Cholesterol increases protein levels of the E3 ligase MARCH6 and thereby stimulates protein degradation

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The E3 ligase membrane-associated ring-CH-type finger 6 (MARCH6) is a polytopic enzyme bound to the membranes of the endoplasmic reticulum. It controls levels of several known protein substrates, including a key enzyme in cholesterol synthesis, squalene monooxygenase. However, beyond its own autodegradation, little is known about how MARCH6 itself is regulated. Using CRISPR/Cas9 gene-editing, MARCH6 overexpression, and immunoblotting, we found here that cholesterol stabilizes MARCH6 protein endogenously and in HEK293 cells that stably express MARCH6. Conversely, MARCH6-deficient HEK293 and HeLa cells lost their ability to degrade squalene monooxygenase in a cholesterol-dependent manner. The ability of cholesterol to boost MARCH6 did not seem to involve a putative sterol-sensing domain in this E3 ligase, but was abolished when either membrane extraction by valosin-containing protein (VCP/p97) or proteasomal degradation was inhibited. Furthermore, cholesterol-mediated stabilization was absent in two MARCH6 mutants that are unable to degrade themselves, indicating that cholesterol stabilizes MARCH6 protein by preventing its autodegradation. Experiments with chemical chaperones suggested that this likely occurs through a conformational change in MARCH6 upon cholesterol addition. Moreover, cholesterol reduced the levels of at least three known MARCH6 substrates, indicating that cholesterol-mediated MARCH6 stabilization increases its activity. Our findings highlight an important new role for cholesterol in controlling levels of proteins, extending the known repertoire of cholesterol homeostasis players.

Cholesterol is essential for mammals, with too little associated with developmental problems (1). However, too much is toxic, leading to diseases such as atherosclerosis and certain cancers. Hence, levels of this lipid are under tight control (2).

To regulate cholesterol levels, cells must balance its efflux, uptake, and synthesis. Statins target an early step in cholesterol synthesis (catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR)[3]), and their success in treating cardiovascular disease clearly demonstrates the clinical significance of this pathway. We previously showed that a critical rate-limiting enzyme in cholesterol synthesis, squalene monooxygenase (SM), is degraded by the proteasome in the presence of excess cholesterol (3). We found that this is mediated through ubiquitination in the first 100 amino acids of SM (SM N100) by the E3 ubiquitin ligase MARCH6 (membrane-associated really interesting new gene (RING) finger (C3HC4) 6) (4). MARCH6 is also one of several E3 ligases implicated in the degradation of HMGCR, another rate-limiting cholesterol synthesis enzyme (4–6). A mutant form of the cholesterol-trafficking protein, NPC1, is targeted to the proteasome by MARCH6-catalyzed ubiquitination (7), and a protein involved in lipid-droplet formation, PLIN2, is also targeted by MARCH6 (8). MARCH6 additionally affects transcriptional regulation in cholesterol homeostasis, ultimately reducing expression of the inducible-degrader of LDL receptor (IDOL) (9), another E3 ligase, which degrades lipoprotein receptors. MARCH6 therefore affects cholesterol homeostasis at several different points.

MARCH6, also known as TEB4 or RNF176, is a 103-kDa, 14-transmembrane domain (TMD) protein (10) localized to the endoplasmic reticulum (ER) (11). The yeast homolog, Doa10, is one of two key E3 ligases that mediate ER-associated degradation. E3 ligases attach ubiquitin moieties to their target substrates to promote their degradation, typically through the proteasome. Substrates of MARCH6, in addition to SM, HMGCR, and mutant NPC1, include a key regulator of thyroid hormone production (type 2 iodothyronine deiodinase, D2) (12), a regulator of G protein signaling involved in diseases such as cancer (RGS2) (13), and a mutant form of a bile salt exporter pump (Bsep*) (14). MARCH6 also autoubiquitinates itself, leading to

[3] The abbreviations used are: HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; D2, type 2 iodothyronine deiodinase; MARCH6, membrane-associated RING finger (C3HC4) 6; RGS2, regulator of G protein signaling-2; SM, squalene monooxygenase; SSD, sterol-sensing domain; TMD, transmembrane domain; VCP, valosin-containing protein; LXR, liver X receptor; 25HC, 25-hydroxycholesterol; ER, endoplasmic reticulum; qRT-PCR, quantitative RT-PCR; Chol/CD, cholesterol complexed to cyclodextrin; PBGD, porphobilinogen deaminase; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; DUB, deubiquitinase.

its own degradation (15); accordingly, a mutation in its RING domain that renders it inactive (C9A) increases its levels (4). MARCH6 is regulated post-translationally by the deubiquitinase USP19 (16), but little else is known about its control. Therefore, we investigated possible mechanisms controlling MARCH6 levels.

Considering that MARCH6 helps control levels of the two rate-limiting enzymes in cholesterol synthesis, HMGCR and SM (4), we reasoned MARCH6 levels may be affected by sterol status through a feedback mechanism, *i.e.* high sterol levels increase MARCH6 levels, thus decreasing SM and HMGCR and consequently cholesterol synthesis. As precedents for sterol-mediated regulation of E3 ligases, both IDOL and RNF145 are transcriptionally up-regulated by sterols through liver X receptor (LXR) (17, 18), and TRC8 is post-translationally down-regulated by sterols (19, 20). Interestingly, TRC8 and MARCH6 were recently found to act together to facilitate degradation of select substrates (21).

One way that sterols are sensed by cells is through a five-TMD region known as a sterol-sensing domain (SSD) (2, 22). HMGCR (23), Scap (24, 25), NPC1 (26, 27), NPC1L1 (28), Patched (29), and Dispatched (30, 31) all have reported SSDs, as do the E3 ligases TRC8 (32) and RNF145 (6, 17). Because MARCH6 also has a large number of TMDs and is involved in cholesterol homeostasis, we hypothesized that it may also contain an SSD.

Here, we report that MARCH6 is stabilized by cholesterol, with subsequent decreases in levels of its substrates. This stabilization appears to be independent of a putative SSD but mediated through inhibition of MARCH6 autodegradation, probably due to a change in its conformation. This is the first time an E3 ligase has been shown to be post-translationally stabilized by cholesterol, introducing a novel mode of controlled protein demolition.

Results

MARCH6 gene expression levels are not affected by sterol status

We first considered whether *MARCH6* gene expression levels are regulated by changing sterol status, as is the case with IDOL (18) and RNF145 (17). To test this, we employed previously generated cDNA sets (33) derived from cells in which the sterol status had been manipulated and levels of SREBP-2 and LXR target genes had been compared with another gene of interest. Here, we measured *MARCH6* mRNA levels and found that these did not change under varying sterol conditions or in response to a synthetic LXR agonist (Fig. 1). To provide context, we have presented the data demonstrating the changing expression levels of SREBP-2 and LXR target genes as observed previously (33). These genes are highly responsive to changing sterol levels, whereas *MARCH6* was unchanged (not statistically significantly different) across all conditions and cell lines, indicating that it is not transcriptionally regulated by sterols.

Cholesterol increases MARCH6 post-translationally

We have previously found that transiently and massively overexpressed proteins do not recapitulate normal regulation (3), and we observed no sterol-mediated change in MARCH6
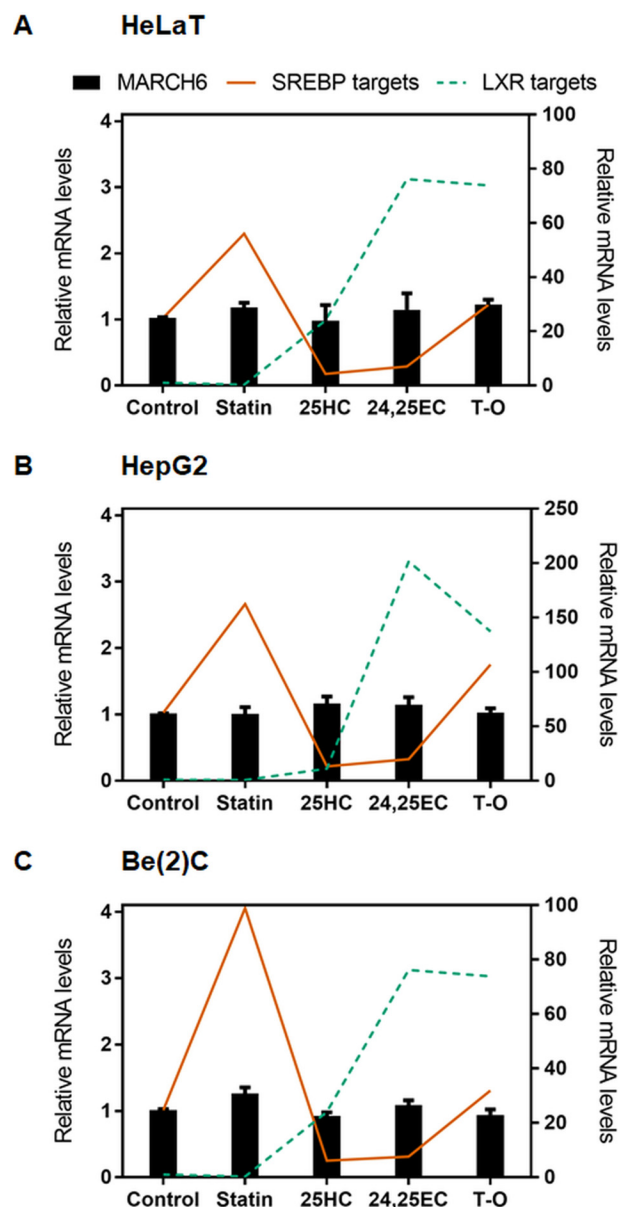

Figure 1. MARCH6 gene expression is not affected by changing sterol status. cDNA samples were from HeLaT (*A*), HepG2 (*B*), and Be(2)C (where Be(2)C are brain neuroblastoma cells) (*C*) cells treated for 24 h with statin, 25HC, 24(*S*),25-epoxycholesterol (*24,25EC*), or the synthetic LXR agonist TO-01317 (*T-O*) as described previously (33). *MARCH6* mRNA levels were measured using qRT-PCR and normalized to *PBGD* housekeeping levels. mRNA levels are relative to the control condition, which was set to 1 in each cell line. Data are presented as mean ± S.E. from three separate experiments for HepG2 and Be(2)C, and two separate experiments for HeLaT, where each experiment was performed with triplicate cultures. SREBP-2 targets (*solid lines*) represents the average values of *HMGCR* and *LDLR* as published previously, and LXR targets (*dashed lines*) represent the average values of *ABCA1* and *ABCG1* (33). *MARCH6* and SREBP-2 targets are plotted on the *left y* axis, and LXR targets are plotted on the *right y* axis.

protein levels when it was transiently overexpressed (4). Here, we instead employed stable expression to examine potential post-translational regulation of MARCH6. We created HEK293 stable cell lines expressing MARCH6 tagged with a Myc or V5 epitope. Under sterol-depleted conditions we typically employ for SM, MARCH6 levels were very low and difficult to visualize, but we noted that addition of cholesterol increased MARCH6 protein (Fig. 2*A*). To maximize MARCH6
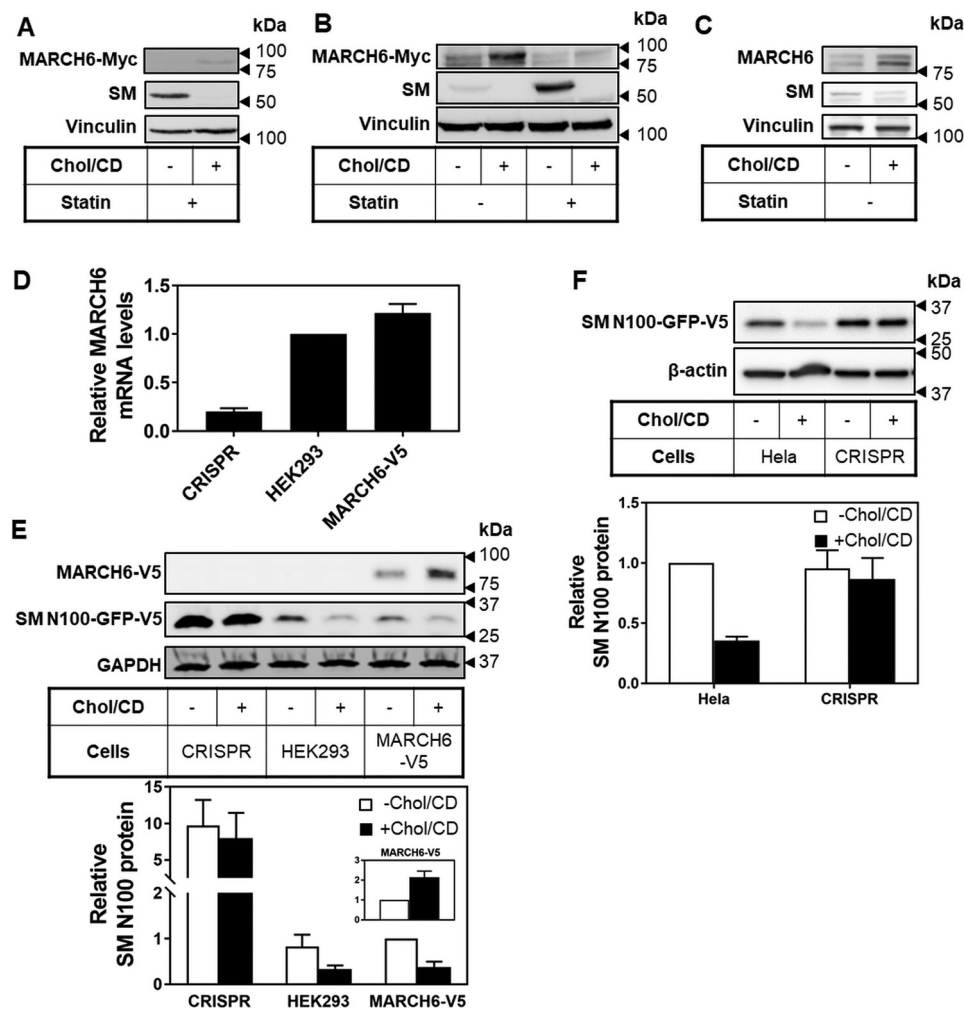
Figure 2. Cholesterol stabilizes MARCH6 post-translationally. *A,* HEK293–MARCH6–Myc cells were pretreated overnight with statin, then treated with or without Chol/CD for 8 h before harvesting. Protein levels were analyzed by Western blotting with Myc (MARCH6), SM, and vinculin as a loading control. *B,* HEK293–MARCH6–Myc cells were pretreated with or without statin overnight, then treated with 10 μM MG132 for 2 h to increase basal levels of MARCH6, then treated for 6 h with or without Chol/CD before harvesting. Protein levels were analyzed by Western blotting with Myc (MARCH6), SM, and vinculin as a loading control. *C,* HEK293 cells were treated for 4 h with or without 20 μg/ml Chol/CD before harvesting. Protein levels were analyzed by Western blotting with endogenous MARCH6 antibody, SM, and vinculin as a loading control. *D,* RNA was harvested from HEK–CRISPR, HEK293, or HEK–MARCH6–V5 cells and cDNA analyzed for gene expression levels of *MARCH6* normalized to the housekeeping gene *PBGD*. HEK293 control levels have been set to 1. Data are presented as mean $\pm$ S.E. from three separate experiments, where each experiment was performed with triplicate cultures. *E,* HEK–CRISPR, HEK293, or HEK–MARCH6–V5 cells transfected for 24 h with 1 μg of pTK-SM N100-GFP-V5 plasmid, then treated for 4 h with or without 20 μg/ml Chol/CD before harvesting. Protein levels were analyzed by Western blotting with V5 (MARCH6 and SM N100) and GAPDH as a loading control. Blots are representative of at least three independent experiments. Relative protein levels were measured using ImageStudio Lite and normalized to the −Chol/CD condition in HEK–MARCH6–V5 cells, which has been set to 1 for each protein. Data are presented as mean $\pm$ S.E. from at least three separate experiments. *F,* HeLa–CRISPR or HeLa cells were transfected for 24 h with 2 μg of pTK-SM N100-GFP-V5 plasmid, pretreated overnight, then treated for 8 h with or without 20 μg/ml Chol/CD before harvesting. Protein levels were analyzed by Western blotting with V5 (SM N100) and β-actin as a loading control. Blots are representative of three independent experiments. Relative protein levels were measured using GeneTools and normalized to the −Chol/CD condition in HeLa cells, which has been set to 1. Data are presented as mean $\pm$ S.E. from at least three separate experiments.

protein levels, we next treated cells with or without statins, which decrease sterol status and also up-regulate SM transcriptionally. We included the proteasomal inhibitor MG132 for 2 h prior to treatment with cholesterol (in the absence of MG132). This result clearly demonstrated that the MARCH6 protein was increased following cholesterol addition (Fig. 2*B*), a first with respect to E3 ligases. The increase in MARCH6 levels was associated with decreased levels of SM, a typical cholesterol-regulated substrate (Fig. 2, *A* and *B*). Similar MARCH6 increases by cholesterol were observed in nine out of 10 clones tested for these MARCH6-expressing stable cell lines (data not shown) with either a Myc or V5 epitope tag.

We next employed a commercially available antibody to endogenous MARCH6. This antibody detected a number of bands by Western blotting (Fig. S1), including a cholesterol-responsive band (Fig. 2*C*) running at approximately the same size as for epitope-tagged MARCH6 (Fig. S1). However, this was the only antibody of three commercially available antibodies and our own in-house–produced antibodies that yielded anything remotely promising. The poor quality of endogenous MARCH6 antibodies has also been observed by others (21), who instead used SM levels as a proxy. Therefore, we instead relied upon our overexpressing cell line, which has only ~20% more *MARCH6* mRNA than its parent cell line (Fig. 2*D*). This
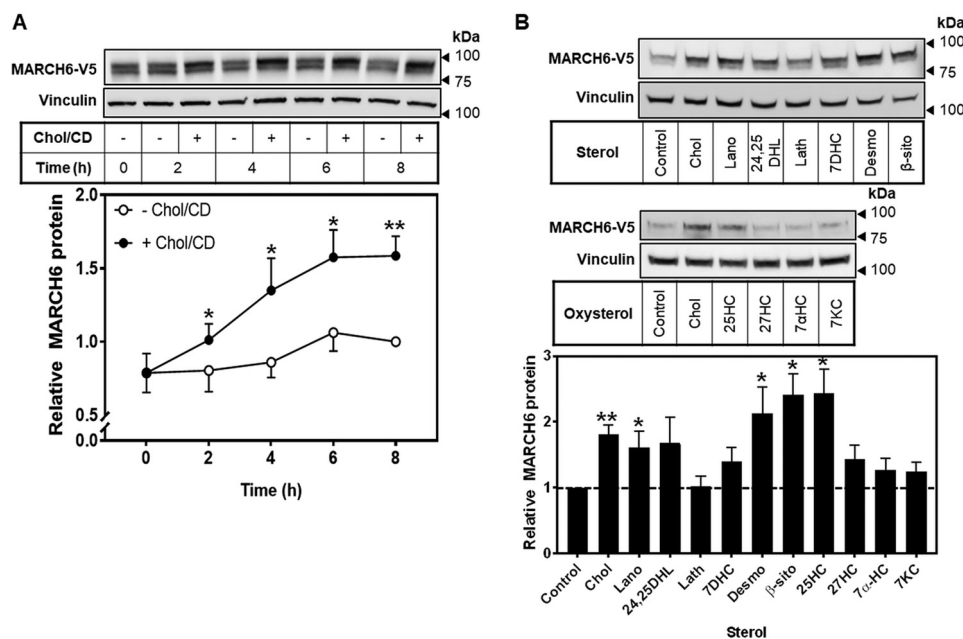
Figure 3. A variety of sterol intermediates and oxysterols stabilize MARCH6. *A,* HEK–MARCH6 –V5 cells were treated with or without 20 μg/ml Chol/CD for the indicated time (0–8 h) before harvesting. Protein levels were analyzed by Western blotting with V5 (MARCH6) and vinculin as a loading control. Blots are representative of at least three independent experiments. Relative MARCH6 protein levels were measured using ImageStudio Lite and normalized to the 8-h −Chol/CD condition, which has been set to 1. Data are presented as mean ± S.E. from at least three separate experiments. *, $p < 0.05$; **, $p < 0.01$, by Student's paired *t* test. *B,* HEK–MARCH6–V5 cells were treated with or without 20 μg/ml Chol/CD or the indicated sterol complexed to cyclodextrin or 1 μg/ml oxysterol for 8 h before harvesting. Protein levels were analyzed by Western blotting with V5 (MARCH6) and vinculin as a loading control. Blots are representative of at least three independent experiments. Relative protein levels were measured using ImageStudio Lite and normalized to the control condition, which has been set to 1. Data are presented as mean ± S.E. from at least three separate experiments. *, $p < 0.05$; **, $p < 0.01$, by Student's paired *t* test. For full sterol names please see Table 1.

means our MARCH6 levels are close to endogenous levels and likely reflect endogenous MARCH6 regulation, as indicated in the endogenous MARCH6 Western blotting, where MARCH6 levels are comparable between the overexpressing and parent cell lines, and both are cholesterol-responsive (Fig. S1).

For additional evidence that the effects we observed were not a result of overexpressed MARCH6, we compared a MARCH6-deficient (CRISPR) cell line and a MARCH6-overexpressing cell line (MARCH6–V5) with the parental HEK293 cell line. We examined cholesterol-mediated degradation of the MARCH6 substrate SM N100 (4). This showed comparable reductions of SM N100 in response to cholesterol in both the HEK293- and MARCH6-expressing cells (Fig. 2E), which was lost in MARCH6-deficient cells. The dependence on MARCH6 of the reduction of SM N100 levels in response to cholesterol was repeated independently in HeLa cells CRISPR modified at exon 1 of the *MARCH6* locus (Fig. 2F). Together, these data are consistent with endogenous levels of MARCH6 being regulated by cholesterol. The relative levels of SM N100 were higher in our modified HEK293 cells than our modified HeLa cells, which could be due to differences in the parental cell lines, as we have observed previously (4), and/or the different media used for treatments.

Sterol intermediates and oxysterols stabilize MARCH6

We next examined the rate of accumulation of MARCH6 over time following addition of cholesterol to cells. We found a significant increase in MARCH6 protein already by 2 h in these cells relative to those without cholesterol added, with relative accumulation increasing up to 8 h (Fig. 3A).

We have previously noted clear differences in the sterol signals for degradation of SM compared with HMGCR (3); there-

fore, we sought to establish which sterols increase MARCH6, a degrader of both of these enzymes. We found that several sterol intermediates and oxysterols tended to increase MARCH6 levels, with lanosterol, desmosterol, β-sitosterol, and 25-hydroxycholesterol reaching statistical significance (Fig. 3B).

Cholesterol inhibits degradation of MARCH6

To examine turnover of MARCH6, we used the protein synthesis inhibitor cycloheximide and followed the rate of MARCH6 disappearance. This showed that MARCH6 is turned over fairly rapidly ($t_{1/2} \sim$2–3 h) (Fig. 4A), but this can be at least partially rescued by addition of cholesterol (Fig. 4B). This result indicates that stabilization of MARCH6 occurs independently of protein synthesis.

To determine whether cholesterol-mediated stabilization of MARCH6 is due to reduced degradation by the ubiquitin–proteasome system, we employed inhibitors targeting the proteasome (MG132), deubiquitinases (PR619), or valosin-containing protein (VCP/p97), which helps extract ubiquitinated proteins from the membrane (VCP inhibitor, CB5083) (Fig. 4C). Inhibition of the proteasome with MG132, or inhibition of VCP with CB5083, raised MARCH6 levels in basal conditions, and cholesterol addition did not further increase these levels, supporting the hypothesis that cholesterol raises MARCH6 levels by preventing its degradation through the proteasome. A different result was found using the general deubiquitinase (DUB) inhibitor PR619; PR619 prevented the increase in MARCH6 protein over its low basal levels in response to cholesterol addition (Fig. 4C). Presumably, autoubiquitination of MARCH6 is no longer reversed by cellular DUB activity, allow-
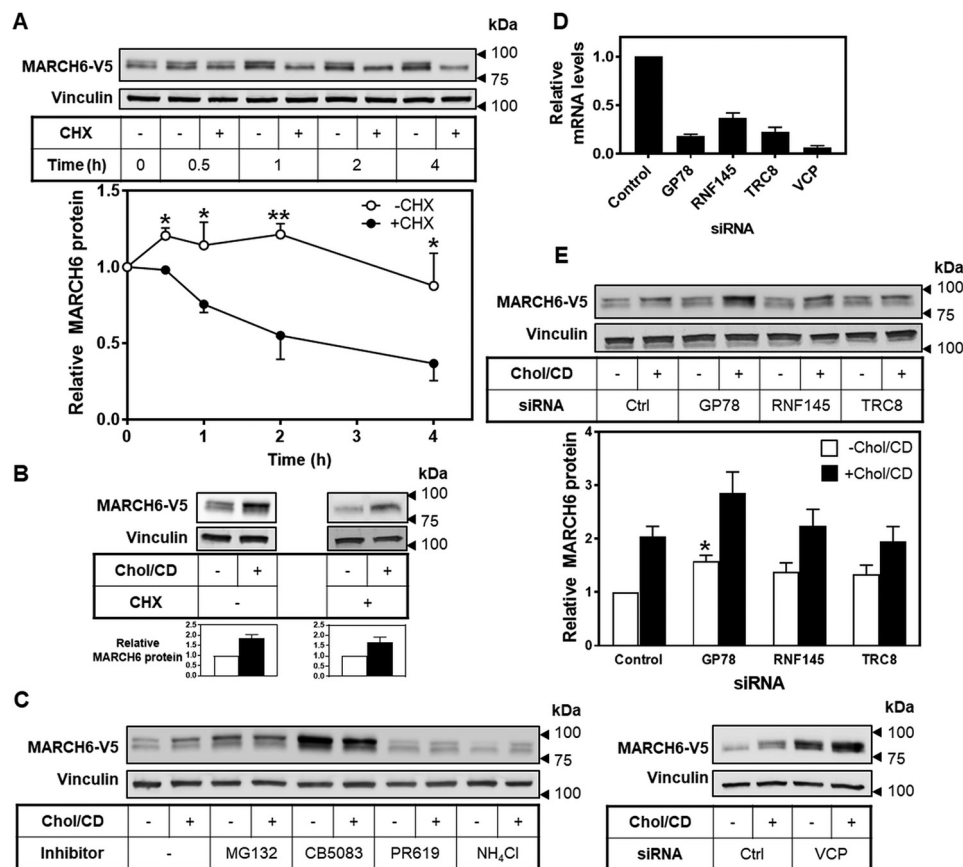
Figure 4. MARCH6 is stabilized by cholesterol by inhibiting its degradation. *A,* HEK–MARCH6–V5 cells were treated with or without 10 μg/ml cyclohex-imide (*CHX*) for the indicated time (0–4 h) before harvesting. *B,* HEK–MARCH6–V5 cells were treated with or without 10 μg/ml cyclohexximide and 20 μg/ml Chol/CD for 4 h before harvesting. *C, left,* HEK–MARCH6–V5 cells were treated with or without 20 μg/ml Chol/CD and inhibitors against the proteasome (MG132;10 μM), VCP inhibitor (CB5083; 5 μM), DUBs (PR619; 10 μM), or lysosomes (NH$_4$Cl, 20 μM) for 4 h before harvesting. *Right,* HEK–MARCH6–V5 cells were transfected with 25 nM indicated siRNA for 24 h and then treated with or without 20 μg/ml Chol/CD for 4 h before harvesting. *D,* HEK–MARCH6–V5 cells were transfected with 25 nM indicated siRNA for 24 h, then RNA harvested and cDNA analyzed for gene expression levels of *GP78, RNF145, TRC8,* and *VCP* normalized to the housekeeping gene *PBGD* and to the control siRNA condition, which has been set to 1. Data are presented as mean ± S.E. from at least three separate experiments, where each experiment was performed with triplicate cultures. *E,* HEK–MARCH6–V5 cells were transfected with 25 nM indicated siRNA for 24 h, then treated with or without 20 μg/ml Chol/CD for 4 h before harvesting. For all blots, protein levels were analyzed by Western blotting with V5 (MARCH6) and vinculin as a loading control. Blots are representative of at least three independent experiments. Relative protein levels were measured using ImageStudio Lite and normalized to the control condition in each blot, which has been set to 1. Data are presented as mean ± S.E. from at least three separate experiments. *A,* *, $p < 0.05$; **, $p < 0.01$, by Student's paired *t* test. *E,* *, $p < 0.01$ by Student's paired *t* test compared with control −Chol/CD condition. All +Chol/CD conditions were significantly different from −Chol/CD conditions, except TRC8, where $p = 0.1$.

ing its rapid degradation by the proteasome even in the presence of cholesterol. The relevant DUB might be USP19 (16).

As part of ER-associated degradation, the AAA ATPase valosin-containing protein (VCP, also known as p97) recognizes polyubiquitinated substrates and extracts them from the ER into the cytosol (34). Interestingly, the VCP inhibitor increased MARCH6 protein levels even further than proteasomal inhibition (Fig. 4C). To confirm this result, we employed VCP siRNA and found similar effects (Fig. 4, *C* and *D*). This could suggest that an additional mode of regulation is involved in controlling MARCH6 levels. Because VCP may also be involved in the degradation of proteins by the lysosome (35), we tested a lysosomal inhibitor (ammonium chloride), but this did not increase MARCH6 levels (Fig. 4C).

We next determined whether ER-associated degradation E3 ligases other than MARCH6 itself are involved in the cholesterol-dependent stabilization of MARCH6. We knocked down GP78 (also known as AMFR), TRC8, or RNF145, which together with INSIG play a role in sterol-mediated HMGCR degradation (5, 6, 36). Furthermore, TRC8 is degraded in

response to sterols (19, 20), and RNF145 is transcriptionally up-regulated by sterols as an LXR target (17, 37). The siRNAs used knocked down their gene targets by at least 63% (Fig. 4D), but there was little effect on MARCH6 protein levels (Fig. 4E). GP78 may play a minor role in degradation of MARCH6, as knocking down this E3 ligase increased MARCH6 above control values by ~56% ($p = 0.039$ by Student's paired *t* test for the −Chol/CD condition). But this increase occurred both with and without cholesterol, and so GP78 is unlikely to be a major contributor to cholesterol-mediated regulation of MARCH6.

MARCH6 may contain a sterol-sensing domain

SSDs are regions of five TMDs that play a role in sterol sensing (2, 22). By comparing sequence homology of the putative SSDs of a number of proteins with the classic SSDs in HMGCR, NPC1, and Scap, we found that our own predicted SSD in MARCH6 (amino acids 330–543; TMD4–8) may be *bona fide* (Fig. 5A), also having similar TMD spacing and orientation with those in other SSD-containing proteins. Furthermore, the SSD
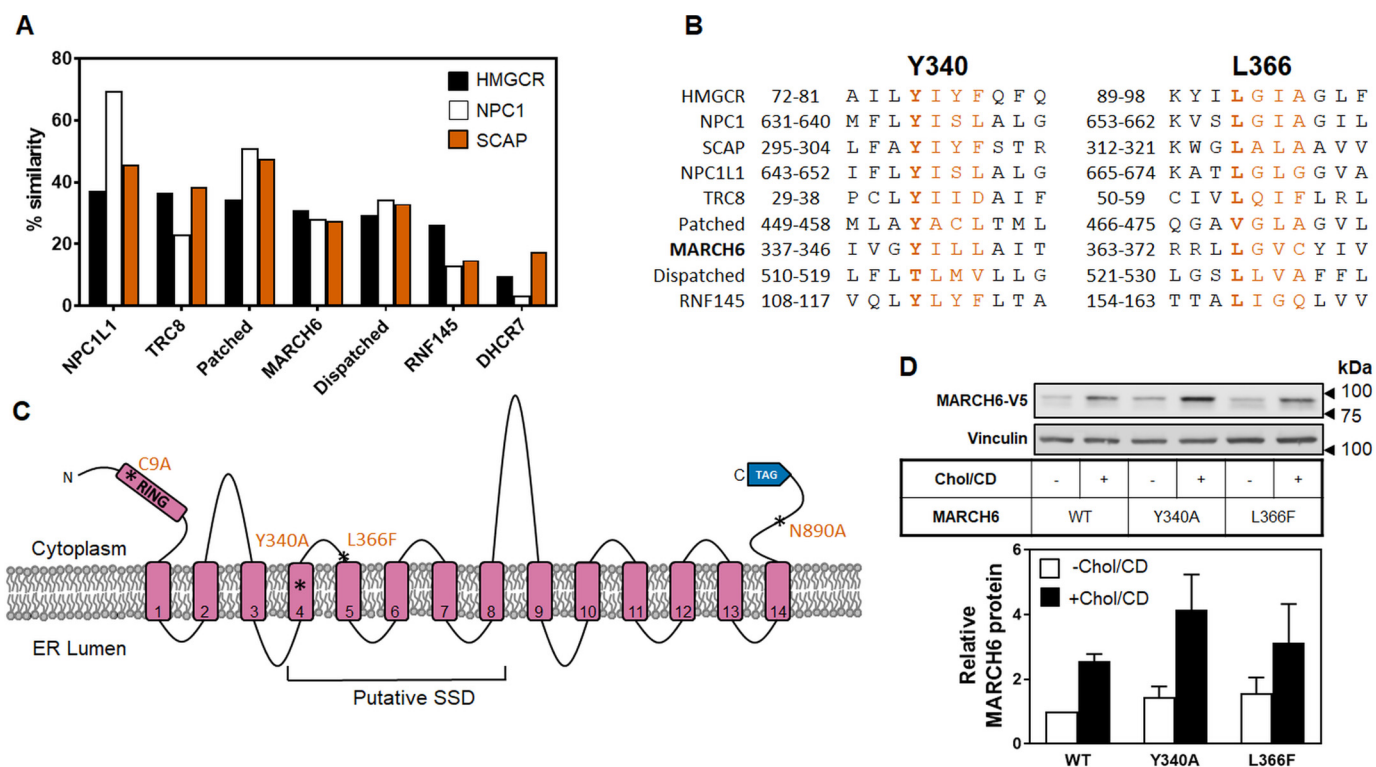
Figure 5. MARCH6 may contain a sterol-sensing domain. *A*, % similarity of SSDs from the indicated proteins with HMGCR, NPC1, or SCAP. *B*, alignment of MARCH6's Tyr-340 and Leu-366 with the indicated proteins. *C*, predicted topology of MARCH6 indicating the putative SSD. *D*, HEK–MARCH6 –V5 or indicated mutant cell lines were treated with or without 20 μg/ml Chol/CD for 4 h and protein lysates subjected to Western blotting for V5 (MARCH6) and vinculin as a loading control. Blots are representative of at least three independent experiments. Relative protein levels were measured using ImageStudio Lite and normalized to the WT −Chol/CD condition, which has been set to 1. Data are presented as mean ± S.E. from at least three separate experiments.

motif YIYF found in HMGCR and Scap revealed a potentially critical tyrosine residue in MARCH6, Tyr-340, which is similarly located within the first TMD of MARCH6's predicted SSD (Fig. 5, *B* and *C*). This sequence (YILL in MARCH6) was similar to the YISL in NPC1 and NPC1L1. There is also a leucine residue (Leu-366) that bears similarities to the critical Leu-315 in the second TMD of Scap (25) (Fig. 5, *B* and *C*).

To determine whether MARCH6 contains a functional SSD, we tested two constructs containing either just the SSD and adjacent loops or also including the RING domain and C-terminal element. However, these constructs were not expressed. This was probably due to misfolding and degradation by the quality control system, as low levels of these proteins could just barely be discerned but only after MG132 treatment (data not shown). Instead, we tested stable cell lines expressing MARCH6 with either a Y340A mutation or an L366F mutation, which we predicted would perturb cholesterol regulation by disrupting the putative SSD. However, both of these mutants were still stabilized by cholesterol (Fig. 5D).

INSIGs are unlikely to be involved in the cholesterol-mediated stabilization of MARCH6

SSDs for ER resident proteins have been proposed as INSIG-interacting domains (2), and because 25HC, which binds INSIGs, stabilized MARCH6 (Fig. 3B), we investigated the requirement for INSIGs in cholesterol-dependent stabilization of MARCH6. Using siRNA to knock down *INSIG-1*, *INSIG-2*, or both (Fig. 6A), we found that MARCH6 was still stabilized by cholesterol (Fig. 6B). However, INSIG-2 plays a role in basal

degradation of MARCH6 ($p < 0.02$ for the −Chol/CD condition by Student's paired *t* test).

Stabilization of MARCH6 by cholesterol occurs via inhibition of self-destruction

MARCH6 catalyzes its own ubiquitination and degradation. To investigate whether the stabilization of MARCH6 by cholesterol occurs via inhibition of this ability to self-destruct, we next tested whether mutations at sites critical for MARCH6 autoubiquitination were similarly stabilized by cholesterol. We tested both a RING mutation, C9A, and a C-terminal element mutation, N890A, which also prevents its own degradation (11). We again employed stable overexpression of these mutants, and we found that both were stabilized in the absence of cholesterol, due to the prevention of self-degradation, but neither was further stabilized in the presence of cholesterol (Fig. 7A), indicating that cholesterol likely prevents degradation of MARCH6 mediated by its ubiquitin ligase activity.

Cholesterol may cause a conformational change in MARCH6

To determine whether cholesterol changes the conformation of MARCH6 to prevent its self-destruction, we employed three chemical chaperones: glycerol, proline, and betaine. These chaperones cause proteins to favor their native conformation (38). Treatment with these chaperones resulted in lower levels of MARCH6 protein, which could be partially overcome by addition of cholesterol (Fig. 7, *B–D*). This suggests that MARCH6 preferentially adopts a conformation that makes it prone to rapid degradation, presumably through enhanced
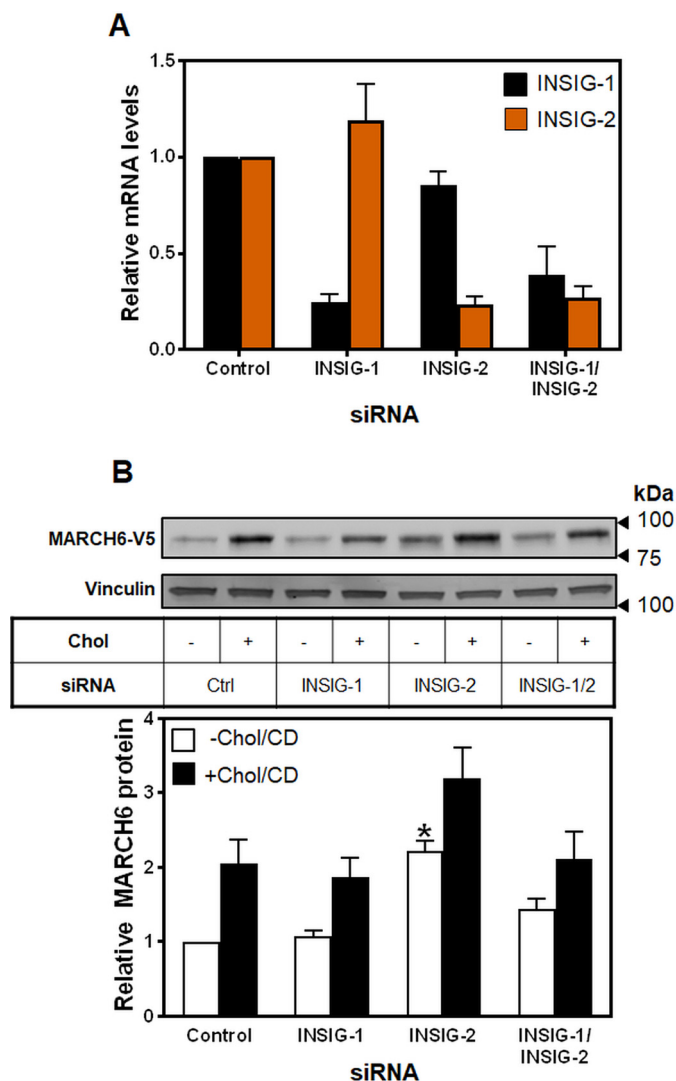
A

B

Figure 6. INSIGs are unlikely to be involved in the sterol-mediated stabilization of MARCH6. *A,* HEK–MARCH6–V5 cells were transfected with 25 nM indicated siRNA for 24 h, then RNA harvested, and cDNA analyzed for gene expression levels of *INSIG-1,* and *INSIG-2,* normalized to the housekeeping gene *PBGD,* and to the control siRNA condition, which has been set to 1. Data are presented as mean ± S.E. from at least three separate experiments, where each experiment was performed with triplicate cultures. *B,* HEK–MARCH6–V5 cells were transfected with 25 nM indicated siRNA for 24 h, then treated with or without 20 μg/ml Chol/CD for 4 h before harvesting. Protein levels were analyzed by Western blotting with V5 (MARCH6) and vinculin as a loading control. Blots are representative of at least three independent experiments. Relative protein levels were measured using ImageStudio Lite and normalized to the control −Chol/CD condition, which has been set to 1. Data are presented as mean ± S.E. from nine separate experiments. *, $p < 0.01$ by Student's paired *t* test. All +Chol/CD conditions were significantly different from −Chol/CD conditions.

auto-ubiquitination, but addition of cholesterol changes this conformation to one that limits auto-degradation. We cannot fully rule out indirect effects of these chaperones, but a direct effect on MARCH6 is the simplest explanation.

Cholesterol-dependent stabilization of MARCH6 is accompanied by a decrease in levels of its substrates

As well as the cholesterol synthesis enzymes SM and HMGCR, MARCH6 has a number of known other substrates, including D2 (12) and RGS2 (13). Considering that increasing cholesterol status stabilizes MARCH6 protein levels, we

hypothesized that this would also result in a decrease in levels of its substrates, as we have shown for one substrate, SM (Fig. 2). To investigate this, we obtained plasmids for RGS2 and D2 expression, and we tested them for cholesterol responsiveness under our previously optimized conditions for SM. D2 and RGS2 levels decreased in response to increased sterol levels (Fig. 8). These changes were small but significant, even in our transient expression system. This is consistent with another group's finding that D2 has increased activity following statin treatment (with sterol deprivation decreasing MARCH6) (39). Endogenous SM has a more pronounced decrease because it is also subjected to transcriptional control by sterols (40). Taken together, these findings identify a new role for cholesterol in the post-translational regulation of D2 and RGS2. It is likely that other substrates of MARCH6 are similarly regulated by cholesterol through its effects on MARCH6 turnover.

Discussion

Here, we have demonstrated that cholesterol increases protein levels of the E3 ligase MARCH6 by preventing its autodegradation, possibly via a sterol-induced conformational change. This in turn increases MARCH6 activity as shown by reduced levels of at least three of its substrates, revealing a new mode of regulation by cholesterol.

Considering that MARCH6 affects cholesterol homeostasis at several points, it seemed plausible that its levels may be affected by sterol status, in a classic feedback mechanism. As precedents, two other RING-containing E3 ligases, IDOL and RNF145, are both LXR gene targets and are up-regulated in response to increasing sterol status (17, 18). Although sterol status does not alter mRNA levels of *MARCH6* (Fig. 1), protein levels are markedly increased (Figs. 2–7). We had previously shown that protein levels were unaffected (4). However, this was likely due to the nature of transient and massive overexpression masking any regulation by cholesterol, as we have previously observed for SM (3).

Here, we instead used stable MARCH6 expression systems (either Myc- or V5-tagged enzyme) expressing MARCH6 at levels comparable with the parent cell line and observed robust cholesterol-mediated stabilization. We have observed this effect in more than 50 experiments, measuring a consistent 2-fold effect. A doubling of the level of an E3 ligase like MARCH6 is biologically significant, as indicated by the reduced levels of two substrates ostensibly unrelated to cholesterol metabolism (Fig. 8).

This cholesterol-mediated reduction in D2 and RGS2 is consistent with cholesterol stabilizing endogenous MARCH6. Indeed, we have presented several lines of evidence that, like ectopic MARCH6, endogenous MARCH6 is very likely to be regulated by cholesterol. Although the utility of current endogenous MARCH6 antibodies is questionable (Fig. S1), we observed cholesterol-mediated stabilization of the probable MARCH6 band. In the absence of a reliable endogenous antibody, we relied on ectopic expression in most experiments, but importantly, *MARCH6* mRNA levels were similar between the parental HEK293 cells and our MARCH6–V5–expressing cells (Fig. 2C). In addition, we found that cholesterol no longer induced degradation of SM N100, a normally cholesterol-re-
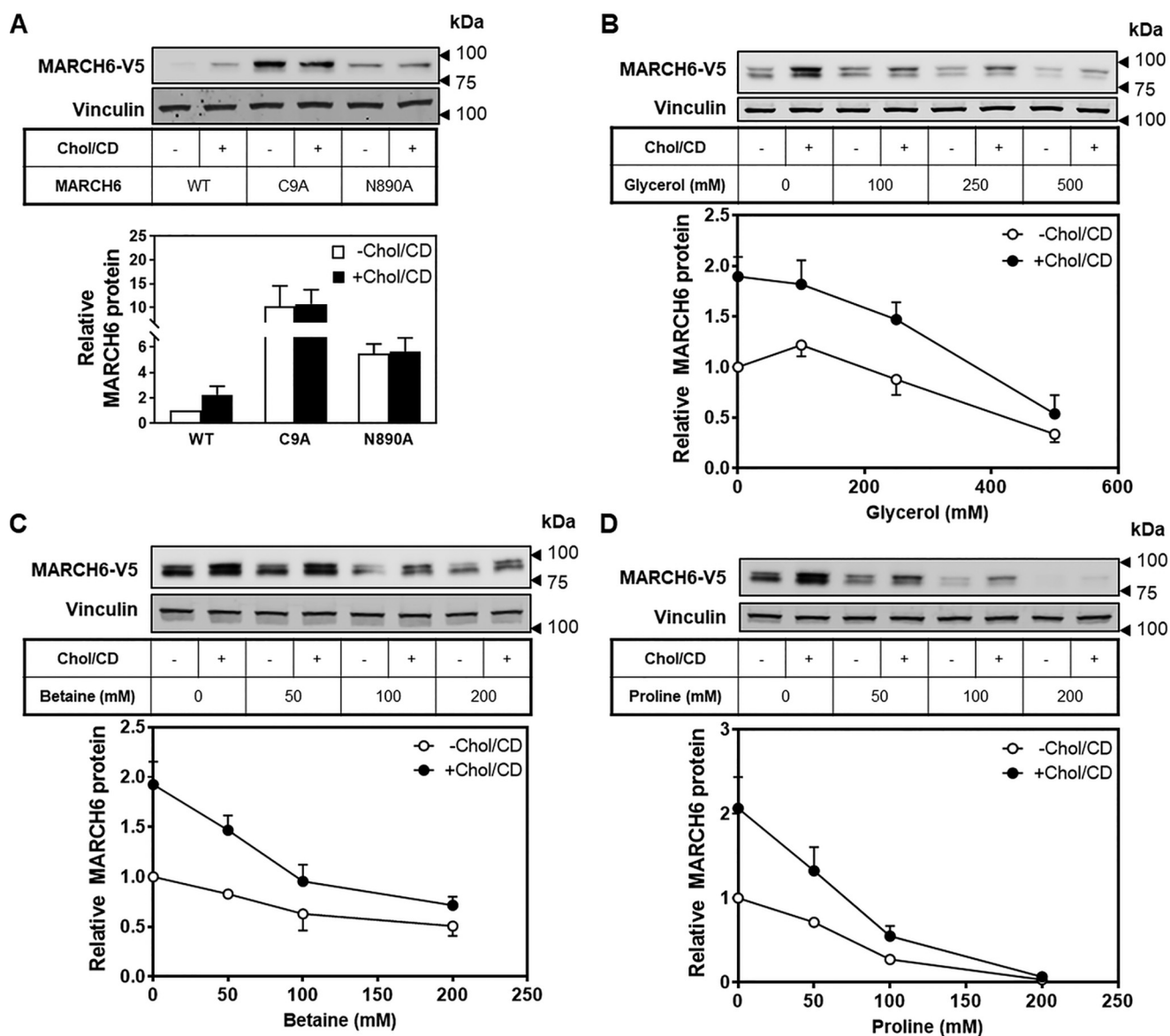
Figure 7. Cholesterol prevents self-destruction of MARCH6, likely through a conformational change. *A,* HEK–MARCH6–V5 or mutant cell lines were treated with or without 20 μg/ml Chol/CD for 4 h before harvesting. Protein lysates were subjected to Western blotting for V5 (MARCH6) and vinculin as a loading control. Blots are representative of at least three independent experiments. Relative protein levels were measured using ImageStudio Lite and normalized to the control −Chol/CD condition, which has been set to 1. Data are presented as mean ± S.E. from at least three separate experiments. *B–D,* HEK–MARCH6–V5 cells were pretreated overnight with the indicated concentration of chemical chaperone (glycerol, betaine, or proline), then treated with or without 20 μg/ml Chol/CD and the indicated concentration of chemical chaperone for 4 h before harvesting. Protein lysates were subjected to Western blotting for V5 (MARCH6) and vinculin as a loading control. Blots are representative of at least three independent experiments. Relative protein levels were measured using ImageStudio Lite and normalized to the control −Chol/CD condition, which has been set to 1. Data are presented as mean ± S.E. from at least three separate experiments.

sponsive derivative of SM, in two MARCH6-deficient cell lines independently generated in two different laboratories by CRISPR–Cas9 technology. We have previously used this technology to generate a HeLa cell line with an epitope-tagged endogenous MARCH6 (11), but less than 1% of the cells had detectable tagged MARCH6 based on immunofluorescence. This cell line is therefore unsuitable for biochemical work.

Although a range of the sterols tested tended to increase MARCH6 levels, about half of them had no significant effect. Notably, lathosterol neither stabilizes MARCH6 nor causes degradation of HMGCR or SM (Fig. 3*B* and Table 1). It could be argued that sterol stabilization of MARCH6 shows less specificity than sterol-mediated degradation of HMGCR and SM

(Table 1). However, considering that these experiments on MARCH6, HMGCR, and SM have been performed across a number of cell types in different laboratories, using variable concentrations and incubation times, it is possible that the differences in sterol specificity are partially explained by these and other confounding factors. For example, the degradation of SM in response to cholesterol may require both a conformational change in SM (41) as well as stabilization of MARCH6 for maximal regulation. Head to head comparisons may be needed to better determine the relative sterol specificities of MARCH6 stabilization and HMGCR and SM degradation.

To further investigate the mechanism by which cholesterol regulates MARCH6, we explored the possibility that MARCH6
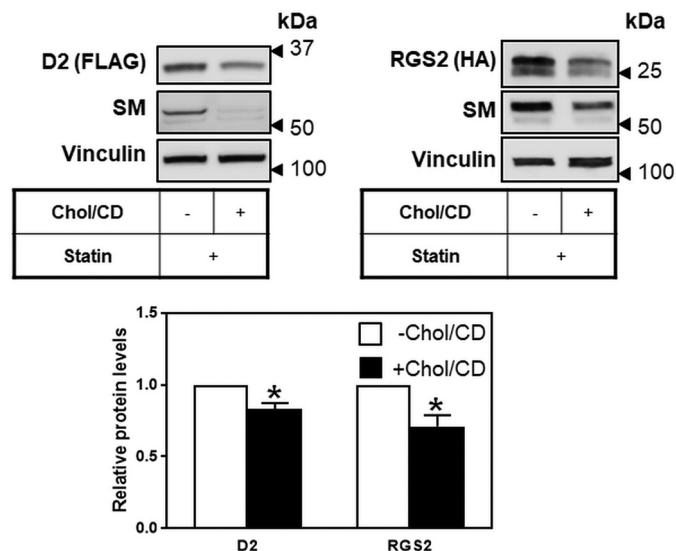
Figure 8. Cholesterol regulates two additional MARCH6 substrates.
HEK293 cells were transfected for 24 h with 0.5 μg of D2-FLAG or 0.5 μg of RGS2-HA plasmid, followed by statin pretreatment overnight, then treated with 10 μM MG132 for 2 h to increase basal levels, and then treated for 6 h with or without 20 μg/ml Chol/CD before harvesting. Protein levels were analyzed by Western blotting with FLAG (D2), HA (RGS2), SM and vinculin as a loading control. Blots are representative of at least three independent experiments. Relative protein levels were measured using ImageStudio Lite and normalized to the −Chol/CD condition, which has been set to 1 in each experiment. Data are presented as mean ± S.E. from at least three separate experiments. *, $p < 0.05$, by Student's *t* test.

Table 1

Sterol specificity

A comparison of the effects of the indicated sterols on degradation of HMGCR and SM and the stabilization of MARCH6 is shown. − indicates no effect; + indicates effect (degradation for HMGCR and SM, stabilization for MARCH6); −/+ indicates nonsignificant effect; ND indicates not determined.

	HMGCR degradation	SM degradation	M6 stabilization
Cholesterol (Chol)	−[a]	+[b]	+
Lanosterol (Lano)	+[c]	−[b]	+
24,25-Dihydrolanosterol (24,25 DHL)	+[c]	−[b]	−/+
Lathosterol (Lath)	−[c]	−[b]	−
7-Dehydrocholesterol (7DHC)	−[c], +[d]	−[b]	−
Desmosterol (Desmo)	−[c]	−[b]	+
β-Sitosterol (β-sito)	−[c]	ND	+
25-Hydroxycholesterol (25HC)	+[a]	−[b]	+
27-Hydroxycholesterol (27HC)	+[a]	−[b]	+
7α-Hydroxycholesterol (7αHC)	−[c]	+[b]	−
7-Ketocholesterol (7KC)	−[c]	+[b]	−

[a] See Ref. 56.
[b] See Ref. 3.
[c] See Ref. 57.
[d] See Ref. 58.

contains an SSD. A number of cholesterol-related proteins contain SSDs, and comparisons of the TMD patterns in these proteins indicated that MARCH6 may similarly possess an SSD comprising 5 of its 14 TMDs (Fig. 5). Importantly, two critical residues from other SSDs were identified in MARCH6, but neither of these residues appears critical for stabilization of MARCH6 by cholesterol, as mutants of either remained sterol-responsive (Fig. 5D). However, it is possible that these tagged mutant proteins oligomerize with endogenous untagged MARCH6 bearing the normal SSD, masking any effect of the mutations.

SSDs have been proposed as INSIG-interacting domains, at least for Scap, HMGCR, and the E3 ligase RNF145 (6). How-

ever, our results indicated that INSIGs are unlikely to be responsible for the cholesterol-dependent stabilization of MARCH6 (Fig. 6). Interestingly, knockdown of *INSIG-2* resulted in a significant increase in MARCH6 protein levels, but knockdown of both *INSIG-1* and *INSIG-2* blunted this increase. It is possible that a complex interplay between the INSIGs and other degradation machinery are exquisitely balanced. For example, GP78 also plays a minor role in basal degradation of MARCH6 (Fig. 4E) and interacts with INSIGs, particularly INSIG-1 (42). In the absence of both INSIGs, GP78 could be more available to degrade MARCH6, blunting the increase in MARCH6 levels observed with *INSIG-2* knockdown alone. Further work is required to determine whether there is a *bona fide* SSD in MARCH6 and whether INSIG-2 directly interacts with this domain.

MARCH6 is rapidly turned over in cells (Figs. 2 and 4), and this appears to be largely due to autodegradation, as mutants that prevent this (C9A and N890A) have much higher expression levels (Fig. 7A) (4, 11) . We found that neither of these mutants was further stabilized by cholesterol (Fig. 7A), indicating that cholesterol likely prevents autodegradation of MARCH6. Because MARCH6's substrate protein levels are decreased following cholesterol treatment (Fig. 8), it is likely that cholesterol alters the specificity of MARCH6 for its substrates, similar to the N890A mutant, which can no longer cause autodegradation but still targets some of its substrates (11). Future work should explore ubiquitination levels on MARCH6 following treatment with cholesterol.

Employing three different small molecule chaperones, we found that cholesterol prevents MARCH6's autodegradation through a conformational change (Fig. 7, B–D), as has been found for other cholesterol-responsive proteins (41, 43). The structure of MARCH6 remains to be solved, and so how precisely cholesterol modulates the shape of this 14-TMD protein in and out of the ER membrane is unknown, although it is worth noting that cholesterol can directly bind to MARCH6 (44).

Two of MARCH6's substrates, D2 and RGS2, were decreased by cholesterol under conditions previously used to assess post-translational degradation of SM (Fig. 8). Endogenous SM is subject to transcriptional regulation by sterols as well as post-translational regulation (3), explaining the larger effect size on this substrate. This finding that two substrates of MARCH6 not directly involved in cholesterol metabolism are degraded in response to cholesterol highlights a new role for cholesterol in regulating other aspects of cell homeostasis beyond the known repertoire. D2 is a deiodinase that activates thyroid hormone by converting the pro-hormone T4 to T3, the biologically active thyroid hormone (12), and RGS2 is a regulator of G proteins that lowers blood pressure by decreasing signaling through $G\alpha_q$ (13). The other established substrates of MARCH6 are itself, a mutant bile salt exporter pump (14), mutant NPC1 (7), the perilipin PLIN2 (8), and the cholesterol synthesis enzymes SM and HMGCR (4). Cholesterol synthesis is especially energy-intensive (45), and interestingly, bile acids induce energy expenditure by promoting intracellular thyroid hormone activation via induction of D2 (46). Also of note, human RGS2 polymorphisms are linked to weight gain and increased susceptibility to metabolic syndrome (47, 48). PLIN2 is a constitutive and ubiq-

uitously expressed lipid droplet protein that is involved in adipocyte differentiation and lipid droplet dynamics. Loss of PLIN2 protects against the development of obesity in both genetic and pharmacological intervention models (49). Therefore, there are likely to be overlapping metabolic networks mediated by MARCH6, and cholesterol may in turn influence these through stabilizing MARCH6.

In the presence of cholesterol, another E3 ligase, TRC8, is degraded, while MARCH6 is stabilized. Considering that these two ER membrane-bound E3 ligases have recently been shown to act together in the degradation of select substrates (21), our new finding of MARCH6 cholesterol regulation needs to be considered. It is possible that TRC8 activity is favored in low cholesterol conditions, while MARCH6 dominates when cholesterol levels are high. Thus, a spectrum of substrate regulation could exist between the two extremes of cholesterol status within cells.

In conclusion, we have identified a new role for cholesterol in controlling protein demolition through stabilization of an E3 ubiquitin ligase, MARCH6. This is likely to affect levels of MARCH6's other substrates, opening up unexplored territory for regulation of cell homeostasis by cholesterol levels. Furthermore, we have shown that MARCH6 is highly entwined with cholesterol and possibly energy metabolism and should be considered a possible candidate E3 ligase for other cholesterol-related and metabolically related proteins that are degraded proteasomally.

Experimental procedures

Plasmids

Our previously described MARCH6–Myc plasmid (4) was subcloned into a pcDNA5/FRT construct, and the Myc tag was replaced with a V5 tag to yield pcDNA5/MARCH6–V5/FRT. Mutations of this construct (C9A, Y340A, L366F, and N890A) were created using site-directed mutagenesis, and primer sequences are available on request. pcDNA3.1 encoding human RGS2-HA was a generous gift from Professor Yaping Tu (50). D10 encoding full-length human D2-FLAG with a double selenocysteine to cysteine mutation and the D15 expression vector were generous gifts from Dr. Ann Marie Zavacki (12). A plasmid expressing the first 100 amino acids of SM (SM N100) fused to GFP, pTK-SM N100-GFP-V5, was generated previously (3). The px459-gRNA1-MARCH6-KO was generated by inserting annealed oligonucleotides JMB414 (CACCGCGC-CGACTTACCTTCCTCCG) and JMB415 (AAACCGGAG-GAAGGTAAGTCGGCGC) into px459 as described previously (11, 51). The px458-gRNA2-MARCH6-KO plasmids containing AGACAAGATGGACACCGCGG and CGGC-GCGGGCGGGAAACGGC were similarly generated.

Cell lines

HEK293 cells stably overexpressing MARCH6 with a Myc or V5 epitope tag (HEK–MARCH6–Myc or HEK–MARCH6–V5) or MARCH6–V5 mutants were created using the FlpIn system as we have done previously (4). Cells were grown in 10% (v/v) fetal calf serum (FCS)/DMEM high glucose with 20 or 50 μg/ml hygromycin B. HeLa cells (ATCC) were cultured in 10% (v/v) FCS/DMEM.

CRISPR/Cas9 modification of chromosomal MARCH6

HEK293 cells—HEK293 cells were seeded at 1.35×10^6 cells/well in 10-cm dishes. The next day, cells were transfected with 10 μg of px458-gRNA2-MARCH6-KO plasmids for 24 h using Lipofectamine/LTX reagent in a 1:4 ratio. Cells were washed and refreshed for a further 24 h before being sorted by FACS into 96-well plates at a density of one cell/well using GFP selection. Single colonies were expanded, and MARCH6 genomic disruption was verified in the selected CRISPR clone by PCR using primers CCTCTCTCGCACCTGAGCGT and GCG-GGGACAAGGGAGGCCAC (Fig. S2).

HeLa cells—HeLa cells were plated at 30–40% confluency in a 6-well plate, transfected after 24 h with px459-gRNA1-MARCH6-KO, and diluted after 48 h at low density in 15-cm² plates to generate single colonies. Isolated single colonies were expanded and screened for MARCH6 gene modification by PCR using primers JMB439 (CGCGGGAGCCTCGTG) and JMB440 (GGGTACGAACCCGGGCG) to amplify a 120-nucleotide fragment disrupted by the isolated CRISPR clone (Fig. S2).

Plasmid transfections

For RGS2 transfections (in a 6-well plate), cells were transfected with 0.5 μg of pcDNA3.1 and 0.5 μg of RGS2-HA using 2 μl of Lipofectamine LTX (ThermoFisher Scientific). For D2 transfections (in a 6-well plate), cells were transfected with 0.5 μg of D2 plasmid and 0.5 μg of D15 plasmid using 2 μl of Lipofectamine LTX. For SM N100 transfections, HEK–MARCH6–CRISPR cells, HEK293 cells, or HEK–MARCH6–V5 cells were seeded in 6-well plates and transfected with 1 μg of pTK-SM N100-GFP-V5, 1.5 μl of Lipofectamine-3000 reagent, and 2 μl of P3000 reagent. Transfections were for 24 h, then treatments as described. HeLa cells and HeLa–MARCH6–CRISPR cells seeded in 6-well plates were transfected with 2 μg of pTK-SM N100-GFP-V5 and 6 μl of X-tremeGENE HP (Roche Applied Science).

Treatments

For treatment with 20 μg/ml sterols, these were complexed to methyl-β-cyclodextrin. Oxysterols were added in ethanol. Inhibitors were added in DMSO. Solvents were kept constant between conditions and did not exceed 0.2% (v/v). Cells were treated in 10% (v/v) FCS/DMEM high glucose, except for statin pretreatments where cells were switched to 10% (v/v) lipoprotein-deficient FCS/DMEM high glucose containing 50 μM mevalonate and 5 μM mevastatin or 10 μM lovastatin. For treatments with chemical chaperones (glycerol, betaine, or proline), cells were pretreated overnight with the indicated concentration of chemical chaperone. Cells were then treated with the indicated concentration of chemical chaperone with or without Chol/CD (20 μg/ml).

siRNA transfection

HEK–MARCH6–V5 cells were transfected for 24 h using 25 nM siRNA targeted to human *INSIG-1* (52) and/or *INSIG-2* (53), *GP78* and *TRC8* (4), *RNF145* (Sigma), and *VCP* (target sequence, GGUAAUCUCUUCGAGGUAU) using Lipo-

fectamine RNAiMAX (ThermoFisher Scientific). Following this, cells were either harvested to measure mRNA levels or treated as indicated and protein harvested for Western blotting.

qRT-PCR

mRNA levels were measured using primers specific to *Insig-1* and *Insig-2* (53), *gp78*, *TRC8*, and *MARCH6* (4), *RNF145* (forward, GGTATATGGCCTTCTCGCCTTGGG, and reverse, GTAAGGAGTGCTGCAGCATTCCGC), *VCP* (forward, GGAAGCGTATCGACCCATCC, and reverse, CTCGTTTGATAGGCTCCCCTT), and porphobilinogen deaminase (*PBGD*) (54) using SYBR Green, as we have described previously (33).

Western blotting

After treatment, protein was harvested from HEK293 cells as we have described previously (4). Briefly, cells were lysed in modified RIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (w/v) SDS, 1.5% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 2 mM $MgCl_2$) supplemented with 2% (v/v) protease inhibitor mixture (Sigma), passed through a 22-gauge needle 20 times, rotated at 4 °C for 30 min, and then centrifuged at 17,000 $\times$ *g* at 4 °C for 15 min. HeLa cells were harvested by scraping in ice-cold PBS, transferred to microcentrifuge tubes, pelleted at 500 $\times$ *g* for 4 min at 4 °C, flash-frozen in liquid nitrogen, and stored at -80 °C. Cell pellets were thawed on ice and then resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% (v/v) Triton X-100, 2 mM EDTA, cOmplete EDTA-free protease inhibitor tablet (Roche Applied Science)). Lysates were rotated for 10 min followed by centrifugation at 11,200 $\times$ *g* for 10 min at 4 °C. The resulting supernatants for both HEK293 and HeLa cells were normalized for protein content and subjected to 10% (w/v) SDS-PAGE and Western blotting with the following: mouse anti-V5 (1:5000, ThermoFisher Scientific, R96025); mouse anti-β-actin (1:10,000, Sigma, A5441); rabbit anti-MARCH6 (1:5000, Bethyl Laboratories, BETHA304-171A); rabbit anti-vinculin (1:2000, Abcam, ab129002); rabbit anti-GAPDH (1:2000, Cell Signaling Technology, 2118L); goat anti-mouse IgG-HRP (1:10,000, Jackson ImmunoResearch, 115-035-003); donkey anti-rabbit IgG-HRP (1:10000, Jackson ImmunoResearch, 711-035-152); or IRDye 680RD donkey anti-rabbit IgG (1:20,000, Millennium Science) as indicated in figure legends. MARCH6 runs at an apparent molecular mass of ~80 kDa during SDS-PAGE, which is lower than its expected mass of ~100 kDa. This is likely due to its 14 TMDs, which confer a greater capacity to bind SDS (55). Blots were imaged using Clarity ECL substrate (Bio-Rad) on a LAS500 imager (GE Healthcare) or, for GAPDH and vinculin, using an Odyssey-CLx imager (LI-COR Bioscience). Densitometry was performed using ImageStudio Lite version 5.2. For HeLa and HeLa–MARCH6–CRISPR experiments, primary antibodies were probed with peroxidase-coupled anti-IgG antibodies (1:10,000, GE Healthcare, NA931V and NA934V), which were activated by enhanced chemiluminescence, imaged on a G:Box system (Syngene), and quantified using Gene Tools (Syngene).

Data presentation

All quantitative data are presented as mean $\pm$ S.E. from at least three independent experiments. Statistical significance ($p < 0.05$) was determined using Student's paired *t* test.

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