





# Hepatic Sel1L-Hrd1 ER-associated degradation (ERAD) manages FGF21 levels and systemic metabolism via CREBH

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## Abstract

Fibroblast growth factor 21 (Fgf21) is a liver-derived, fasting-induced hormone with broad effects on growth, nutrient metabolism, and insulin sensitivity. Here, we report the discovery of a novel mechanism regulating *Fgf21* expression under growth and fasting-feeding. The Sel1L-Hrd1 complex is the most conserved branch of mammalian endoplasmic reticulum (ER)-associated degradation (ERAD) machinery. Mice with liver-specific deletion of Sel1L exhibit growth retardation with markedly elevated circulating Fgf21, reaching levels close to those in Fgf21 transgenic mice or pharmacological models. Mechanistically, we show that the Sel1L-Hrd1 ERAD complex controls *Fgf21* transcription by regulating the ubiquitination and turnover (and thus nuclear abundance) of ER-resident transcription factor Crebh, while having no effect on the other well-known Fgf21 transcription factor Ppar $\alpha$ . Our data reveal a physiologically regulated, inverse correlation between Sel1L-Hrd1 ERAD and Crebh-Fgf21 levels under fasting-feeding and growth. This study not only establishes the importance of Sel1L-Hrd1 ERAD in the liver in the regulation of systemic energy metabolism, but also reveals a novel hepatic “ERAD-Crebh-Fgf21” axis directly linking ER protein turnover to gene transcription and systemic metabolic regulation.

**Keywords** ER quality control; FGF21; gene transcription; metabolism; Sel1L-Hrd1 ERAD

**Subject Categories** Membrane & Intracellular Transport; Metabolism; Post-translational Modifications, Proteolysis & Proteomics

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## Introduction

The liver regulates growth and systemic energy homeostasis through inter-organ communication via the secretion of growth factors, hormones, and peptides. The “starvation hormone” Fgf21 is predominantly secreted from the liver, activating energy-saving pathways that reduce activity, growth, and anabolic reactions, while increasing insulin sensitivity, lipolysis, and adipose tissue browning (BonDurant *et al*, 2017; Fisher *et al*, 2012; Inagaki *et al*, 2008). Recent reports have shown the therapeutic potential of Fgf21 in a variety of human disease conditions such as diabetes, obesity, and cardiovascular disorders (Kharitonov & Adams, 2014; Zhao *et al*, 2012). Expression of *Fgf21* is primarily known to be induced in response to metabolic cues such as fasting by a synergistic interaction between the nuclear receptor peroxisome proliferator-activated receptor  $\alpha$  (Ppar $\alpha$ ) and the recently identified ER-resident transcription factor cAMP-responsive element-binding protein 3-like protein 3, hepatocyte-specific (Crebh3l3 or Crebh) (Badman *et al*, 2007; Hondares *et al*, 2010; Inagaki *et al*, 2007; Kim *et al*, 2014; Omori *et al*, 2001). Unlike Ppar $\alpha$ , Crebh encodes an ER membrane-anchored transcription factor, which requires regulated intramembrane proteolysis at the Golgi to generate the Crebh N-terminal fragment (Crebh-N) (Vecchi *et al*, 2009; Zhang *et al*, 2012a,b; Zhang *et al*, 2006). Subsequently, Crebh-N goes to the nucleus and is believed to recruit and form a complex with Ppar $\alpha$ , which induces transcriptional activation of the *Fgf21* gene (Kim *et al*, 2014; Nakagawa *et al*, 2014). While it is well known that *Fgf21* expression is induced by the nuclear receptor Ppar $\alpha$  during fasting, how *Fgf21*

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expression is regulated and the importance of CREBH under physiological contexts remains to be established.

In this study, we identified a surprising link between Fgf21 gene transcription and a principal ER quality control machinery, known as ER-associated degradation (ERAD). ERAD is responsible for recognizing and retro-translocating protein substrates, misfolded or not, from the ER for cytosolic proteasomal degradation (Guerriero & Brodsky, 2012; Qi *et al*, 2017). The most conserved ERAD system in mammals is the Sel1L-Hrd1 protein complex: Hrd1 being the E3 ubiquitin ligase, and Sel1L being its essential cofactor. Studies from yeast and mammalian systems have suggested that the single-spanning ER transmembrane protein Sel1L is required for the stability of Hrd1 as well as substrate recruitment and selection (Mehnert *et al*, 2015; Mueller *et al*, 2008; Sun *et al*, 2014; Vashistha *et al*, 2016). Germline deletion of *Sel1L* or *Hrd1* in mice leads to embryonic lethality (Francisco *et al*, 2010; Yagishita *et al*, 2005), and acute deletion of either gene in adult mice leads to premature death (Fujita *et al*, 2015; Sun *et al*, 2014).

Subsequent characterization of cell type-specific Sel1L-knockout (Ji *et al*, 2016; Sha *et al*, 2014; Shi *et al*, 2017; Sun *et al*, 2016, 2014, 2015) and Hrd1-knockout (Fujita *et al*, 2015; Kong *et al*, 2016; Wu *et al*, 2014; Xu *et al*, 2016; Yang *et al*, 2014) mouse models, including gene deletion in adipocytes, immune cells, enterocytes, and various neurons, has revealed the significance of Sel1L-Hrd1 ERAD in a cell type- and substrate-specific manner *in vivo* (Qi *et al*, 2017). For example, mice with Sel1L deficiency in adipocytes exhibit lipodystrophy and postprandial hyperlipidemia due to the ER retention of lipoprotein lipase (LPL) (Sha *et al*, 2014). Further, mice with Sel1L ablation in arginine-vasopressin (AVP) neurons progressively develop polyuria and polydipsia—characteristics of diabetes insipidus, due to a maturation defect of AVP precursor, proAVP, in the ER (Shi *et al*, 2017). Similarly, mice with Sel1L ablation in proopiomelanocortin (POMC) neurons develop hyperphagia and obesity even under low-fat chow diet due to ER retention of POMC prohormone (Kim *et al*, 2018).

In the characterization of hepatocyte-specific Sel1L-deficient mice, we discovered that the Sel1L-Hrd1 ERAD complex plays a key role in regulating *Fgf21* transcription and growth in a Crebh-dependent manner. ER-resident Crebh is an unstable protein with a half-life of about 1 h and is targeted for proteasomal degradation by the Sel1L-Hrd1 ERAD complex. In the absence of Sel1L, Crebh accumulates intracellularly, leading to a marked elevation of *Fgf21* gene transcription in the liver and circulating Fgf21 levels. Our data further show that physiological effect of hepatic Sel1L-Hrd1 ERAD is indeed mediated, at least partially, through the CREBH-FGF21 axis. Pointing to the physiological significance of Sel1L-Hrd1 ERAD, we further showed that hepatic Sel1L-Hrd1 protein complex represses the expression of the Crebh-*Fgf21* axis during growth and fasting-feeding. Thus, this study identifies the Sel1L-Hrd1 ERAD complex as a key repressor of *Fgf21* transcription in the liver.

## Results

### Expression of Sel1L-Hrd1 ERAD in the liver is responsive to physiological signals

As the liver plays a vital role in nutrient metabolism, we speculate that the levels of Sel1L-Hrd1 ERAD in the liver may fluctuate in

response to metabolic signals during growth and fasting-feeding. Indeed, protein levels of hepatic Sel1L and Hrd1 were steadily elevated during growth from 3 to 24 weeks of age (Fig 1A) and were significantly higher during feeding than after an overnight fast (Fig 1B). mRNA level of Hrd1, but not Sel1L, was also elevated during growth and feeding (Appendix Fig S1A and B).

### Growth retardation of *Sel1L<sup>Alb</sup>* mice

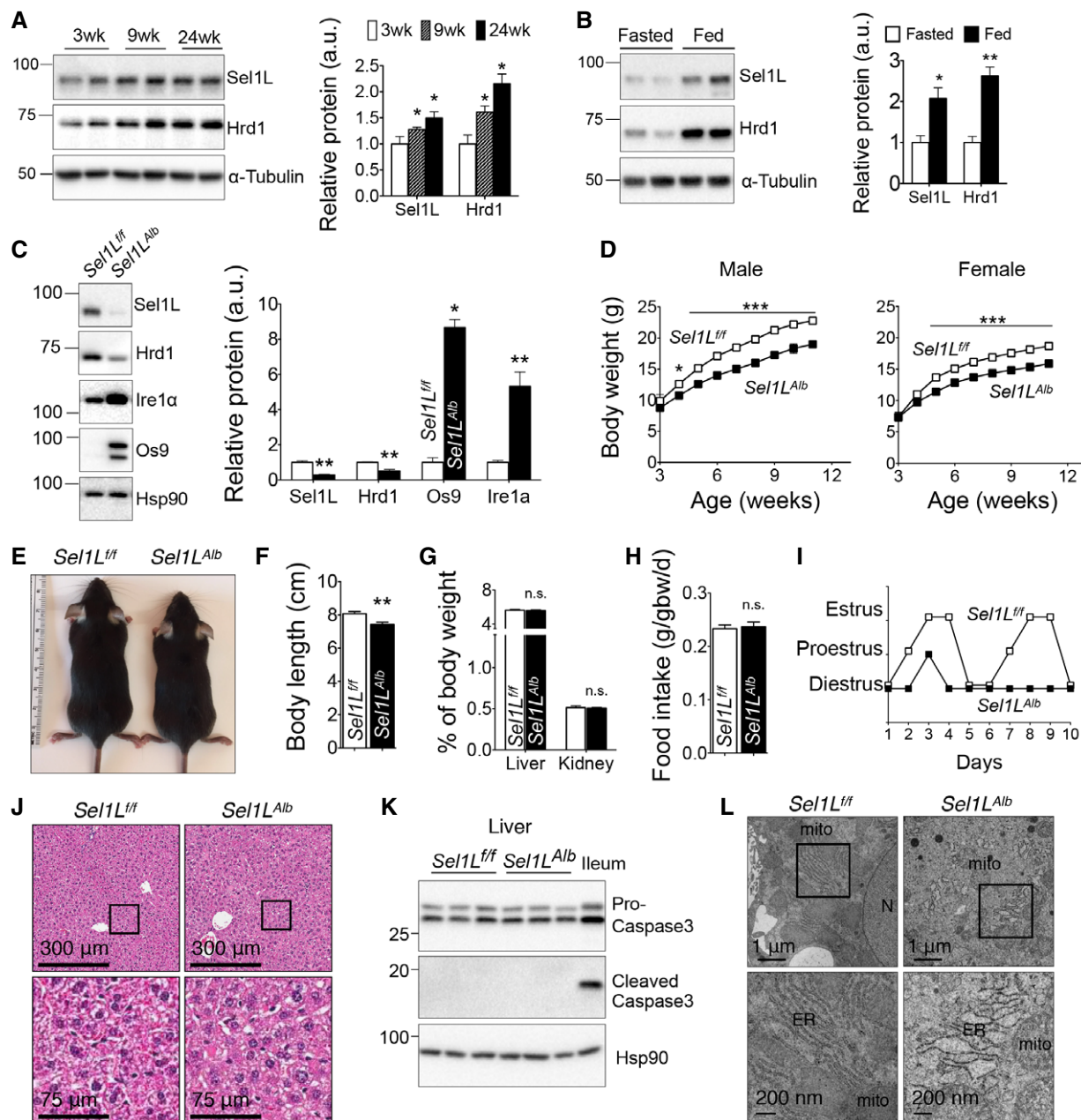
To explore the role of hepatic Sel1L-Hrd1 ERAD *in vivo*, we generated liver-specific Sel1L knockout mice (*Sel1L<sup>Alb</sup>*) by crossing *Sel1L<sup>fl/fl</sup>* with the hepatocyte-specific driver mouse line expressing Cre recombinase under the albumin promoter (Appendix Fig S1C). Sel1L was specifically deleted in the liver (Fig 1C), but not in other tissues such as small intestine (Appendix Fig S1D–F). The protein level of the E3 ligase Hrd1 was significantly reduced by 50% in the absence of Sel1L, while its mRNA level was increased by 2.5-fold (Fig 1C and Appendix Fig S1G). On the other hand, protein levels of the previously published Sel1L-Hrd1 ERAD substrate Ire1 $\alpha$  and Os9 (Sha *et al*, 2014; Sun *et al*, 2015) were increased by 5- to 10-fold in the *Sel1L<sup>Alb</sup>* liver (Fig 1C), while their mRNA levels were only modestly upregulated (Appendix Fig S1G).

Interestingly, both male and female *Sel1L<sup>Alb</sup>* mice showed significant growth retardation postweaning compared to their WT littermates on regular chow diet (Fig 1D). This growth retardation was due to shorter stature as demonstrated by body length measurements (Fig 1E and F), while ratios of organ to body weights for the liver and kidneys were unaffected (Fig 1G). Daily food intake was comparable (Fig 1H) between the two genotypes. Female *Sel1L<sup>Alb</sup>* mice at 2–4 months of age had reduced estrous cycle, which normally lasted 4–5 days in *Sel1L<sup>fl/fl</sup>* littermates (Fig 1I) and hence were largely infertile, but not the males.

Histological examination of the *Sel1L<sup>Alb</sup>* livers revealed no obvious abnormalities (Fig 1J), and there was no increase in cell death as measured by TUNEL staining (Fig EV1A) and caspase-3 cleavage (Fig 1K), nor inflammation as measured by the expression of various inflammatory genes (Fig EV1B). ER stress was only moderately increased in the Sel1L-deficient hepatocytes, as measured by Ire1 $\alpha$  phosphorylation and *Xbp1* mRNA splicing in the livers of 9-week-old *Sel1L<sup>Alb</sup>* mice (Fig EV1C and D) as well as in *Sel1L<sup>Alb</sup>* primary hepatocytes (Fig EV1E). Morphologically, the ER in *Sel1L<sup>Alb</sup>* hepatocytes was dilated (Fig 1L). These observations are in line with our previous reports in several cell types such as adipocytes and enterocytes that Sel1L deficiency *in vivo* is associated with a mild adaptive UPR, and uncoupled from inflammation and cell death (Qi *et al*, 2017; Sha *et al*, 2014; Sun *et al*, 2016, 2014, 2015). Taken together, these data demonstrate that Sel1L deficiency in the liver leads to growth retardation without overt inflammation or cell death.

### Elevated Fgf21 levels in the absence of Sel1L

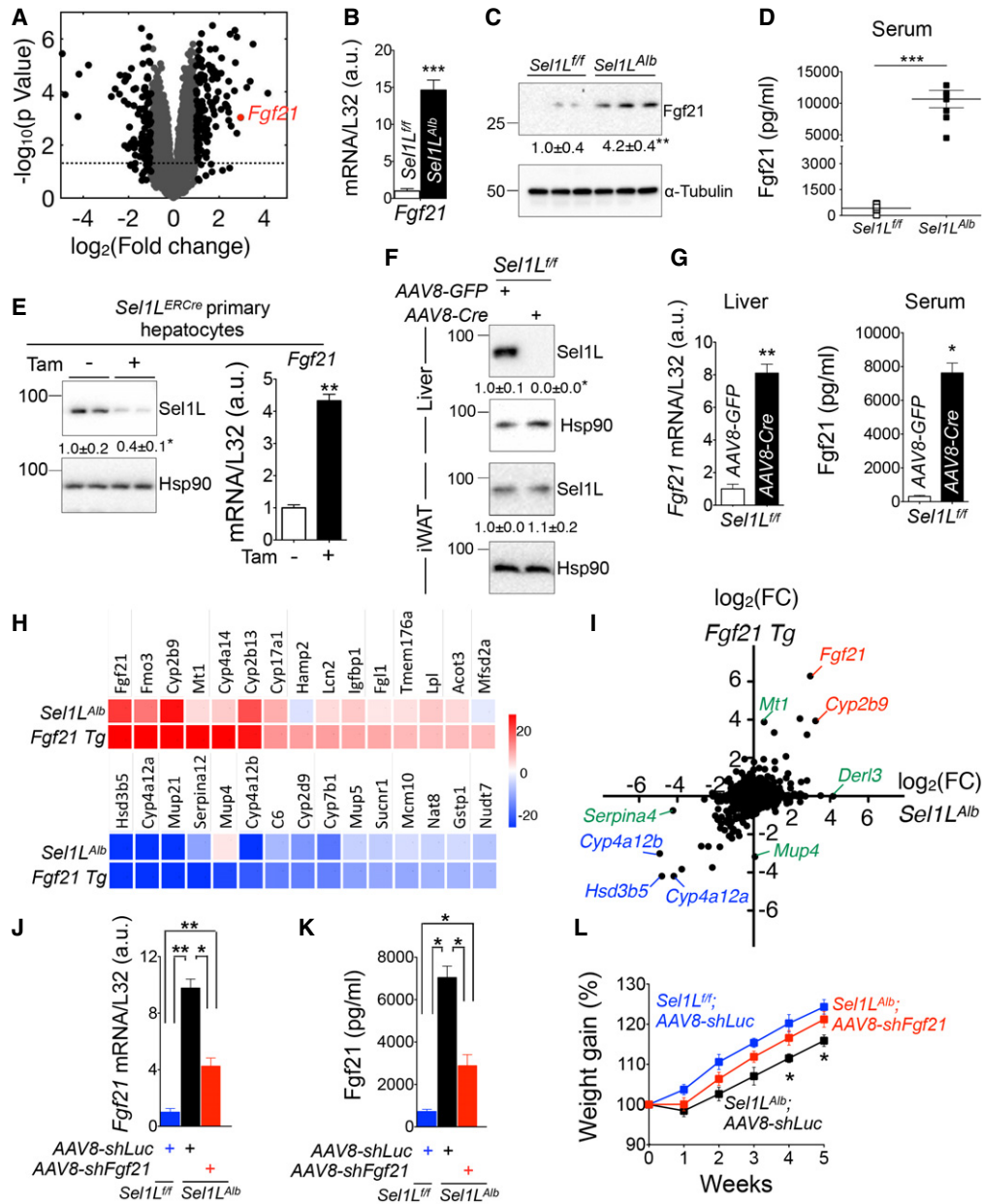
To explore how deletion of Sel1L in the liver has such a profound effect on growth, we performed transcriptomics analysis of the livers from *Sel1L<sup>Alb</sup>* mice and their control littermates at 9 weeks of age. A total of 169 genes were significantly upregulated, while 129 genes were significantly downregulated ( $P < 0.05$ , black dots represent fold change  $> 2$ ) in *Sel1L<sup>Alb</sup>* livers (Fig 2A). Gene set enrichment analysis confirmed significant upregulation of “protein



**Figure 1. Liver-specific Sel1L deficiency in mice (*Sel1L<sup>Alb</sup>*) causes growth retardation.**

- A Western blot analysis and quantitation of Sel1L-Hrd1 ERAD proteins in the livers of WT mice at 3, 9 and 24 weeks of age ( $n = 3-6$  per group, 2 independent repeats).
- B Western blot analysis and quantitation of Sel1L-Hrd1 ERAD proteins in the livers of 10-week-old WT mice under fasted (overnight) or *ad libitum* fed conditions ( $n = 3-6$  per group, 2 independent repeats).
- C Western blot analysis and quantitation of ERAD proteins in *Sel1L<sup>fl/fl</sup>* and *Sel1L<sup>Alb</sup>* livers ( $n = 4$  per group, 3 independent repeats).
- D Growth curves of male ( $n = 10$  each) and female ( $n = 7$  each) mice.
- E, F Representative images (E) and nose-to-anus length (F) of male mice at 6 weeks of age ( $n = 6-10$  per group).
- G Organ-to-body weight ratios of liver and kidney in 6-week-old male mice ( $n = 6$  per group, 3 independent repeats).
- H Daily food intake (g/day) normalized to gram of body weight (gbw) ( $n = 3$  per group, measured over 3 days).
- I Representative estrus cycle mapping in 2- to 4-month-old females ( $n = 6$  per group).
- J H&E images of paraffin-embedded liver sections from 9-week-old mice ( $n = 4$  per group, 3 independent repeats).
- K Western blot analysis of cell death (cleaved and pro-caspase-3) in livers of 9-week-old mice, with WT ileum as positive control ( $n = 3$  per group, 3 independent repeats).
- L Representative transmission electron microscope (TEM) images obtained from 9-week-old female mice livers ( $n = 10-12$  cells from one mouse each). N, nucleus; mito, mitochondria; ER, endoplasmic reticulum.

Data information: Hsp90 and  $\alpha$ -tubulin, loading controls for Western blot analysis. Values are mean  $\pm$  SEM; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , n.s., non-significant by Student's *t*-test.



**Figure 2. Elevated *Fgf21* expression in the liver as well as circulating *Fgf21* in the absence of *Sel1L*.**

- A Volcano plot depicting transcriptomics data from the livers of 9-week-old *Sel1L<sup>fl/fl</sup>* and *Sel1L<sup>Alb</sup>* mice ( $n = 3$  per group); dotted line marks  $P = 0.05$ ; black dots represent fold change  $> 2$ .
- B, C qPCR (B) and Western blot (C) analyses of *Fgf21* expression in 9-week-old livers ( $n = 3-6$  per group, 3 independent repeats).
- D ELISA analysis of *Fgf21* in serum from 8- to 9-week-old mice ( $n = 6-7$  per group).
- E Protein levels of *Sel1L* (left panel) and mRNA levels of *Fgf21* (right panel) in primary mouse hepatocytes isolated from the tamoxifen-inducible *Sel1L*-knockout *Sel1L<sup>ERCre</sup>* mice (2 independent repeats).
- F, G Acute loss-of-function model where 8-week-old *Sel1L<sup>fl/fl</sup>* mice were injected i.v. with either AAV8-Cre or control AAV8-GFP: (F) Western blot analysis of hepatic and control adipose *Sel1L* protein ( $n = 3$  per group); and (G) qPCR analysis of hepatic *Fgf21* expression and ELISA analysis of serum *Fgf21* ( $n = 3$  per group, 2 independent repeats).
- H Heatmaps of top 15 significantly upregulated and downregulated genes in *Fgf21 Tg* livers and their expression levels in *Sel1L<sup>Alb</sup>* livers ( $n = 3$  per group).
- I Scatter plot depicting the logarithmic fold change (FC) for 16,402 genes in *Sel1L<sup>Alb</sup>* and *Fgf21 Tg* livers ( $n = 3$  per group); genes that are highly upregulated or downregulated in both datasets are marked in red and blue, respectively; genes that are upregulated unique to each data set (e.g., *Derl3* for *Sel1L*-Hrd1 ERAD-deficient liver) are marked in green.
- J-L Data from rescue experiments where 5-week-old *Sel1L<sup>fl/fl</sup>* and *Sel1L<sup>Alb</sup>* mice were injected i.v. with AAV8-sh*Fgf21* or control AAV8-sh*Luc*: (J-K) qPCR analysis of *Fgf21* mRNA (J) and ELISA analysis of *Fgf21* in serum (K) 3 weeks after injection ( $n = 4$  per group). (L) Weight gain curve after injection ( $n = 7$  per group).

Data information: Hsp90 and  $\alpha$ -tubulin, loading control for Western blot analysis. Ribosomal *L32*, loading control for qPCR analysis. Values are mean  $\pm$  SEM; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , n.s., non-significant by Student's  $t$ -test (B-G) or two-way ANOVA analysis (J-L).

processing in the ER”, in keeping with the notion that Sel1L deficiency triggers cellular adaptation in hepatocytes. Additionally, various key metabolic pathways, including “insulin sensitivity”, “PI3K-Akt pathways”, “steroid hormone synthesis”, and more, were significantly affected in *Sel1L<sup>Alb</sup>* livers (Appendix Fig S2A).

Interestingly, *Fgf21*, a fasting-induced hormone, was one of the top hits among the most highly upregulated genes in *Sel1L<sup>Alb</sup>* mice (Fig 2A). Quantitative PCR (qPCR) and Western blot analyses showed a 13- and 4-fold increase of *Fgf21* mRNA and protein levels, respectively, in the *Sel1L<sup>Alb</sup>* livers (Fig 2B and C). Strikingly, serum levels of *Fgf21* were elevated by over 20-fold in *Sel1L<sup>Alb</sup>* mice, reaching to ~ 10 ng/ml vs. ~ 0.5 ng/ml in WT littermates (Fig 2D). As a control, *Fgf21* expression in brown and white adipose was not changed (Appendix Fig S2B).

To exclude potential developmental defects associated with chronic *Sel1L* deletion, we performed acute *Sel1L* deletion in the following two experiments. First, we cultured primary hepatocytes from a tamoxifen-inducible *Sel1L*-floxed mouse model (*Sel1L<sup>ERCre</sup>*) and found that *Fgf21* expression was significantly increased upon the addition of tamoxifen (Fig 2E). Second, we injected 8-week-old *Sel1L<sup>ff</sup>* mice once i.v. with adeno-associated virus serotype 8 (AAV8)-expressing Cre recombinase (or control GFP). Acute injection of AAV8-Cre abolished *Sel1L* protein level specifically in the liver, not in white adipose tissue (Fig 2F) 2–3 weeks’ postinjection. Similar to the chronic *Sel1L<sup>Alb</sup>* model, acute *Sel1L* deletion led to an 8-fold increase of hepatic *Fgf21* mRNA, and a profound 14-fold elevation of circulating *Fgf21* levels (Fig 2G). Hence, these data firmly establish the key role of hepatic *Sel1L*-Hrd1 ERAD in regulating *Fgf21* levels in both chronic and acute *Sel1L*-deficient models.

We next performed an unbiased comparison of genome-wide expression analyses of genes that were altered in *Sel1L<sup>Alb</sup>* livers to genome-wide expression analysis of genes altered in *Fgf21*-overexpressing transgenic mouse livers (*Fgf21 Tg*; GEO dataset series GSE39313). Indeed, the two datasets showed a large overlap among the top 15 upregulated or downregulated hepatic genes (Fig 2H and Appendix Fig S2C). This analysis further revealed a positive correlation between the genes altered in both datasets, as evident from the polarization of the scatter plot toward the top-right and bottom-left quadrants (Fig 2I). Several examples of shared genes are highlighted in red (upregulated) and blue (downregulated) while those unique to each dataset, i.e., close to either axis, are marked in green (Fig 2I). Therefore, *Sel1L* deficiency in the liver results in elevated *Fgf21* expression and circulating *Fgf21*.

### Systemic metabolic changes of *Sel1L<sup>Alb</sup>* mice are in part mediated by *Fgf21*

To further establish the causal link between elevated *Fgf21* levels and *Sel1L* deficiency-associated systemic changes, we injected *Sel1L<sup>Alb</sup>* mice with AAV8 encoding shRNA against *Fgf21* or control luciferase (*luc*). As a result of this knockdown, both the hepatic mRNA and circulating levels of *Fgf21* in these mice were reduced by around 60% (Fig 2J–K) while having no effect on the *Sel1L*-Hrd1 protein levels in the liver (Appendix Fig S3A). Interestingly, this knockdown partially reversed the growth retardation (Fig 2L) and rescued the reduced female fertility (Appendix Fig S3B) of the *Sel1L<sup>Alb</sup>* mice.

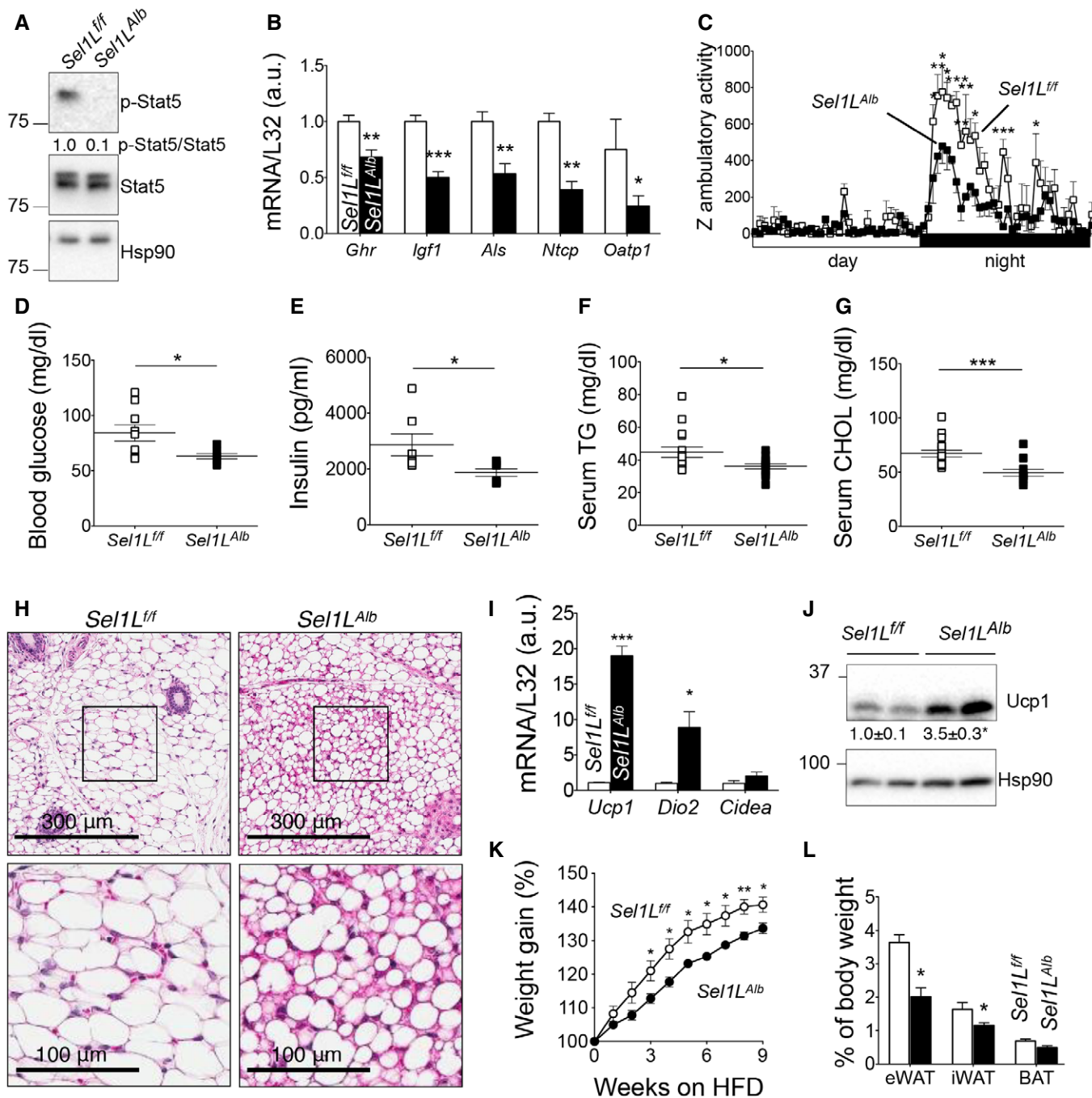
To provide further evidence for the *Sel1L*-*Fgf21* link, we next asked whether *Sel1L<sup>Alb</sup>* mice phenocopy *Fgf21* gain-of-function

models. *Fgf21* has many metabolic effects aimed at promoting organismal adaptation to starvation (BonDurant *et al*, 2017; Potthoff *et al*, 2012). Importantly, similar to our *Sel1L<sup>Alb</sup>* mice, *Fgf21* transgenic mice also exhibited growth retardation, which was partially mediated by Stat5-dependent signaling pathways (Inagaki *et al*, 2008; Kharitononkov *et al*, 2005; Owen *et al*, 2014; Zhang *et al*, 2012a,b). Indeed, in line with *Fgf21* gain-of-function models, Stat5 phosphorylation was markedly decreased in *Sel1L<sup>Alb</sup>* livers (Fig 3A) with diminished expression of various growth-related, p-Stat5-associated genes such as *Growth hormone receptor (Ghr)*, *Insulin-like growth factor-1 (Igf1)*, *Acid labile subunit (Als)*, *Na<sup>+</sup>-taurocholate cotransporting polypeptide (Ntcp)*, and *Organic anion transporter family member-1 (Oatp1)* (Fig 3B). Moreover, in line with the suppressive effect of *Fgf21* on hepatic lipogenesis (Chau *et al*, 2010; Zhu *et al*, 2014), many genes involved in hepatic lipid synthesis and transport, including *Sterol regulatory element-binding protein-1c (Sreb1c)*, *3-Hydroxy-3-methylglutaryl-CoA reductase (Hmgcr)*, *Acetyl-CoA carboxylase-1/2 (Acc1/2)*, *Low-density lipoprotein receptor (Ldlr)*, *ATP binding cassette subfamily G member-5/8 (Abcg5/8)*, and *ATP binding cassette subfamily A member-1 (Abca1)*, were significantly downregulated in *Sel1L<sup>Alb</sup>* livers compared to those of their WT littermates (Fig EV2A).

In mice, *Fgf21* is known to induce torpor, a hibernation-like energy-conserving, low-activity physical state (Bookout *et al*, 2013; Inagaki *et al*, 2007). Indeed, *Sel1L<sup>Alb</sup>* mice exhibited lower ambulatory activity compared to that of WT littermates (Fig 3C). Moreover, similar to *Fgf21* gain-of-function mouse models (Inagaki *et al*, 2007; Kharitononkov *et al*, 2005; Owen *et al*, 2014), *Sel1L<sup>Alb</sup>* mice exhibited reduced blood glucose levels (Fig 3D), lower serum insulin levels (Fig 3E) with comparable serum glucagon levels (Fig EV2B), and significantly decreased serum triglyceride and cholesterol levels (Fig 3F and G) compared to wild-type littermates. Additionally, *Sel1L<sup>Alb</sup>* mice exhibited improved insulin sensitivity, but comparable glucose tolerance (Fig EV2C and D). This improved insulin sensitivity phenotype was also partially reversed upon AAV-mediated *Fgf21* knockdown (Fig EV2E) in *Sel1L<sup>Alb</sup>* mice.

*Fgf21* is known to affect white adipose tissue browning (BonDurant *et al*, 2017; Fisher *et al*, 2012; Hondares *et al*, 2010; Owen *et al*, 2014; Park *et al*, 2016a). Histological assessment of inguinal white adipose tissue depots from *Sel1L<sup>Alb</sup>* mice revealed smaller lipid droplets (Fig 3H), which was confirmed by immunostaining for the lipid droplet marker perilipin (Appendix Fig S4A and B). Indeed, in the inguinal white adipose tissues of *Sel1L<sup>Alb</sup>* mice, expression levels of genes associated with browning such as *Uncoupling protein-1 (Ucp1)* and *Iodothyronine deiodinase-2 (Dio2)* were significantly elevated by ~ 20- and ~ 10-fold, respectively (Fig 3I). Protein levels of *Ucp1* were tripled in inguinal white adipose tissues of *Sel1L<sup>Alb</sup>* mice (Fig 3J).

*Fgf21* is also considered as an anti-obesity hormone owing to its ability to confer resistance to diet-induced weight gain (Kharitononkov *et al*, 2005). In keeping with this, *Sel1L<sup>Alb</sup>* mice exhibited significantly reduced weight gain (Fig 3K) upon being fed a 60% high-fat diet, along with significantly reduced fat depots (Fig 3L and Appendix Fig S4C) and improved insulin sensitivity (Appendix Fig S4D), overall suggestive of an ameliorated response to diet-induced obesity. Therefore, we conclude that the metabolic phenotypes of *Sel1L<sup>Alb</sup>* mice are in part mediated through *Fgf21*.



**Figure 3. *Sel1L<sup>Alb</sup>* mice phenocopy *Fgf21*-gain-of-function mice.**

- A Western blot analysis of p-Stat5 in livers of 9-week-old mice ( $n = 3$  per group, 3 independent repeats), with quantitation of the ratio of p-Stat5 to total Stat5 shown below the blot.
- B qPCR analysis of p-Stat5-associated growth genes in the livers of 9-week-old mice ( $n = 6$  per group, 2 independent repeats).
- C Z ambulatory activity of 9-week-old male mice as measured over 24 h ( $n = 4$  males per group).
- D–G Blood glucose (D), serum insulin (E), serum triglyceride (TG) (F), serum cholesterol (CHOL) (G) levels in 9-week-old mice after 6 h fasting ( $n = 6$ –10 per group).
- H H&E images of inguinal white adipose tissue (iWAT) from 8-week-old mice ( $n = 3$  per group, 2 independent repeats).
- I qPCR analysis of browning-related genes in iWAT ( $n = 3$ –6 per group).
- J Western blot analysis of Ucp1 in iWAT of 8-week-old mice ( $n = 3$ –4 per group, 3 independent repeats).
- K Weight gain curve of male mice after 60% high-fat diet (HFD) starting at 5 weeks of age ( $n = 4$  per group, 2 independent repeats).
- L Adipose tissue weight normalized to body weight in male mice following 9 weeks of HFD feeding ( $n = 4$  per group).

Data information: Hsp90, loading control for Western blot analysis. Ribosomal L32, loading control for qPCR analysis. Values are mean  $\pm$  SEM; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  by Student's *t* test or two-way ANOVA, as needed.

### Crebh, not Ppar $\alpha$ , accumulates intracellularly in the absence of Sel1L

We next explored how Sel1L or Sel1L-Hrd1 ERAD is linked to *Fgf21* in the liver. *Fgf21* expression is transcriptionally controlled synergistically by nuclear receptor Ppar $\alpha$  and Crebh. The schematic model depicting the intracellular trafficking of Crebh from ER to Golgi, proteolytic cleavage at Golgi and nuclear entry is shown in Fig EV3A. Additionally, *Fgf21* expression may also be regulated via transcription factors such as Xbp1s in response to ER stress (De Sousa-Coelho et al, 2012, 2013; Kim et al, 2013b; Laeger et al, 2014; Wan et al, 2014); however, the physiological significance of these regulators remains to be established.

Excitingly, Crebh protein levels were significantly elevated (> 20-fold) in the livers of *Sel1L<sup>Ab</sup>* mice at 9 weeks of age (Fig 4A and quantitated in Fig 4C). By contrast, the protein levels of Ppar $\alpha$  and Xbp1s were unchanged (Fig 4B and quantitated in Fig 4C). Importantly, this Crebh protein accumulation was due to a post-transcriptional mechanism as the mRNA levels of *Crebh* were not affected by Sel1L deficiency (Fig 4D). Moreover, the nuclear, active form of Crebh (Crebh-N) was also significantly upregulated by ~ 8-fold in *Sel1L<sup>Ab</sup>* livers (Fig 4B and quantitated in Fig 4C). Similar induction of Crebh protein was observed in the livers of adult mice with acute deletion of Sel1L (Fig 4E).

qPCR analysis further confirmed that, in addition to *Fgf21*, several other known Crebh target genes, such as *Amyloid P component, serum (Apcs)*, *Apolipoprotein-A4 (Apoa4)*, and *Cell death inducing DFFA like effector c (Cidec)* (Park et al, 2016a,b; Xu et al, 2014; Zhang et al, 2006), were also significantly upregulated in *Sel1L<sup>Ab</sup>* livers (Fig EV3B). In contrast, canonical Ppar $\alpha$  targets such as *Acyl-CoA synthetase long-chain family member 1 (Acs1)*, *Carnitine palmitoyltransferase 1A (Cpt1a)*, *Acyl-CoA oxidase 1 (Acox1)*, and *Microsomal triglyceride transfer protein (Mtp)* were significantly downregulated in *Sel1L<sup>Ab</sup>* livers (Fig EV3C).

As *Fgf21* expression is reportedly induced by ER stress (Jiang et al, 2014; Kim et al, 2013a; Schaap et al, 2013), we next compared the effect of Sel1L-Hrd1 ERAD deficiency to pharmacological UPR activation on Crebh accumulation and *Fgf21* expression. First, the Crebh protein accumulation in *Sel1L<sup>Ab</sup>* livers was much higher than that in the livers of wild-type mice challenged with the potent pharmacological ER stress inducer tunicamycin (Fig 4F). Additionally, the transcriptional induction of *Fgf21* in *Sel1L<sup>Ab</sup>* livers or primary hepatocytes was much more pronounced compared to that in tunicamycin-challenged WT liver or in thapsigargin-treated WT primary hepatocytes (Fig EV3D and E), in spite of much higher ER stress observed in the latter (Fig EV1C–E). We conclude that hepatic Sel1L-Hrd1 ERAD regulates *Fgf21* induction through a mechanism involving Crebh, but independently of Ppar $\alpha$ - or ER stress.

Like the other recently characterized Sel1L-Hrd1 ERAD substrate Ire1 $\alpha$  (Sun et al, 2015), the accumulated Crebh protein in Sel1L-deficient hepatocytes remained soluble in mild detergent NP-40 (Fig 4G), suggesting that Crebh does not form insoluble aggregates in the absence of Sel1L-Hrd1 ERAD. Moreover, confocal immunostaining of Crebh further demonstrated the intracellular accumulation of Crebh protein including in the nucleus of *Sel1L<sup>Ab</sup>* hepatocytes when compared to WT livers (Fig 4H and zoomed-out images shown in Fig EV3F). Chromatin immunoprecipitation (ChIP) of

Crebh revealed increased binding of Crebh onto the proximal promoter of the *Fgf21* gene in *Sel1L<sup>Ab</sup>* liver vs. WT liver (Fig 4I).

Finally, to demonstrate a similar mechanism in human hepatocytes, we knocked out Sel1L via the CRISPR/Cas9 system in the human hepatoma cell line Hep3B. Acute deletion of Sel1L significantly increased CREBH protein level and *Fgf21* gene expression (Fig 4J–K). Taken together, we conclude that Sel1L deficiency in the liver leads to intracellular and nuclear accumulation of Crebh in both mice and humans.

### ER-resident Crebh is an endogenous Sel1L-Hrd1 ERAD substrate

A marked accumulation of Crebh protein in Sel1L-deficient cells prompted us to test the hypothesis that Crebh is a bona fide endogenous Sel1L-Hrd1 ERAD substrate in the liver. In keeping with our hypothesis, a previous study showed that Crebh is degraded by proteasomes (Bailey et al, 2007), but the nature and significance of this degradation event remains unknown. Indeed, Crebh protein was unstable with a half-life of about 45 min in Crebh-transfected HEK293T cells treated with the translation inhibitor cycloheximide (Fig 5A). Crebh protein became greatly stabilized with half-lives of over 4 h in *SEL1L<sup>-/-</sup>* and *HRD1<sup>-/-</sup>* cells (Figs 5A and EV4A). Notably, accumulation and stabilization of Crebh protein were more pronounced in *HRD1<sup>-/-</sup>* cells than in *SEL1L<sup>-/-</sup>* cells, potentially owing to the residual Hrd1 protein in *SEL1L<sup>-/-</sup>* cells (Fig 1C; Kim et al, 2018).

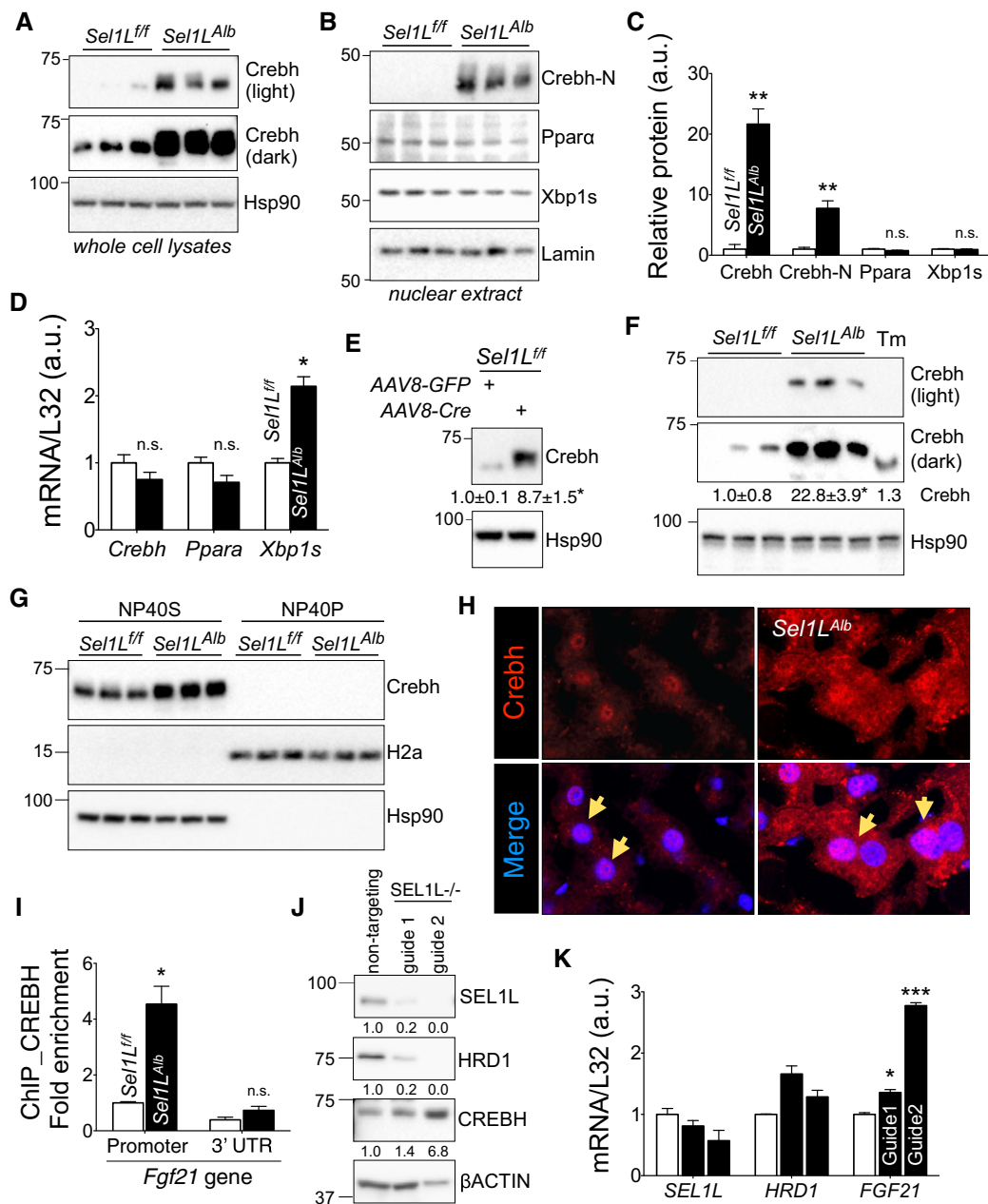
As Crebh is cleaved in the Golgi to generate N-terminal fragment Crebh-N, we next generate cleavage-defective Crebh by mutating its S1P and S2P cleavage sites (Zhang et al, 2006). Indeed, similar to wild-type Crebh, non-cleavable Crebh mutant was unstable and became stabilized in the absence of Sel1L and Hrd1 (Fig EV4B). Moreover, unlike Crebh, steady-state protein levels and half-life of Crebh-N protein were not affected by Hrd1 deficiency (Fig EV4C and D). Hence, these data point to the specificity of the Sel1L-Hrd1 ERAD effect toward ER-resident Crebh, not cleaved the Crebh-N fragment.

To further examine the contribution of proteasomes vs. lysosomes in the clearance of Crebh protein, we treated Crebh-transfected cells with proteasome inhibitor bortezomib (BTZ) or lysosomal inhibitor chloroquine (CHQ) in the presence of cycloheximide (CHX). BTZ, but not CHQ, stabilized Crebh protein (Fig 5B). Moreover, the effect of BTZ on Crebh protein was dampened in HRD1-deficient cells (Fig 5C), further supporting the notion that degradation of Crebh is largely mediated by the Sel1L-Hrd1 ERAD complex.

Crebh interacted strongly with both Hrd1 and Sel1L under regular native immunoprecipitation conditions (Fig EV4E and F and Lane 2, Fig 5D). Additionally, following lysate denaturing prior to immunoprecipitation, robust ubiquitination of Crebh protein was detected and blocked upon expression of catalytically inactive Hrd1 C2A mutant (top panel Lanes 1–3, Fig 5D). In contrast, Crebh-N did not interact strongly with, nor was ubiquitinated by, Hrd1 (Lanes 4–5, Fig 5D). Together, these data demonstrate that Crebh is targeted for proteasomal degradation by Sel1L-Hrd1 ERAD, requiring the activity of Hrd1 E3 ligase.

### Sel1L-Hrd1 ERAD deficiency does not affect the ER-to-Golgi trafficking of Crebh

Although ER stress was previously reported to be required for the ER exit of Crebh (Zhang et al, 2006), a recent study suggested that

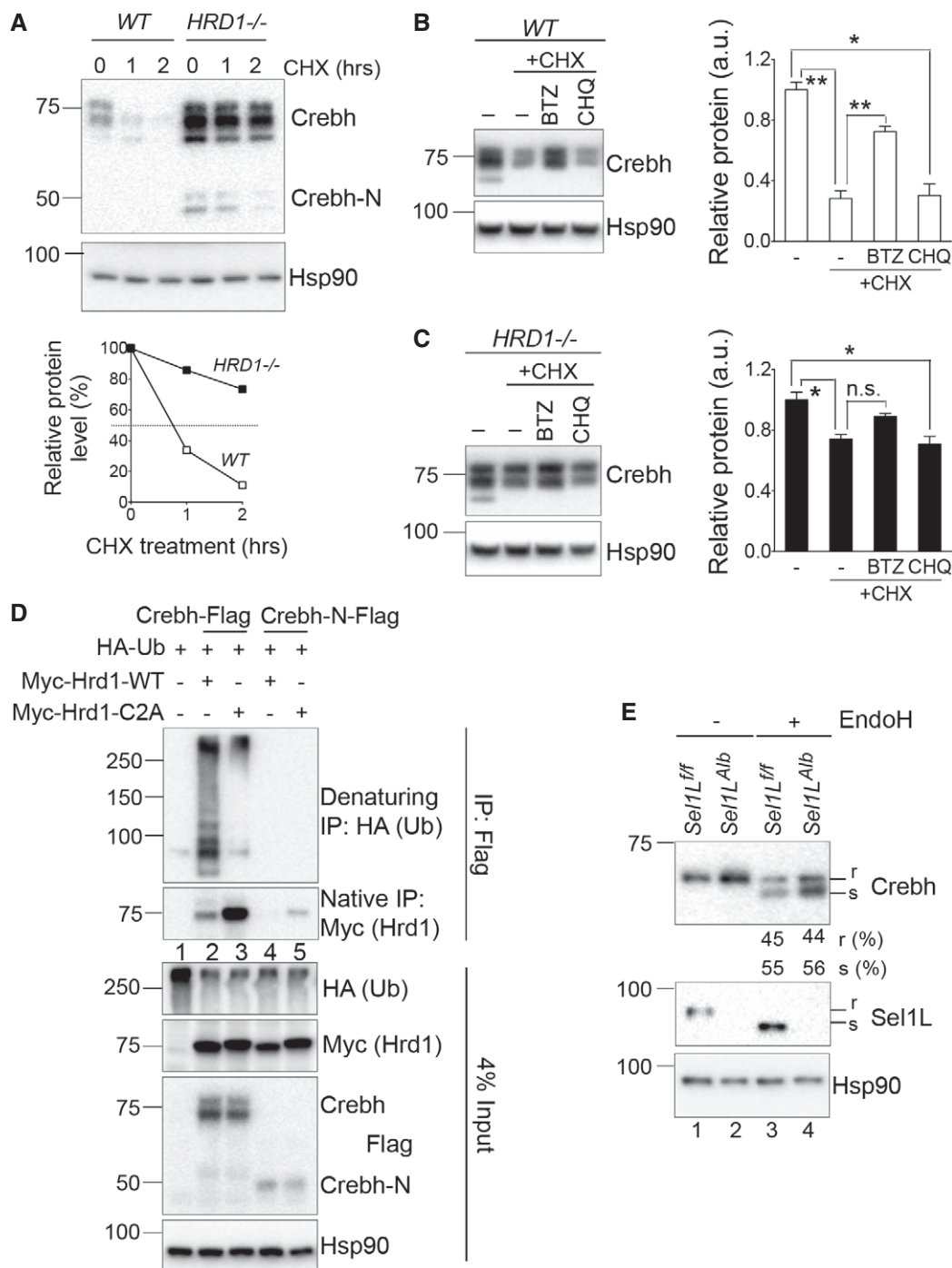


**Figure 4. Sel1L deficiency leads to the accumulation of Crebh in the liver.**

A–C Western blot analysis of Crebh, Crebh-N, Ppara, and Xbp1s in whole cell lysates (A) and nuclear extracts (B) in WT and *Sel1L<sup>Alb</sup>* livers (*n* = 3 per group, 3 independent repeats) with quantitation shown in (C).  
 D qPCR analysis of *Crebh*, *Ppara*, and *Xbp1s* in WT and *Sel1L<sup>Alb</sup>* livers (*n* = 4–6 per group, 2 independent repeats).  
 E Western blot analysis of Crebh in acute Sel1L loss-of-function model as described in Fig 2F and G (*n* = 3 per group).  
 F Western blot analysis of Crebh protein in 9-week-old mice (*n* = 3 per group, 2 independent repeats). WT mice injected i.p. with tunicamycin (Tm, 1.5 μg/g body weight) for 72 h were included as a control.  
 G Western blot analysis of Crebh in the livers of 9-week-old mice after NP-40-detergent fractionation into NP-40 soluble (NP-40S) and pellet (NP-40P) (*n* = 3 per group, 2 independent repeats).  
 H Representative confocal images of Crebh in the liver cryosections of 8-week-old mice (zoomed out versions in Fig EV3F). Note that a fraction of hepatocytes is binucleated. yellow arrows represent Crebh staining inside in the hepatocyte nucleus.  
 I ChIP analysis of Crebh binding onto the *Fgf21* promoter in the livers of 9-week-old mice, normalized first to 5% input group and then to no-antibody ChIP samples (*n* = 3 pooled per group, 2 independent repeats).  
 J, K Western blot analysis of SEL1L, HRD1, and CREBH proteins (J) and qPCR analysis of *SEL1L*, *HRD1*, and *FGF21* (K) in human Hep3B hepatocytes upon CRISPR deletion of SEL1L with two different guides.

Data information: Hsp90, H2A, β-actin, and lamin, loading controls for Western blot analysis. Ribosomal L32, loading control for qPCR analysis. Values are mean ± SEM; \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; n.s., non-significant by Student’s *t*-test.





**Figure 5. Crebh is an Sel1L-Hrd1 ERAD substrate.**

- A Western blot analysis of Crebh protein half-life in transfected WT and HRD1<sup>-/-</sup> HEK293T cells treated with cycloheximide (CHX) for indicated times. The decay of protein from one experiment is shown below.
- B, C Western blot analysis and quantitation of Crebh in Crebh-transfected WT (B) and HRD1<sup>-/-</sup> (C) HEK293T cells pre-treated with the proteasomal inhibitor bortezomib (BTZ) or lysosomal inhibitor chloroquine (CHQ) for 2 h and then with CHX for additional 1 h ( $n = 2$  per group, 2 independent repeats).
- D Western blot analysis of Crebh ubiquitination following immunoprecipitation (IP) of Crebh-Flag and Crebh-N-Flag in HEK293T cells transfected with indicated plasmids. Samples were boiled with SDS before IP for denaturing IP and not so for native IP. These cells were treated with proteasomal inhibitor BTZ for the last 6 h prior to immunoprecipitation.
- E Western blot analysis of endoglycosidase H (EndoH) sensitivity of Crebh in the livers of 9-week-old mice; “r” refers to endoH-cleavage-resistant species and “s” refers to endoH-cleavage-sensitive species ( $n = 3$  per group, 2 independent repeats).

Data information: Hsp90, loading control for Western blot analysis. Values are mean  $\pm$  SEM; \* $P < 0.05$ ; \*\* $P < 0.01$ ; n.s., non-significant by two-way ANOVA analysis.

may not be the case, but rather Crebh constitutively migrates to the Golgi (Xu *et al*, 2014). Does Sel1L-Hrd1 ERAD affect ER-to-Golgi trafficking of Crebh? As Crebh undergoes N-linked glycosylation in the ER, a step required for its ER exit to Golgi (Bailey *et al*, 2007; Chan *et al*, 2010), we next tested the endoglycosidase H (endoH) sensitivity of Crebh protein to distinguish ER- vs. Golgi-resident Crebh. Interestingly, despite having elevated total protein level at the basal state, Sel1L-deficient liver exhibited a similar percent of endoH resistant to sensitive Crebh protein to that in WT liver (Lanes 3 vs. 4, Fig 5E). Indeed, at the steady state, nearly 50% of CREBH were endoH resistant, i.e., at the Golgi, regardless of ERAD status, suggesting that a large portion of CREBH constitutively exits the ER. As a control, Sel1L as an ER-resident protein was completely endoH sensitive (Lanes 1 vs. 3, Fig 5E). Hence, our data suggest that Sel1L-Hrd1 ERAD does not regulate the trafficking of Crebh from the ER to Golgi.

### Crebh links hepatic Sel1L-Hrd1 ERAD to Fgf21 and systemic metabolic regulation

To demonstrate whether Crebh indeed links Sel1L-Hrd1 ERAD to Fgf21 and systemic metabolic regulation, we deleted Crebh in the livers of *Sel1L<sup>Alb</sup>* mice by i.v. injecting GFP-encoding AAV8 expressing shRNA against *Crebh* (*shCrebh*) or control luciferase (*shLuc*). Five weeks after AAV injection, GFP-positive livers were observed in all cohorts (Fig EV5A). *shCrebh* injection resulted in a ~60% reduction of Crebh protein and mRNA levels in *Sel1L<sup>Alb</sup>* livers (Fig 6A and B), and in conjunction, both mRNA and circulating levels of Fgf21 in *Sel1L<sup>Alb</sup>* mice were reduced by ~75% (Fig 6B and C). Importantly, the ER stress response markers such as *Xbp1* mRNA splicing and *Ire1a/Perk* pathway activation were not affected by Crebh knockdown in *shCrebh*-injected *Sel1L<sup>Alb</sup>* livers compared to the *shLuc*-injected *Sel1L<sup>Alb</sup>* livers (Fig EV5B–D), further underscoring that the effect of Sel1L-Hrd1 ERAD on Fgf21 is largely uncoupled from ER stress.

Upon Crebh deletion, the expression levels of p-Stat5-dependent, growth-associated genes were also elevated in *Sel1L<sup>Alb</sup>* mice compared to those receiving *shLuc* (Fig 6D). Indeed, *Sel1L<sup>Alb</sup>* mice with Crebh deletion showed better weight gain than *Sel1L<sup>Alb</sup>* mice injected with *shLuc* control (Figs 6E and EV5E). Moreover, deletion of Crebh in *Sel1L<sup>Alb</sup>* mice also partially reversed the insulin sensitivity of *Sel1L<sup>Alb</sup>* mice (Fig 6F). Lastly, Crebh knockdown markedly reduced the *Ucp1* expression in white adipose tissue that was observed in *Sel1L<sup>Alb</sup>* mice (Fig 6G and H). Hence, hepatic Sel1L-Hrd1 ERAD regulates growth and systemic metabolism, at least in part, via the suppression of the “Crebh-Fgf21” axis.

### Sel1L-Hrd1 ERAD suppresses “Crebh-Fgf21” axis under multiple physiological contexts

The above results pointed to Sel1L-Hrd1 ERAD as a key regulator of the “Crebh-Fgf21” axis with significant impact on systemic metabolism. We next asked whether there is a correlation between Sel1L-Hrd1 ERAD and Crebh-Fgf21 under two physiological settings—fasting-feeding and growth.

During feeding, hepatic Sel1L and Hrd1 levels were markedly higher than during fasting (Fig 1B and Lanes 3 vs. 4, Fig 7A), while protein levels of Crebh and Crebh-N exhibited the opposite trend (Lanes 3 vs. 4 and 1 vs. 2, Fig 7A). Indeed, there was negative

correlation between Sel1L-Hrd1 proteins and Crebh protein/*Fgf21* mRNA levels under fasting-feeding (Fig 7B). Furthermore, Crebh protein level was highly elevated and remained largely constant during fasting-feeding in Sel1L-deficient liver (Lanes 7–8 vs. 3–4, Fig 7A and quantitated in Fig 7C), suggesting that Sel1L-Hrd1 ERAD is largely responsible for the decline of Crebh protein level in the fed state. Consequently, the circulating levels of Fgf21 were also greatly elevated in both fasted and fed states of Sel1L-deficient mice (Fig 7D).

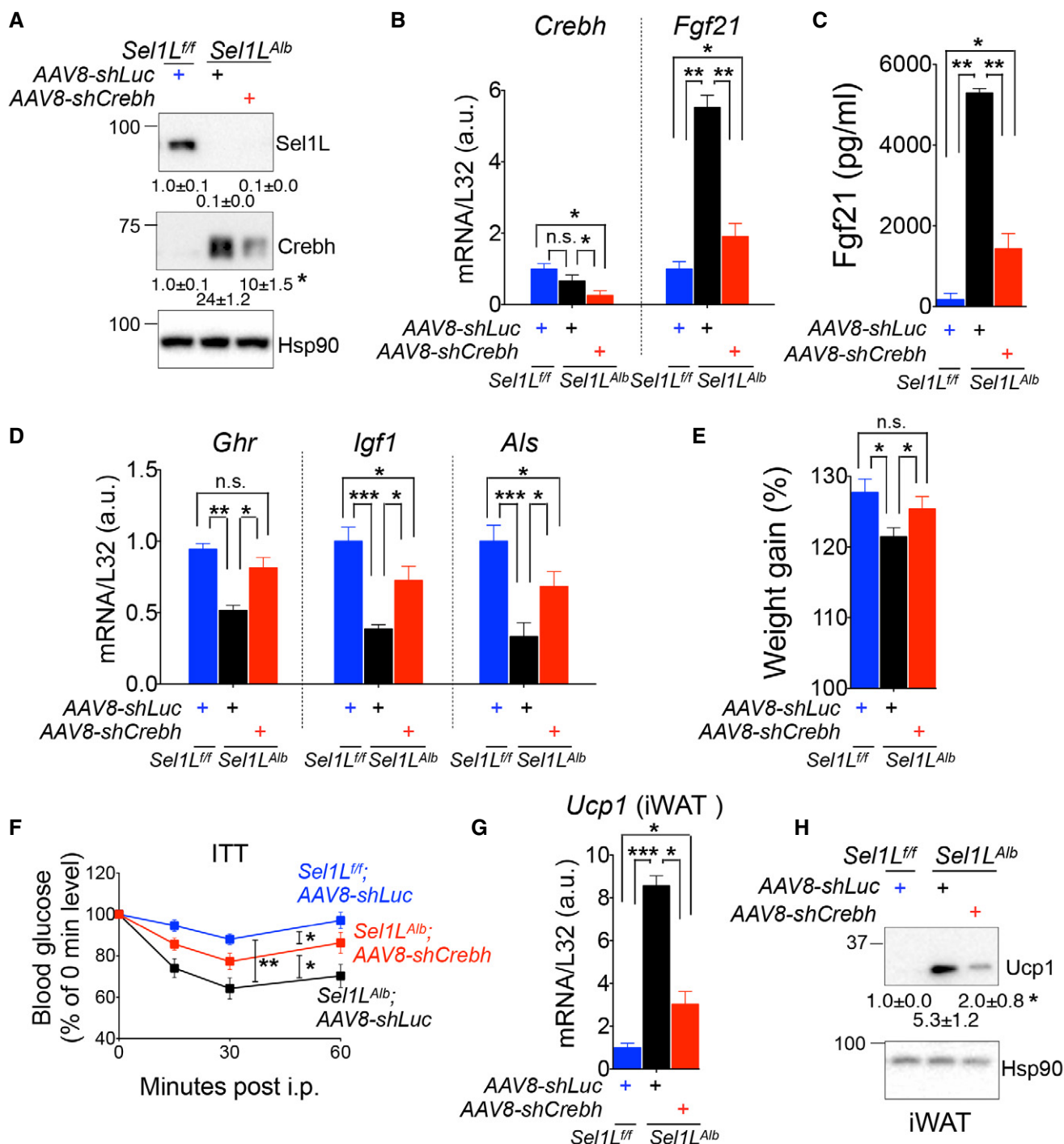
During growth, hepatic Sel1L and Hrd1 proteins also steadily increased with age from 3 to 24 weeks (Fig 1A and Lanes 4–6, Fig 7E), while protein levels of both Crebh and Crebh-N steadily decreased (Lanes 1–6, Fig 7E). There was a negative correlation between Sel1L-Hrd1 proteins and Crebh protein/*Fgf21* mRNA levels in WT mice during growth (Fig 7F). Importantly, in the *Sel1L<sup>Alb</sup>* mice, the decline of hepatic Crebh protein was markedly blunted by the Sel1L deficiency (Lanes 7–12 vs. 1–6, Fig 7E and quantitated in Fig 7G). This results in highly elevated circulating Fgf21 levels over the different ages of the *Sel1L<sup>Alb</sup>* mice, unlike the gradual decline with age in WT mice (Fig 7H).

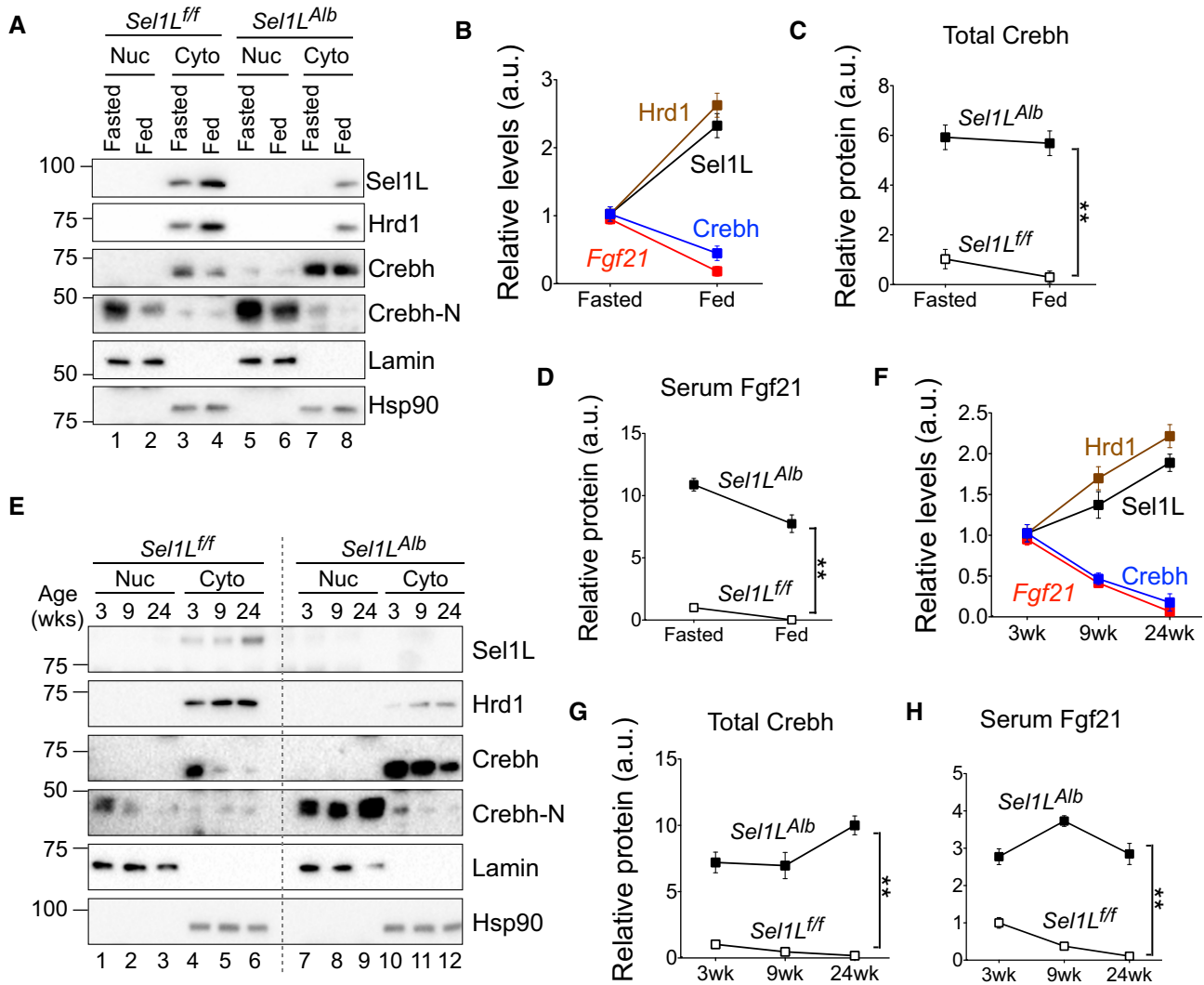
Therefore, these data not only demonstrate an inverse association between hepatic Sel1L-Hrd1 ERAD and Crebh protein levels under various physiological setting, but also establish Sel1L-Hrd1 ERAD as a key repressor of the “Crebh-Fgf21” axis during growth and fasting-feeding.

## Discussion

The liver controls overall energy metabolism and regulates a wide range of key processes in the body, including growth and fasting-feeding cycle (Lajtha *et al*, 1976). The hepatokine Fgf21 is a key metabolic endocrine hormone involved in inter-organ crosstalk and is considered as a potent therapeutic agent for the treatment of obesity and obesity-associated diabetes (Fisher & Maratos-Flier, 2016). This study reports Sel1L-Hrd1 ERAD as a key physiological repressor of Fgf21 gene transcription in the nucleus under various metabolic states in the body. Indeed, our data reveal a common mechanism by which diverse physiological contexts such as fasting-feeding and growth integrate at the level of Sel1L-Hrd1 ERAD via the control of Crebh protein abundance on the ER membrane to regulate *Fgf21* gene transcription (Fig 8). This metabolic tuning of Fgf21 levels by Sel1L-Hrd1 ERAD plays a key role in setting the normal growth and physiological paradigm in the body, as evident from the strong metabolic phenotypes of *Sel1L<sup>Alb</sup>* mice.

Our study reveals that growth and feeding can both increase the expression of the Sel1L-Hrd1 ERAD protein complex in the ER, which in turn negatively regulates the protein level of Crebh and *Fgf21* gene transcription. ER-resident Crebh protein is unstable, and its abundance is tightly controlled by the Sel1L-Hrd1 protein complex. Indeed, Crebh or *Fgf21* deletion in Sel1L-deficient mice significantly reverses or attenuates some growth and metabolic phenotypes of the hepatic Sel1L-deficient mice, suggesting that hepatic Sel1L-Hrd1 ERAD effect is at least partially mediated through the “Crebh-Fgf21” axis. The impact of Sel1L-Hrd1 ERAD deficiency on Crebh and *Fgf21* induction is profound, as demonstrated by the dramatic elevation of serum Fgf21 levels (~10 ng/ml), nearly reaching those in models of transgenic Fgf21-overexpression or pharmacological administration (~10–25 ng/ml).

**Figure 6. Crebh links hepatic Sel1L-Hrd1 ERAD to Fgf21.**Five-week-old *Sel1L<sup>ff/ff</sup>* and *Sel1L<sup>Alb</sup>* mice were injected i.v. once with AAV8-shCrebh or control AAV8-shLuc.A Western blot analysis of hepatic Sel1L and Crebh 5 weeks postinjection ( $n = 3$  mice each, 2 independent repeats).B, C qPCR analysis of *Crebh* and *Fgf21* mRNA (B) and ELISA analysis of circulating Fgf21 (C) 5 weeks postinjection ( $n = 3$  per group, 2 independent repeats).D qPCR analysis of hepatic growth-associated genes 5 weeks after injection ( $n = 6$  per group).E Weight gain 6 weeks postinjection ( $n = 10$  per group).F Insulin tolerance test (ITT) 5 weeks after injection ( $n = 10$  per group).G, H qPCR (G) and Western blot analysis (H) of *Ucp1* levels in inguinal white adipose tissue (iWAT) 5 weeks postinjection ( $n = 3$  per group, 2 independent repeats).Data information: Hsp90, loading control for Western blot analysis. Ribosomal L32, loading control for qPCR analysis. Values are mean  $\pm$  SEM; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., non-significant by two-way ANOVA analysis (B–G) and Student's  $t$ -test (A, H).



**Figure 7. Sel1L-Hrd1 ERAD represses Crebh and Fgf21 under fasting-feeding and growth.**

Analysis of correlation between Sel1L-Hrd1 ERAD and Crebh-Fgf21 during fasting-feeding (A–D) and growth (E–H).

**A** Western blot analysis of hepatic Sel1L-Hrd1 ERAD and Crebh of nuclear (Nuc) and cytosolic (Cyto) fractions from 10-week-old *Sel1L<sup>ff</sup>* and *Sel1L<sup>Alb</sup>* mice under overnight fasted or fed states.

**B** Quantitation of levels of Sel1L/Hrd1/Crebh proteins and *Fgf21* mRNA in the livers of 10-week-old *Sel1L<sup>ff</sup>* mice.

**C** Quantitation of total Crebh (Crebh+Crebh-N) protein levels in the livers of 10-week-old *Sel1L<sup>ff</sup>* and *Sel1L<sup>Alb</sup>* mice.

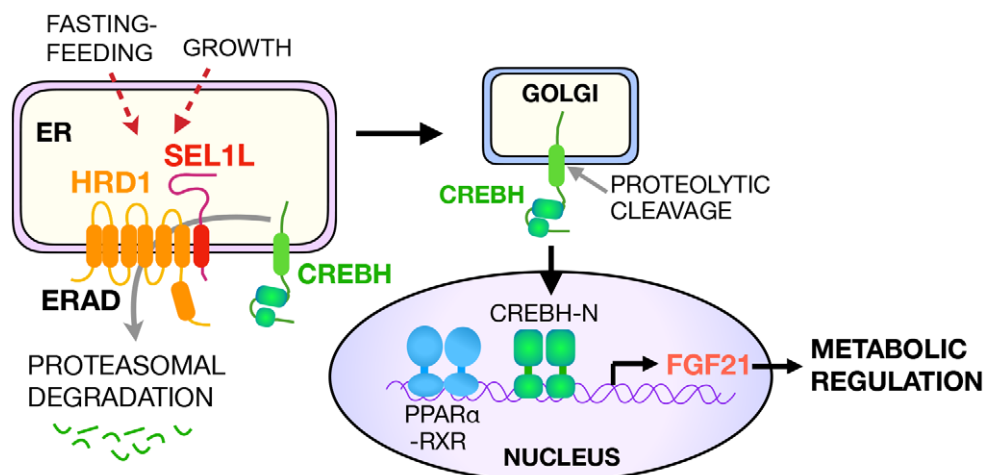
**D** Serum Fgf21 levels in 10-week-old *Sel1L<sup>ff</sup>* and *Sel1L<sup>Alb</sup>* mice.

**E–H** Similar to (A–D) with the exception that these experiments were done with the livers from *Sel1L<sup>ff</sup>* and *Sel1L<sup>Alb</sup>* mice at 3, 9, and 24 weeks of age.

Data information: Hsp90 and lamin, loading controls for cytosolic and nuclear fractions. Values are mean ± SEM; \*\**P* < 0.01 by two-way ANOVA analysis. *n* = 3–4 per group, 2 independent repeats.

Therefore, systemic energy homeostasis and *Fgf21* expression, both in the liver and circulation, is tightly linked to the activity of hepatic Sel1L-Hrd1 ERAD, the expression of which, in turn, may be responsive to the metabolic state of the body. For example, specific hormones and metabolites (such as insulin, glucagon, leptin, corticosterone, glucose, fatty acids) associated with fasting-feeding and/or growth may be involved in the transcriptional regulation of Sel1L and/or Hrd1. In this regard, further investigation will be needed to dissect the molecular pathways that link physiological cues to hepatic ERAD gene expression.

An outstanding question remains as to which transcription factor—Crebh or Pparα—is the more pertinent regulator of *Fgf21* expression in the liver in response to physiological cues. A recent study showed that Crebh is required for transcriptional activation of *Fgf21* by Pparα by forming the Crebh-Pparα complex and also by regulating the expression of *Pparα* (Kim *et al*, 2014). Our study demonstrates a profound effect of Sel1L-Hrd1 ERAD on *Fgf21* gene transcription via Crebh, but not Pparα, during growth and fasting-feeding. Although our study does not exclude the importance of Pparα in *Fgf21* transcription, it reveals another layer of regulation of



**Figure 8. Model: Hepatic Sel1L-Hrd1 ERAD regulates systemic metabolism via modulation of the “Crebh-Fgf21” axis in physiological contexts.**

Model depicting how physiological signals such as growth and fasting-feeding are integrated at the ER membrane via the Sel1L-Hrd1 ERAD complex in liver to regulate CREBH protein turnover at the ER membrane, and *Fgf21* transcription and metabolic states in the body. New findings elucidated by this study are highlighted in red arrows.

*Fgf21* expression during fasting-feeding and growth, centered at the level of ER membrane.

ERAD is often considered to be responsible for clearing “misfolded” proteins from the ER, and hence, ERAD deficiency is mostly known to cause the accumulation of misfolded proteins and thereby trigger ER stress. Indeed, one recurrent question associated with ERAD-deficient models is whether the presumptive induction of ER stress in ERAD deficiency is the true cause of observed phenotypes. Our data show that the effect of ER stress on Crebh and *Fgf21* expression pales when compared to that of Sel1L-Hrd1 ERAD deficiency in the liver or hepatocytes. Moreover, our data showed that Sel1L deficiency only triggers very moderate ER stress response, likely due to cellular adaptation as shown in other cell types (Kim *et al*, 2018; Shi *et al*, 2017). Hence, while modest but tonic ER stress may contribute somewhat to the overall phenotype of the Sel1L-deficient mice, the marked elevation of *Fgf21* is likely a direct result of Crebh stabilization and accumulation in the absence of Sel1L-Hrd1 ERAD. Indeed, a recent study reported that ER stress is dispensable for Crebh activation in the liver (Xu *et al*, 2014). Providing further support to our model, none of the previous hepatic UPR mouse models showed metabolic phenotypes similar to our hepatocyte-specific Sel1L-Hrd1 ERAD-deficient system or to the *Fgf21* gain-of-function mouse models (Hur *et al*, 2012; Jurczak *et al*, 2012; Lee *et al*, 2008; Olivares & Henkel, 2015; So *et al*, 2012; Zhang *et al*, 2011). For example, genetic ablation of the UPR sensor *Ire1α* alters lipid metabolism and ketogenic programs in the liver (So *et al*, 2012; Zhang *et al*, 2011). Silencing a major *Ire1α* effector *X-box Binding Protein-1 (Xbp1)* in the liver leads to marked disequilibrium in lipid homeostasis and impaired recovery from pharmacologically induced ER stress (Olivares & Henkel, 2015). Thus, we believe that mammalian Sel1L-Hrd1 ERAD in the liver plays an intimate and critical role in growth and fasting-feeding by directly regulating the activity of the “Crebh-Fgf21” axis. Additionally, liver-specific deletion of *Autophagy-related-7 (Atg7)* appears to lead to improved energy metabolism

in the body owing to mitochondrial defect-associated *Fgf21* production (Kim *et al*, 2013a). While we did not find any mitochondrial structural defects in our Sel1L-deficient livers, further investigation will be done to assess the relative importance and potential crosstalk between these two, distinct cellular quality control pathways in modulating *Fgf21* expression under physiological contexts.

Overall, this study delineates a novel role of Sel1L-Hrd1 ERAD in the liver and, importantly, establishes Sel1L-Hrd1 ERAD-mediated Crebh degradation as an important regulatory mechanism underlying *Fgf21* transcription regulation. This novel “ERAD-Crebh-Fgf21” cascade from the ER to the nucleus (Fig 8) represents another beautiful example of the regulation of cellular, and in this case, systemic response by Sel1L-Hrd1 ERAD and may provide with multiple components that could be utilized for therapeutic intervention in diseases associated with growth and metabolic defects.

## Materials and Methods

### Mice

The *Sel1L<sup>fl/fl</sup>* mice (Sun *et al*, 2014) were crossed with mice expressing Albumin-promoter-driven Cre on the C57BL/6J background (JAX 003574, B6.Cg-Tg(Alb-Cre)21Mgn/J) to generate hepatocyte-specific Sel1L-deficient mice (*Sel1L<sup>Alb</sup>*) and its WT littermates *Sel1L<sup>fl/fl</sup>*. Due to the infertility of *Sel1L<sup>Alb</sup>* female mice, only male *Sel1L<sup>Alb</sup>* mice were used for breeding. Mice were fed a low-fat diet (13% fat, 57% carbohydrate, and 30% protein, LabDiet 5LOD) or a high-fat diet (60% fat, 20% carbohydrate, and 20% protein) where indicated. Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments) was used with 1-day acclimatization period followed by 1-day measurement for metabolic cage analyses studies. Mice were housed on a regular 12-h light/dark cycle and regulated temperature (22°C). All animal procedures were done in accordance with the IACUC at Cornell University

(2007-0051) and University of Michigan Medical School (PRO00006888). Mice were routinely fasted in the morning for 5–6 h before sacrifice except as whenever stated (e.g., longer fasting or *ad libitum*). Mice were fasted for 6 h before ITT and 16 h overnight before GTT experiments. Cervical dislocation was used for euthanasia, and tissues were immediately either fixed or frozen in liquid nitrogen upon collection. Vaginal smears were examined on a daily basis for estrus cycle analysis.

Based on sample size formula of power analysis, 4–6 mice per group is the minimal number of animals to obtain statistical significance. A total of 4–10 mice was routinely used in each study to ensure adequate power. No samples or animals were excluded from analyses. Mice in each group were randomly chosen for experiment, matching in age, genotype, and gender. The investigators were not blinded during experiments and result assessment.

### Serum metabolite analysis

Multiplex analysis was carried out with appropriate standards and blanks for serum insulin and glucagon measurement. Serum triglycerides (TG) and cholesterol (CHOL) were measured by IDEXX Laboratories. Blood glucose was measured via the TrueTest Glucometer. Serum levels of Fgf21 were measured by ELISA analyses as per the protocol provided by the mouse Fgf21 ELISA kit (R&D Systems MF2100).

### Western blot analysis

Protein extraction from cell and tissue lysates and Western blotting following SDS–PAGE separation (and phos-tag gel for p-Ire1 $\alpha$ ) were performed as previously described (Qi *et al*, 2011; Sha *et al*, 2009; Yang *et al*, 2010). The quantification of signals was done using Bio-Rad ImageLab software, and protein levels among samples were normalized to Hsp90,  $\beta$ -actin, or  $\alpha$ -tubulin as loading controls. The commercially available antibodies used are as follows: Flag (1:500 for staining, 1:5,000 for Western blot; Sigma F1804); HA (1:3,000; Sigma H9658); Myc (1:1,000; Sigma); Ucp1 (1:10,000; Sigma U6382); Hsp90 (1:4,000; Santa Cruz sc-7947);  $\alpha$ -tubulin (1:2,000; Santa Cruz sc-5286); lamin (1:1,000; Santa Cruz sc-6215); Xbp1 (1:1,000; Santa Cruz sc-7160); BiP (1:1,000; Santa Cruz sc-1051); KDEL (1:500; Abcam ab50601); Sel1L (1:2,000; Abcam ab78298); Os9 (1:5,000; Abcam ab109510);  $\beta$ -actin (1:10,000, Abcam ab20272); Crebh (1:1,000 for Western, 1:100 for immunostaining; Kerfast EWS101); Fgf21 (1:1,000; R&D AF3057); Ppara $\alpha$  (1:500; Millipore MAB3890); Perilipin (1:200; Cell Signaling 9349); phospho-Stat5 (1:1,000; Cell Signaling 9359); Stat5 (1:1,000; Cell Signaling #94205); Ire1 $\alpha$  (1:2,000; Cell Signaling 3294); Perk (1:1,000; Cell Signaling 3192); p-elf2 $\alpha$  (1:1,000; Cell Signaling 9721); eIF2 $\alpha$  (1:2,000; Cell Signaling 9722); caspase-3 (1:1,000; Cell Signaling 9665); cleaved-caspase-3 (1:1,000; Cell Signaling 9661). Hrd1 antibody (1:300) was kindly provided by Dr. Richard Wojcikiewicz (SUNY Upstate Medical University); H2a antibody (1:5,000) was kindly provided by Dr. Yihong Ye (NIDDK). Secondary antibodies for Western blot were goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP (1:5,000; Bio-Rad), and donkey anti-goat IgG-HRP (1:5,000; Jackson ImmunoResearch). Secondary antibodies for immunostaining were anti-mouse IgG Cy3 and FITC; anti-rabbit IgG Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647; and anti-goat

IgG Alexa Fluor 594, Alexa Fluor 680 (all 1:500; Jackson ImmunoResearch).

### Immunoprecipitation

Cells/tissue were lysed in a buffer containing 150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 1% NP-40, protease inhibitors and phosphatase inhibitors, and 10 mM N-ethylmaleimide. A total of 2–6 mg protein lysates was incubated with antibody-coated agarose beads overnight with gentle rocking at 4°C. Immuno-complexes were washed in a buffer containing 137 mM NaCl, 20 mM Tris–HCl pH 7.5, 2 mM EDTA, and 10% glycerol and eluted by boiling at 95°C for 5 min in SDS sample buffer. For “denaturing” immunoprecipitation conditions in Fig 5D, lysis was first carried out in 200  $\mu$ l of above stated buffer with 5 mM DTT and 1% SDS, and denatured for 10 min at 95°C before diluting 1:5 with lysis buffer and continuing with antibody incubation.

### Detergent solubility analysis

Frozen liver tissue was weighed and homogenized as previously described in NP-40 lysis buffer. The lysate volume was then normalized by tissue weight and protein concentration via Bradford assay. The lysate was then centrifuged for 10 min at 12,000 g, and the supernatant collected was the NP-40-Soluble fraction after addition of 5 $\times$  SDS-containing sample buffer and heating at 65°C for 15 min. The pellet obtained was re-suspended in 1 $\times$  SDS sample buffer with a volume normalized to its corresponding soluble fraction and boiled at 95°C for 30 min to form the NP-40-Insoluble/Pellet fraction.

### Nuclear fractionation

Nuclear fractionation of the liver was performed as previously described (Sha *et al*, 2009).

### RNA extraction, RT, and qPCR

Total RNA was extracted from liver tissues and cells using Trizol and BCP phase separation reagent, and RNA quality was measured via OD. RT–PCR for Xbp1 splicing was performed as previously described (Sha *et al*, 2009). qPCR analysis was carried out using Taq polymerase, oilgo-dT primer, SYBR-Green-based master mix, and Applied Biosystems qPCR machine. All PCR data were normalized to the ribosomal *L32* gene expression level. qPCR primer sequences are as follows: *Sel1L* F: TGGGTTTTCTCTCTCCTCTG, R: CCTTTGTTCCGGTACTTCTTG; *Hrd1* F: AGCTACTTCAGTGAACCCACT, R: CTCCTTACAATGCCCACTGAC; *Os9* F: GCTGCTGACTGATGAGGAT, R: CGGTAGTTGCTCTCCAGCTC; *Ire1 $\alpha$*  F: CTGTGGTCAAGATGGACTGG, R: GAAGCGGAAGTGAAGTAGC; *BiP* F: TGTGGTACCCACCAAGAAGTC, R: TTCAGTGTCACTCGGA GAAT; *Chop* F: TATCTCATCCCCAGGAAACG, R: GGGCACTGACCA CTCTGTTT; *Fgf21* F: CTGGGGGTCTACCAAGCATA, R: CACC CAGGATTTGAATGACC; *Ghr* F: AGCTCGATTACAAGTGTCG, R: GATGACCAATTCTTGCAGCTTG; *Igf1* F: GCTCTTCAGTTCGTGTGTT GGAC, R: TTGGGCTGTCAGTGTGGCGC; *Als* F: AACCTCAGGAA TAACTCCTTGC, R: CACCGGCTGGCAGTCATCTCCC; *Oatp1* F: CCT

CAGCTGTACAATGATTGCC, R: TTTTGGTTCAATGCAGGGTTG; *Ntcp* F: GAAGTCCAAAAGGCCACACTATGT, R: ACAGCCACAGA GAGGGAGAAAAG; *Abcg5* F: TCAATGAGTTTTACGGCCTGAA, R: GCACATCGGGTGATTTAGCA; *Abcg8* F: TGCCACCTTCACAT GTC, R: ATGAAGCCGGCAGTAAGGTAGA; *Ldlr* F: AGGCTGTGGG CTCCATAGG, R: TGCCGTCCAGGGTCATCT; *Hmgcr* F: CTTGTGGA ATGCCTTGATG, R: AGCCGAAGCAGCACATGAT; *Srebp1c* F: GGAGCCATGGATTGCACATT, R: CCTGTCTACCCCCAGCATA; *Abca1* F: GCTTGTTGGCCTCAGTTAAGG, R: GTAGTTCAGGCGTA CAGAGAT; *Crebh* F: CAGCTCAAGAAAGCAGGAAG, R: AGCTGCTC CAGAAGAGACAA; *Ppara* F: GCGTACGGCAATGGCTTTAT, R: GA ACGGCTTCCTCAGGTTCTT; *Ucp1* F: CCAGTGGATGTGGTAAAAA CAA, R: TCAAAGCACACAAAATGATGA; *Dio2* F: CAGTGTGGT GCACGTCTCCAATC, R: TGAACCAAAGTTGACCACCAG; *Cidea* F: TGCTCTTGTATCGCCAGT, R: GCCGTGTTAAGGAATCTGCTG; *Apcs* F: ACAGTCCGTGGTATGGAAGA, R: TGGGGCTTTCACAGTG TATT; *Apoa4* F: CCAGTAAGCAACAATGCCA, R: TGGAAGAGGG TACTGAGCTGC; *Cidec* F: ATGGACTACCCATGAAGTCT, R: CGG TGCTAACACGACAGGG; *Acc1* F: ATGGGCGGAATGGTCTCTTTC, R: TGGGGACCTGTCTTCATCAT; *Acc2* F: CACCATCCGTGAAAA CATCA, R: AGCAGCTGAGCCACTGTAT; *Xbp1* F: ACACGCTGG GAATGGACAC, R: CCATGGGAAGATGTTCTGGG; *Acs1l*: F: AA CGATGTACGATGGCTTCC, R: CATATGGCTGGTTGGCTTT; *Acox1*: F: GATGTGACCCTTGGCTCTGT, R: AGAGATTCGGCCTCTCTGTG; *Cpt1a* F: ATGACGGCTATGGTGTTC, R: GGCTTGCTCAAGTG CTTC; *Mttp*: F: TACCCGTTCTGGTCTGCAT, R: TCTGGCTG AAGTGGAATAC.

### Primary hepatocyte culture

Mice (6–8 weeks of age) were euthanized with isoflurane inhalation, and the liver was perfused first with warm calcium/magnesium-free HBSS containing 25 mM HEPES, EDTA, 1% penicillin-streptomycin, and then with warm calcium/magnesium-free HBSS containing 25 mM HEPES, 2 mM CaCl<sub>2</sub>, 1% penicillin-streptomycin, and 0.75 mg/ml collagenase (Sigma-Aldrich C8051). After removal and mechanical dispersion of the swollen, digested liver, cells were suspended in ice-cold plating medium (DMEM with 2% sodium pyruvate, 2% penicillin-streptomycin, 10% heat-inactivated FBS, 1 μM dexamethasone, and 0.1 μM insulin) and passed through a 70-μm cell strainer. The cells were then pelleted by centrifugation for 3 min at 60 × g at 11°C and re-suspended buffered Percoll before pelleting by centrifugation for 4 min at 800 × g at 11°C. The final cells were washed thrice in plating medium (centrifugation for 3 min at 60 × g at 11°C), viability-checked (via trypan blue exclusion as > 80%), and plated on 6-well plates. Once the cells attached well (2–4 h after plating), the plating medium was replaced with culture medium (DMEM containing 2% sodium pyruvate, 2% penicillin-streptomycin, 0.2% BSA, 0.1 μM dexamethasone, and 1 nM insulin). Before treatment with nutrients/hormones, the cells were kept in starvation media (no serum/growth factors/hormones) for 4 h.

### Chromatin immunoprecipitation

Chromatin IP from liver tissue was carried out as per the protocol previously described (Murgatroyd *et al.*, 2012) using 1:250–500 dilution of the Crebh antibody, regular Protein A agarose beads

(Invitrogen) instead of magnetic beads, and phenol–chloroform-based DNA extraction in place of columns. The final precipitated DNA was amplified via qPCR using following primers: *Fgf21* promoter (positions –8 to –125 relative to transcribed region) F: ATCCCCAGCTGAGAAGACAC, R: GCCCTTTTCATTTCAGACCCC; end of *Fgf21* coding sequence (positions +1,214 to +1,439 relative to transcribed region) F: AAGGCTCTACCATGCTCAGG, R: CGT CTGCCTCAGAAGGACTC.

### Immunostaining and histology

For H&E staining, tissues upon dissection were directly placed in 10% neutral-buffered formalin, stored at 4°C, and processed by the Michigan Histology Core for paraffin embedding, section cutting and H&E staining on a fee-for-service basis. For other *in vivo* staining, livers on anesthetized mice were perfused first with PBS and then with 4% paraformaldehyde for fixation. After further overnight fixation, the tissues were dehydrated overnight in 15% sucrose solution and then frozen in Tissue-Tek O.C.T. Compound. 5-μm sections were cut using a cryotome for further staining. During immunocytochemistry, cells were cultured on poly-L-lysine-coated coverslips and were fixed in 4% formalin for 10 min. For paraffin-embedded sections, boiling in 1 mM EDTA for 25 min was done for antigen retrieval. For cryosections and cells, permeabilization was carried out in 0.3% Triton X-100 and 0.3% glycine for 10 min at room temperature. Thereafter, the sections/cells were incubated in blocking solution (1% donkey serum, 0.03% Triton X-100 in PBS) for 40–60 min at room temperature, and then incubated with primary antibodies for 24–72 h, as needed at 4°C in humidified chambers. Thereafter, they were washed thrice with PBST (0.03% Triton X-100 in PBS) and incubated with secondary antibodies for 2 h at room temperature. Counterstaining and mounting were performed using hard-set mounting medium containing DAPI (Vector H-1200). Fluorescent samples were imaged with Nikon A1 Confocal Microscope at the University of Michigan Imaging Core using identical imaging parameters within each experiment. H&E-stained samples were scanned using Aperio Scanscope (Leica Biosystems). Images were processed using ImageJ plugin (FIJI).

### TUNEL assay

Paraffin-embedded liver sections were subjected to TUNEL assay as per manufacturer’s protocol using the In-Situ Cell Death detection kit (Roche, 11684795910). Images were obtained by Zeiss LSM710 Confocal Microscope at the Cornell University Biotechnology Resource Center Imaging Facility.

### Transmission electron microscopy

The livers of anesthetized mice were perfused via the hepatic portal vein first with Sorenson’s buffer to wash out the blood and then with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M Na-cacodylate buffer as fixative. The liver was then cut into tiny pieces (about 1–2 mm cubes) and soaked overnight in the same fixative. Thereafter, the samples were submitted to the University of Michigan Histology and Imaging Core for washing, embedding, sectioning, and imaging via transmission electron microscopy (TEM).

## Cell lines and transfection

Cells lines were cultured in DMEM (Corning, NY) containing 1% penicillin/streptomycin, 1% sodium pyruvate, and heat-inactivated 10% FBS (GIBCO). Cells were transfected within 16–22 h after plating with Lipofectamine 2000 and harvested around 24 h after. Human hepatoma Hep3B cells were used to generate CRISPR knockouts of SEL1L via lentiviral packaging system with guide sequences ACTGCAGGCA GAGTAGTTGC and GACATCAGATGAGTCAGTAA. *HRD1*<sup>-/-</sup> HEK293T cells were previously described (Shi *et al*, 2017), and *Sel1L*<sup>-/-</sup> and *Hrd1*<sup>-/-</sup> N2a cells were previously described (Kim *et al*, 2018). An attempt to overexpress Sel1L in cells resulted in complications in cell survival, an issue currently under further investigation.

## Plasmids

All Crebh plasmid constructs (Crebh, Crebh-N) were generated as described (Zhang *et al*, 2006). The pAAV8-D(+)-U6-siRNA-CMV-GFP vector was purchased from Addgene. Myc-tagged WT and mutant (C2A) Hrd1 constructs were kindly provided by Dr. Yihong Ye (NIDDK). pcDNA3-HA-Ub was a kind gift from Dr. Hideki Nishitoh (University of Miyazaki, Japan). Sequences used for mutagenesis to generate cleavage-defective Crebh (mutating to Alanines the RNNNRNL of the S1P cleavage site to ANNNANA and the LP of the S2P cleavage site to AA) are as follows, with the new Alanines underlined: CCTCATCATCCGCCGCTCATCAGCCCTTTGGCCCCA ACAAACCGAGAGCCCTGGGGACTTTGCGCCTGTAGCAGTGTTCT CCGCAACTCGCCACAACGATG.

## AAV-mediated gene delivery

siRNA sequences against mouse *Fgf21* (GGGATTCAACACAGGAG AAAC) encoding siRNA, *Crebh* (TCGAGAAAAAAGACATAGCGG CTGGAAAGATCTCTTGAATCTTTCCAGCCGCTATGTCA) encoding the hairpin shRNA, and control luciferase (GTTGCGCGGAG GAGTTGTG) were cloned into pAAV8-D(+)-U6-siRNA-CMV-GFP vector via BamHI and EcoRI restriction sites. AAV was generated from these plasmids at the Harvard Children’s Hospital Virus Core (Boston, MA) on a fee-for-service basis. AAV8 was injected once into 5- to 8-week-old mice via the tail vein at the dose of 5–10 × 10<sup>11</sup> viral genome copies/mouse. Blood was collected via a small nick in the tail for subsequent ELISA analysis of circulating Fgf21 levels after 2 or more weeks’ postinjection.

## Microarray analysis

Liver tissues were snap-frozen in liquid nitrogen. RNA was extracted as described above followed by DNaseI (New England Biolabs) digestion. RNA quality and concentration were measured using an Agilent 2100 bioanalyzer. Microarray analysis was carried out as previously described (Sun *et al*, 2012) and is deposited as GSE118658.

## Statistical analysis

All results have been expressed as mean ± SEM unless otherwise stated. Comparisons between groups are done by paired two-tailed Student’s *t*-test or two-way ANOVA as needed. All experiments have been repeated at least twice or thrice and performed with multiple

independent biological samples from which representative data have been shown.

**Expanded View** for this article is available online.

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## Author contributions

AB designed and performed most experiments; SS designed and performed experiments with human hepatocytes; HW and ML provided technical assistance; QL provided reagents; SK performed microarray analyses; LY and KZ provided reagents and discussion; AB and LQ wrote the manuscript; everybody commented on and approved the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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