degradation (54–57) and serines are disorder-promoting residues (58), these serines may also contribute to the degron through increasing disorder.

Direct evidence for serine ubiquitination using mass spectrometry is scarce in the literature. Although indirect, mutagenesis has proved useful in identifying non-canonical ubiquitination in several ERAD substrates (24, 30, 32–34), though none of these include components of the complex cholesterol homeostatic machinery that resides in the ER. Using mass spectrometry, we identified ubiquitination at Ser+83 (Fig. 5B), making SM the first reported protein in the cholesterol homeostatic machinery to undergo serine ubiquitination. Our mass spectrometry experiments revealed Ser+83 ubiquitination was sub-stoichiometric. While we were unable to obtain mass spectrometric evidence for ubiquitination of Ser+59, Ser+61 and Ser+87, ubiquitination of these residues certainly cannot be ruled out. Sensitivity is one issue but also of note, the tryptic peptides covering Ser+59 and Ser+61 are too long (26 amino acids) to be identified using conventional proteomic approaches.

The regulatory serine residues (Ser+59, Ser+61, Ser+83 and Ser+87) are conserved in mammalian SM N100 (10), but only one is conserved in chicken SM N100 (Fig. 6A), which lacks cholesterol regulation (21). Introducing serine residues into chicken SM N100 progressively decreases protein levels but does not affect cholesterol regulation (Fig. 6, B and C), implying that the nature of the amphipathic helix is also important. Perhaps the gain of serine residues in SM N100 during evolution has allowed the emergence of more exquisite regulated protein degradation to help fine-tune cholesterol homeostasis.

The degradation machinery regulating yeast and mammalian SM is evolutionarily conserved. Ube2J2 and MARCH6 are needed for degradation of SM (22, 49, 50). The yeast orthologs of Ube2J2 and MARCH6 are Ubc6 (47) and Doa10 (43, 46), which are required for degrading yeast SM (commonly known as Erg1) (45). However, there are differences in the degron architecture. Yeast SM lacks the N100 region of mammalian SM (10). The proposed ubiquitinated residue, Lys+311, essential for ERAD of yeast SM (45), is not conserved as it corresponds to His+384 in human SM. Mutating
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conserved lysine residues into arginine in human full-length SM does not prevent SM cholesterol-accelerated degradation (10). Although the degradation machinery is conserved, adaptations were possibly required to deal with the more sophisticated substrate in mammals with its additional regulatory domain (SM N100).

If the identified serine residues (Ser-59, Ser-61, Ser-83 and Ser-87) can each be ubiquitinated by MARCH6, how is site selectivity achieved? Different E3 ubiquitin ligases have distinct mechanisms to select residues for ubiquitination (59–62). With 14 predicted transmembrane domains (63), MARCH6 is likely to have substantial contact with membrane-bound SM N100, allowing multiple ubiquitination events on the closely gathered critical serine residues. As E2 ubiquitin-conjugating enzymes are essential binding partners of the E3 ubiquitin ligases (64, 65), interaction between MARCH6 and Ube2J2 may influence site selectivity for ubiquitination. Further biochemical and structural studies are needed, and these may reveal general mechanistic features applicable to other substrates in ERAD.

Considering that we lack direct evidence for ubiquitination of Ser-59, Ser-61 and Ser-87, could these residues perhaps play another role in facilitating degradation? For example, serine residues can be phosphorylated and in the case of phosphodegrons can cross-talk with ubiquitination to regulate substrate degradation (66–68). In silico predictions revealed Ser-59 and Ser-83 are putative phosphorylation sites for four kinases, PKA, CaMKII, AMPK and GSK3. Preliminary experiments revealed that inhibiting these kinases individually does not affect SM N100-GFP-V5 protein levels or cholesterol-accelerated degradation (data not shown). The catalytic activity of some cholesterol synthesis enzymes is regulated by phosphorylation (69–71), although it is not immediately obvious how phosphorylation on the SM N100 regulatory domain would affect SM catalytic activity.

The newly identified regulatory serine residues are unlikely to have major effects on the interaction between MARCH6 and SM N100. A construct expressing the second half of SM N100 (SM N100 Δ48) cannot be rescued with MARCH6 knockdown (data not shown), indicating MARCH6 binding sites may be present in the first half of SM N100. This suggests our serine residues in the second half are unlikely to be critical for MARCH6 binding beyond ubiquitination.

SM N100 requires four critical serine residues for cholesterol-accelerated degradation (Fig. 2), whereas full-length SM seems to require more residues (Fig. 4, D and E). Substituting the catalytic domain of SM with GFP may have placed SM N100 in a slightly different context for ubiquitination. However, both SM N100 and full-length SM are degraded by MARCH6 during cholesterol excess (22). Perhaps SM N100-GFP is more readily ubiquitinated than full-length SM, suggesting the catalytic domain may hinder ubiquitination to some extent.

It is unlikely that Ser-59, Ser-61, Ser-67, Ser-71, Ser-83 and Ser-87 bind to cholesterol directly as these residues are in the cytosol (20). Ser-67 and Ser-71 are located in the amphipathic helix, but like most amphipathic helices, it probably does not bury itself deeply enough to strongly bind to cholesterol (72–74). Structural insights of SM N100 would reveal if the re-entrant loop and amphipathic helix form favorable cholesterol contacts, or if cholesterol binding could even occur outside the ER membrane. In addition, use of a cholesterol probe and mass spectrometry (75, 76) may reveal the cholesterol binding sites in SM N100.

In conclusion, we identified key serine residues, Ser-59, Ser-61, Ser-67 and Ser-83, and Ser-87, which are likely to act as serine ubiquitination sites governing the cholesterol-accelerated degradation of SM, a rate-limiting enzyme important in disease (77–79) and biotechnology (80, 81). This work advances our understanding of degron architecture and provides a model whereby excess cholesterol deforms the SM N100 amphipathic helix, adding to the disorder of the flanking regions where the key serines reside, allowing these residues to be ubiquitinated by MARCH6 (Fig. 7). We have built on our previous findings (20, 21) to enrich our understanding of how cells respond to excess cholesterol, introducing a role for serine ubiquitination in the interplay between membrane cholesterol sensing and regulated protein degradation.
EXPERIMENTAL PROCEDURES

Cell culture – Cells were maintained as monolayers at 37°C in 5% CO₂. CHO-7 cells were cultured and maintained in DMEM/Ham’s F-12 (DF-12) medium supplemented with 5% (v/v) LPDS, penicillin (100 U/ml) and streptomycin (100 µg/ml). HEK-293 Flp-In™ T-REx™ cells stably expressing SM N100-GFP-V5 (HEK-SM N100-GFP-V5) (22) were cultured and maintained in DMEM (high-glucose) supplemented with 10% (v/v) FCS, 200 µg/ml hygromycin B and penicillin (100 U/ml) and streptomycin (100 µg/ml).

Cholesterol and MARCH6 regulation – For plasmid transfections in 6-well plates, cells were transfected for 24 h with 0.75 µg pTK-empty vector plasmid DNA and 0.25 µg expression plasmid DNA using 1.5 µL Lipofectamine 3000 and 2 µL P3000 reagent. For siRNA and plasmid co-transfections in 6-well plates, cells were transfected for 24 h with 0.75 µg pTK-empty vector DNA, 0.25 µg expression plasmid DNA and 25 nM siRNA using 4.5 µL Lipofectamine 2000. All transfection components in 12-well plates are the same as 6-well plates except the amount for each component is reduced by 2.5 times. All transfections were performed in maintenance media lacking penicillin and streptomycin. After 24 h transfection, CHO-7 cells were pre-treated in maintenance media containing compactin (5 µM) and mevalonate (50 µM) overnight to reduce basal cholesterol status. Cells were then treated with cholesterol complexed with methyl-β-cyclodextrin (CD) (20 µg cholesterol/mL) for 8 h before harvesting cell lysates. The complexing process was performed as described previously (82). Cells were then harvested as described in Western blotting.

Ube2J2 overexpression – For plasmid transfections in 6-well plates, cells were transfected for 24 h with 0.10 µg pTK-empty vector plasmid DNA, 0.15 µg Ube2J2-FLAG plasmids (kindly gifted by Dr. John V (Eoin) Fleming, University College Cork, Ireland), and 0.75 µg pTK-SM N100-GFP-V5 plasmid, using 1.5 µL Lipofectamine 3000 and 2 µL P3000 reagent. After 24 h transfection, cells were then harvested as described in Western blotting.

Western blotting – Lysates from transiently transfected cells were harvested by scraping in 2% SDS lysis buffer [2% (w/v) SDS, 10 mM Tris-HCl, pH 7.6, 100 mM NaCl] supplemented with protease inhibitors. Cells were passed 20 times through a 21-gauge needle and then vortexed for 20 min at room temperature. Protein concentration was quantified using the bicinchoninic acid assay (Thermo Fisher) and normalized. Lysates were equally loaded for SDS-PAGE separation and transferred onto nitrocellulose membranes. Membranes were blocked with 5% (w/v) skim milk/PBST and probed with mouse anti-V5 (1:5,000 dilution in 5% (w/v) skim milk/PBST; Life Technologies, Inc., catalog no. R960-25), mouse anti-FLAG (1:10,000 dilution in 5% (w/v) BSA/PBST; Merck, catalog no. F3165), rabbit anti-GAPDH (1:2,000 dilution in 5% (w/v) BSA/PBST; Cell Signaling Technology, catalog no. 2118L, lot no. 10) and rabbit anti-β-tubulin (1:2,500 dilution in 5% (w/v) BSA/PBST; Abcam, ab6046). After incubations with primary antibodies, blots were incubated with IRDye® 680RD donkey anti-rabbit IgG (1:10,000 dilution in 5% (w/v) skim milk/PBST; LI-COR, product no. 925-68073) and IRDye® 800CW donkey anti-mouse IgG (1:10,000 dilution in 5% (w/v) skim milk/PBST; LI-COR, product no. 926-32212). Membranes were then visualized using the Odyssey CLx (LI-COR). Western blots were quantified by densitometry using Image Studio Lite (version 5.2.5). Locations of molecular mass standards are indicated on the blots.

Immunoprecipitation for ubiquitination site determination via mass spectrometry – HEK-SM N100-GFP-V5 cells were pre-treated in DMEM (high-glucose) supplemented with 10% (v/v) FCLPDS, penicillin (100 U/mL) and streptomycin (100 µg/mL) media containing compactin (5 µM) and mevalonate (50 µM) overnight to reduce basal cholesterol status. Cells were then treated with cholesterol complexed with methyl-β-cyclodextrin (CD) (20 µg cholesterol/mL) and 10 µM MG132 for 8 h before harvesting cell lysates. Cells were scraped in cold PBS and pelleted by centrifugation at 1,000 x g for 10 minutes at 4°C. Pellets were lysed in RIPA buffer [20 mM Tris-HCl (pH 7.4), 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, and 1 mM sodium orthovanadate] containing 10 mM NEM, 10 µM MG132 and eOmplete™ ULTRA...
Protease Inhibitor Cocktail Tablets (1 tablet per 10 mL of RIPA buffer). Lysates were passed 40 times through a 22-gauge needle, then centrifuged at 20,000 × g for 20 minutes at 4°C. Supernatant protein concentration was quantified using the bicinchoninic acid assay. Normalized supernatant was immunoprecipitated for 16–18 h at 4°C with anti-V5 conjugated to magnetic Dynabeads Protein G. Beads were washed 3 times with RIPA buffer by rotating at 4°C. Bound proteins were eluted by boiling beads at 95°C for 10 min in elution buffer (1 × Laemmli Sample Buffer, 0.4 × RIPA buffer, 4% (w/v) SDS). Eluted proteins were subjected to SDS-PAGE followed by staining with EZ-Run™ Protein Gel Staining Solution to visualize bands prior to proteolytic peptide sample preparation.

**Proteolytic peptide sample preparation** – Gel lanes were excised into 6 slices according to protein mass, which were destained, reduced and alkylated following standard procedures (83) with the following alteration: alkylation was performed using 50 mM chloroacetamide instead of iodoacetamide, thereby avoiding the generation of lysine adducts which mimic diglycine ubiquitin remnants (84). In-gel tryptic digestions and peptide extractions were performed following procedures described previously (85). Peptide extraction solutions were dried in a SpeedVac™ (Thermo Scientific) and reconstituted in 20 µL 0.1% (v/v) formic acid.

**Mass spectrometry** – Proteolytic peptide samples were subjected to LC-MS/MS analysis on a Tribrid Fusion Lumos mass spectrometer (Thermo Scientific, Bremen, Germany) interfaced with an UltiMate 3000 HPLC and autosampler system (Dionex, Amsterdam, The Netherlands). Proteolytic peptides were separated by nano-LC following procedures described previously (86), and eluting peptides were ionized using positive ion mode nano-ESI as described previously (87). Survey scans m/z 350–1500 (MS AGC, = 4 × 10^5; maximum injection time = 50 ms) were recorded in the orbitrap (resolution = 120,000 at m/z 200). Peptide ions (>2.5 × 10^4 counts, charge states +2 to +5) were sequentially selected for MS/MS using an inclusion list employed with open retention time windows (discussed below) followed by data dependent acquisition, with the total number of dependent scans maximized within 2 s cycle times. Product ions were generated via HCD and mass analyzed in the orbitrap using the following parameters: HCD collision energy = 30; maximum injection time = 250 ms; orbitrap resolution = 30,000 at m/z 200; MSn AGC = 5 × 10^4; inject ions for all available parallelizable time enabled; 1 microscan collected per scan; and monoisotopic precursor selection placed in peptide mode. Dynamic exclusion was enabled and set to: n times = 1, exclusion duration 20 s, ± 10 ppm. To enhance signal-to-noise ratios in MS/MS spectra collected from targeted ions, additional experiments were performed as above with the following alterations: peptide ions were selected for MS/MS using an inclusion list only and 2 microscans were collected per MS/MS scan.

To preferentially target peptides with potential diglycine mass shifts for MS/MS, inclusion lists were generated with the aid of the MS-Digest utility (University of California, San Francisco). Theoretical m/z values were calculated for singly charged tryptic SM N100 peptide ions (with up to 2 missed cleavages), from which all possible combinations of diglycine mass shifts (+114.0429 Da) on serine, lysine, cysteine and threonine residues were added. For these modified peptide ions, theoretical m/z values for ions of charge states +2 to +4 were calculated. Values falling in the range m/z 350–1500 were incorporated into the inclusion lists used in the LC-MS/MS experiments described above.

**Sequence database searches** – Sequence database searches were performed using the Proteome Discoverer mass informatics platform (version 1.4, Thermo Scientific), using the search program Mascot (versions 2.5, Matrix Science). Peak lists derived from LC-MS/MS were searched using the following parameters: instrument type was ESI-TRAP; precursor ion and peptide fragment mass tolerances were ±5 ppm and ±0.02 Da respectively; variable modifications included in each search were GlyGly (S) and GlyGly (K); additional variable modifications included in separate searches were carbamidomethyl (C), oxidation (M), GlyGly (C) and GlyGly (T); enzyme specificity was trypsin with up to 2 missed cleavages; and the UniProt database (September 2018 release, 558,125
sequence entries) was searched using human sequences only.

**Bioinformatics predictions and sequence alignments** – Sequence alignments were generated using Geneious 9.1.5 with default settings (88). Helical wheel diagrams were generated using HeliQuest (89). To identify disordered regions, a total of 13 predictors obtained from DisEMBL (90), DISOPRED3 (91), DisProt (92), GlobPlot2.3 (58), IUPred (93), PONDR (94–96), and PrDOS (97) were used to analyze the SM N100-GFP sequence.

**Data presentation and statistical analysis** – Relative protein levels for all SM N100, full-length SM, and chicken SM N100 constructs were determined by normalizing to WT (human SM N100 or human full-length SM), which was set to 1. So, for instance, a value of 2 would indicate the construct had double the protein level of WT (human SM N100 or human full-length SM).

Cholesterol regulation was given as the proportion of protein degraded for each construct, normalized to the proportion of protein degraded for WT, which was set to 1. Values approaching 0 on the cholesterol regulation scale indicate little or no cholesterol regulation, which means no degradation in the presence of excess cholesterol. A value closer to 1 would indicate similar levels of degradation in the presence of excess cholesterol when compared to WT (human SM N100 or human full-length SM).

For contribution of residues to cholesterol regulation (Fig. 2D), this data was generated using data collected to produce cholesterol regulation comparisons (Fig. 2C). The values are presented in an opposite manner, in that the data is presented as 1 minus cholesterol regulation values (Fig. 2C). In this scale (Fig. 2D), higher values mean the protein is not degraded effectively in the presence of excess cholesterol. Lower values approaching 0 mean more degradation in the presence of excess cholesterol. On the x-axis, the pooled data refers to cholesterol regulation data containing alanine mutations omitted from the cholesterol regulation data contributed by S43/C46 (cluster 2), S59/S61 (cluster 3), S67/S71 (cluster 4) and S83/S87 (cluster 5). As a result, by observing a drop closer to 0 which reflects more effective degradation, this will show which of the initial five clusters (T3/T9/T11, cluster 1; S43/C46, cluster 2; S59/S61, cluster 3; S67/S71, cluster 4; S83/S87, cluster 5) contributes more to cholesterol regulation.

The relative MARCH6 response (Fig. 3, A and B) was determined similarly to the cholesterol regulation. After densitometric analyses, the fold-increase for each construct after MARCH6 siRNA knockdown was normalized to the fold-increase for WT, which was set to 1. Values approaching 0 on the MARCH6 response scale indicate less of a rescue compared to WT when MARCH6 was knocked down. A value closer to 1 would indicate the MARCH6 knockdown rescue effect on SM N100 mutants is similar to that of WT.

For the heat map of disordered region frequency (Fig. 6E), numbers on the scale refer to the number of times a residue was recognized as being part of a disordered region. The lowest value 0 would mean that the residue was never recognized as being part of an observed disordered region in any of the 13 predictors whereas a value of 13 would mean the residue was part of a disordered region in all 13 predictors.

All Western blots are representative of at least three independent experiments with the number (n) of independent experiments presented in the figure legend. Densitometry data from at
least three independent experiments are presented as bar graphs. Densitometry data in bar graphs are presented as mean + S.E., Statistical differences were determined by the Student's paired t test (two-tailed), where p values of $p < 0.05$ (*) and $p < 0.01$ (**) were considered statistically significant.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTION
All authors contributed to the experimental design. NKC carried out all the research and data analyses in this study except for mass spectrometry experiments. GHS performed the mass spectrometry experiments and data analyses. NKC and AJB wrote the manuscript. The final version of the manuscript was read and approved by all authors.
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FIGURE LEGEND

Figure 1. Lysine residues and the N-terminus are not key ubiquitination sites in the cholesterol-accelerated degradation of SM N100. A, Lysine (K) residues were mutated to arginine (R), with the amino acid position numbers of SM N100 indicated in the lysine-less (K-less) construct (left). A bulky mCherry protein was cloned onto the N-terminus of SM N100 (right). B and D, CHO-7 cells transiently expressing SM N100 constructs were tested for cholesterol regulation as described in ‘Experimental Procedures’ (n = 3–4). C and E, Densitometric quantification of the Western blots in B and D. Graphs show mean + S.E. (n = 3–4), *, p < 0.05, or **, p < 0.01.

Figure 2. Serine residues in SM 100 are required for the cholesterol-accelerated degradation of SM N100. A, Schematic of the SM N100 degron topology in the endoplasmic reticulum (ER) membrane. SM N100 possesses numerous potential ubiquitination sites including five lysine (K) residues, three threonine (T) residues, seven serine (S) residues, one cysteine (C) residue and the free N-terminus. The site-directed mutagenesis mutants generated are listed in the table (right) and are given a cluster number. The initial five clusters are numbered from 1 to 5 (left), before then combined to generate more cluster mutations (right). A schematic of blue (indicating native unmutated residue cluster residues) and yellow (mutated cluster residues to alanine) is presented for identifying which clusters are mutated in the constructs used. B, CHO-7 cells transiently expressing SM N100 constructs were tested for cholesterol regulation as described in ‘Experimental Procedures’ (n = 3–7). The five boxes below each of the clusters correspond to the scheme in A. C, Densitometric quantification of the Western blots in B. Graphs show mean + S.E. (n = 3–7), *, p < 0.05, or **, p < 0.01. The blue and yellow schematic is the same as in A and B. D, Data for cholesterol regulation from C was analyzed and represented as residue effects on cholesterol regulation. On the x-axis, clusters were removed one at a time to observe for what effect it has on cholesterol regulation. Losing residues Ser+59 and Ser+61 (S59/S61) for example has the greatest effect on cholesterol regulation. The numbers on the bars represent the number of independent experiments (see ‘Experimental Procedures’ for a more detailed description of data presentation).

Figure 3. Serine to alanine mutations in the second half of SM N100 blunt MARCH6-dependent degradation. A and B, CHO-7 cells transiently expressing SM N100 constructs were tested for MARCH6 regulation as described in ‘Experimental Procedures’ (n = 3–7). Densitometric quantification of the Western blots are presented as mean + S.E. (n = 3–7), *, p < 0.05, or **, p < 0.01. The five boxes below each of the clusters correspond to the colour scheme in Fig. 2A.

Figure 4. SM N100 and SM cholesterol-accelerated degradation rely on different sets of serine residues. A, In the ‘Cholesterol responsive’ (left) section are constructs known to retain cholesterol regulation. In the ‘Unknown cholesterol response’ (right) section are sites of interests where alanine mutations are introduced. B and D, CHO-7 cells transiently expressing SM N80 and SM constructs were tested for cholesterol regulation as described in ‘Experimental Procedures’ (n = 3–5). C and E, Densitometric quantification of the Western blots in B and D are presented as mean + S.E. (n = 3–5), *, p < 0.05, or **, p < 0.01.

Figure 5. SM N100 is regulated by Ube2J2 and serine ubiquitination. A, CHO-7 cells were transfected for 24 h with the indicated constructs. The asterisk (*) denotes a non-specific band from the anti-FLAG antibody and the black triangle is pointed at the band of interest. Densitometric quantification of the Western blot is presented as mean + S.E. (n = 3), *, p < 0.05. B, When subjected to higher energy collisional dissociation, dissociation of the doubly charged diglycine-modified tryptic peptide S(GlyGly)PPESENKEQLEAR, observed at m/z 864.4136 and identified with a Mascot ion score of 55 and expectation value of 1.2×10^{-4} (above), matches that of the doubly charged unmodified tryptic peptide SPPPESENKEQLEAR, observed at m/z 807.3921 and identified with a Mascot ion score of 99 and expectation value of 4.1×10^{-9} (below). Precursor ions and product ions with m/z values matching those of theoretical b and y ions (+40 ppm) are annotated in each spectrum.
Figure 6. Serine residues in human SM N100 confer instability to chicken SM N100. A, first 100 amino acids of human and chicken were aligned with Geneious 9.1.5 using default settings. Black shades indicate identical residues and grey shades indicate similarity as determined by Blosum62 score matrix with a threshold of 1. The red asterisks indicate the four residues of interest which were identified as critical in human SM N100. B, CHO-7 cells transiently expressing SM N100 constructs were tested for cholesterol regulation as described in ‘Experimental Procedures’ (n = 7–14). C, Densitometric quantification of the Western blot in B is presented as mean + S.E. (n = 3–5), *, p < 0.05, or **, p < 0.01. D, Helical wheel representations of amphipathic helices generated from HeliQuest. The arrows indicate the magnitude and direction of the hydrophobic moment. The hydrophobicity (H) and hydrophobic moment (µH) from HeliQuest are also shown. E, Heat map shows frequency of each residue in SM N100 being assigned as part of a disordered region. More yellow refers to higher frequency the residue has been assigned as disordered while black refers to lower frequency. Red arrows point to residues Ser-59, Ser-61, Ser-83 and Ser-87, the four critical residues identified in this study. The heat map in the lower part is a closer view of residues 50 to 100, compared to the upper part which is a view of the entire SM N100.

Figure 7. Current model of cholesterol-accelerated degradation of SM N100. SM N100 senses cholesterol levels in the ER membrane via a re-entrant loop and an amphipathic helix. With excess cholesterol, the amphipathic helix is pushed out of the ER membrane, exposing a hydrophobic patch contributing to increased disorder. The lengthened disordered region includes residues Ser-59, Ser-61, Ser-83 and Ser-87 (in yellow stars), which may all serve as ubiquitination sites, although Ser-83 was the only non-canonical ubiquitination site formally identified in this work. The MARCH6 E3 ubiquitin ligase most likely ubiquitinates Ser-83, and perhaps also Ser-59, Ser-61 and Ser-87, to mediate the cholesterol-accelerated degradation of the SM N100 degron.
FIGURE 1

A

Wild-type
K-less mutant
N-terminal blocking with mCherry

B

| Construct      | SM N100-GST-V5 | β-Tubulin |
|----------------|----------------|-----------|
| WT             | -              | -         |
| K-less         | +              | +         |

C

Relative Cholesterol Levels

D

| Construct      | SM N100-GFP-V5 | mCherry-SM N100-GFP-V5 | mCherry-V5 |
|----------------|----------------|------------------------|------------|
| Chol/CD        | -              | +                      | -          |

E

Relative Protein Levels
FIGURE 2

Serine ubiquitination and cholesterol regulation
FIGURE 3

A

SM N100-GFP-V5
β-Tubulin

Cluster
MARCH6 siRNA

| Cluster | 0 | 6 | 12 | 15 |
|---------|---|---|----|----|
| MARCH6 siRNA | - | + | - | + |

B

SM N100-GFP-V5
β-Tubulin

Construct
F35A/S37A/L65A/L69A
Δ62–73
L42A
MARCH6 siRNA

| Construct | WT | F35A/S37A/L65A/L69A | Δ62–73 | L42A |
|-----------|----|--------------------|--------|------|
| MARCH6 siRNA | - | + | - | + | + |
Serine ubiquitination and cholesterol regulation

FIGURE 4

A

Cholesterol responsive

Wild-type

SM N80 → GFP → V5

Mutant

SM N80 → GFP → V5

Wild-type

SM → V5

B

SM N80-GFP-V5

β-Tubulin

Cluster

0 3 4 9

Chol/CD

- + - + - + - +

C

Relative Protein Levels

Constructs

0 3 4 9

D

SM-V5

β-Tubulin

Cluster

0 15

Chol/CD

- + - +

E

Relative Protein Levels

Constructs

0 15

Cholesterol Regulation

Constructs

0 15

More degraded

More degraded

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FIGURE 5

A

SM N100-GFP-V5

Ube2J2-FLAG

β-Tubulin

| Protein            | WT   | C94S |
|--------------------|------|------|
| Ube2J2-FLAG        |      |      |
| SM N100-GFP-V5     | +    |      |

B

Relative abundance (a.u.)

Relative SM N100 Levels

WT   C94S

Ube2J2-FLAG

---

Serine ubiquitination and cholesterol regulation
FIGURE 6

A

B

C

D

E

Serine ubiquitination and cholesterol regulation
Non-canonical ubiquitination of the cholesterol-regulated degron of squalene monooxygenase
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