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Differential use of E2 ubiquitin conjugating enzymes for regulated degradation of the rate-limiting enzymes HMGCR and SQLE in cholesterol biosynthesis

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HIGHLIGHTS

- Loss of the E2 ubiquitin conjugating enzyme UBE2J2 prevents sterol-initiated degradation of SQLE in multiple human cell types.
- Different E2s mediate the degradation of cholesterol biosynthetic enzymes HMGCR and SQLE.
- UBE2J2 is a new regulator of cellular cholesterol homeostasis in mammalian cells.

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ABSTRACT

Background and aims: Cholesterol is an essential lipid for cellular function and membrane integrity, and hence its cellular levels and distribution must be tightly regulated. Biosynthesis of cholesterol is ramped when its cellular levels are low. Herein, the ER-resident and rate-limiting enzymes 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and squalene monooxygenase (SQLE) play a prominent role. We have recently reported that MARCH6, an E3 ubiquitin ligase, specifically promotes cholesterol-stimulated ubiquitylation and subsequent proteasomal degradation of SQLE, but not of HMGCR. To further delineate how post-translational regulation of SQLE and HMGCR is differentially achieved, we hypothesized that their sterol-dependent degradation machinery makes use of distinct E2 ubiquitin conjugating enzymes.

Methods: To study this possibility, we therefore used a CRISPR/Cas9-based approach to screen for ER-associated degradation (ERAD)-associated E2 enzymes that are essential for MARCH6-dependent degradation of SQLE.

Results: We report here the identification of UBE2J2 as the primary E2 ubiquitin conjugating enzyme essential for this process in mammalian cells, in contrast to UBE2G2, which is essential for sterol-stimulated degradation of HMGCR. We demonstrate that ablating UBE2J2 disturbs cholesterol-accelerated SQLE degradation in multiple human cell types, including cells of hepatic origin, and that the ability of UBE2J2 to support SQLE degradation critically depends on its enzymatic activity.

Conclusions: Our findings establish UBE2J2 as an important partner of MARCH6 in cholesterol-stimulated degradation of SQLE, thereby contributing to the complex regulation of cellular cholesterol homeostasis.

1. Introduction

Cholesterol plays a pivotal role as a constituent of biological membranes and as a precursor for vitamins, hormones and bile acids. Accordingly, its production, distribution, and elimination must be tightly regulated at the cellular and organismal level. Accordingly, dysregulated cholesterol metabolism is extensively linked to a wide variety of human diseases, including atherosclerosis and the metabolic

Abbreviations: LDL, low-density lipoprotein; LDLR, LDL-receptor; SREBP, sterol-regulatory element binding protein; β-MCD, β-methyl cyclodextrin; SQLE, squalene monooxygenase; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; CRISPR, clustered regularly interspaced short palindromic repeats; ERAD, endoplasmatic reticulum associated degradation

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syndrome [1,2].

Cells can acquire cholesterol by taking up low-density lipoprotein (LDL) via the LDL-receptor (LDLR) pathway, or alternatively via *de novo* cholesterol biosynthesis through the mevalonate pathway. The synthesis of cholesterol by the mevalonate pathway requires the coordinated activity of > 20 enzymes and is transcriptionally regulated by the sterol-regulatory element binding protein (SREBP) family of transcription factors [3]. However, next to transcriptional regulation, post-transcriptional control exquisitely governs abundance of the rate limiting enzymes Squalene Monooxygenase (SQLE) and 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMGCR) [4]. Both enzymes are subject to robust degradation by the ubiquitin-proteasome system in response to metabolic cues [5–7]. Intriguingly, these cues differ for the two enzymes. Whereas proteasomal degradation of HMGCR is stimulated by lanosterol, intermediate metabolites of the biosynthetic pathway, and oxysterols [8–10], that of SQLE is primarily triggered by cholesterol, the end product of the pathway itself [5]. Another distinguishing feature of these processes is that whereas sterol-stimulated degradation of HMGCR requires INSIG proteins, that of SQLE does not [5,8,11]. The ability to differentially control the regulated degradation of these two rate-limiting enzymes may allow fine tuning of synthesis of important products of the isoprenoid branch of the mevalonate pathway, such as ubiquinone, dolichol and isoprenoids, independently from that of cholesterol itself [6,12]. Finally, the enzymes required for HMGCR and SQLE ubiquitylation and proteasomal degradation are different. Ubiquitylation follows the mandatory sequence of enzymatic reactions consisting of activating ubiquitin by an E1 enzyme, binding of ubiquitin to an E2 ubiquitin conjugating enzyme, and finally the ligation of ubiquitin onto the target protein that is supported by the E3 ubiquitin ligase [13]. Extensive research into the ubiquitylation cascade of HMGCR has implicated the E2 enzyme UBE2G2, as well as the E3 ubiquitin ligases gp78, TRC8 and RNF145 in its degradation [4,14–18], while the RING-type ubiquitin ligase MARCH6 has been shown to promote the degradation of SQLE [19]. However, the E2 enzyme(s) governing this process remains elusive.

In yeast, the SQLE homologue Erg1 is degraded by the MARCH6 homologue Doa10, in cooperation with the E2 conjugating enzymes Ubc6 and Ubc7 [20]. However, a marked difference in complexity can be observed between ERAD in yeast versus mammalian cells [13,21–23]. We therefore used a CRISPR/Cas9-based screening approach to delineate the ERAD machinery responsible for MARCH6-dependent degradation of SQLE in mammalian cells and report here the identification of the E2 UBE2J2 as a critical determinant of this process.

2. Materials and methods

2.1. Cell culture

HepG2, a hepatoma cell line and a commonly used human hepatocyte cell model, and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). Hap1 cells, a unique mammalian haploid cell line [14], was grown in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco). Media were supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/mL). Cells were grown at 37 °C with 5% CO_2. Where indicated, cells were sterol-depleted by culture in DMEM or IMDM supplemented with 10% lipoprotein-deficient serum (LPDS), 2.5 μg/mL simvastatin (Calbiochem) and 100 μM mevalonate (Sigma) for 24 h. β-methyl cyclodextrin (βMCD)-cholesterol (βMCD-C) was purchased from Sigma and prepared as a 10 mg/mL stock in water. LPDS was prepared as previously described [24].

2.2. UBE2J2 expression constructs

The UBE2J2 cDNA was amplified from a plasmid encoding UBE2J2-HA [25] and cloned into pDONR221. Site-directed mutagenesis was used to introduce a C94S mutation in UBE2J2. The wildtype (WT) and C94S UBE2J2 cDNA were subsequently cloned into the pLenti-V5 vector using the gateway cloning system (ThermoFisher). All plasmids used in this study were isolated using the Direct-zol DNA isolation kit (Zymo Research), and their correctness verified by Sanger sequencing.

2.3. Transfection of mammalian cells

Cells were seeded at 50% confluency 24 h before transfection. Subsequently, cells were transfected as indicated in the Fig. legends using the JetPrime transfection reagent (Polyplus). For RNA silencing experiments, HepG2 cells were transfected with 40 nM ON-TARGETplus SMARTpool UBE2J2 siRNA (L-008614-00-0005) and non-targeting control siRNA (D-001810-10-20) (Dharmacon) with Lipofectamine RNAiMAX (Life Technologies) for 72 h.

2.4. CRISPR/Cas9-mediated ablation of ERAD-associated E2s

HEK293T cells lacking the E2 ubiquitin-conjugating enzymes *UBE2J1*, *UBE2J2* and *UBE2G2* were generated using a limited CRISPR/Cas9-based library. The plasmids encoding for the guide RNAs were generously provided by Emmanuel Wiertz [25]. The guide RNA sequences are listed in Table 1. Cells were transfected with two plasmids encoding for two independent guides per gene. Cells were subsequently selected with 1 μg/mL Puromycin (Sigma). Hap1 cells lacking *UBE2J2* were generated by CRISPR/Cas9-mediated genome editing, as described recently [26]. Briefly, cells were co-transfected with pSC-TIA-p2A-Blast and a guide RNA targeting the 3rd exon of *UBE2J2,* which was cloned as a *BbsI* fragment into px330 (Addgene #42230). Subsequently, cells were selected with 5 μg/mL Blasticidin and single-cloned, as we have previously reported [26]. Correct targeting of *UBE2J2* was verified by analysis of genomic DNA and qPCR of independent clones.

2.5. SQLE and HMGCR degradation assays

To assess the (chole)sterol-stimulated degradation of SQLE and HMGCR, cells were cultured in sterol-depletion medium for 24 h. Subsequently, degradation of SQLE was initiated by treatment with 50 μg/mL βMCD-C (corresponding to 2 μg/mL cholesterol). HMGCR degradation was stimulated by addition of 10 μM 25- hydroxycholesterol (25-HC) to the culture medium. Where indicated in the Fig. legends, cells were treated with 25 μM MG132 (Calbiochem) to inhibit proteasomal degradation.

2.6. Immunoblot analysis

Cells were lysed in radio-immunoprecepitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 100 mM Tris-HCl, pH 7.4) supplemented with a cocktail of protease inhibitors (Roche). The lysates were cleared by centrifugation at 4 °C for 10 min at 10,000 × *g*. Proteins were separated on NuPAGE Novex 4–12% Bis-Tris gels and transferred to nitrocellulose membranes. Immunoblot analysis was performed using the following antibodies: ß-actin (Merck, 1:10000), SQLE (Protein Tech Group, 1:1000), FLAG (Sigma Aldrich; 1:1000), V5 (Invitrogen, 1:1000), HMGCR (ATCC CRL-1811; IgG-A9). Quantifications of indicated immunoblots are presented in Supplementary Fig. 3.

Table 1
CRISPR/Cas9 guide RNAs used in the study.

Gene	Guide RNA 1	Guide RNA 2
UBE2J1	GTTGTAGCGGGTCTCCATGG	GAATGGCACTTCACGGTTAG
UBE2J2	GCTGAATCCCGTTCTGGACG	TATAGAGACGTCGGACTTCA
UBE2G2	GGAGAAGATCCTGCTGTCGG	GGACTTAACGGGTAATCAAG

2.7. RNA isolation and quantitative PCR

Cells were lysed in Tri-ZOL reagent and RNA isolated using the Direct-zol RNA kit (Zymo Research). RNA was reverse transcribed into cDNA using the Biotool cDNA synthesis kit (Biotool). Real-time quantitative PCR (qPCR) was performed on a LightCycler 480 II system (Roche) using the SensiFAST SYBR reagent (Bioline) and gene expression was normalized to expression of *36B4* as a reference gene. qPCR primer sequences are available on request.

2.8. Data presentation and statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism version 6.0. The values were tested for normality and equal variance, and subsequently subjected to unpaired and, where necessary, an unpaired repeated *t*-test with Holm-Sidak correction for multiple testing. Densitometric analysis of immunoblots was performed with the ImageJ software package (NIH). Intensity of protein bands were normalized to the loading control actin.

3. Results

To identify the E2 conjugating enzyme responsible for MARCH6-dependent degradation of SQLE, we used a CRISPR/Cas9-based candidate-gene screening approach. We generated HEK293T cells lacking the E2 ubiquitin conjugating enzymes implicated in ERAD: UBE2J1, UBE2J2, and UBE2G2. We confirmed reduced transcript levels of the three E2s (Fig. 1A) and observed expression of FLAG-tagged Cas9 in the edited cells (Fig. 1B and C). In wild-type (WT) cells, HMGCR and SQLE are subject to rapid sterol-stimulated degradation (Supplementary Fig. 1). However, in *UBE2J2*^{KO}- cells, sterol-stimulated degradation of SQLE, but not that of HMGCR, was largely attenuated (Fig. 1B). Of note, cells lacking UBE2G2 exhibited moderate attenuation of SQLE degradation, indicating that although UBE2J2 is the primary E2 necessary for MARCH6-dependent SQLE degradation in HEK293T cells, some redundancy between E2 enzymes in SQLE degradation may exist.

In contrast to SQLE, HMGCR could be efficiently degraded in cells lacking UBE2J2. However, this was not the case in cells devoid of UBE2G2, as these cells were unable to support 25-hydroxycholesterol (25-HC)-stimulated degradation of HMGCR (Fig. 1C). This finding is consistent with our recently reported genome-wide haploid mammalian genetic screen, which interrogated sterol-stimulated degradation of endogenous HMGCR and reported on the role of UBXD8 in this process in Hap1 cells [14]. Additionally, this screen allowed us to investigate the E2 ubiquitin conjugating enzyme(s) required for this process (Supplementary Fig. 2A). A total of 34 E2s were identified in the screen, of which only two ERAD E2s were identified as being significantly involved in HMGCR degradation: *UBE2G2* and, less prominently, *UBE2J2* (Supplementary Fig. 2B). Consistent with *UBE2G2* being the strongest hit in the haploid screen, we show here that cells lacking this E2 are unable to efficiently support sterol-stimulated degradation of HMGCR (Fig. 1C). Collectively, our results suggest that the degradation of SQLE and HMGCR depends on different E2 enzymes. While sterol-stimulated degradation of HMGCR in HEK293T cells requires UBE2G2, that of SQLE is predominantly mediated by UBE2J2.

As the primary aim of this study was to characterize the ERAD machinery responsible for sterol-mediated SQLE degradation, and to further generalize the role of UBE2J2 herein, we wanted to extend this finding to other mammalian cell types. We therefore generated Hap1-*UBE2J2*^{KO} cells by targeting the endogenous *UBE2J2* locus using a CRISPR/Cas9-based approach. The editing of the *UBE2J2* locus was confirmed by sequencing, and by the reduction of *UBE2J2* mRNA expression (Fig. 2A). Control Hap1 cells showed cholesterol-stimulated breakdown of SQLE, which could be attenuated by blocking the proteasome with the inhibitor MG132. In line with the results obtained in

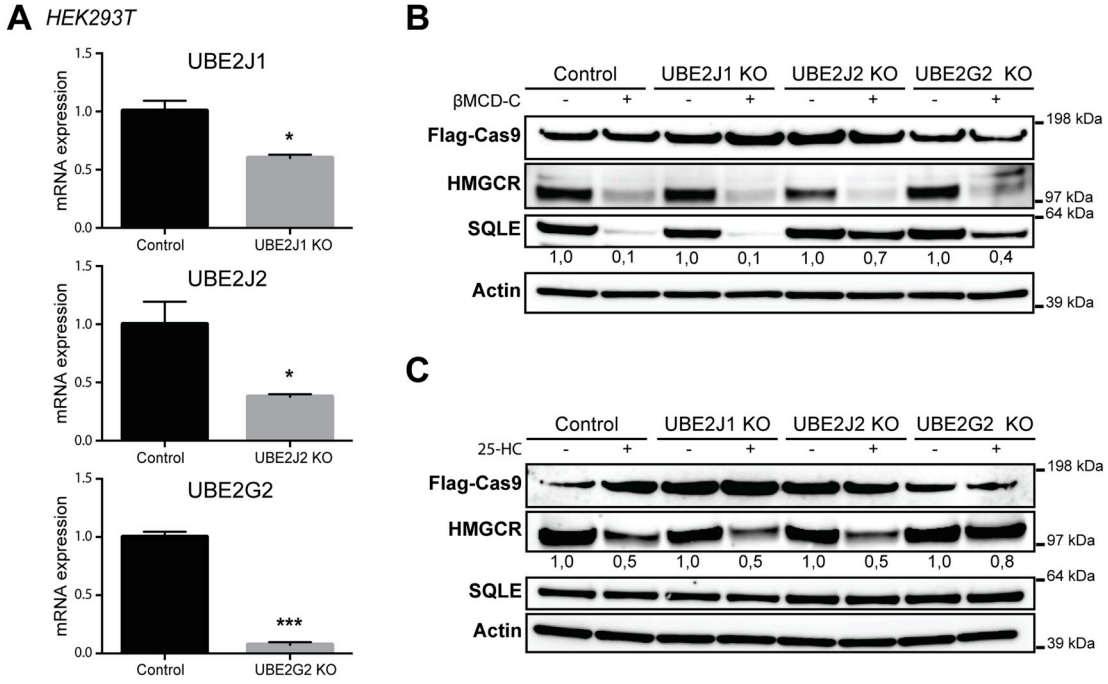

Fig. 1. Function of various E2s in SQLE and HMGCR degradation.
(A) Total RNA was isolated from control-, UBE2J1^{KO}-, UBE2J2^{KO}- and UBE2G2^{KO}-HEK293T cells and expression of the indicated genes was determined by qPCR. Each bar and error represent the mean ± SEM (n = 3). *$p < 0.05$, ***$p < 0.001$. (B and C) Control-, UBE2J1^{KO}-, UBE2J2^{KO}- and UBE2G2^{KO}-HEK293T cells were cultured in sterol-depletion medium for 24 h and subsequently treated with (B) 50 μg/mL βMCD-cholesterol (βMCD-C) for 4 h, or (C) 10 μM 25-hydroxycholesterol (25-HC) for 1 h. Total cell lysates were immunoblotted as indicated. Immunoblots are representative of 3 independent experiments, and numbers between the blots indicate the mean SQLE- or HMGCR-intensity from three independent experiments. Detailed quantification is provided in Supplementary Fig. 3A and B.

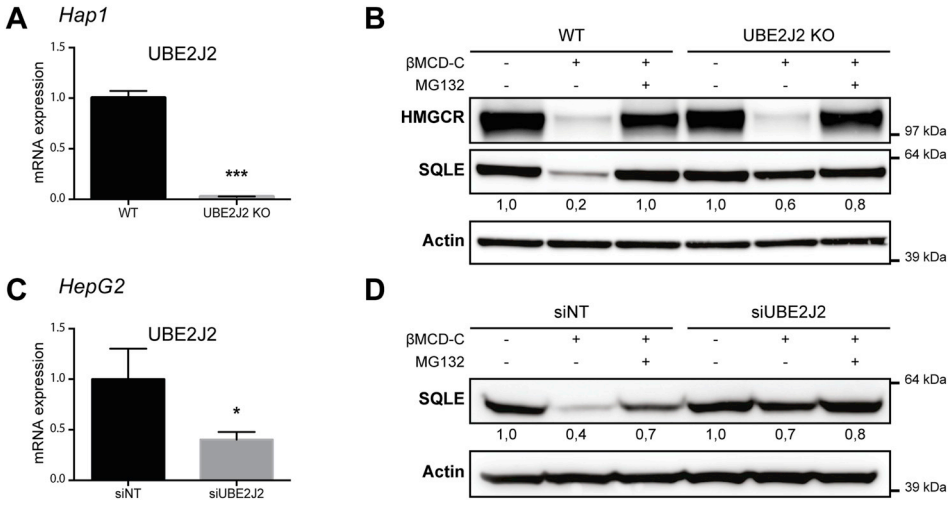

Fig. 2. UBE2J2 promotes SQLE degradation in different cell types.
(A and B) Wildtype (WT) and UBE2J2^{KO}-Hap1 cells and (C and D) HepG2 cells transfected with control siRNA (non-targeting; siNT) or UBE2J2 siRNA (siUBE2J2) were cultured in sterol-depletion medium for 24 h, before treatment with 50 μg/mL βMCD-cholesterol (βMCD-C) and 25 μM MG132 for 4 h (B) or 8 h (D) as indicated in the figures. (B and D) Total cell lysates were immunoblotted as indicated. Immunoblots are representative of three independent experiments, and numbers between the blots indicate the mean SQLE intensity from three independent experiments. Detailed quantification is provided in Supplementary Fig. 3C, D and E. (A and C) Total RNA was isolated and expression of UBE2J2 was determined by qPCR. Each bar and error represent the mean ± SEM (n = 3). $*p < 0.05$, $***p < 0.001$.

HEK293T cells, absence of UBE2J2 markedly prevented degradation of SQLE, but not that of HMGCR (Fig. 2B). Furthermore, RNAi-mediated silencing of UBE2J2 expression in HepG2 cells, a commonly used human hepatocyte-like cell model, also attenuated sterol-stimulated degradation of SQLE in a proteasome-dependent manner (Fig. 2C and D).

Having established the critical necessity of UBE2J2 for degradation of SQLE, we considered the possibility that its expression may be sensitive to the cellular sterol-status, as this may allow coupling of its activity to metabolic demand. However, while expression of the SREBP targets HMGCR and SQLE increased and that of IDOL decreased by sterol-depletion as anticipated, expression of UBE2J2 itself or of MARCH6, UBE2J1 and UBE2G2 remained unchanged in HepG2 cells (Fig. 3A) and HEK293T cells. Moreover, ablation of UBE2J2 did not result in significant changes of UBE2J1 and UBE2G2 in these cells (Fig. 3B), or in Hap1 cells (Fig. 3C). In aggregate, these results suggest that SQLE degradation by MARCH6 and UBE2J2 is a selective post-transcriptional mechanism, which does not rely on sterol-dependent transcriptional regulation. Moreover, our results indicate that ablating UBE2J2 does not change the expression levels of the other ERAD E2s as a compensatory mechanism.

To demonstrate that the catalytic activity of UBE2J2 is essential for its function in MARCH6-dependent SQLE degradation, we generated a catalytically inactive UBE2J2 mutant (UBE2J2$_{C94S}$ [27]) and tested its ability to rescue cholesterol-stimulated degradation of SQLE in *UBE2J2^{KO}* HEK293T cells, which otherwise are unable to support this process. Whereas reintroducing wildtype *UBE2J2* largely restored sterol-mediated SQLE degradation in *UBE2J2^{KO}* cells, SQLE remained refractory to degradation in cells in which the mutant was reintroduced (Fig. 4A). In fact, reintroducing wildtype *UBE2J2* decreased the basal levels of SQLE, while this was not the case for the catalytically inactive mutant (Fig. 4B). These findings indicate that the catalytic domain of UBE2J2 is essential for its function in MARCH6-dependent SQLE degradation. Collectively, our results support a model in which MARCH6 acts in concert with UBE2J2 in a metabolic feedback network to promote the cholesterol-stimulated and basal degradation of SQLE (Fig. 4C).

4. Discussion

The regulation of intracellular cholesterol levels is a biological process of vital importance for cellular function and integrity, and involves robust transcriptional and post-transcriptional mechanisms that are sensitive to metabolic feedback control. Amongst post-transcriptional mechanisms, the ubiquitin proteasome system plays a prominent role as it allows rapid coupling of metabolic cues to fine tuning of metabolic flux through the mevalonate pathway. As such, the levels of the two rate-limiting biosynthetic enzymes of the mevalonate pathway, HMGCR and SQLE, are subject to tight and well-coordinated regulation by the ubiquitin proteasome system [4,10], and are robustly degraded in response to sterol-derived cues. We recently identified the RING-type E3 ligase MARCH6 as the E3 responsible for the cholesterol-stimulated

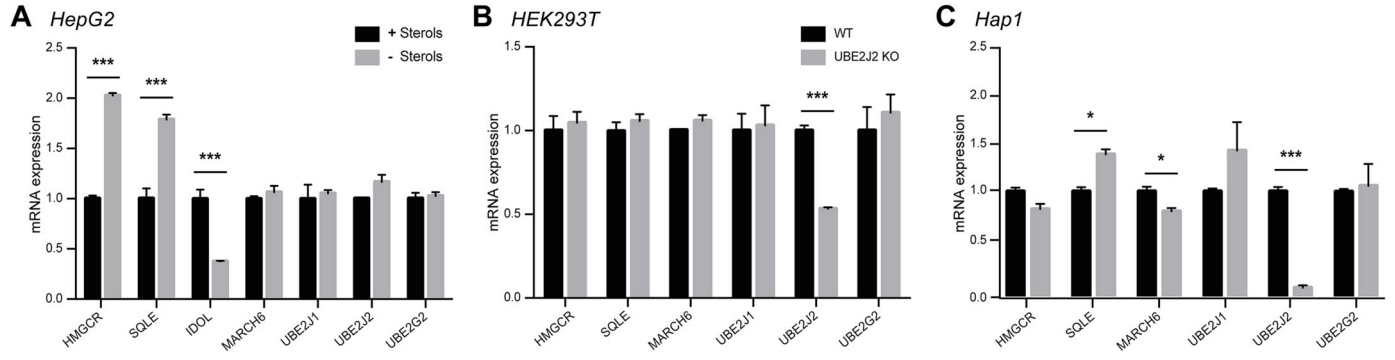

Fig. 3. E2s are not regulated by the sterol status of the cells.
Total RNA was isolated from (A) HepG2 cells cultured in sterol-containing- (black bars) or sterol-depletion medium (grey bars) for 24 h, (B) HEK293T wild type (WT; black bars) and UBE2J2^{KO} (grey bars) cells, and (C) Hap1 wild type (WT; black bars) and UBE2J2^{KO} (grey bars) cells. Expression of the indicated genes was determined by qPCR. Each bar and error represent the mean ± SEM (n = 3). $*p < 0.05$, $***p < 0.001$.

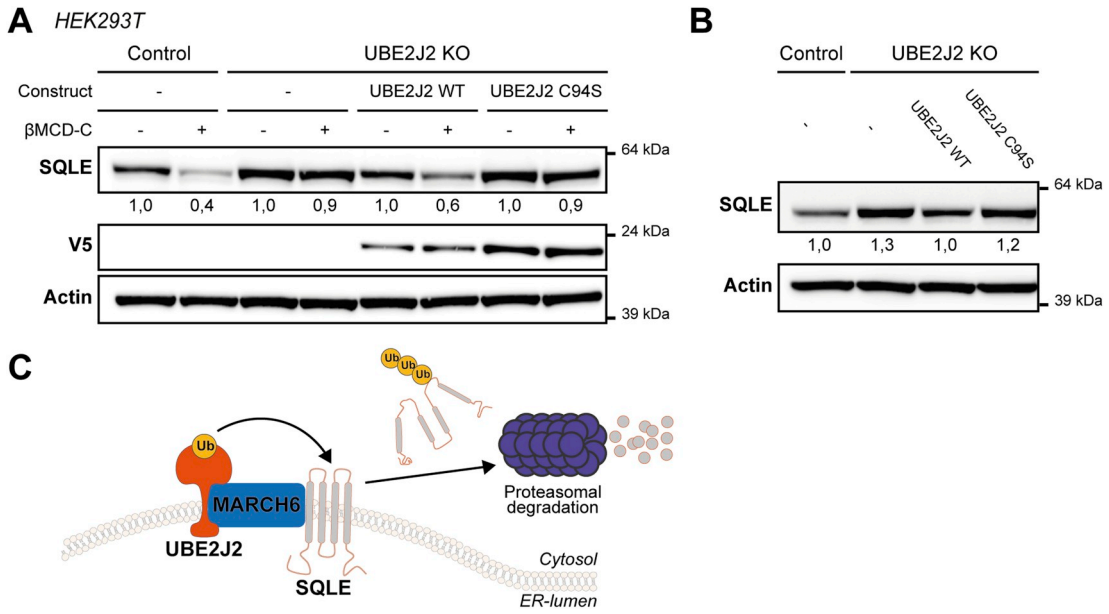

Fig. 4. UBE2J2 promotes degradation of SQLE in a ubiquitin-binding-domain (UBD)-dependent manner.
Control HEK293T, or HEK293T-UBE2J2^{KO} cells in which the V5-tagged wildtype (WT) or mutant (C94S) UBE2J2 were transiently reintroduced were cultured in sterol-depletion medium for 24 h, before (A) treated with 50 μg/mL βMCD-cholesterol (βMCD-C) for 4 h. (A and B) Total cell lysates were immunoblotted as indicated. Immunoblots are representative of three independent experiments, and numbers between the blots indicate the mean SQLE intensity from three independent experiments. Detailed quantification is provided in Supplementary Fig. 3F and G. (C) Schematic representation of UBE2J2- and MARCH6-dependent SQLE ubiquitylation and subsequent proteasomal degradation.

degradation of SQLE [19]. In this study, by using a candidate-gene genetic approach, we now identify the E2 ubiquitin conjugating enzyme UBE2J2 as a partner of MARCH6 in cholesterol-stimulated degradation of SQLE in mammalian cells.

Our finding that MARCH6 specifically uses UBE2J2 to promote SQLE degradation raises the intriguing question as to how this selectivity is achieved. One possibility is that expression of either MARCH6 or UBE2J2 is sensitive to the cellular sterol status, and that this may underlie their ability to act in concert to promote SQLE degradation. This would be akin to what we have previously reported for the E3 ubiquitin ligase Inducible degrader of the LDLR (IDOL), that promotes ubiquitylation of the LDLR in response to an increase in cellular sterol content and thus limits cholesterol uptake [28,29]. However, we show that, unlike SQLE, expression of both *MARCH6* and *UBE2J2* was refractory to manipulation of the cellular sterol status. As such, the determinants that govern the selective recruitment of UBE2J2 to MARCH6 for SQLE degradation remain to be determined.

The issue of specificity within the ubiquitin proteasomal system extends beyond E2-E3 selective pairing and pertains also to the substrate specificity of E3 ubiquitin ligases. The ER-resident ligase MARCH6 is one out of several hundred E3 ubiquitin ligases present in mammalian cells [30]. These ligases stimulate ubiquitylation of their targets in response to a plethora of signals. Importantly, the substrate specificity of some ligases may overlap, as evident in the case of ubiquitylation and degradation of HMGCR for which GP78, TRC8, and more recently RNF145, have been implicated [4,14–18]. Intriguingly, SQLE seems to be solely recognized by MARCH6 [19]. The question of redundancy also extends to the E2 arm of the ubiquitin system. In contrast to the existence of multiple ERAD-associated E3s [30,31], only 3 ERAD-associated E2 ubiquitin conjugating enzymes are known [32]. This implies that regulation of substrate degradation must also exist at the level of E2-E3 complex formation [13,32]. Indeed, we find that while degradation of HMGCR requires UBE2G2, as previously reported [17], that of SQLE relies predominantly on the use of UBE2J2. However, interestingly, UBE2G2^{KO} cells also show a minor decrease in their ability to degrade SQLE in response to cholesterol. This may represent a vestigial function maintained in mammalian UBE2G2, as its yeast homolog Ubc7 acts as a promiscuous E2 ligase and interacts with gp78, Hrd1 and the yeast MARCH6-homologue Doa10 [20,33].

ERAD has been extensively studied in yeast and our findings highlight an additional divergence between yeast and mammalian ERAD. When comparing ERAD in yeast versus mammalian cells, a marked difference in complexity can be observed. While in yeast only three E3s (Hrd1, Asi and Doa10) and two E2s (Ubc6 and Ubc7) govern all ERAD [32], the array of E3 ligases is more diversified in mammalian cells, with up to 30 E3s, and 3 E2s [13,30]. While ERAD of the yeast homolog of SQLE, Erg1, is governed by Doa10 and the E2s Ubc6 and (promiscuous) Ubc7, our study demonstrates that UBE2J1 and UBE2J2, the two yeast Ubc6 homologues in mammalian cells, have distinct roles in ERAD of SQLE; Only Ube2J2 is implicated in SQLE degradation. Collectively, our findings shed more light on the physiological roles of ERAD E2s and underlines the importance of conducting more research to clarify the mechanisms by which ERAD contributes to the regulation of cholesterol homeostasis in mammalian cells.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

AL and NZ designed the research, JT, EC, MvdB, SS and AL performed research, JT, MvdB, AL and NZ analyzed data, and JT, AL and NZ wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2018.12.008.

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