

1 **The sterol-responsive RNF145 E3 ubiquitin ligase mediates the degradation of HMG-  
2 CoA reductase together with gp78 and Hrd1**

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20 E3 ubiquitin ligase

21 **ABSTRACT**

22 HMG-CoA reductase (HMGCR), the rate-limiting enzyme of the cholesterol biosynthetic  
23 pathway and the therapeutic target of statins, is post-transcriptionally regulated by sterol-  
24 accelerated degradation. Under cholesterol-replete conditions, HMGCR is ubiquitinated and  
25 degraded, but the identity of the E3 ubiquitin ligase(s) responsible for mammalian HMGCR  
26 turnover remains controversial. Using systematic, unbiased CRISPR/Cas9 genome-wide  
27 screens with a sterol-sensitive endogenous HMGCR reporter, we comprehensively map the  
28 E3 ligase landscape required for sterol-accelerated HMGCR degradation. We find that  
29 RNF145 and gp78, independently co-ordinate HMGCR ubiquitination and degradation.  
30 RNF145, a sterol-responsive ER-resident E3 ligase, is unstable but accumulates following  
31 sterol depletion. Sterol addition triggers RNF145 recruitment to HMGCR and Insig-1,  
32 promoting HMGCR ubiquitination and proteasome-mediated degradation. In the absence of  
33 both RNF145 and gp78, Hrd1, a third UBE2G2-dependent ligase partially regulates HMGCR  
34 activity. Our findings reveal a critical role for the sterol-responsive RNF145 in HMGCR  
35 regulation and elucidate the complexity of sterol-accelerated HMGCR degradation.

## 36 INTRODUCTION

37 Cholesterol plays a critical role in cellular homeostasis. As an abundant lipid in the  
38 eukaryotic plasma membrane, it modulates vital processes including membrane fluidity and  
39 permeability (Hannich et al., 2011; Haines, 2001) and serves as a precursor for important  
40 metabolites including steroid hormones and bile acids (Payne and Hales, 2004; Chiang,  
41 2013). The cholesterol biosynthetic pathway in mammalian cells also provides intermediates  
42 for essential non-steroid isoprenoids and therefore requires strict regulation (Goldstein and  
43 Brown, 1990). The endoplasmic-reticulum (ER) resident, polytopic membrane glycoprotein  
44 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) is central to this pathway,  
45 catalysing the formation of mevalonate, a crucial isoprenoid precursor. As the rate-limiting  
46 enzyme in mevalonate metabolism, HMGCR levels need to be tightly regulated, as dictated  
47 by intermediates and products of the mevalonate pathway (Johnson and DeBose-Boyd,  
48 2017). The statin family of drugs, which acts as competitive inhibitors of HMGCR, represents  
49 the single most successful approach to reducing plasma cholesterol levels and therefore  
50 preventing atherosclerosis related diseases (Heart Protection Study Collaborative Group,  
51 2002). Understanding how HMGCR is regulated is therefore of fundamental biological and  
52 clinical importance.

53 Cholesterol, together with its biosynthetic intermediates and isoprenoid derivatives, regulates  
54 HMGCR expression at both the transcriptional and posttranscriptional level (Johnson and  
55 DeBose-Boyd, 2017). Low cholesterol induces transcriptional activation of HMGCR through  
56 the sterol response element binding proteins (SREBPs) which bind SREs in their promoter  
57 region (Osborne, 1991). In a cholesterol rich environment, SREBPs are inactive and held in  
58 the ER in complex with their cognate chaperone SREBP cleavage-activating protein (SCAP)  
59 in association with the ER-resident Insulin-induced genes 1/2 (Insig-1/2) anchor proteins  
60 (Dong and Tang, 2010; Yabe et al., 2002). A decrease in membrane cholesterol triggers  
61 dissociation of the SCAP-SREBP complex from Insigs and translocation to the Golgi  
62 apparatus, where the SREBP transcription factor is proteolytically activated by Site-1 and

63 Site-2 proteases, released into the cytosol and trafficked to the nucleus (reviewed in Horton,  
64 Goldstein, & Brown, 2002). Low sterol levels therefore dramatically increase both HMGCR  
65 mRNA and extend HMGCR protein half-life, ensuring the resultant elevated enzyme levels  
66 stimulate the supply of mevalonate to re-balance cholesterol homeostasis (Goldstein and  
67 Brown, 1990; Brown et al., 1973). Once cholesterol levels are restored, excess HMGCR is  
68 rapidly degraded by the ubiquitin proteasome system (UPS) in a process termed sterol-  
69 accelerated degradation (Hampton et al., 1996; Ravid et al., 2000; Sever et al., 2003a). This  
70 joint transcriptional and translational regulation of HMGCR is therefore controlled by a host  
71 of ER-resident polytopic membrane proteins and represents a finely balanced homeostatic  
72 mechanism to rapidly regulate this critical enzyme in response to alterations in intracellular  
73 cholesterol. While the ubiquitin-mediated, post-translational regulation of HMGCR is well-  
74 established, the identity of the critical mammalian ER-associated degradation (ERAD) E3  
75 ubiquitin ligase(s) responsible for sterol-accelerated HMGCR ERAD remains controversial.

76 In yeast, *S cerevisiae* encodes three ERAD E3 ligases, of which Hrd1p (HMG-CoA  
77 degradation 1), is named for its ability to degrade yeast HMGCR (Hmg2p) in response to  
78 non-sterol isoprenoids (Hampton et al., 1996; Bays et al., 2001). The marked expansion and  
79 diversification of E3 ligases in mammals makes the situation more complex, as in human  
80 cells there are 37 putative E3 ligases involved in ERAD, few of which are well-characterised  
81 (Kaneko et al., 2016). Hrd1 and gp78 represent the two mammalian orthologues of yeast  
82 Hrd1p. Hrd1 was not found to regulate HMGCR (Song et al., 2005; Nadav et al., 2003).  
83 However, gp78 was reported to be responsible for the sterol-induced degradation of  
84 HMGCR as (i) gp78 associates with Insig-1 in a sterol-independent manner, (ii) Insig-1  
85 mediates a sterol-dependent interaction between HMGCR and gp78, (iii) overexpression of  
86 the transmembrane domains of gp78 exerted a dominant-negative effect and inhibited  
87 HMGCR degradation, and (iv), siRNA-mediated depletion of gp78 resulted in decreased  
88 sterol-induced ubiquitination and degradation of HMGCR (Song et al., 2005). The same  
89 laboratory subsequently suggested that the sterol-induced degradation of HMGCR was

90 mediated by two ERAD E3 ligases, with TRC8 involved in addition to gp78 (Jo et al., 2011).  
91 However, these findings remain controversial as, despite confirming a for gp78 in the  
92 regulation of Insig-1 (Lee et al., 2006; Tsai et al., 2012), an independent study found no role  
93 for either gp78 or TRC8 in the sterol-induced degradation of HMGCR (Tsai et al., 2012).  
94 Therefore, the E3 ligase(s) responsible for the sterol-accelerated degradation of HMGCR  
95 remains disputed.

96 The introduction of systematic forward genetic screening approaches to mammalian systems  
97 (Carette et al., 2009; Wang et al., 2014) has made the unbiased identification of ubiquitin E3  
98 ligases more tractable, as demonstrated for the viral (Greenwood et al., 2016; Van den  
99 Boomen and Lehner, 2015; van de Weijer et al., 2014; Stagg et al., 2009) and endogenous  
100 regulation of MHC-1 (Burr et al., 2011; Cano et al., 2012).

101 To identify the E3 ligases governing HMGCR ERAD we applied a genome-wide forward  
102 genetic screen to a dynamic, cholesterol-sensitive reporter cell line, engineered to express a  
103 fluorescent protein fused to endogenous HMGCR. This approach identified cellular genes  
104 required for sterol-induced HMGCR degradation, including UBE2G2 and the RNF145 ERAD  
105 E3 ligase. The subtle phenotype observed upon RNF145 depletion alone, suggested  
106 redundant ligase usage. A subsequent, targeted ubiquitome CRISPR/Cas9 screen in  
107 RNF145-knockout cells showed RNF145 to be functionally redundant with gp78, the E3  
108 ligase originally implicated in HMGCR degradation. We confirmed that loss of gp78 alone  
109 showed no phenotype, while loss of both E3 ligases significantly inhibited the sterol-induced  
110 ubiquitination and degradation of HMGCR. Complete stabilisation required additional  
111 depletion of a third ligase - Hrd1. We find that endogenous RNF145 is an auto-regulated,  
112 sterol-responsive E3 ligase which is recruited to Insig proteins under sterol-replete  
113 conditions, thus promoting the regulated ubiquitination and sterol-accelerated degradation of  
114 HMGCR. Our data resolve the controversy of the E3 ligases responsible for the post-  
115 translational regulation of HMGCR and emphasise the complexity of the mammalian  
116 ubiquitin system in fine-tuning sterol-induced HMGCR turnover and cholesterol homeostasis.

117 **RESULTS**

118 **Targeted knock-in at the endogenous HMMGCR locus creates a dynamic, cholesterol-  
119 sensitive reporter**

120 To identify genes involved in the post-translational regulation of HMGCR, we engineered a  
121 cell line in which Clover, a bright fluorescent protein (Lam et al., 2012), was fused to the C-  
122 terminus of endogenous HMGCR, generating an HMGCR-Clover fusion protein (**Figure 1A**).  
123 The resulting HMGCR-Clover Hela single-cell clone expresses a dynamic, cholesterol-  
124 sensitive fluorescent reporter that is highly responsive to fluctuations in intracellular  
125 cholesterol. Basal HMGCR-Clover levels in sterol-replete tissue culture media were  
126 undetectable by flow cytometry (**Figure 1B**) and phenocopy endogenous WT HMGCR  
127 expression (**Figure 1C**, compare lanes 1 and 4). Following overnight sterol depletion, a ~  
128 25-fold increase in HMGCR-Clover expression was detected (shaded grey to blue histogram  
129 in **Figure 1D**, **Figure 1C** (lanes 2 and 5)), representing a combination of increased SREBP-  
130 induced transcription and decreased sterol-induced HMGCR degradation. Reintroduction of  
131 sterols induced the rapid degradation of HMGCR-Clover (~ 80% decrease within 2h),  
132 confirming the sterol-dependent regulation of the reporter (blue to red histogram in **Figure**  
133 **1D**). Residual, untagged HMGCR detected by immunoblot in the reporter cells under sterol-  
134 depleted conditions suggested that at least one HMGCR allele remained untagged (**Figure**  
135 **1C**, compare lanes 2 and 5). This unmodified allele allowed us to monitor both tagged and  
136 untagged forms of HMGCR. Inhibiting the enzymatic activity of HMGCR with mevastatin also  
137 stabilised HMGCR-Clover expression, as did inhibition of the proteasome (bortezomib) or  
138 p97 (NMS-873) (**Figure 1E**), confirming the rapid, steady-state degradation of the HMGCR  
139 reporter. Furthermore, we showed that CRISPR/Cas9-mediated ablation of both Insig-1 and  
140 -2 together induced a dramatic increase in HMGCR-Clover expression, equivalent to levels  
141 seen following sterol depletion (**Figure 1F**). Under these conditions, the SREBP-SCAP  
142 complex is not retained in the ER, leading to constitutive SREBP-mediated transcription of  
143 HMGCR-Clover, irrespective of the sterol environment. CRISPR-mediated gene disruption of

144 either Insig-1 or -2 alone caused only a small, steady-state rescue of HMGCR-Clover  
145 (**Figure 1F**), which was more pronounced with the loss of Insig-1 than Insig-2. While Insig-1-  
146 deficient cells were unable to completely degrade HMGCR upon sterol addition, only a minor  
147 defect in HMGCR degradation was seen in the absence of Insig-2 (**Figure 1F**), suggesting  
148 that Insig-1 is dominant over Insig-2 under these conditions. Finally, we confirmed that  
149 HMGCR-Clover was appropriately localised to the ER by confocal microscopy (**Figure 1G**).  
150 Thus, HMGCR-Clover is a dynamic, cholesterol-sensitive reporter, which rapidly responds to  
151 changes in intracellular cholesterol and is regulated in a proteasome-dependent manner.

152

153 **A genome-wide CRISPR/Cas9 screen identifies RNF145 as an E3 ligase required for**  
154 **HMGCR degradation**

155 To identify genes required for the sterol-induced degradation of HMGCR, we performed a  
156 genome-wide CRISPR/Cas9 knockout screen in HMGCR-Clover cells. We took advantage  
157 of the rapid decrease in HMGCR-Clover expression following sterol addition to cells starved  
158 overnight (16h) of sterols (**Figure 1D**), and enriched for rare genetic mutants with reduced  
159 ability to degrade HMGCR-Clover in response to sterols. To this end, HMGCR-Clover cells  
160 were transduced with a genome-wide CRISPR/Cas9 knockout library comprising 10 sgRNAs  
161 per gene (Morgens et al., 2017). Mutagenised cells were first depleted of sterols overnight;  
162 sterols were then reintroduced for 5h, at which point rare mutant cells with reduced ability to  
163 degrade HMGCR-Clover upon sterol repletion were enriched by fluorescence-activated cell  
164 sorting (FACS) (**Figure 2A**, gating shown in **Figure 2 – figure supplement 1A**). This  
165 process was repeated again eight days later to further purify the selected cells. The enriched  
166 population contained only a small percentage of cells (1.96% after sort 1, 24.49% after sort  
167 2) with increased steady-state HMGCR-Clover expression (green filled histogram in **Figure**  
168 **2B**). However, the majority of sterol-starved cells from this selected population showed  
169 impaired degradation of HMGCR-Clover after addition of sterols (compare red *versus* orange  
170 histogram (**Figure 2B**, compare lanes 6 and 9 in **Figure 2 – figure supplement 1B**)). The

171 broad distribution of this histogram (**Figure 2B** red histogram) suggested that the enriched  
172 cell population contains a variety of mutants which differ in their ability to degrade HMGCR-  
173 Clover.

174 The sgRNAs in the selected cells, and an unselected control library, were sequenced on the  
175 Illumina HiSeq platform (**Figure 2A (viii)**). Using the RSA algorithm, we identified a set of 11  
176 genes (-logP > 5), which showed significant enrichment in the selected cells. Many of these  
177 are known to be required for the sterol-induced degradation of HMGCR (**Figure 2C**) (König  
178 et al. 2007). The screen identified the E2 conjugating enzyme *UBE2G2* and its accessory  
179 factor *AUP1*, which recruits *UBE2G2* to lipid droplets and membrane E3 ubiquitin ligases  
180 (Klemm et al., 2011; Jo et al., 2013; Spandl et al., 2011; Christianson et al., 2012), as well as  
181 both Insig-1 and -2 (Yabe et al., 2002; Yang et al., 2002; Sever et al., 2003a). The role of the  
182 remaining hits is summarized (**Figure 2 – figure supplement 2**) and validation of selected  
183 hits as shown (Insig-1/2, **Figure 1D**; *UBE2G2*, *EHD1*, *GALNT11*, *LDLR* and *TECR*, **Figure 2**  
184 – **figure supplement 1C/D**).

185 Strikingly, the only ER-resident ubiquitin E3 ligase to emerge from the screen is RNF145, a  
186 poorly characterised ER-resident ubiquitin E3 ligase. RNF145 shares 27% amino acid  
187 identity with TRC8, which is one of the E3 ligases (together with gp78) previously suggested  
188 to ubiquitinate HMGCR (Jo et al., 2011). Interestingly, RNF145 also harbours a YLYF motif  
189 at its N-terminus, which is similar to the YIYF motif present in the sterol-sensing domain  
190 (SSD) of SCAP and HMGCR required for their binding to the Insig proteins (Yang et al.,  
191 2002; Sever et al., 2003a; Jiang et al., 2018; Cook et al., 2017; Zhang et al., 2017). The  
192 presence of the YLYF motif suggested that RNF145 might itself interact with the Insig  
193 proteins and therefore represented a promising candidate from our genetic screen.

194 To examine the role of RNF145 in HMGCR degradation, we designed four independent  
195 sgRNAs, either targeting RNF145 individually or as a pool. Under cholesterol-replete  
196 conditions, no accumulation of the HMGCR-reporter was observed in RNF145-depleted cells  
197 (top and middle rows, **Figure 2D**), but a small and highly reproducible decrease in HMGCR-

198 Clover degradation was seen following re-introduction of sterols (red histograms, bottom row  
199 **Figure 2D**), emphasising the utility of the endogenous fluorescent reporter in identifying  
200 subtle phenotypes. Since the identity of the E3 ligases regulating HMGCR turnover remains  
201 controversial, the modest effect of RNF145 loss on HMGCR-Clover sterol-induced  
202 degradation suggested the involvement of additional ligase(s). Our screen therefore  
203 identified both known and novel components implicated in sterol-dependent HMGCR ERAD.

204

### 205 **RNF145 together with gp78 are required for HMGCR degradation**

206 If a second E3 ligase is partially redundant with RNF145, its effect should be unmasked in  
207 RNF145-deficient cells. We therefore generated a focussed subgenomic sgRNA library  
208 targeting 1119 genes of the ubiquitin-proteasome system as described in **Materials and**  
209 **Methods**, including 830 predicted ubiquitin E3 ligases, and used this library to screen for  
210 genes required for the degradation of HMGCR in RNF145-deficient HMGCR-Clover cells  
211 (**Figure 3 – figure supplement 4B**, lane 2 for knockout validation). Due to the reduced  
212 complexity of this focussed library, only a single FACS enrichment step was used (**Figure**  
213 **3A**, red histogram).

214 Strikingly, this screen identified gp78 (gene name: *AMFR*) (**Figure 3B**, **Figure 3 – figure**  
215 **supplement 5**), the E3 ligase previously implicated in HMGCR degradation (Jo et al., 2011;  
216 Song et al., 2005; Fang et al., 2001). Taking a combined knockout strategy we asked  
217 whether gp78 and RNF145 are together responsible for HMGCR degradation. As predicted  
218 by the genetic approach (**Figure 3C(ii)**), there was no difference in sterol-induced HMGCR-  
219 Clover degradation between control and gp78-depleted HMGCR-Clover cells. Gp78 was not,  
220 therefore, a false-negative from our initial, genome-wide CRISPR/Cas9 screen (**Figure 2C**).  
221 Individual knockout of RNF145 again showed that sterol-induced HMGCR-Clover  
222 degradation was mildly impaired in RNF145-depleted cells (**Figure 3C (iii)**). However,  
223 sgRNA-mediated targeting of gp78 together with RNF145 (**Figure 3C (iv)**, see **Figure 3 –**

224 **figure supplement 4A** and **B** lane 3 for knockout validation), resulted in a significant  
225 increase in both steady-state HMGCR-Clover (**Figure 3C (iv)** grey to green filled  
226 histograms) and an inability to degrade HMGCR-Clover upon addition of sterols to sterol-  
227 starved cells (**Figure 3C (iv)** blue to red histogram), a phenotype comparable to UBE2G2  
228 deletion (**Figure 3C (v)**). Our results therefore suggest a partial functional redundancy  
229 between gp78 and RNF145 and imply that both ligases can independently regulate the  
230 sterol-induced degradation of HMGCR.

231

232 **RNF145 and gp78 regulate endogenous wild type HMGCR**

233 To confirm that the phenotypes observed in RNF145- and gp78-deficient HMGCR-Clover  
234 cells were representative of endogenous, wild type HMGCR regulation, we deleted RNF145  
235 and/or gp78 from WT HeLa cells and monitored endogenous HMGCR by immunoblot  
236 analysis. The sterol-induced degradation of HMGCR was assessed in four RNF145  
237 knockout clones, derived from two different sgRNAs (validation in **Figure 3 – figure**  
238 **supplement 1A, B**). No difference in the sterol-induced degradation of HMGCR was seen in  
239 these RNF145 knockout clones (**Figure 3 – figure supplement 2**, compare lanes 6 and 7-  
240 10). The subtle effect on HMGCR-Clover expression revealed by flow cytometry (**Figures**  
241 **2D and 3C**) may not be detected by the less sensitive immunoblot analysis. Similarly, loss of  
242 gp78 alone (**Figure 3 – figure supplement 3A** for sgRNA validation) did not affect HMGCR  
243 degradation (**Figure 3D**, compare lanes 6 and 7-10), but loss of gp78 together with RNF145  
244 resulted in a significant rescue of steady state HMGCR (**Figure 3E**, **Figure 3 – figure**  
245 **supplement 3C**). Following sterol addition, gp78/RNF145 double-knockout clones showed a  
246 marked (although still incomplete) reduction in sterol-induced HMGCR degradation (**Figure**  
247 **3F**, compare lanes 7+8 with 9-12). These data validate the phenotypes exhibited by the  
248 HMGCR-Clover reporter cell line and confirm a role for both gp78 and RNF145 in the sterol-  
249 induced degradation of endogenous HMGCR.

250

251 **RNF145 E3 ligase activity is required for HMGCR degradation**

252 To determine whether RNF145 E3 ligase activity is required for HMGCR degradation, we  
253 complemented a mixed population of gp78/RNF145 double-knockout HMGCR-Clover cells  
254 (**Figure 3 – figure supplement 4B**, lane 3 for knockout validation) with either epitope-  
255 tagged wild type RNF145, or a catalytically-inactive RNF145 RING domain mutant (C552A,  
256 H554A) (**Figure 4A**). The pronounced block in the sterol-induced degradation of HMGCR-  
257 Clover was rescued by expression of wild-type, but not the RNF145 RING domain mutant  
258 (**Figure 4B**, compare blue to red histogram). The E3 ligase activity of RNF145 is therefore  
259 critical for HMGCR ERAD.

260

261 **Endogenous RNF145 is an unstable E3 ligase, whose transcription is sterol-regulated**

262 Endogenous RNF145 has a short half-life (~ 2h) and displayed rapid, proteasome-mediated  
263 degradation (**Figure 5A (i)**), an observation confirmed in multiple cell lines (**Figure 5 –**  
264 **figure supplement 1A**). This rapid turnover of endogenous RNF145 contrasts sharply with  
265 the stability of endogenous gp78, which shows little degradation over the 10 hour chase  
266 period (**Figure 5A (i)**). Although RNF145 and gp78 both target HMGCR for degradation, the  
267 two ligases did not appear to be co-regulated as RNF145 stability was unaffected by gp78  
268 and vice-versa (**Figure 5A (i, ii)**, **Figure 5 – figure supplement 1B**). However, endogenous  
269 RNF145 was stabilised by deletion of its cognate E2 enzyme UBE2G2 (**Figure 5B**), and,  
270 furthermore, the catalytically-inactive RING domain mutant expressed in RNF145-deficient  
271 cells ( $\Delta$ R145#4 + R145-V5 (mut)) exhibited greater abundance at steady-state compared  
272 with its wild type counterpart (**Figure 3 – figure supplement 4C**). Together these data show  
273 that RNF145 is intrinsically unstable and rapidly turned over in an auto-regulatory manner.

274 Since RNF145 is rapidly turned over, we aimed to determine whether RNF145 gene  
275 transcription was sterol-responsive. Sterol depletion induced RNF145 ( $\sim 2.99 \pm 0.65$  fold

276 increase,  $p = 0.0009$ ) mRNA expression as well as HMGCR ( $\sim 12.26 \pm 3.16$  fold increase,  $p =$   
277 0.0004) mRNA expression (**Figure 5C**). This accumulation of endogenous RNF145 was  
278 suppressed following the addition of MBCD-complexed cholesterol (chol:MBCD) to the  
279 starvation media (**Figure 5D**), whereas gp78 abundance remained unaltered (**Figure 5 –**  
280 **figure supplement 1D**). RNF145 is therefore a unique, sterol-regulated E3 ligase whose  
281 expression is dependent on the cellular sterol status.

282

### 283 **Endogenous RNF145 shows a sterol-sensitive interaction with HMGCR and Insig-1**

284 The Insig proteins provide an ER-resident platform for sterol-dependent interactions between  
285 HMGCR and its regulatory components (Dong et al., 2012). RNF145 is sterol regulated and  
286 degrades HMGCR, making it important to determine whether it interacts directly with  
287 HMGCR, or via the Insig proteins. In sterol-replete but not sterol-deplete conditions,  
288 endogenous HMGCR co-immunoprecipitates both epitope-tagged RNF145 (**Figure 6A**,  
289 **Figure 3 – figure supplement 4C** lane 3 for relative RNF145-V5 levels upon reconstitution),  
290 as well as endogenous RNF145 (**Figure 6B**).

291 The low expression levels of endogenous RNF145 made any interaction with endogenous  
292 Insig-1 challenging to detect. We circumvented this problem by repeating the co-  
293 immunoprecipitation in UBE2G2 knockout cells, which express increased levels of  
294 endogenous RNF145 (**Figure 5B**). Under these conditions, RNF145 showed a sterol-  
295 dependent interaction with Insig-1, correlating with RNF145's association with HMGCR  
296 (**Figure 6C**). Importantly, endogenous RNF145 is not, therefore, continually bound to Insig-  
297 1, but, like HMGCR, associates with Insig-1 in a sterol-dependent manner.

298

299

300

301 **In the absence of RNF145 and gp78, Hrd1 targets HMGCR for degradation**

302 Despite our two genetic screens identifying a requirement for RNF145 and gp78 in HMGCR  
303 degradation (**Figure 2C** and **3B**), the combined loss of these two ligases failed to completely  
304 inhibit sterol-induced HMGCR degradation (**Figure 3C (iv)**; **Figure 7A (ii)**). Furthermore,  
305 ablation of UBE2G2 in RNF145/gp78 double-knockout cells further exacerbated the sterol-  
306 dependent degradation defect (**Figure 7A (iv)**), predicting the role for an additional E3  
307 ligase(s) utilising UBE2G2 in HMGCR degradation. We therefore assessed whether ablation  
308 of either of the two remaining ER-resident E3 ligases that use UBE2G2, TRC8 (van de  
309 Weijer et al., 2017) and Hrd1 (Kikkert et al., 2004), exacerbated the HMGCR-degradation  
310 defect in RNF145/gp78 double-knockout cells (**Figure 7**, **Figure 7 – figure supplement 1B**  
311 **and 2B** for knockdown validation). While the loss of TRC8 had no effect on HMGCR-Clover  
312 expression, the loss of Hrd1 in RNF145/gp78 double-knockout cells increased steady-state  
313 HMGCR-Clover expression and caused a complete block in the sterol-accelerated  
314 degradation of HMGCR-Clover (**Figure 7B (ii)**, **Figure 7 – figure supplement 1A** for  
315 validation with individual independent sgRNAs). The additive effect of Hrd1 depletion on the  
316 sterol-induced turnover of endogenous HMGCR was independently confirmed by  
317 immunoblot analysis (**Figure 7C**, compare lanes 2, 4 and 6) and was observed as early as  
318 60 minutes after sterol addition (**Figure 7 – figure supplement 1D**, compare lanes 7 and 9).  
319 Importantly, depletion of Hrd1, either alone or in combination with depletion of either gp78 or  
320 RNF145, did not affect HMGCR-Clover degradation (**Figure 7 – figure supplement 1C**).  
321 Moreover, TRC8 depletion affected neither steady-state HMGCR-Clover expression, nor  
322 sterol-induced HMGCR-Clover degradation (**Figure 7B (iii)**). Indeed, despite a functional  
323 TRC8 depletion (**Figure 7 – figure supplement 2B** for validation) (Stagg et al., 2009), we  
324 could detect no role for TRC8, depleted either alone or in combination with RNF145, in the  
325 sterol-induced degradation of HMGCR (**Figure 7 – figure supplement 2A**).

326 In summary, gp78 with RNF145 are the only combination of ligases whose loss inhibited  
327 HMGCR degradation. Hrd1 depletion also delays sterol-induced HMGCR degradation, but  
328 only in the absence of RNF145 and gp78.

329

330 **RNF145, gp78 and Hrd1 are required for sterol-accelerated HMGCR ubiquitination**

331 As a complete block of sterol-accelerated HMGCR degradation required the depletion of all  
332 three UBE2G2-dependent ligases, we determined how the sequential depletion of these  
333 ligases affected the ubiquitination status of HMGCR. The combined loss of RNF145 with  
334 gp78 showed a dramatic reduction in HMGCR ubiquitination, but a complete loss of  
335 ubiquitination required the depletion of all three ligases (**Figure 7D**). As predicted, depletion  
336 of UBE2G2 also caused a marked decrease in HMGCR ubiquitination. Taken together,  
337 these results demonstrate the remarkable plasticity of the HMGCR-degradation machinery.

338 **DISCUSSION**

339 The generation of a dynamic, cholesterol-sensitive endogenous HMGCR reporter cell line  
340 allowed an unbiased genetic approach to identify the cellular machinery required for sterol-  
341 accelerated HMGCR degradation. This reporter cell line has the advantage of being able to  
342 identify both complete and partial phenotypes and helps explain why the identity of the E3  
343 ligases responsible for the sterol-accelerated degradation of HMGCR has remained  
344 controversial. We find that three E3 ligases - RNF145, gp78 and Hrd1 - are together  
345 responsible for HMGCR degradation (**Figure 8**). The activity of the two primary ligases,  
346 RNF145 and gp78 is partially redundant as the loss of gp78 alone did not affect HMGCR  
347 degradation, while loss of RNF145 showed only a small reduction on HMGCR degradation.  
348 In the absence of both RNF145 and gp78, a third ligase, Hrd1, can compensate and partially  
349 regulate HGMCR degradation, but this effect of Hrd1 is only revealed in the absence of both  
350 RNF145 and gp78, and in no other identified combination.

351

352 Initial reports of a role for gp78 in HMGCR degradation, either alone (Song, Sever, &  
353 DeBose-Boyd, 2005) or in combination with TRC8 (Jo et al., 2011), were not reproduced in  
354 an independent study (Tsai et al., 2012) and so this important issue has remained  
355 unresolved. Our initial genome-wide screen successfully identified many of the components  
356 known to be required for sterol-accelerated HMGCR degradation (e.g. Insig-1/2, and AUP1,  
357 **Figure 2C**) (Sever et al., 2003b; Miao et al., 2010; Jo et al., 2013), thus validating the  
358 suitability of this genetic approach. The screen also identified the E2 conjugating enzyme  
359 UBE2G2 and the E3 ligase RNF145. Depletion of UBE2G2 prevented HMGCR degradation,  
360 implying that all ligases involved in HMGCR degradation utilise this E2 enzyme. In contrast,  
361 and despite being a high confidence hit in our screen, depletion of RNF145 caused a highly  
362 reproducible but small inhibition of sterol-accelerated degradation, confirming the sensitivity  
363 of the screen to detect partial phenotypes and predicting the requirement for at least one  
364 additional UBE2G2-dependent ligase. A subsequent, targeted ubiquitome library screen in

365 an RNF145-knockout reporter cell line confirmed a role for gp78 in HMGCR degradation.  
366 Gp78 has previously been shown to use UBE2G2 as its cognate E2 enzyme in the  
367 degradation of ERAD substrates (Chen et al., 2006). During preparation of this manuscript,  
368 the combined involvement of RNF145 and gp78 in HMGCR degradation in hamster (CHO)  
369 cells was also reported (Jiang et al., 2018), confirming the role for these ligases in other cell  
370 lines.

371

372 The availability of an RNF145-specific polyclonal antibody provides further insight into the  
373 expression and activity of endogenous RNF145, without the concerns of overexpression  
374 artefacts. RNF145 is an ER-resident E3 ligase with several unique features that make it well-  
375 suited for HMGCR regulation. A challenge facing all proteins responsible for cholesterol  
376 regulation is that the target they monitor, cholesterol, resides entirely within membranes. We  
377 find that RNF145, like SCAP and HMGCR, two key proteins involved in cholesterol  
378 regulation, is both sterol regulated and associates with its Insig binding partner in a sterol-  
379 responsive manner. Under sterol deplete conditions, endogenous RNF145 is associated with  
380 neither HMGCR nor Insig1, but the addition of sterols triggers RNF145 binding to the ER-  
381 resident Insig-1 protein (**Figure 5C**). Like HMGCR and SCAP, RNF145 contains a sterol-  
382 sensing domain in its transmembrane region (Cook et al., 2017), suggesting that sterols  
383 facilitate its association with Insigs. Similarly, the association of HMGCR with Insigs and  
384 gp78 is also sterol-dependent through its SSD (Lee et al., 2007). Therefore, sterols trigger  
385 the recruitment of RNF145 to HMGCR, leading to Insig-dependent HMGCR ubiquitination  
386 and degradation. This ability of RNF145 to rapidly bind Insigs following sterol availability is a  
387 feature shared with the related sterol-responsive proteins including HMGCR and SCAP and  
388 further supports a key role for this ligase in HMGCR regulation.

389

390 A striking feature of RNF145 is its short half-life and rapid proteasome-mediated  
391 degradation, which contrasts with the long-lived gp78 (**Figure 6A, Figure 5 – figure**  
392 **supplement 1B**). RNF145 is an intrinsically unstable ligase whose half-life is regulated  
393 through autoubiquitination and was not prolonged on binding to Insig proteins (data not  
394 shown). Its stability and turnover is RING- and UBE2G2-dependent, but independent of  
395 either the gp78 (**Figure 6A-C**) or Hrd1 ligase (**Figure 5 – figure supplement 1C**). As cells  
396 become sterol-depleted, the transcriptional increase in RNF145 (**Figure 6E**) likely  
397 anticipates the need to rapidly eliminate HMGCR, once normal cellular sterol levels are  
398 restored.

399 While it is not unusual for more than one ligase to be required for substrate ERAD  
400 degradation (Christianson and Ye, 2014; Morito et al., 2008; Stefanovic-Barrett et al., 2018),  
401 the redundancy in HMGCR turnover is intriguing. This may simply reflect the central role of  
402 HMGCR in the mevalonate pathway and the importance of a fail-safe mechanism of  
403 HMGCR regulation to both maintain substrates for non-sterol isoprenoid synthesis and  
404 prevent cholesterol overproduction. Alternative explanations can also be considered,  
405 particularly as the properties of RNF145 and gp78 are so different. Under sterol-deplete  
406 conditions gp78 also regulates the degradation of Insig-1, but following addition of sterols,  
407 the association of Insigs with SCAP displaces its binding to gp78 (Yang et al., 2002; Lee et  
408 al., 2006). Different Insig-associated complexes are therefore likely to co-exist within the ER  
409 membrane, under both sterol-replete and -deplete conditions, and therefore reflect the sterol  
410 microenvironment of the ER (Goldstein et al., 2006). Under these circumstances it might be  
411 advantageous to have more than one ligase regulating HMGCR. Alternatively, gp78 may  
412 provide basal control of the reductase, which can then be ‘fine-tuned’ by the sterol-  
413 responsive RNF145, reflecting the sterol concentration of the local ER environment. It will  
414 therefore be important to further understand the stoichiometry and nature of the different  
415 Insig complexes within the ER membrane. While all cells need to regulate their intracellular  
416 cholesterol, the contribution of each ligase to sterol regulation may also depend on their

417 differential tissue expression. In this regard, liver-specific ablation of gp78 in mice has been  
418 reported to lead to increased steady-state levels of hepatocyte HMGCR (Liu et al., 2012),  
419 whereas, gp78 knockout MEFs show no apparent impairment in HMGCR degradation (Tsai  
420 et al., 2012). Further delineation of the contribution of each ligase to HMGCR degradation in  
421 different tissues and cell types will be important.

422 A role for the Hrd1 E3 ligase in HMGCR regulation was unanticipated, and both orthologues  
423 (gp78 and Hrd1) of yeast Hrd1p, which regulates yeast HMGCR (Hmg2p), are therefore  
424 involved in mammalian HMGCR turnover. The best recognised function of Hrd1 is the  
425 ubiquitination of misfolded or unassembled ER-luminal and membrane proteins targeted for  
426 ERAD (Sato et al., 2009; Tyler et al., 2012; Christianson et al., 2008). Our finding that Hrd1  
427 is only involved in HMGCR regulation when the other two ligases are absent, suggests that  
428 under sterol-rich conditions, and in the absence of RNF145 or gp78, conformational changes  
429 in the sterol sensing domains of HMGCR to a less ordered state are recognised and  
430 targeted by the Hrd1 quality control pathway.

431 In summary, our unbiased approach to identify proteins involved in sterol-regulated HMGCR  
432 degradation resolves the ambiguity of the E3 ligases responsible, and further unveils  
433 additional control points in modulating the activity of this important enzyme in health and  
434 disease.

435

436 **CONFLICT OF INTEREST**

437 The authors declare no conflict of interest.

438

439 **AUTHOR CONTRIBUTIONS**

440 PJL, SAM, NV and RTT conceived the project. Experiments were carried out by SAM, NV,  
441 DJB and ASD. The CRISPR/Cas9 ubiquitin library was designed by JAN and SAM and  
442 generated by JAN and DJB. SAM, NV and PJL analysed the data. SAM and NV prepared  
443 the figures. NV, PJL and SM wrote the manuscript.

444

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460 **ABBREVIATIONS**

461	AMFR	-	Autocrine Motility Factor Receptor
462	B2M	-	beta-2-microglobulin
463	Chol	-	cholesterol
464	CHX	-	cycloheximide
465	CRISPR	-	clustered regularly interspaced short palindromic repeats
466	CTR	-	control
467	ER	-	endoplasmic reticulum
468	ERAD	-	ER-associated degradation
469	FCS	-	fetal calf serum
470	Gp78	-	glycoprotein 78
471	HMGCR	-	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
472	Hrd1	-	HMG-CoA Reductase Degradation 1
473	IAA	-	iodoacetamide
474	Insig-1/2	-	Insulin-induced gene-1/2
475	LPDS	-	lipoprotein-deficient serum
476	MBCD	-	methyl- $\beta$ -cyclodextrin
477	RNF145	-	RING finger protein 145
478	SD	-	sterol-depleted
479	SREBP2	-	Sterol Regulatory Element Binding transcription factor 2
480	TRC8	-	Translocation in renal carcinoma on chromosome 8

481	UBE2G2	-	Ubiquitin-conjugating enzyme E2 G2
482	UPS	-	ubiquitin proteasome system
483	VCP	-	Valosin-containing protein
484	WT	-	wild-type

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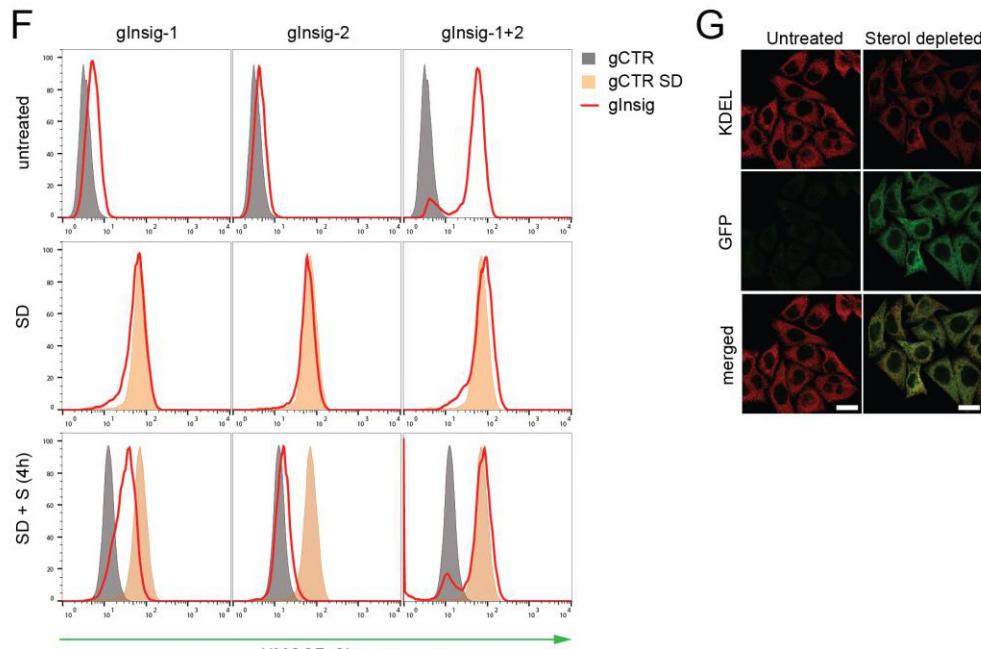
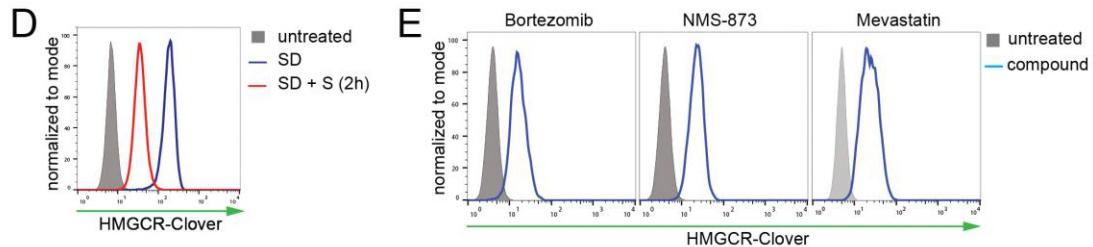
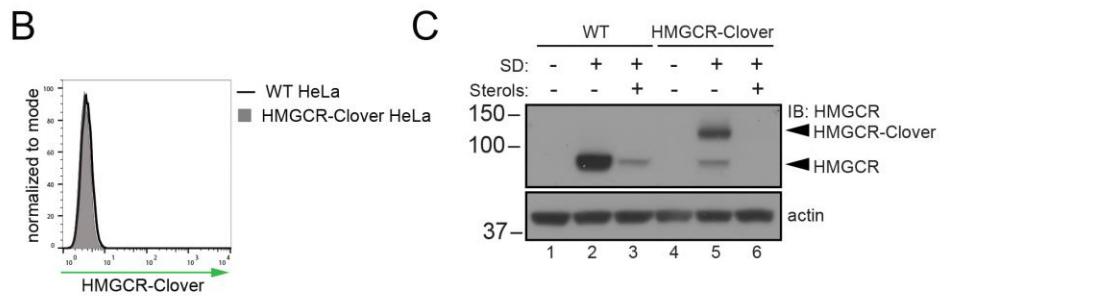
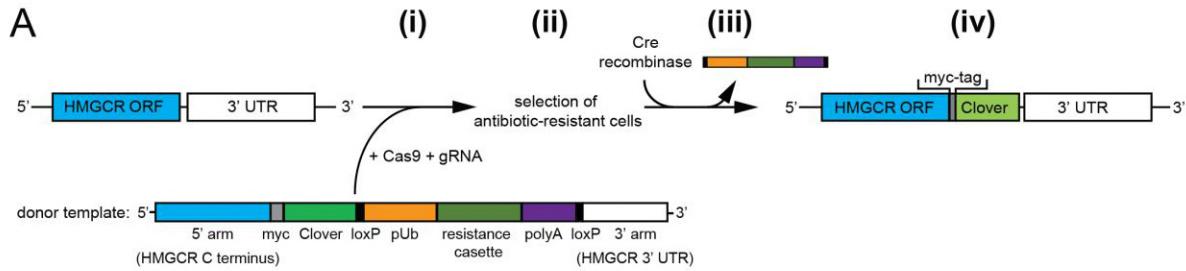
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677

678

## FIGURES

### FIGURE 1



679  
680

681 **Figure 1. Fluorescent protein tagging of endogenous HMGCR generates a cholesterol-  
682 sensitive dynamic reporter.**

683 **(A)** Schematic showing generation of the HMGCR-Clover reporter. (i) The endogenous  
684 HMGCR locus of HeLa cells was modified by transfection of Cas9, gRNA and a donor  
685 template. The 5' and 3' arm of the donor template were designed as homologous sequences  
686 encoding the C-terminal region and 3' UTR of the HMGCR gene. The C-terminal Clover  
687 (green) was appended in frame to the ORF of HMGCR (blue) including a myc-tag (grey) as  
688 spacer and an antibiotic resistance cassette flanked by loxP sites. (ii) Cells having stably  
689 integrated the recombination construct were enriched by antibiotic selection. (iii) The  
690 resistance cassette was removed by transient transfection of Cre recombinase to yield  
691 endogenous, C-terminally modified HMGCR (iv). ORF, open reading frame; UTR,  
692 untranslated region.

693 **(B – E)** HMGCR-Clover reporter phenocopies untagged HMGCR.

694 **(B)** HMGCR-Clover expression (shaded histogram) as detected by flow cytometry under  
695 sterol-replete conditions.

696 **(C)** Immunoblot of HMGCR in sterol-depleted (SD) HeLa WT vs. HMGCR-Clover cells -/+  
697 sterols (S) for 2h. For sterol depletion (SD), cells were switched to SD medium for 16h.  
698 Whole-cell lysates were separated by SDS-PAGE and HMGCR(-Clover) detected with an  
699 HMGCR-specific antibody.

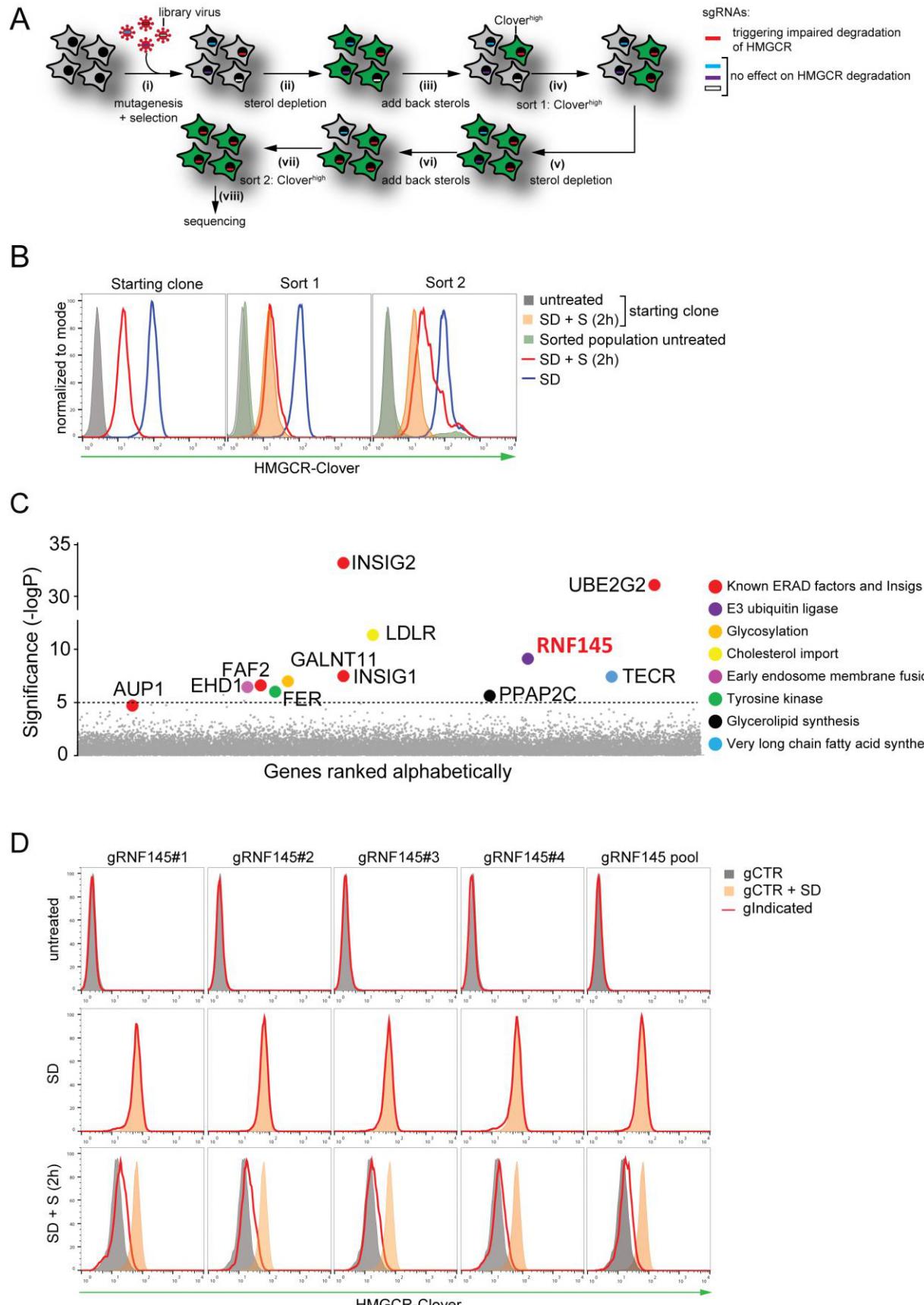
700 **(D)** Cytofluorometric analysis of HeLa HMGCR-Clover cells cultured in sterol-replete  
701 (shaded histogram) vs. sterol-depleted medium (SD) (16h, blue line histogram). Sterols (S)  
702 (2 µg/ml 25-hydroxycholesterol, 20 µg/ml cholesterol) were added back for 2h (red line  
703 histogram). **(E)** Flow cytometric analysis of HMGCR-Clover cells treated overnight with  
704 Bortezomib (25 nM), mevastatin (10 µM), or NMS-873 (10 µM) for 8h.

705 **(F)** CRISPR/Cas9-mediated depletion of Insig-1 and -2 together induce a dramatic increase  
706 in HMGCR-Clover expression, equivalent to sterol depletion (SD). HMGCR-Clover cells

707 transiently expressing the indicated Insig-1/2 specific gRNAs (4 sgRNAs per gene) were  
708 treated as in (D) and, where indicated, sterols (S) added back for 4h (SD + S, bottom row).  
709 Representative of  $\geq 3$  independent experiments.

710 **(G)** Immunofluorescence analysis of HMGCR-Clover and KDEL (ER marker) expression,  
711 showing co-localisation in sterol-depleted (SD, 16h) cells. Scale bar = 20  $\mu$ m.

## FIGURE 2



714 **Figure 2. Genome-wide CRISPR knockout screen identifies a role for RNF145 in the**  
715 **sterol-dependent degradation of HMGCR.**

716 **(A - B)** Schematic view of the CRISPR/Cas9 knockout screen workflow and FACS  
717 enrichment.

718 **(A)** HMGCR-Clover cells transduced with a genome-wide gRNA library targeting 19930  
719 genes (i) were subjected to sterol-starvation/repletion (ii, iii, v, vi). Mutants unable to degrade  
720 HMGCR-Clover despite sterol repletion (Clover<sup>high</sup>) were enriched by two sequential rounds  
721 of FACS (iv, vii) and candidate genes identified by deep sequencing (viii).

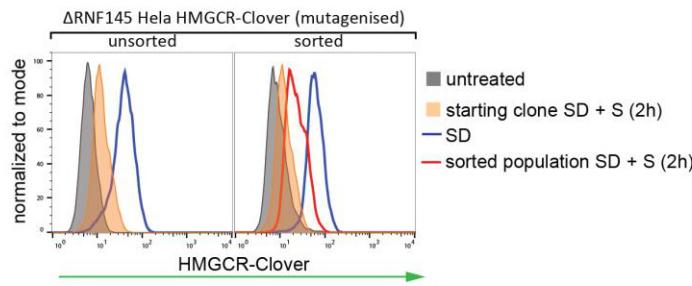
722 **(B)** Enrichment of HMGCR-Clover mutants after sort 1 and sort 2 (red line histograms,  
723 corresponding to steps 'iv' and 'vii' in Figure 2A) as determined by flow cytometry. Cells were  
724 treated as described in Figure 1D. SD, sterol-depleted; S, sterols.

725 **(C)** Candidate genes identified in the genome-wide knockout screen. Genes scoring above  
726 the significance threshold of  $-\log P \geq 5$  (dotted line) and AUP1 ( $-\log P = 4.7$ ) are highlighted.

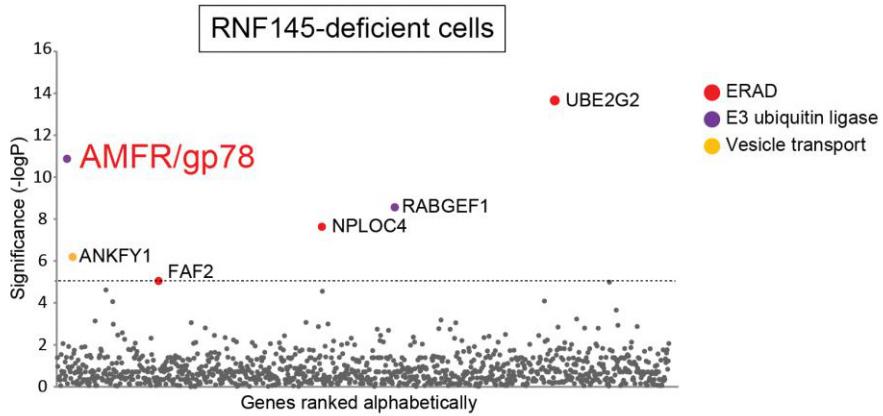
727 **(D)** RNF145 depletion mildly impairs sterol-accelerated HMGCR-Clover degradation.  
728 HMGCR-Clover cells transiently expressing 4 independent RNF145-specific sgRNAs  
729 (gRNF145#1-4, red line histogram), individually or as a pool, vs. gB2M (gCTR) were sterol-  
730 depleted overnight (middle row, SD) and re-examined by flow cytometry following 2h sterol  
731 addition (bottom row, SD + S). Representative of  $\geq 3$  independent experiments.

## FIGURE 3

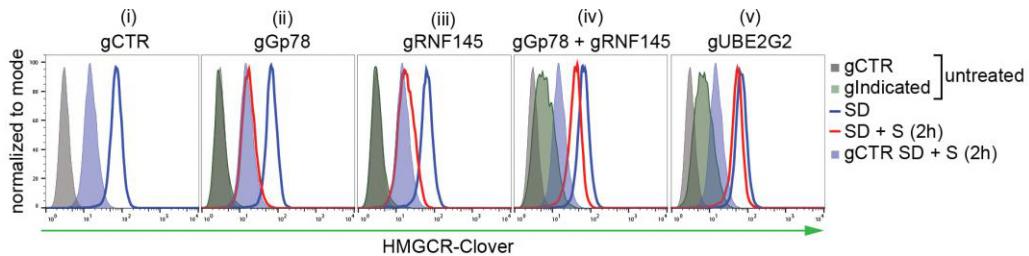
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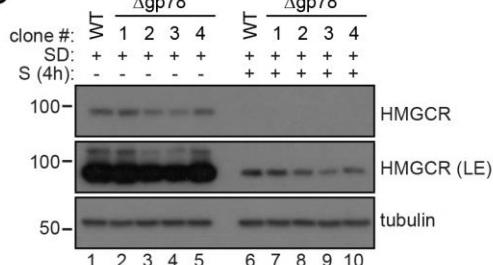
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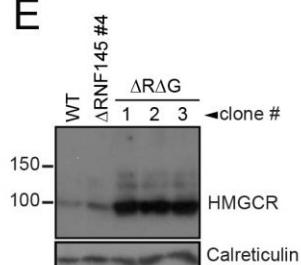
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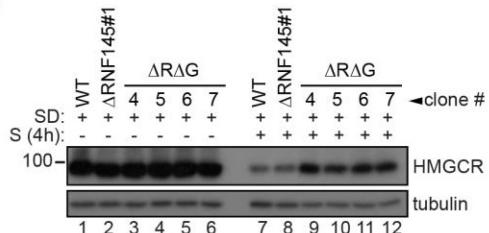
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F



733 **Figure 3. RNF145 together with gp78 are required for HMGCR degradation**

734 **(A - B)** FACS enrichment and scatter plot of candidate genes identified in the ubiquitome-  
735 targeted knockout screen. **(A)** HeLa HMGCR-Clover  $\Delta$ RNF145#5 cells were mutagenized  
736 using a targeted ubiquitome-specific sgRNA library and mutant cells showing impaired  
737 sterol-dependent degradation of HMGCR-Clover were enriched by FACS. Enrichment is  
738 represented by a broad population of Clover<sup>high</sup> cells in the presence of sterols (S, 2h) after  
739 overnight sterol depletion (blue to red histogram).

740 **(B)** Genes scoring above the significance threshold of  $-\log P \geq 5$  (dotted line) are highlighted.

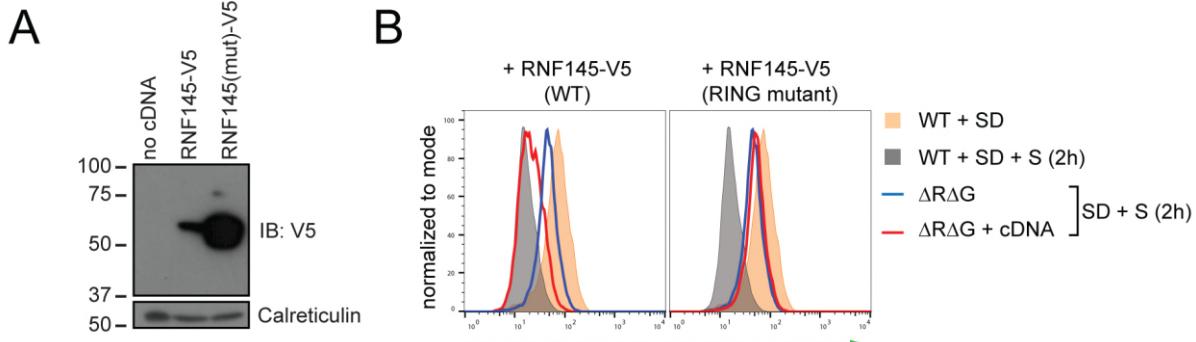
741 **(C - F)** sgRNA targeting of gp78 together with RNF145 increases steady-state HMGCR-  
742 Clover and inhibits sterol-accelerated degradation of sterol-starved HMGCR-Clover. **(C)**  
743 HMGCR-Clover cells transiently transfected with indicated sgRNAs were sterol-depleted  
744 (SD) overnight (blue line histogram) and sterols (2  $\mu$ g/ml 25-hydroxycholesterol, 20  $\mu$ g/ml  
745 cholesterol) added back (SD+S) for 2h (red line histogram or blue shaded histogram for  
746 gCTR). Representative of  $\geq 3$  independent experiments.

747 **(D)** Four independent gp78 knockout clones (#1-4) or WT cells were sterol-depleted (16h)  $\pm$   
748 S (4h) and HMGCR levels monitored by immunoblotting. LE, long exposure.

749 **(E)** HMGCR steady-state levels in three RNF145/gp78 double knockout clones ( $\Delta$ R $\Delta$ G #1-  
750 3).

751 **(F)** Four RNF145/gp78 double-knockout clones ( $\Delta$ R $\Delta$ G #4 - 7), RNF145 knockout, and WT  
752 cells were sterol-depleted (SD) overnight and HMGCR expression assessed  $\pm$  sterols (4h)  
753 by immunoblot analysis.

## FIGURE 4



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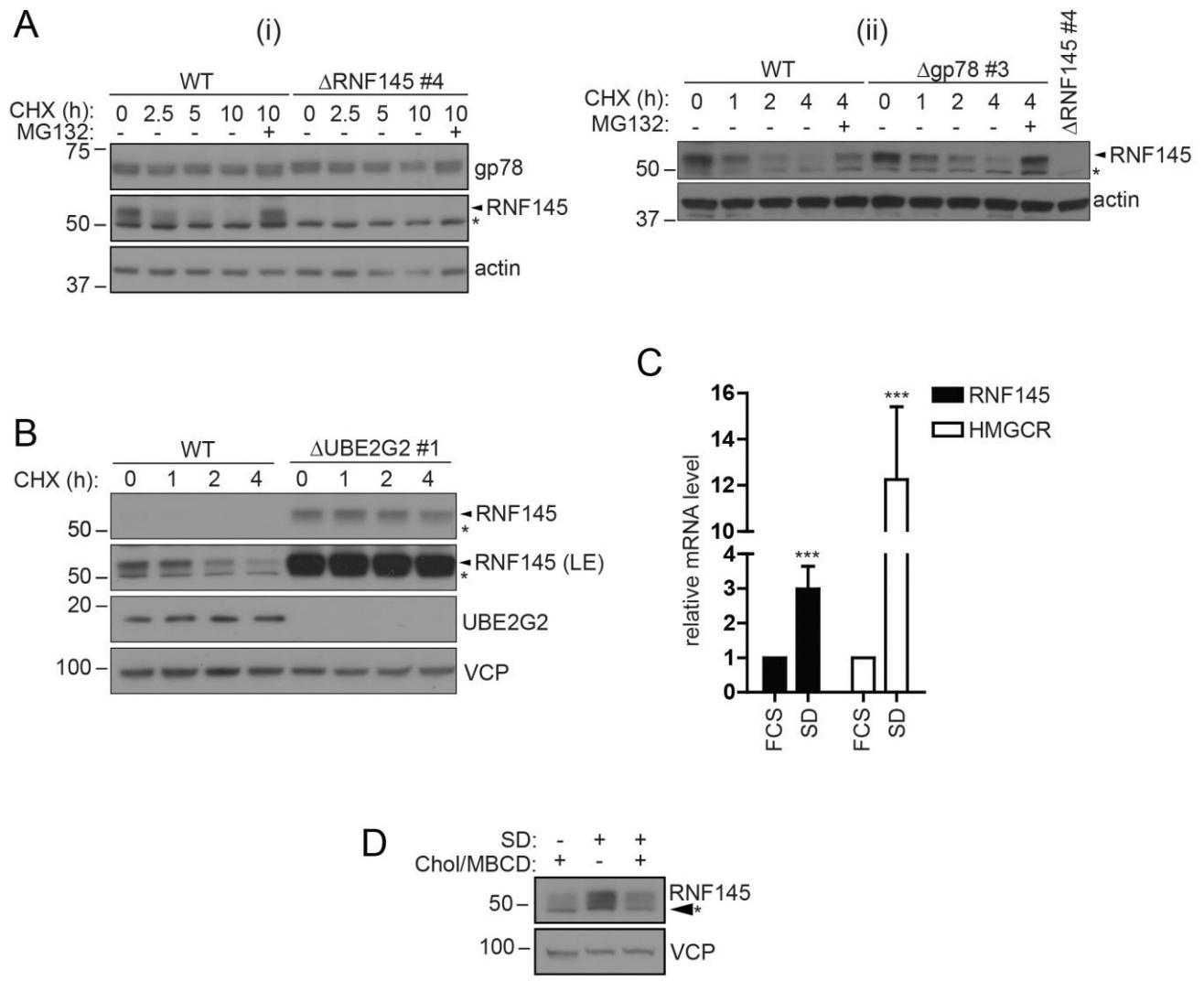
755 **Figure 4. RNF145 E3 ligase activity is required for HMGCR degradation.**

756 **(A)** Exogenous expression of RNF145 and RING-mutant RNF145 in HMGCR-Clover cells.

757 RNF145/gp78 double-knockout HMGCR-Clover cells were transduced with lentivirus  
758 expressing either RNF145-V5 or a catalytically inactive RING domain mutant  
759 RNF145(C552A, H554A)-V5 cDNA and cell lysates separated by SDS-PAGE and  
760 subsequent immunoblot analysis. IB, immunoblot.

761 **(B)** Wildtype (WT) but not RING mutant RNF145 complements the RNF145-deficient  
762 phenotype. RNF145/gp78 double-knockout HMGCR-Clover cells ( $\Delta R\Delta G$  #11) were  
763 transduced with lentivirus expressing either RNF145-V5 or a catalytically inactive RING  
764 domain mutant RNF145(C552A, H554A)-V5 cDNA. Cells were sterol-depleted (16h) and  
765 after sterol repletion (2h), HMGCR-Clover levels were assessed by flow cytometry.

## FIGURE 5



766  
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768 **Figure 5. RNF145 is an intrinsically unstable, sterol-responsive E3 ligase.**

769 **(A and B)** RNF145 has a short half-life and is auto-regulated by UBE2G2.

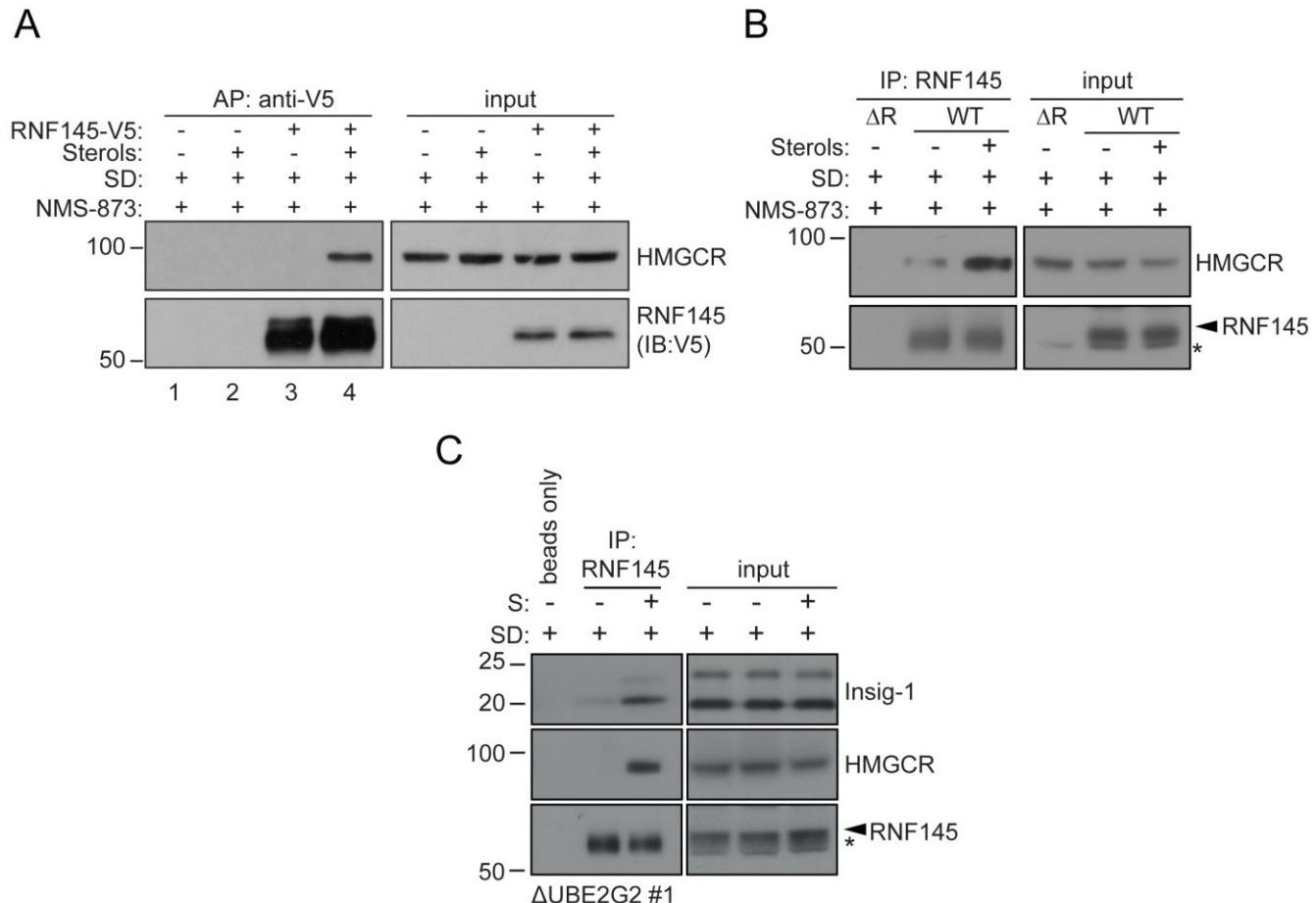
770 **(A)** Translational shutoff analysis of gp78 in WT *versus*  $\Delta$ RNF145 #4 (i) or RNF145 in  $\Delta$ gp78  
771 #3 cells (ii) treated with cycloheximide (CHX, 1  $\mu$ g/ml)  $\pm$  MG-132 (20  $\mu$ g/ml) for the indicated  
772 times. Non-specific bands are indicated by an asterisk (\*). Representative of  $\geq 2$  independent  
773 experiments.

774 **(B)** Immunoblot analysis of WT and  $\Delta$ UBE2G2 cells treated with CHX (1  $\mu$ g/ml) for the  
775 indicated times. VCP serves as a loading control. LE, long exposure.

776 **(C and D)** Sterol depletion induces transcriptional activation and increased levels of RNF145  
777 protein. **(C)** Relative RNF145 and HMGCR mRNA levels as measured by qRT-PCR in HeLa  
778 cells grown in 10% FCS (FCS) or sterol-depleted (SD, 10% LPDS + 10  $\mu$ M mevastatin + 50  
779  $\mu$ M mevalonate) for 48 h. Mean  $\pm$  S.D. (n = 4) and significance are shown, unpaired  
780 Students t-test: \*\*\*p  $\leq$  0.001.

781 **(D)** HeLa cells were grown under sterol-rich or sterol-deplete conditions (as indicated) for 48  
782 h in the presence of mevastatin (10  $\mu$ M) and mevalonate (50  $\mu$ M)  $\pm$  complexed cholesterol  
783 (chol:MBCD, 37.5  $\mu$ M). Whole cell lysates were separated by SDS-PAGE and underwent  
784 immunoblot analysis. Non-specific bands are indicated (\*). Representative of  $\geq 2$   
785 independent experiments.

## FIGURE 6



786  
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788 **Figure 6. Endogenous RNF145 shows sterol-sensitive binding to Insig-1 and HMGCR.**

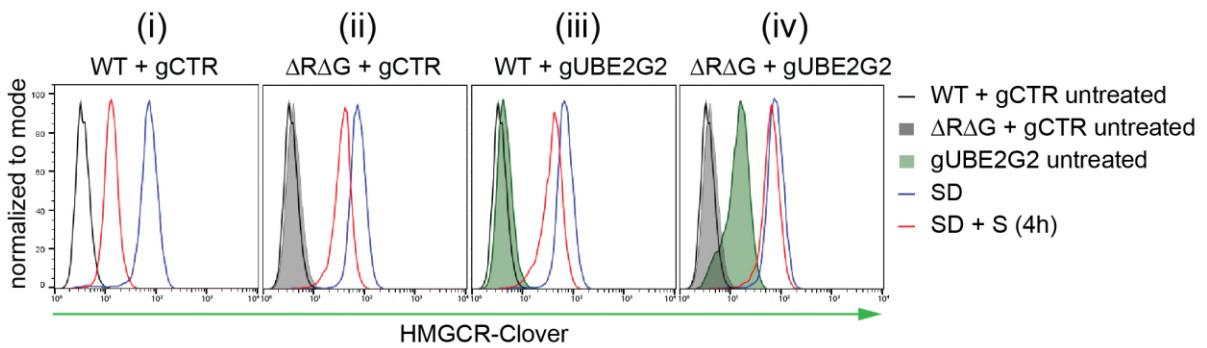
789 **(A)** Exogenous RNF145 shows sterol-sensitive binding to HMGCR. RNF145 knockout cells  
790 stably reconstituted with RNF145-V5 ( $\Delta$ R145 #4 + R145-V5, as shown in Figure 3 – figure  
791 supplement 4 C, lane 3) were sterol-depleted (SD, 20h) and sterols (S) added back for 1h in  
792 the presence of NMS-873 (10  $\mu$ M, 1.5h). RNF145-V5 was affinity-purified (AP) and HMGCR  
793 detected by immunoblotting. Representative of  $\geq$  3 independent experiments.

794 **(B - C)** Endogenous RNF145 shows sterol-sensitive binding to HMGCR and Insig-1. **(B)**  
795 HeLa WT or  $\Delta$ RNF145 #4 ( $\Delta$ R) cells were treated as in (A) and endogenous RNF145 was  
796 immunoprecipitated (IP), and RNF145 and HMGCR detected by immunoblot analysis. Non-  
797 specific bands are designated by an asterisk (\*). Representative of  $\geq$  3 independent  
798 experiments.

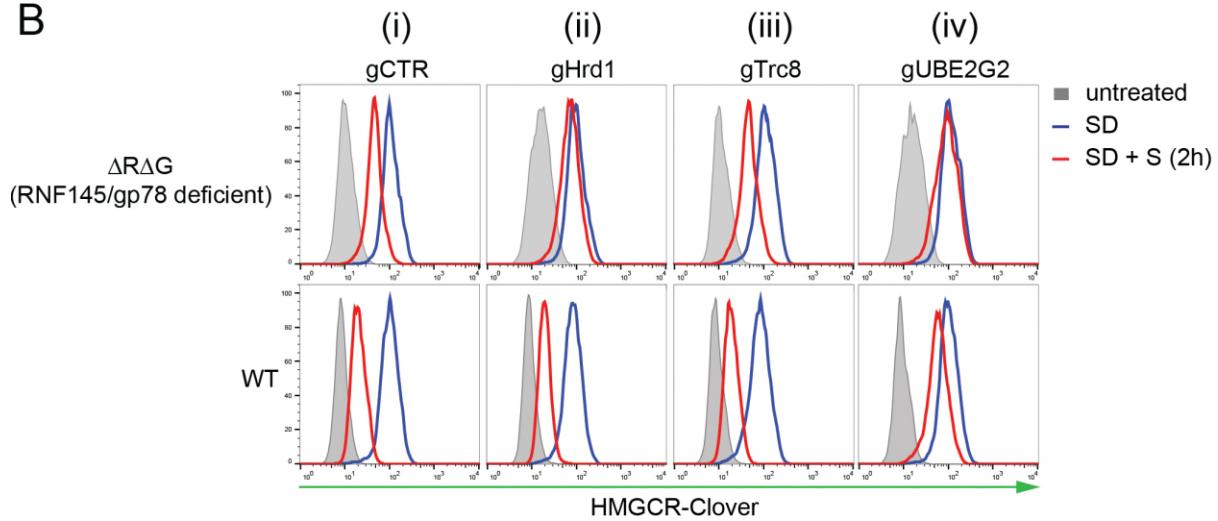
799 **(C)** HeLa UBE2G2 knockout cells ( $\Delta$ UBE2G2 #1) were sterol-depleted (SD, 20h) and sterols  
800 (S) added for 1h. Endogenous RNF145 was affinity-purified and following SDS-PAGE  
801 separation, Insig-1 and HMGCR detected by immunoblot analysis. Representative of  $\geq$  2  
802 independent experiments.

## FIGURE 7

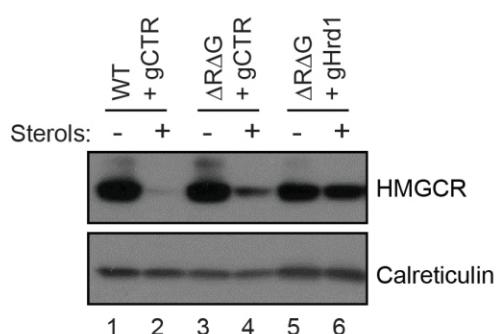
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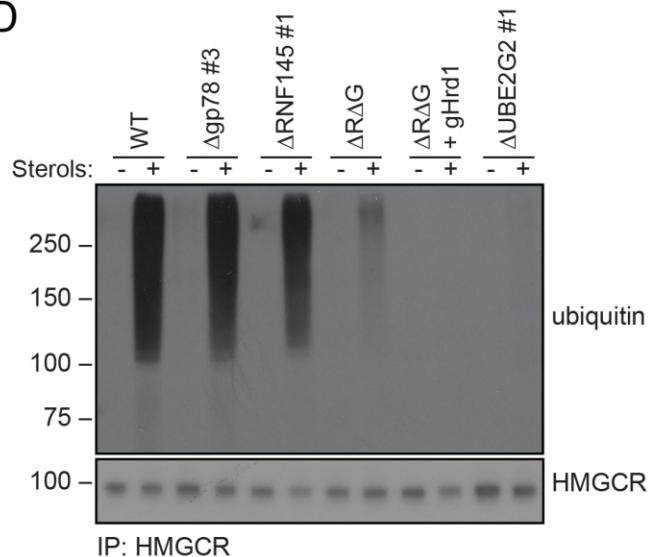
B



C



D



805 **Figure 7. In the absence of RNF145 and gp78, Hrd1 targets HMGCR for ubiquitination**  
806 **and degradation**

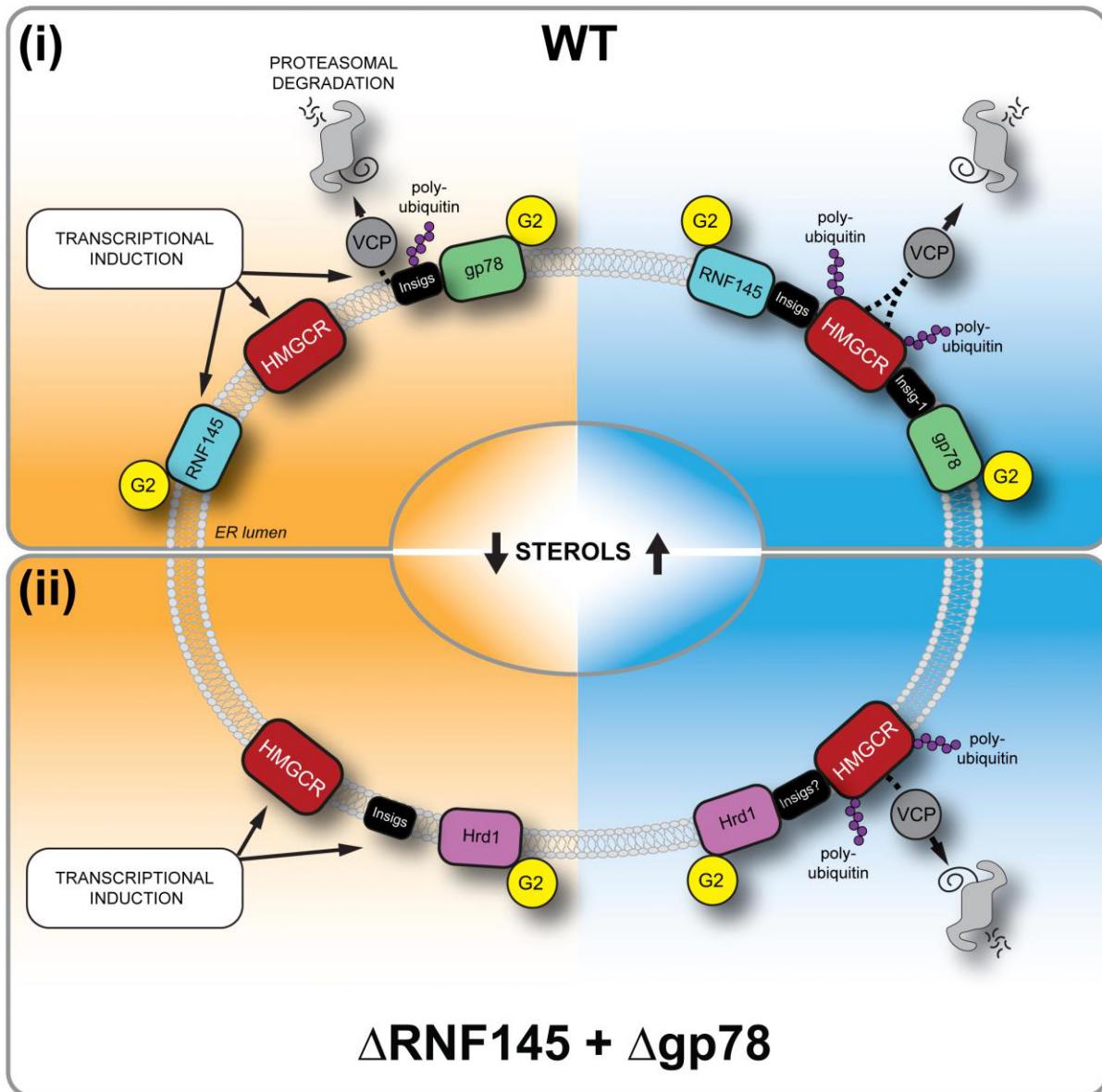
807 **(A)** Loss of gp78, RNF145 and UBE2G2 exert an additive effect on HMGCR degradation.  
808 WT or RNF145/gp78 double knockout ( $\Delta R\Delta G$  #11) HeLa HMGCR-Clover cells transiently  
809 expressing gRNAs targeting UBE2G2 (gUBE2G2) or B2M (gCTR) were enriched by  
810 puromycin selection, sterol-depleted (SD) overnight and HMGCR-Clover expression  
811 assessed  $\pm$  sterols (S, 4h). Representative of 3 independent experiments.

812 **(B and C)** A targeted gene approach shows loss of Hrd1 from RNF145/gp78 double  
813 knockout cells blocks sterol-accelerated degradation of HMGCR. **(B)** WT and  $\Delta RNF145$   
814  $\Delta gp78$  ( $\Delta R\Delta G$ ) HMGCR-Clover cells transfected with gCTR, a pool of four sgRNAs targeting  
815 either Hrd1 (gHrd1), or TRC8 (gTRC8) or gUBE2G2, were sterol-depleted (SD, 20h) and  
816 HMGCR-Clover expression assessed  $\pm$  sterols (S, 2h).

817 **(C)** WT and RNF145+gp78 double knockout cells ( $\Delta R\Delta G$  #7) HMGCR cells were transfected  
818 with a pool of four Hrd1-specific sgRNAs or gCTR, and sterol-depleted overnight before  
819 addition of sterols (4h) and analysis by SDS-PAGE and immunoblotting. RNF145 and gp78  
820 knockout validation is shown in **Figures 3 – figure supplement 1B ( $\Delta RNF145$ #1)** and  
821 **Figure 3 – figure supplement 3B ( $\Delta R\Delta G$ #7)**, respectively.

822 **(D)** RNF145, gp78 and Hrd1 are required for sterol-accelerated HMGCR ubiquitination.  
823 HMGCR was immunoprecipitated (IP) from the indicated cell lines grown in sterol-depleted  
824 media (20h)  $\pm$  sterols (2h). MG-132 (50  $\mu$ M) was added 30 minutes before sterol  
825 supplementation. Ubiquitinated HMGCR was detected using an anti-ubiquitin antibody.

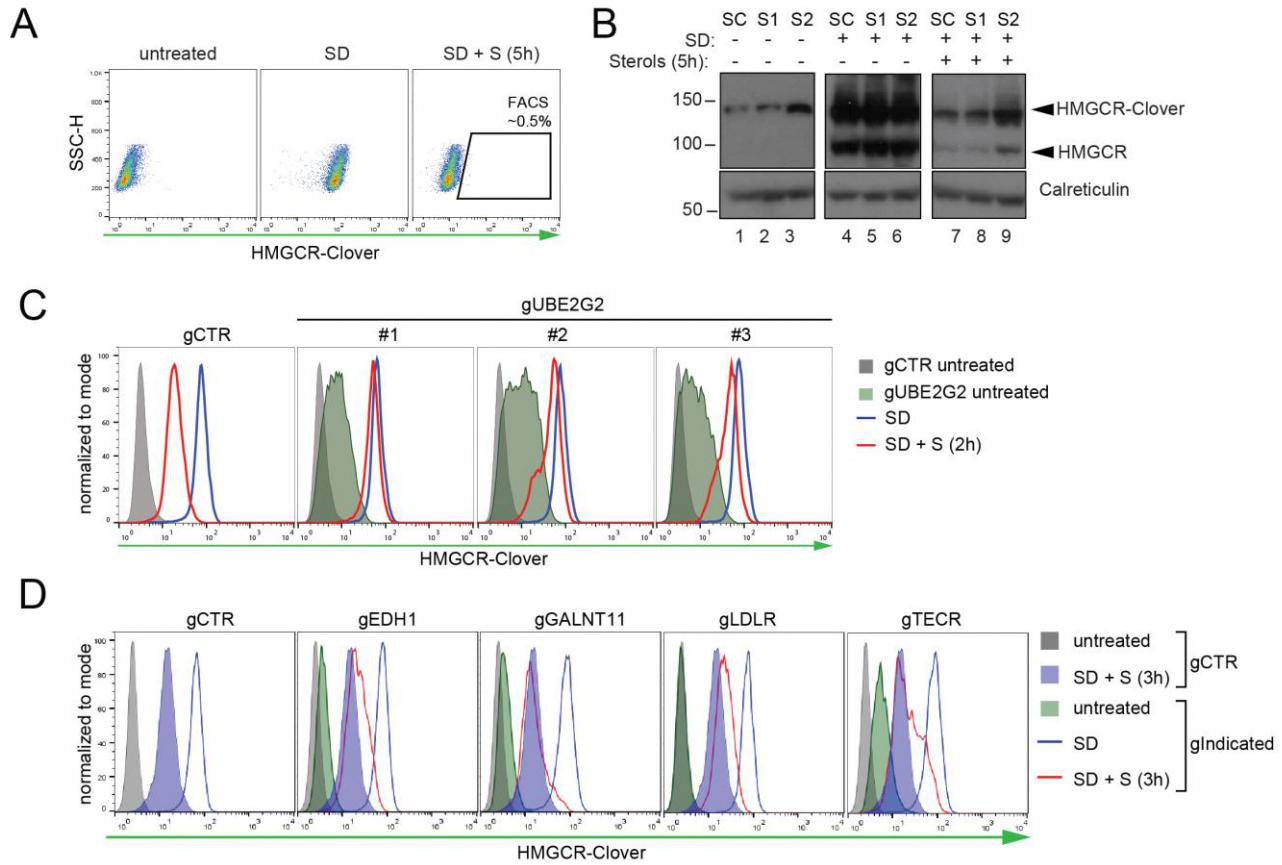
## FIGURE 8



826  
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828 **Figure 8. Sterol-induced degradation by RNF145, gp78, and Hrd1. (i)** Under sterol-  
829 depleted conditions (shaded orange), HMGCR, Insig1, and RNF145 are transcriptionally  
830 induced leading to accumulation of RNF145 and HMGCR. Insigs are continually turned over  
831 by gp78-mediated polyubiquitination, extracted from the membrane by VCP and degraded  
832 by the 26S proteasome. HMGCR stability is dramatically increased as it is not engaged by  
833 either RNF145, gp78 or their shared E2 ubiquitin ligase UBE2G2 (G2). In the presence of  
834 sterols (shaded blue), RNF145 and gp78 are recruited to HMGCR in an Insig-assisted  
835 fashion, mediating the sterol-accelerated and UBE2G2-dependent degradation of HMGCR  
836 by the UPS. Under these conditions both RNF145 and gp78 can independently ubiquitinate  
837 HMGCR, which is then extracted from the ER membrane in a VCP-dependent manner. The  
838 stoichiometry and make-up of the different Insig complexes within the ER membrane are  
839 unknown **(ii)** When RNF145 and gp78 are not available, Hrd1 and UBE2G2 can promote  
840 removal of HMGCR in the presence of sterols.

## FIGURE 2 - FIGURE SUPPLEMENT 1



841

842 **Figure 2 – figure supplement 1. Genome-wide screen for proteins involved in HMGCR**

843 **ERAD. (A)** Gating strategy to enrich for Clover<sup>high</sup> mutants with impaired sterol-induced  
844 HMGCR-Clover degradation. HeLa HMGCR-Clover cells mutagenized with sgRNA library  
845 were subjected to overnight sterol depletion (SD) before adding back sterols (SD + S) for 5h.  
846 Typically, the highest ~ 0.5% of Clover<sup>high</sup> cells were selected for enrichment (indicated).

847 **(B)** Immunoblot analysis for HMGCR-Clover enrichment after sort 1 (S1), sort (S2) as  
848 compared to the starting clone (SC). Cells were sterol-depleted (SD) overnight ± sterols (2h).

849 **(C)** Flow cytometric analysis of HMGCR-Clover cells transiently expressing three  
850 independent sgRNAs (gUBE2G2 #1-3) versus gB2M (gCTR) after overnight sterol-depletion  
851 ± sterols (2h).

852 (D) HMGCR-Clover cells were transfected with a pool of four sgRNAs for each indicated  
853 gene or a sgRNA against B2M (gCTR) and treated as in (C).

854 **Figure 2 – figure supplement 2.** Candidate genes ( $-\log(p) \geq 5$ ) identified in a genome-wide  
855 CRISPR/Cas9 screen for proteins involved HMGCR degradation.

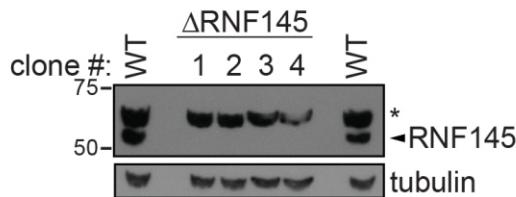
Gene	Full name	$-\log(p)^*$	Function
AUP1	Ancient Ubiquitous Protein 1	4.70	ERAD
EHD1	EH Domain Containing 1	6.50	Early endosome membrane fusion
FAF2	Fas Associated Factor Family Member 2	6.63	ERAD
FER	Tyrosine-protein kinase Fer	6.05	Tyrosine kinase
GALNT11	Polypeptide N-acetylgalactosaminyltransferase 11	7.03	Protein glycosylation
INSIG1	Insulin Induced Gene 1	7.50	Cholesterol metabolism
INSIG2	Insulin Induced Gene 2	33.24	Cholesterol metabolism
LDLR	Low Density Lipoprotein Receptor	11.44	Cholesterol metabolism
PPAP2C	Phospholipid phosphatase 2	5.67	Glycerolipid synthesis
RNF145	RING finger protein 145	9.18	E3 ubiquitin ligase
TECR	Very-long-chain enoyl-CoA reductase	7.45	Very-long chain fatty ester synthesis
UBE2G2	Ubiquitin Conjugating Enzyme E2 G2	31.14	E2 ubiquitin ligase

856 \*Only statistically significant hits ( $-\log(p) > 5$ ) are shown.

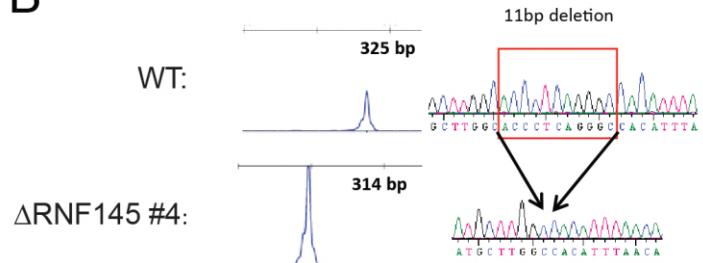
857

## FIGURE 3 - FIGURE SUPPLEMENT 1

A



B



858

859

860 **Figure 3 – figure supplement 1. Validation of RNF145 knockout clones.**

861 **(A)** Immunoblot for RNF145 in WT vs. four RNF145 HeLa knockout ( $\Delta\text{RNF145}$ ) clones,

862 generated with 2 independent sgRNAs. Non-specific bands are indicated (\*).

863 **(B)** Confirmation of RNF145 knockout clone #4 ( $\Delta\text{RNF145} \#4$ ). The genomic region

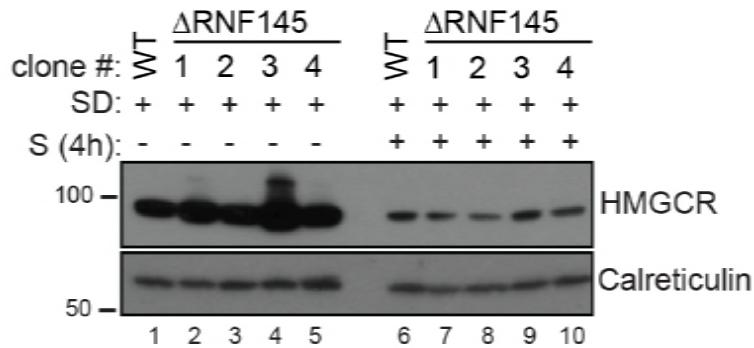
864 surrounding the predicted sgRNA annealing site was amplified using fluorescent primers and

865 amplicon size was determined capillary electrophoresis (Agilent Bioanalyzer 2100).

866 Fluorescent traces are shown alongside amplicon sequences as obtained by Sanger

867 sequencing, confirming an 11 bp deletion (indicated).

## FIGURE 3 - FIGURE SUPPLEMENT 2

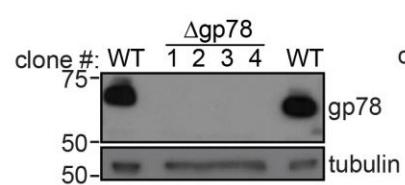


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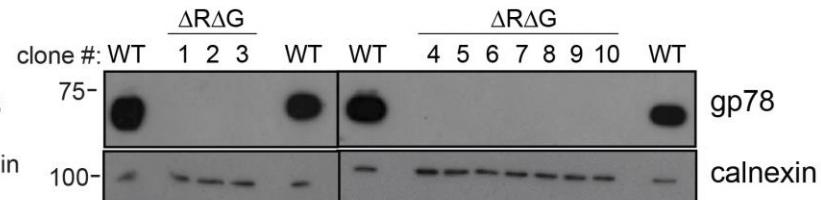
869 **Figure 3 – figure supplement 2. RNF145 loss is insufficient to block sterol-induced**  
870 **HMGR degradation.** Cells were sterol-depleted (SD) overnight before addition of sterols  
871 (S, 4h). Whole-cell lysates from WT and four RNF145 knockout clones (#1-4) were  
872 separated by SDS-PAGE and HMGR levels visualised by immunoblot analysis. Calreticulin  
873 serves as a loading control.

## FIGURE 3 - FIGURE SUPPLEMENT 3

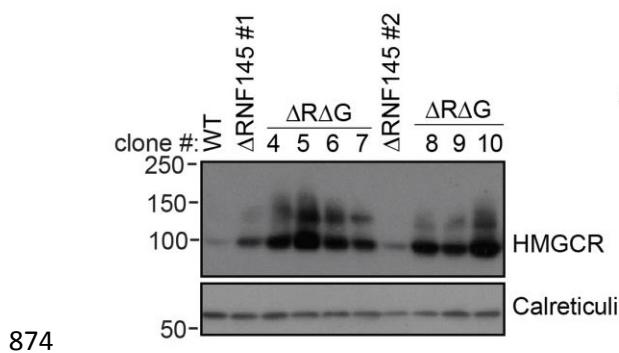
A



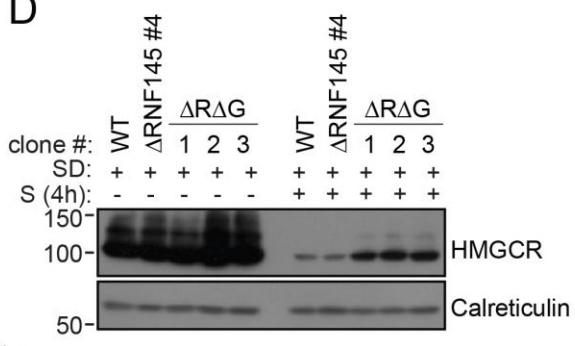
B



C



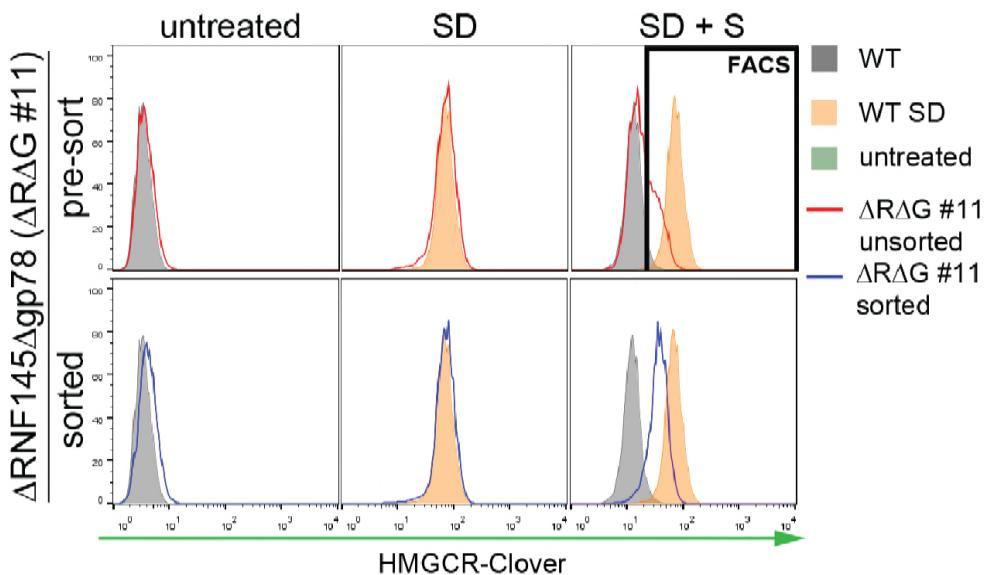
D



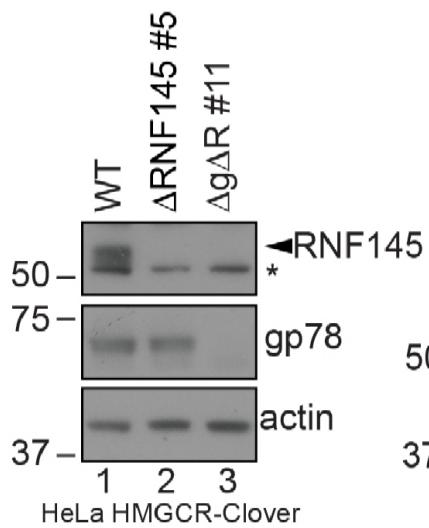
875 **Figure 3 – figure supplement 3. RNF145/gp78 double-knockout cells show increased**  
876 **HMGCR at steady-state and impaired sterol-induced HMGCR degradation. (A)**  
877 Immunoblot of four gp78 knockout HeLa clones derived by transfection with two independent  
878 gp78 sgRNAs.  
  
879 **(B)** Confirmation of gp78 loss in RNF145/gp78 knockout HeLa cells derived from ΔRNF145  
880 clones #1, #2, and #4 (for validation of RNF145 knockout see Figure 3 – figure supplement  
881 1). Calnexin serves as a loading control.  
  
882 **(C)** Steady-state expression of HMGCR in HeLa WT, ΔRNF145, and RNF145/gp78 double  
883 knockout clones (ΔRΔG) was determined by immunoblotting.  
  
884 **(D)** Indicated cell lines were sterol-depleted (SD) overnight ± sterols (S, 4h) and HMGCR  
885 detected by immunoblotting. Calreticulin serves as a loading control. LE, long exposure.

# FIGURE 3 - FIGURE SUPPLEMENT 4

A

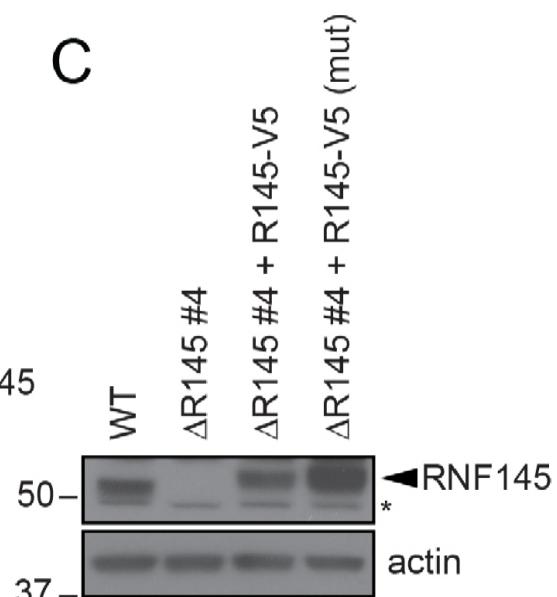


B



886  
887

C



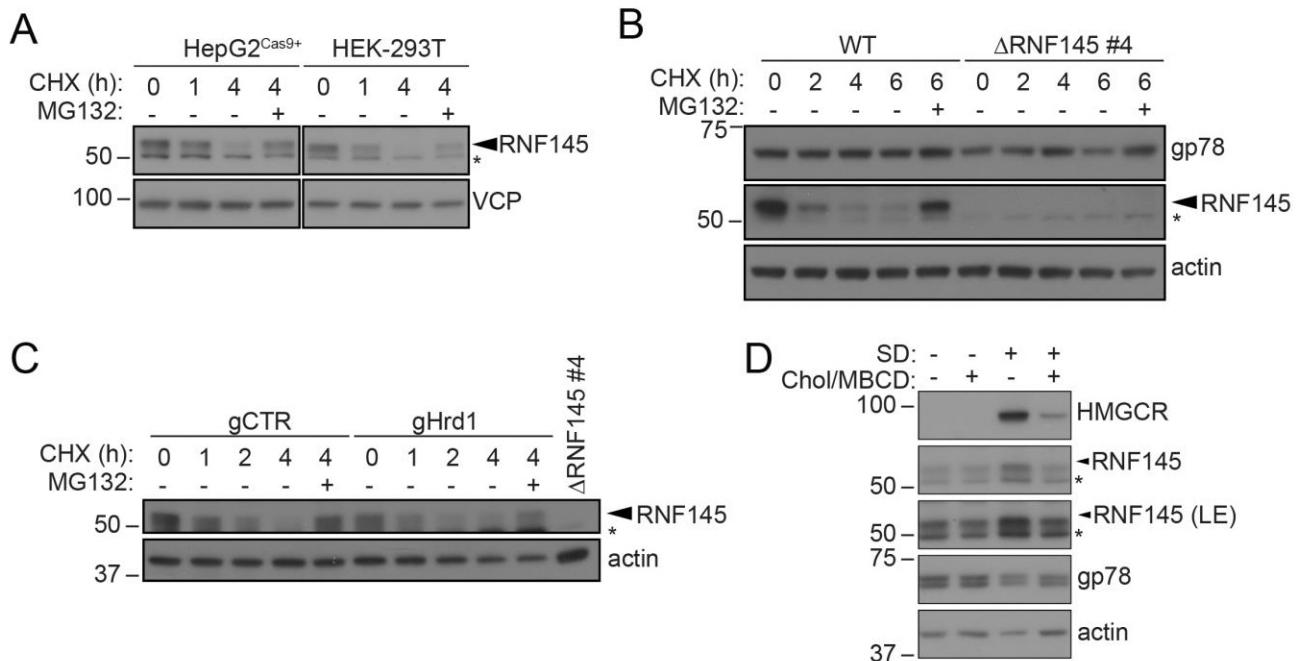
888 **Figure 3 – figure supplement 4. Establishment of RNF145/gp78 knockout HeLa HMCR-**  
889 **Clover and RNF145 complementation cell lines. (A)** HMGCR-Clover cells were  
890 transfected with RNF145 sgRNA#8 and gp78 sgRNA#4 ( $\Delta R\Delta G$  #11, for sgRNAs used see  
891 **Supplementary Files 4 and 5**) and knockout pools were enriched with puromycin. Eight  
892 days *post* transfection, cells were sterol-depleted (SD) overnight  $\pm$  sterols (S, 2h). HMGCR-  
893 Clover<sup>high</sup> cells were enriched by FACS.  
894 **(B)** RNF145 and gp78 levels in WT *versus*  $\Delta$ RNF145 clones #5, and RNF145/gp78 depleted  
895 ( $\Delta R\Delta G$  #11) HeLa HMGCR-Clover cells.  
896 **(C)** Stable genetic complementation of  $\Delta$ RNF145 (clone #4) cells by transduction with  
897 constructs encoding either RNF145-V5 ( $\Delta R145$  #4 + R145-V5) or RNF145(C552A, H554A)-  
898 V5 ( $\Delta R145$  #4 + R145-V5 (mut)). RNF145 variants were detected using an RNF145-specific  
899 antibody. Non-specific bands are indicated (\*).

900 **Figure 3 – figure supplement 5.** Candidate genes (-log(p)>5) identified in a ubiquitome  
901 CRISPR/Cas9 screen for proteins mediating HMGCR degradation in RNF145 deficient cells.

Gene	Full name	-log(p)*	Function
AMFR	Gp78/Autocrine Motility Factor Receptor	10.87	ER ubiquitin E3 ligase
ANKFY1	Ankyrin Repeat And FYVE Domain Containing 1	6.19	Proposed Rab5 effector
FAF2	Fas Associated Factor Family Member 2	5.05	ERAD
NPLOC4	NPL4 Homolog	7.63	Ubiquitin Recognition Factor
RABGEF1	RAB Guanine Nucleotide Exchange Factor 1	8.56	Nucleotide exchange factor, E3 ubiquitin ligase
UBE2G2	Ubiquitin Conjugating Enzyme E2 G2	13.66	E2 ubiquitin ligase

902 \*Only statistically significant hits (-log(p)>5) are shown.

## FIGURE 5 - FIGURE SUPPLEMENT 1



903

904 **Figure 5 – figure supplement 1. RNF145 is rapidly degraded by the ubiquitin  
905 proteasome system. (A)** Translation shutoff assay in HepG2<sup>Cas9+</sup> and HEK-293T cells. Cells  
906 were treated with cycloheximide (CHX, 1  $\mu$ M)  $\pm$  MG132 (20  $\mu$ g/ml) for the indicated times  
907 and endogenous RNF145 levels determined by immunoblotting.

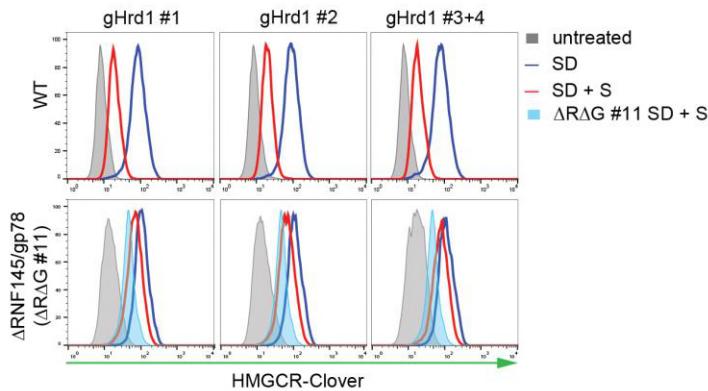
908 **(B)** Gp78 is stable in the absence of RNF145. WT and  $\Delta$ RNF145 #4 HeLa cells were  
909 cultured in the presence of CHX, (1  $\mu$ M)  $\pm$  MG132 (10  $\mu$ g/ml) for 0-6 h and gp78/RNF145  
910 levels monitored by Western blotting. The asterisk (\*) indicates a non-specific band.

911 (C) HeLa<sup>Cas9+</sup> HMGCR-Clover were transiently transfected with a pool of 4 Hrd1-specific  
912 sgRNAs (gHrd1) or a B2M targeting control sgRNA (gCTR) and sgRNA containing cells  
913 enriched by puromycin selection. Cells were treated as in (B) for the indicated times.

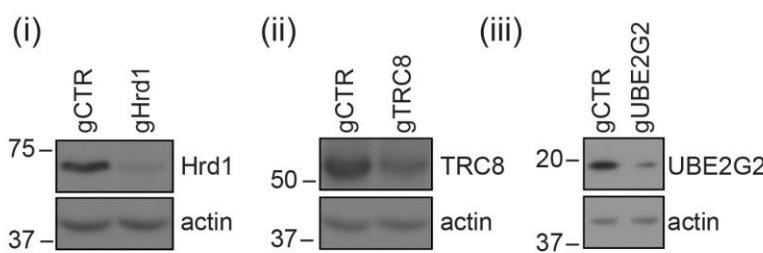
914 (D) HeLa cells were grown under sterol-rich or sterol-deplete conditions for 48 h in the  
915 presence of mevastatin (10  $\mu$ M) and mevalonate (50  $\mu$ M)  $\pm$  complexed cholesterol  
916 (chol:MBCD, 25  $\mu$ M). SDS-PAGE and immunoblot analysis was performed on whole-cell  
917 lysates. Non-specific bands are indicated (\*). LE, long exposure.

## FIGURE 7 - FIGURE SUPPLEMENT 1

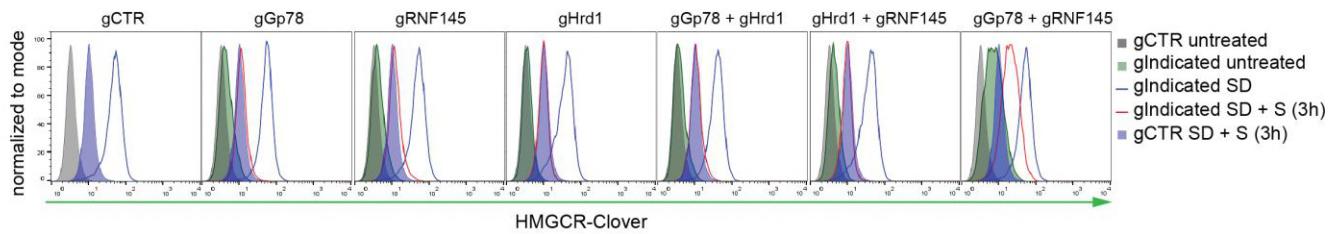
A



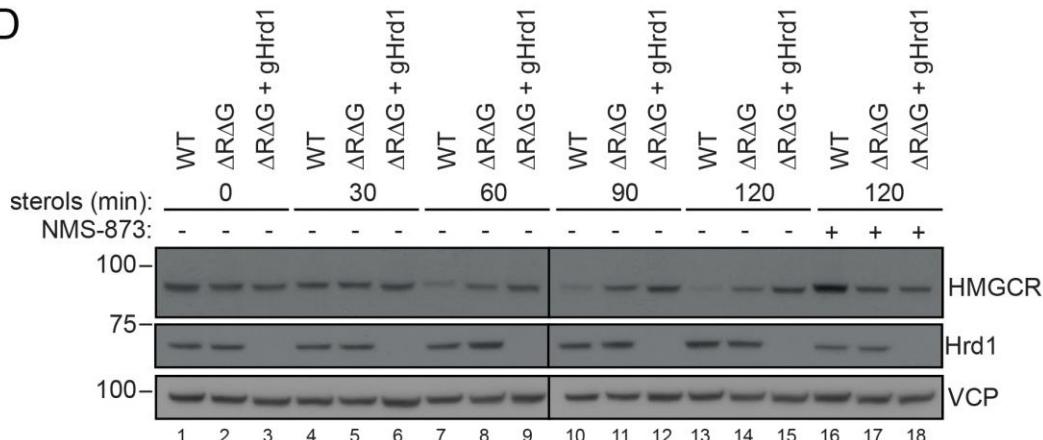
B



C



D



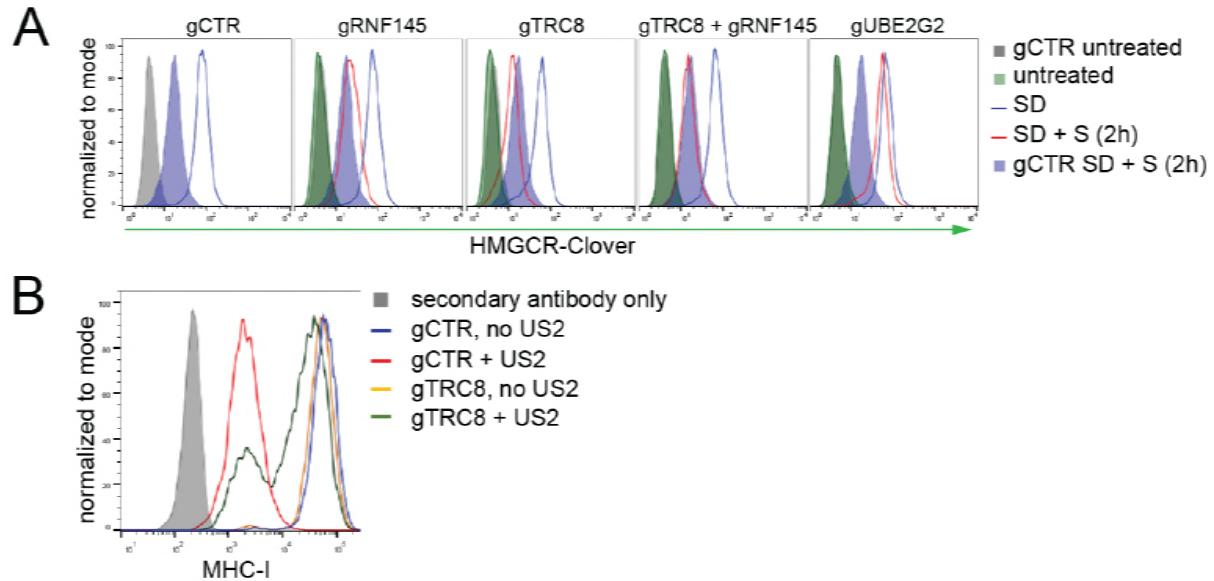
920 **Figure 7 – figure supplement 1. Combinatorial depletion of E3 ligases. (A)** WT or  
921 RNF145/gp78 knockout ( $\Delta R\Delta G$  #11) HeLa HMGCR-Clover cells transiently transfected with  
922 a B2M-specific (gCTR) or Hrd1-specific (gHrd1 #1-4) sgRNAs were sterol-starved (SD, 20h)  
923  $\pm$  sterols (S, 2h). HMGCR clover expression was detected by FACS analysis. Cells  
924 transfected with gCTR are from the same experiment shown in **Figure 7B** and histograms  
925 (gCTR SD+S) were therefore re-plotted.

926 **(B)** Validation of Hrd1 (i), TRC8 (ii), and UBE2G2 (iii) depletion in  $\Delta R\Delta G$  #11 cells. Cell lines  
927 used in **Figure 7B** and **Figure 7 – figure supplement 1A** were collected at steady-state and  
928 indicated proteins detected from whole-cell lysate by immunoblotting. A sgRNA targeting  
929 B2M (gCTR) served as a control.

930 **(C)** WT HMGCR-Clover transfected with indicated guide pools were sterol-depleted (SD)  
931 overnight  $\pm$  sterols (S, 3h). Reporter expression was measured by FACS.

932 **(D)** WT and  $\Delta R\Delta G$ #7 HeLa cells transfected with gCTR or a pool of four Hrd1-specific  
933 sgRNAs were sterol-depleted (20h) before addition of sterols for the indicated times  $\pm$  NMS-  
934 873 (10  $\mu$ M). Validation of RNF145 ( $\Delta RNF145$ #1) and gp78 knockout ( $\Delta R\Delta G$ #7) can be  
935 found in **Figure 3 – figure supplement 1A** and **3B**, respectively.

## FIGURE 7 - FIGURE SUPPLEMENT 2



936

937 **Figure 7 – figure supplement 2. TRC8 depletion does not affect HMGCR-Clover**  
938 **degradation. (A)** Overnight sterol depletion (SD) ± sterols (S, 2h) in HeLa HMGCR-Clover  
939 cells transiently transfected with pools of indicated guides as described in Materials and  
940 Methods.

941 **(B)** TRC8 knockdown was confirmed by US2-mediated TRC8-dependent downregulation of  
942 MHC-I. HeLa cells transiently expressing either control sgRNA (gCTR) or gTRC8 were  
943 selected for puromycin resistance and transduced with a lentiviral US2 and/or TRC8  
944 construct 5 days *post* transfection. Cell-surface MHC-I staining and FACS analysis were  
945 performed on day 10 *post* transfection.

946 **MATERIALS AND METHODS**

947 **Plasmids and expression constructs**

948 Single guide RNAs (sgRNAs) were cloned into pSpCas9(BB)-2A-Puro V1 (Addgene #48139,  
949 deposited by Dr. Feng Zhang), pSpCas9(BB)-2A-Puro V2 (Addgene #62988, deposited by  
950 Dr. Feng Zhang) as previously described (Ran et al., 2013). To generate the ubiquitome  
951 sgRNA library, sgRNAs (sgRNA sequences in **Supplementary File 1**) were cloned into  
952 pKLV-U6gRNA(BbsI)-PGKpuro2ABFP (Addgene # 50946) as reported previously (Doench  
953 et al., 2016). The RNF145 CDS, PCR amplified from an RNF145 IMAGE clone (Source  
954 Bioscience, Nottingham, UK), was cloned into pHRSIN-P<sub>SFFV</sub>-GFP-P<sub>PGK</sub>-Hygromycin<sup>R</sup>  
955 (BamHI, NotI) (Demaison et al., 2002), replacing GFP with the transgene. To generate  
956 RNF145-V5, RNF145 CDS was Gibson cloned into pHRSIN-P<sub>SFFV</sub>-P<sub>PGK</sub>-Hygromycin<sup>R</sup>  
957 containing a downstream in-frame V5 tag. RNF145-V5 RING domain mutations (C552A,  
958 H554A) were introduced by PCR amplification of RNF145-V5 fragments with primers  
959 encoding for C552A and H554A mutations and RNF145(C552A, H554A)-V5 was introduced  
960 into pHRSIN-P<sub>SFFV</sub>-P<sub>PGK</sub>-Hygromycin<sup>R</sup> by Gibson assembly. FLAG-NLS-Cas9 was cloned  
961 from the lentiCRISPR v2 (Sanjana et al., 2014) (Addgene #49535, deposited by Feng  
962 Zhang) into pHRSIN.pSFFV MCS(+) pSV40 Blast (BamHI, NotI).

963

964 **Compounds**

965 The following compounds were used in this study: Dulbecco's Modified Eagle's Medium high  
966 glucose (DMEM; Sigma-Aldrich, 6429-500ml), foetal calf serum (FCS; Seralab (catalogue  
967 no: EU-000, SLI batch: E8060012, Supplier batch: A5020012) and Life Technologies  
968 (catalogue no: 10270, lot: 42G4179K)), lipoprotein-deficient serum (LPDS; biosera, FB-  
969 1001L/100), mevastatin (Sigma-Aldrich, M2537-5MG), mevalonolactone (Sigma-Aldrich,  
970 M4467-1G), cholesterol (Sigma-Aldrich, C3045-5G), 25-hydroxycholesterol (Sigma-Aldrich,  
971 H1015-10MG), methyl-β-cyclodextrin (MBCD; Sigma-Aldrich, 332615-1G), bortezomib/PS-  
972 341 (BostonBiochem, I-200), (S)-MG132 (Cayman Chemicals, 10012628), NMS-873  
973 (Selleckchem, s728501), digitonin (Merck, 300410-5GM), cycloheximide (Sigma-Aldrich, C-  
974 7698), IgG Sepharose<sup>TM</sup> 6 Fast Flow (GE Healthcare, 17-0969-01), ProLong<sup>TM</sup> Gold  
975 Antifade Mountant with DAPI (Thermo Fisher), bovine serum albumin (BSA; Sigma-Aldrich,  
976 A4503-10G), Protein A-Sepharose<sup>R</sup> (P3391-1.5G), iodoacetamide (IAA; Sigma-Aldrich,  
977 I1149-5G), cOmplete protease inhibitor (EDTA-free; Roche, 27368400), phenylmethylsulfonyl  
978 fluoride (PMSF; Roche, 20039220), V5 peptide (Sigma-Aldrich, V7754-  
979 4MG), N-ethylmaleimide (NEM; Sigma-Aldrich, E3876-5G), puromycin (Cayman Chemicals,

980 13884), hygromycin B (Invitrogen, 10687010), Penicillin-Streptomycin (10,000 U/mL;  
981 Thermo Fisher, 15140122).

982

### 983 **Antibodies**

984 Antibodies specific for the following targets were used for immunoblotting analysis: Insig-1  
985 (rabbit; Abcam, ab70784), Hrd1 (rabbit; Abgent, AP2184a), TRC8 (rabbit; Santa Cruz, sc-  
986 68373), tubulin (mouse; Sigma, T9026), VCP (mouse; abcam, ab11433),  $\beta$ -actin (mouse;  
987 Sigma-Aldrich, A5316), calnexin (mouse; AF8, kind gift from M Brenner, Harvard Medical  
988 School), calreticulin (rabbit; Pierce, PA3-900), HMGCR (mouse; Santa Cruz, sc-27195),  
989 HMGCR (rabbit; Abcam, ab174830), gp78 (rabbit; ProteinTech, 16675-1-AP), Insig-1 (rabbit;  
990 Abcam, ab70784), RNF145 (rabbit; ProteinTech, 24524-I-AP), V5 (mouse; Abcam,  
991 ab27671), VU-1 ubiquitin (mouse; Life Sensors, VU101), UBE2G2 (mouse; Santa Cruz, sc-  
992 100613), GFP (rabbit; Life technologies, A11122), KDEL (mouse; Enzo, 10C3), HRP-  
993 conjugated anti-mouse and anti-rabbit (goat; Jackson ImmunoResearch), TrueBlot® Anti-  
994 Rabbit-HRP (Rockland, 18-8816-31), TrueBlot® Anti-Mouse-HRP ULTRA (Rockland, 18-  
995 8817-30). Alexa Fluor 488 (goat anti-rabbit; Thermo Fisher), Alexa Fluor 568 (goat anti-  
996 mouse; Thermo Fisher) were used as secondary antibodies for immunofluorescence  
997 microscopy. Anti-MHC-I (W6/32; mouse) and Alexa Fluor 647 (rabbit anti-mouse; Thermo  
998 Fisher) were used for cytofluorometric analysis.

999

### 1000 **Cell Culture**

1001 HeLa, HEK-293T, Huh-7 and HepG2 cells were maintained in DMEM + 10% FCS +  
1002 penicillin/streptomycin (1:100) (5% CO<sub>2</sub>, 37°C). Transfection of HeLa cells was performed  
1003 using the TransIT-HeLa MONSTER kit (Mirus) according to the manufacturer's instructions.  
1004 Cells were seeded at low confluence in 12-well tissue culture plates and the next day  
1005 transfection mix (1  $\mu$ g DNA, 3  $\mu$ l TransIT-HeLa reagent + 2  $\mu$ l MONSTER reagent in  
1006 OptiMEM (Gibco)) was added. Alternatively, reverse transfection was performed by seeding  
1007 3.5\*10<sup>5</sup> cells per well of a 12-well plate to the transfection mix on the day of transfection. For  
1008 co-transfection of multiple plasmids, equal amounts of each plasmid were added up to 1  $\mu$ g.

1009

### 1010 **CRISPR/Cas9-mediated gene knockout**

1011 CRISPR/Cas9-mediated genomic editing was performed according to Ran *et al.* (Ran *et al.*,  
1012 2013). For generation of knockout cell lines, cells were transfected with pSpCas9(BB)-2A-

1013 Puro (PX459) V1.0 or V2.0 (Addgene #48139, and #62988 respectively; deposited by Dr.  
1014 Feng Zhang) containing a sgRNA specific for the targeted gene of interest. Guide RNA  
1015 sequences are listed in **Supplementary File 4**. Single cell clones were derived from cells  
1016 transfected with a single sgRNA, whereas mixed knockout populations were generated by  
1017 introducing 1 – 4 sgRNAs (**Supplementary File 5** for cell lines used in this study). Cells  
1018 were cultured for an additional 24h before selection with puromycin (2 µg/ml) at low  
1019 confluence for 72h. The resulting mixed knockout populations were used to generate single-  
1020 cell clones by limiting dilution. Gene disruption was validated by immunoblotting,  
1021 immunoprecipitation and/or targeted genomic sequencing.

1022

### 1023 **CRISPR/Cas9-mediated gene knock-in**

1024 An HMGCR-Clover knock-in donor template was created by Gibson assembly of ~ 1 kb  
1025 flanking homology arms, PCR-amplified from HeLa genomic DNA, and the Nsil and Pcil  
1026 digested backbone from pMAX-GFP (Amaxa), into the loxP-Ub-Puro cassette from pDonor  
1027 loxP Ub-Puro (kind gift from Prof Ron Kopito, Stanford University). Each arm was amplified  
1028 using nested PCR. The 5' arm was amplified using 5'-GATGCAGCACAGAATGTTGGTAG-3'  
1029 and 5'-CAATGCCATGTTCCAGTTCAG-3', followed by 5'-  
1030 CAATGCCATGTTCCAGTTCAG-3' and 5'-CAGCTGCACCAGCCATCTATAG-3'. The 3'  
1031 arm was amplified using the following primer pairs: 5'-CCAAGGAGCTTGCACCAAGAAG-3'  
1032 and 5'-CTAAGGTCCCAGTCTTGCTTG-3'. The product served as template for a subsequent  
1033 PCR step using the primers 5'-CCAAGGAGCTTGCACCAAGAAG-3' and 5'-  
1034 GTCACCCTCATCTAAGCAAC-3'. Overhangs required for Gibson assembly were  
1035 introduced by PCR. HeLa cells were co-transfected with Cas9, sgRNA targeting immediately  
1036 downstream of the HMGCR stop codon and donor template. Three different donor templates  
1037 were simultaneously transfected, each differing in the drug resistance marker (puromycin,  
1038 hygromycin and blasticidin). The transfected cells were treated with the three antibiotics five  
1039 days post-transfection until only drug-resistant cells remained. The resulting population was  
1040 transfected with Cre-recombinase in pHRSIN MCS(+) IRES mCherry pGK Hygro. mCherry  
1041 positive cells were single-cell cloned by FACS.

1042

### 1043 **Lentivirus production and transductions**

1044 HEK-293T cells were transfected with a lentiviral expression vector, the packaging vectors  
1045 pCMVΔR8.91 and pMD.G at a ratio of 1:0.7:0.3 using TransIT-293 (Mirus) as recommended  
1046 by the manufacturer. For production of CRISPR library virus, HEK-293T cells were

1047 transfected as above in 15 cm tissue culture plates. 48 h post transfection, virus-containing  
1048 media was collected, filtered (0.45  $\mu$ m pore size) and directly added to target cells or frozen  
1049 (-80°C) for long-term storage. Cells were transduced in 6-well tissue culture plates at an  
1050 M.O.I. < 1 and selected with puromycin (2  $\mu$ g/ml) or hygromycin B (200  $\mu$ g/ml). To generate  
1051 HeLa HMGCR-Clover stably expressing Cas9, HeLa HMGCR-Clover cells were transduced  
1052 with pHRSIN-P<sub>SFFV</sub>-Cas9-P<sub>PGK</sub>- Hygromycin<sup>R</sup> and stable integrants selected with hygromycin  
1053 B. Cas9 activity was confirmed by transduction with pKLV encoding a  $\beta$ -2-microglobulin  
1054 (B2M)-targeting sgRNA followed by puromycin selection. MHC-I surface expression was  
1055 assessed by flow cytometry in puromycin-resistant cells five days post transduction.  
1056 Typically, ~ 90% reduction of cell surface MHC-I expression was observed.

1057

### 1058 **Fluorescent PCR**

1059 To identify CRISPR-induced frame-shift mutations, genomic DNA was extracted from wild  
1060 type HeLa cells and RNF145 CRISPR clones using the Quick-gDNA MicroPrep kit (Zymo  
1061 Research) followed by nested PCR of the genomic region 5' and 3' of the predicted sgRNA  
1062 binding site. One in each primer pair for the second PCR was 5' modified with 6-FAM<sup>TM</sup>  
1063 (fluorescein, Sigma-Aldrich). Primer sequences were as follows: For sgRNA #8  
1064 PCR1\_Forward: CAGAATGCTCACTAGAAGATTAG, PCR1\_Reverse:  
1065 GTAGTATACGTTCTCACATAG, PCR2\_Forward: GTGATGTAGACACTCACCTAC and  
1066 PCR2\_Reverse GTGACAACCTATTAGATTCTGTG. PCR products were detected using an  
1067 ABI 3730xl DNA Analyser.

1068

### 1069 **Flow cytometry and Fluorescence-activated cell sorting (FACS)**

1070 Cells were collected by trypsinisation and analysed using a FACS Calibur (BD) or an LSR  
1071 Fortessa (BD). Flow cytometry data was analysed using the FlowJo software package. Cells  
1072 resuspended in sorting buffer (PBS + 10 mM HEPES + 2% FCS) were filtered through a 50  
1073  $\mu$ m filter, and sorted on an Influx machine (BD), or, for the ubiquitome CRISPR/Cas9 screen,  
1074 on a FACS Melody (BD). Sorted cells were collected in DMEM + 50% FCS and  
1075 subsequently cultured in DMEM + 10% FCS + penicillin/streptomycin. For MHC-I flow  
1076 cytometric analysis, cells resuspended in cold PBS were incubated with W6/32 (20 min,  
1077 4°C), washed twice and then incubated with Alexa-647-labelled anti-mouse antibody (15  
1078 min, 4°C). Cells were washed twice and resuspended in PBS.

1079

1080 **CRISPR/Cas9 knockout screens**

1081 For genome-wide and ubiquitome CRISPR/Cas9 knockout screens,  $10^8$  and  $1.2 \times 10^7$  HeLa  
1082 HMGCR-Clover (Cas9) or  $\Delta$ RNF145 #6 (Cas9), respectively, were transduced at M.O.I. ~  
1083 0.3 by spinfection (750xg, 60 min, 37°C). Transduction efficiency was determined via flow-  
1084 cytometry-based measurement of BFP expression 48-72h post infection. Transduced cells  
1085 were enriched by puromycin (1  $\mu$ g/ml). On day 9 (genome-wide screen) or day 7  
1086 (ubiquitome-library screen) post transduction, cells were rinsed extensively with PBS and  
1087 cultured overnight in starvation medium (DMEM + 10% LPDS + 10  $\mu$ M mevastatin +  
1088 penicillin/streptomycin) before sterol addition (2  $\mu$ g/ml 25-hydroxycholesterol and 20  $\mu$ g/ml  
1089 cholesterol for 5h). An initial FACS selection ('sort 1') on cells expressing high levels (~0.3-  
1090 0.6% of overall population) of HMGCR-Clover (HMGCR-Clover<sup>high</sup>) was performed.  $2 \times 10^5$   
1091 (genome-wide screen) and ~  $10^5$  (ubiquitome screen) sorted cells were pelleted and DNA  
1092 was extracted using the Quick-gDNA MicroPrep kit (Zymo Research). To gauge sgRNA  
1093 enrichment, DNA was extracted from  $3 \times 10^6$  (genome-wide library screen) or  $6 \times 10^6$   
1094 (ubiquitome library screen) cells pre-sort using the Gentra Puregene Core kit A (Qiagen).  
1095 Cells in the genome-wide screen were subjected to a second round of sterol deprivation and  
1096 sort (see above) after expansion of initially  $2.5 \times 10^5$  sorted cells for 8 days. Sorted cells were  
1097 cultured until  $5 \times 10^6$  cells could be harvested for genomic DNA extraction using the Gentra  
1098 Puregene Core kit A (Qiagen). Individual integrated sgRNA sequences were amplified by  
1099 two sequential rounds of PCR, the latter introducing adaptors for Illumina sequencing  
1100 (**Supplementary File 3**). Sequencing was carried out using the Illumina HighSeq (genome-  
1101 wide screen) and MiniSeq (ubiquitome screen) platforms. Illumina HiSeq data was analysed  
1102 as described previously (Timms et al., 2016). Guide RNA counts were analysed with the  
1103 RSA algorithm under default settings (König et al., 2007). Of note, a gene's calculated high  
1104 significance value and therefore high enrichment in the selected population does not  
1105 necessarily reflect its importance relative to genes with lower significance values/enrichment,  
1106 since gene disruption can be incomplete or lethal phenotypes might evade enrichment.

1107

1108 **Quantitative PCR**

1109 Whole-cell RNA was isolated with the RNeasy Plus Mini Kit (Qiagen, Venlo, Netherlands)  
1110 and reverse transcribed using Oligo(dT)15 primer (Promega, C110A) and SuperScript™ III  
1111 reverse transcriptase (Invitrogen). Transcript levels were determined in triplicate using  
1112 SYBR® Green PCR Master Mix (Applied Biosystems) in a real time PCR thermocycler (7500  
1113 Real Time PCR System, Applied Biosystems). Primers used for target amplification can be

1114 found in **Supplementary file 2**. RNA quantification was performed using the  $\Delta\Delta CT$  method.  
1115 GAPDH transcript levels were used for normalization.

1116

### 1117 **Sterol depletion assays**

1118 HeLa cells at ~ 50% confluence were washed five times with PBS and cultured for 16-20h in  
1119 starvation medium (DMEM + 10% LPDS + 10  $\mu$ M mevastatin + penicillin/streptomycin)  
1120 before addition of 25-hydroxycholesterol (2  $\mu$ g/ml) and cholesterol (20  $\mu$ g/ml) to analyse  
1121 sterol-accelerated protein degradation. For experiments in **Figures 5C, D and 6**, sterol-  
1122 depletion was performed in starvation medium + 50  $\mu$ M mevalonate.

1123

### 1124 **Chol:MBCD complex preparation**

1125 Complexation of cholesterol (2.5 mM) with MBCD (25 mM) was performed according to  
1126 Christian *et al.* (Christian et al., 1997). An emulsion of cholesterol powder (final: 2.5 mM) and  
1127 an MBCD solution (25 mM) was produced by vortexing and tip sonication (1 min in 10 s  
1128 intervals), and continuously mixed for 16h at 37°C. The solution was sterile filtered (0.45  $\mu$ m  
1129 PVDF pore size) and stored at -20°C.

1130

### 1131 **Preparation of sterols and mevalonate**

1132 Sterols were prepared by resuspension in ethanol or complexation with MBCD (see above).  
1133 Mevalonate was prepared by adding 385  $\mu$ l 2.04M KOH to 100 mg mevalonolactone  
1134 (Sigma). The solution was heated (1h, 37°C) and adjusted to a 50 mM stock solution.

1135

### 1136 **SDS-PAGE and immunoblotting**

1137 Cells were collected mechanically in cold PBS or by trypsinisation, centrifuged (1000xg, 4  
1138 min, 4°C), and cell pellets resuspended in lysis buffer (1% (w/v) digitonin, 1x cOmplete  
1139 protease inhibitor, 0.5 mM PMSF, 10 mM IAA, 2 mM NEM, 10 mM TRIS, 150 mM NaCl, ph  
1140 7.4). After 40 min incubation on ice, lysates were centrifuged (17.000xg, 15 min, 4°C), the  
1141 post-nuclear fraction isolated and protein concentration determined by Bradford assay.  
1142 Samples were adjusted with lysis buffer and 6 x Laemmli buffer + 100 mM dithiothreitol  
1143 (DTT) and heated at 50°C (15 min). Samples were separated by SDS-PAGE and transferred  
1144 to PVDF membranes (Merck) for immunodetection. Membranes were blocked in 5% milk +  
1145 PBST (PBS + 0.2% (v/v) Tween-20) (1h) and incubated with primary antibody in PBST + 2%

1146 (w/v) BSA at 4°C overnight. For detection from whole-cell lysate, membranes were  
1147 incubated in peroxidase (HRP)-conjugated secondary antibodies. For detection of  
1148 immunoprecipitated proteins, TrueBlot® HRP-conjugated secondary antibodies (Rockland)  
1149 were used. Immunoprecipitated RNF145 was detected using Protein A-conjugated HRP.

1150

### 1151 **Immunoprecipitation**

1152 Cells were seeded to 15 cm tissue culture plates (4\*10<sup>6</sup> cells per plate). The following day,  
1153 cells were washed five times with PBS and cultured in starvation medium (DMEM + 10%  
1154 LPDS + 10 µM mevastatin + 10 µM mevalonate + penicillin/streptomycin) for 20h. To  
1155 prevent HMGCR membrane extraction and degradation, starved cells were treated with  
1156 NMS-873 (50 µM) 0.5h prior to sterol addition (2 µg/ml 25-hydroxycholesterol and 20 µg/ml  
1157 cholesterol for 1h) and collection in cold PBS. Cells were lysed in IP buffer 1 (1% (w/v)  
1158 digitonin, 10 µM ZnCl<sub>2</sub>, 1x cOmplete protease inhibitor, 0.5 mM PMSF, 10 mM IAA, 2 mM  
1159 NEM, 10 mM TRIS, 150 mM NaCl, ph 7.4), post-nuclear fractions isolated by centrifugation  
1160 (17.000×g, 4°C, 15 min) adjusted to 0.5% (w/v) digitonin and pre-cleared with IgG  
1161 Sepharose™ 6 Fast Flow (1h). Endogenous RNF145 and V5-tagged RNF145 were  
1162 immunoprecipitated at 4°C overnight from 3 - 6 mg whole-cell lysate using Protein A-  
1163 Sepharose® and anti-RNF145 or V5 antibody, respectively. Beads were collected by  
1164 centrifugation (1500xg, 4 min, 4°C), washed for 5 min with IP buffer 2 (0.5% (w/v) digitonin,  
1165 10 µM ZnCl<sub>2</sub>, 10 mM Tris, 150 mM NaCl, ph 7.4) and 4 x 5 min with IP buffer 3 (0.1% (w/v)  
1166 digitonin, 10 µM ZnCl<sub>2</sub>, 10 mM TRIS, 150 mM NaCl, ph 7.4). Proteins whose interaction with  
1167 RNF145 was labile in the presence of 1% (v/v) Triton X-100 were recovered by eluting twice  
1168 with 20 µl TX100 elution buffer (1% (v/v) Triton X-100 + 2x cOmplete protease inhibitor in 10  
1169 mM TRIS, 150 mM NaCl pH 7.4) at 37°C under constant agitation. Immunoprecipitated  
1170 RNF145 was subsequently eluted in 30 µl 2x Laemmli buffer + 3% (w/v) DTT. RNF145-V5  
1171 and associated complexes were recovered by two sequential elutions with V5 elution buffer  
1172 (1 mg/ml V5 peptide + 2x cOmplete protease inhibitor in 10 mM TRIS, 150 mM NaCl pH 7.4)  
1173 for 30 min under continuous agitation. Eluted samples were adjusted Laemmli buffer and  
1174 denatured at 50°C for 15 min.

1175

### 1176 **Ubiquitination assays**

1177 Cells were sterol-depleted (20h), treated with 20 µM MG132 and left for 30 min before  
1178 addition of sterols (2 µg/ml 25-hydroxycholesterol and 20 µg/ml cholesterol for 1h) or EtOH  
1179 (vehicle control). Immunoprecipitation of ubiquitinated HMGCR was performed as described

1180 above from 1 mg whole-cell lysate and using rabbit  $\alpha$ -HMGCR (Abcam, ab174830). Proteins  
1181 were eluted in 30  $\mu$ l 2x Laemmli buffer + 100 mM DTT at 50°C for 15 min. For  
1182 immunoblotting of ubiquitin with mouse VU-1  $\alpha$ -ubiquitin (Life Sensors, VU101), the PVDF  
1183 membrane was incubated with 0.5% (v/v) glutaraldehyde/PBS pH 7.0 (20 min) and washed  
1184 3x with PBS prior to blocking in 5% (w/v) milk + PBS + 0.1% (v/v) Tween-20.

1185

1186 **Indirect immunofluorescence confocal microscopy**

1187 Cells were grown on coverslips, fixed in 4% PFA (15 min), permeabilised in 0.2% (v/v) Triton  
1188 X-100 (5 min) and blocked with 3% (w/v) BSA/PBS (30 min). Cells were stained with primary  
1189 antibody diluted in 3% (w/v) BSA/PBS (1h), washed with 0.1% (w/v) BSA/PBS, followed by  
1190 staining with secondary antibody in 3% BSA/PBS (1h), an additional washing step (0.1%  
1191 (w/v) BSA/PBS) and embedded using ProLong™ Gold Antifade Mountant with DAPI  
1192 (Thermo Fisher). Images were acquired using an LSM880 confocal microscope (Zeiss) at  
1193 64x magnification.

1194

1195 **Statistical analysis**

1196 Statistical significance was calculated using the unpaired Student's t-test.

1197

1198 **Data deposition**

1199 Sequencing data from CRISPR/Cas9 knockout screens presented in this study have been  
1200 deposited at SRA (genome-wide screen: SUB4198636; ubiquitome screen: SUB4183663).

1201

1202 **SUPPLEMENTARY FILES**

1203

1204 **Supplementary File 1.** sgRNA sequences and genes targeted by the CRISPR/Cas9  
1205 ubiquitome library.

1206 **Supplementary File 2.** Primer sequences used for qPCR.

1207 **Supplementary File 3.** Primers used in CRISPR/Cas9 screens.

1208 **Supplementary File 4.** sgRNA sequences for generation of knockout cell lines.

1209 **Supplementary File 5.** Genetically modified cell lines used in this study.