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ARTICLE

Ubiquitin C-terminal hydrolase L1 is required for pancreatic beta cell survival and function in lipotoxic conditions

K. Y. Chu · H. Li · K. Wada · J. D. Johnson

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Abstract
Aims/hypothesis Ubiquitin C-terminal hydrolase L1 (UCHL1) is associated with neurodegenerative diseases and has been suggested to have roles in pancreatic beta cells. Our proteomic analysis revealed that UCHL1 was the most increased protein in MIN6 cells exposed to palmitate. The present study used a genetic loss-of-function model to test the hypothesis that UCHL1 is required for normal beta cell function and fate under lipotoxic conditions.
Methods Human islets, mouse islets and MIN6 cells were used to analyse UCHL1 protein levels and regulation of UCHL1 by palmitate. The levels of free mono-ubiquitin and poly-ubiquitinated proteins were assessed. Gracile axonal dystrophy (GAD) mutant mice lacking UCHL1 were fed a normal or lipotoxic high-fat diet. Glucose tolerance, insulin tolerance and insulin secretion were assessed in vivo. Beta cell death and proliferation were assessed by TUNEL and proliferating cell nuclear antigen (PCNA) staining. Insulin secretion, calcium signalling, endoplasmic reticulum (ER) stress, apoptosis and SNARE protein levels were assessed in vitro.
Results UCHL1 protein, which was highly specific to beta cells, was increased by palmitate at basal glucose, but not in the context of hyperglycaemia associated with frank diabetes. Although islet development and function were initially normal in *Uchl1*$^{-/-}$ mice, a 4-week high-fat diet caused glucose intolerance and impaired insulin secretion. *Uchl1*$^{-/-}$ mice had increased ER stress and beta cell apoptosis. The levels of SNARE proteins were dysregulated in *Uchl1*$^{-/-}$ islets. Palmitate-stimulated vesicle-associated membrane protein 2 (VAMP2) ubiquitination was modulated by a chemical UCHL1 inhibitor.
Conclusions/interpretation Together, these data suggest that UCHL1 has essential functional and anti-apoptotic roles in beta cells under stress conditions associated with lipotoxicity.

Keywords Apoptosis · Parkinson's disease · Type 2 diabetes · Ubiquitin proteasome system

Abbreviations

BiP	Binding immunoglobulin protein
CHOP	C/EBP homologous protein
ER	Endoplasmic reticulum
GAD	Gracile axonal dystrophy
HA	Haemagglutinin
PCNA	Proliferating cell nuclear antigen
SNAP25	Synaptosomal-associated protein 25
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein
STX4	Syntaxin 4
VAMP2	Vesicle-associated membrane protein 2
UCHL1	Ubiquitin C-terminal hydrolase L1

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Introduction

Type 2 diabetes results from relative deficiency in functional beta cell mass, the product of beta cell function and survival [1, 2]. Multiple cellular functions and cell fate decisions are modulated by ubiquitination, a key reversible post-translational modification that can target proteins for degradation or modify their activity [3, 4]. Emerging evidence implies essential roles for ubiquitination in both beta cell survival and insulin secretion [5–8]. The ubiquitin proteasome system and ubiquitin-mediated autophagy have been reported to be regulated by stress conditions, such as glucotoxicity and oxidative stress, resulting in decreased insulin secretion and beta cell apoptosis [6, 7]. The roles of specific components of the ubiquitin proteasome system in beta cells are poorly understood.

Ubiquitin C-terminal hydrolase L1 (UCHL1; also known as PGP9.5, GAD and PARK5) was first identified as a deubiquitinating enzyme that hydrolyses the peptide bond at the C terminus of ubiquitin. It is involved in the processing of ubiquitin precursors and the poly-ubiquitin chains [9–11]. UCHL1 has also been shown to stabilise free ubiquitin and prevent its degradation [12]. In addition, it has been shown that dimerised UCHL1 can exhibit ubiquitin ligase activity, opposing its hydrolase function, to promote protein aggregation [13]. *Uchl1* expression is enriched in the brain, testis/ovary and pancreatic islets, whereas the closely related *Uchl3* gene is ubiquitously expressed [7]. Mutations and modifications of *UCHL1* are associated with human neurodegenerative diseases such as Parkinson's and Alzheimer's diseases [14, 15]. In mice, the gracile axonal dystrophy (GAD) mutation in *Uchl1* leads to neurodegeneration [16–18]. Neurons and beta cells have a large number of developmental and functional similarities, and UCHL1 has been proposed as a marker for both neuronal [19] and endocrine pancreatic precursor cells [20, 21]. Nevertheless, the in vivo roles of UCHL1 in islet cell development, function and fate have not been defined using a loss-of-function genetic approach.

Type 2 diabetes and neurodegenerative diseases have common risk factors and links with obesity [22, 23]. It is possible that these diseases have similar degenerative mechanisms in response to common risk factors. Type 2 diabetes, where obesity is a major risk factor [24, 25], is characterised by beta cell death in the lipotoxic milieu, as well as pathology associated with protein misfolding and accumulation [26], reminiscent of neurodegenerative diseases. Moreover, endoplasmic reticulum (ER) and oxidative stress have been suggested as common mediators of both beta cell [27–29] and neuronal [30, 31] death. Obesity and hyperlipidaemia cause ER [27, 32] and oxidative stress, and may promote both diabetes and neurodegenerative diseases [30, 31]. As part of our efforts to determine the mechanisms of lipotoxicity, UCHL1 was identified in a proteomic screen as the most upregulated protein in MIN6 beta cells treated with the fatty acid, palmitate [27]. Here, we test the hypothesis that UCHL1 is essential for beta cell function and survival under lipotoxic conditions using a combination of in vivo and in vitro studies and a mouse model lacking Uchl1.

Methods

Reagents Reagents were from Sigma unless otherwise indicated. Palmitic acid was complexed with 20% essentially fatty acid-free BSA as described [33]. All experiments were performed in the presence of 10% FBS. UCHL1 and UCHL3 inhibitors were from Calbiochem/EMD (Gibbstown, NJ, USA). Primary antibodies anti-proliferating cell nuclear antigen (PCNA), anti-UCHL1 and anti-syntaxin 4 (STX4), anti-insulin and anti-glucagon were from were from Millipore (Billerica, MA, USA), anti-ubiquitin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-C/EBP homologous protein (CHOP) was from Affinity BioReagents (Rockford, IL, USA), anti-vesicle-associated membrane protein 2 (VAMP2) was from Synaptic Systems (Goettingen, Germany), anti-synaptosomal-associated protein 25 (SNAP25), anti-K48 linked-ubiquitin, anti-ß-actin, anti-binding immunoglobulin protein (BiP) and anti-cleaved caspase-3 were from Cell Signaling Technology (Danvers, MA, USA). DRAQ5 far-red fluorescent DNA dye was from Biostatus (Shepshed, UK).

In vivo mouse studies Studies were approved by the University of British Columbia Animal Care Committee in accordance with national guidelines. The GAD mice used in the present study possess an intragenic deletion mutation in the *Uchl1* gene, which leads to the neuronal degeneration of the gracile tract and hindlimb paralysis at about 12 weeks of age [34]. Since this affects food intake and therefore blood glucose, mice were only used between 4 and 9 weeks of age, when these symptoms were not seen. In some experiments, mice were fed a high-fat diet (58% of energy from fat; OpenSource, New Brunswick, NJ, USA) or a control diet (11% of energy from fat; LabDiet5015) for 4 weeks after 4 weeks of age. Intraperitoneal glucose tolerance tests (IPGTTs; 2 g glucose/kg body weight), insulin secretion tests (2 g glucose/kg body weight) or insulin tolerance tests (0.1 unit/kg body weight) or glucagon secretion tests were performed after a 6 h fast. Plasma insulin and glucagon were measured with the mouse insulin and glucagon ELISA kits from Alpco (Salem, NH, USA).

Pancreatic islet isolation and cell culture Mouse islets were isolated and cultured using a previously described protocol

[27]. Islets were lysed immediately after isolation for Western blot analysis or subjected to other treatments in RPMI medium as indicated or dispersed into single cells for intracellular Ca^{2+} measurement. MIN6 cells were cultured in DMEM containing 25 mmol/l glucose, 10% FBS, 100 U/ml penicillin and 100 mg/l streptomycin at 37°C and 5% CO_2.

Quantitative real-time PCR Total RNA was extracted from cells using the Qiagen RNeasy Mini Kit (Mississauga, Ontario, Canada). cDNA was synthesised using the SuperScript III Reverse Transcriptase (Invitrogen, Burlington, ON, Canada). Real-time PCR quantification of expressed genes used PerfeCT SYBR Green (Quanta Biosciences, Gaithersburg, MD, USA) and the StepOnePlus System (Applied Biosystems, Foster City, CA, USA). The primer sequences are listed in Electronic supplementary material (ESM) Table 1.

Ca^{2+} imaging Islets were dispersed into single cells, plated on coverslips and imaged using Fura-2 [35, 36]. Ca^{2+} responses were quantified as maximal amplitude above baseline and integrated area.

Immunoblotting and immunoprecipitation Western blots were performed as described [33]. Protein was extracted from cells with lysis buffer (Cell Signaling Technology) containing protease inhibitors. Lysates (30 μg) were separated in 12% (wt/vol.) polyacrylamide gels before transfer on to a polyvinylidene fluoride (PVDF) membrane. After blocking, membranes were incubated with primary antibodies overnight, and then with peroxidase-labelled secondary antibodies. Band intensity was quantified using Photoshop (Adobe, San Jose, CA, USA).

To immunoprecipitate ubiquitinated proteins, MIN6 cells were transfected with a haemagglutinin (HA)-tagged ubiquitin construct (kindly provided by T. Mayor, UBC, Canada). The next day, cells were treated for 24 h before lysis in buffer with 10 mmol/l chloroacetamide. Lysates (500 μg) were incubated with anti-HA agarose resin from the Pierce HA-tag IP kit (Thermo, Rockford, IL, USA). Eluted proteins were subjected to 8% (wt/vol.) polyacrylamide gel electrophoresis, transferred to membranes, and probed with antibodies.

Immunofluorescent staining Human islets and pancreas samples were obtained from Dr Garth Warnock (Vancouver General Hospital) after informed consent. Mouse pancreases were collected and fixed in 4% paraformaldehyde (PFA). Pancreatic sections (5 μm) were immunostained as described [27]. The TUNEL assay (Roche, Mississauga, ON, Canada) was performed according to the manufacturer's instructions. Sections were mounted in Vectashield with DAPI (Vector Laboratories, Ontario, Canada) and imaged through a ×100 Zeiss objective and a CoolSnap HQ2 Camera. The percentage of TUNEL or PCNA-positive beta cells was determined using Fiji (http://pacific.mpi-cbg.de).

Statistical analysis Data are expressed as mean±SEM. Multiple comparisons between groups were performed using ANOVA followed by Tukey's post hoc test. Unpaired *t* tests were used when the differences between two groups were analysed. A p value of <0.05 was considered significant.

Results

Palmitate and high-fat diet increase UCHL1 protein levels UCHL1 was abundant in human pancreatic beta cells and was exclusive to the islets, with virtually no detection in the exocrine cells (Fig. 1a). UCHL1 was found in both cytoplasm and nucleus of human and mouse beta cells (Fig. 1a). UCHL1 levels were significantly increased by palmitate at basal glucose in a study of the MIN6 proteome [27] (details in ESM Fig. 1). In the present study, we confirmed that UCHL1 protein levels were augmented by palmitate at basal glucose in MIN6 cells. Further, we demonstrated that palmitate did not increase UCHL1 levels in the context of high glucose (Fig. 1b). The effects of palmitate on UCHL1 were dose-dependent (Fig. 1c). Palmitate-induced UCHL1 protein increase was abolished by cycloheximide, an inhibitor of protein translation (Fig. 1d), and not due to upregulation of its mRNA (Fig. 1e), implicating increased translation of *Uchl1*. To test whether UCHL1 was increased in an in vivo model of lipotoxicity, we fed wild-type and GAD mice a high-fat diet for 4 weeks. Indeed, UCHL1 protein was significantly elevated in wild-type mouse islets (Fig. 1f). UCHL1 was not detectable in GAD mouse islets with a null intragenic deletion mutation in the *Uchl1* gene (Fig. 1f), validating both the antibodies used and the GAD strain of mice as a null model for our studies. The GAD mice will be referred to as *Uchl1* null (*Uchl1*$^{-/-}$) in this article. We briefly examined whether other members of the *UCH* gene family might be upregulated in lipotoxic or glucolipotoxic conditions, and found that *Uchl3* and *Bap1* were increased by palmitate in the context of low glucose (Fig. 1e).

Regulation of ubiquitination by UCHL1 Ubiquitination requires a pool of free ubiquitin monomers, which is dynamic in cells. Free mono-ubiquitin levels can be exhausted by at least four mechanisms: (1) reduced amount of ubiquitin precursor; (2) loss of enzymes (e.g. UCHL1) that process it into monomers; (3) increased free mono-ubiquitin degradation; (4) or increased use of monomers in

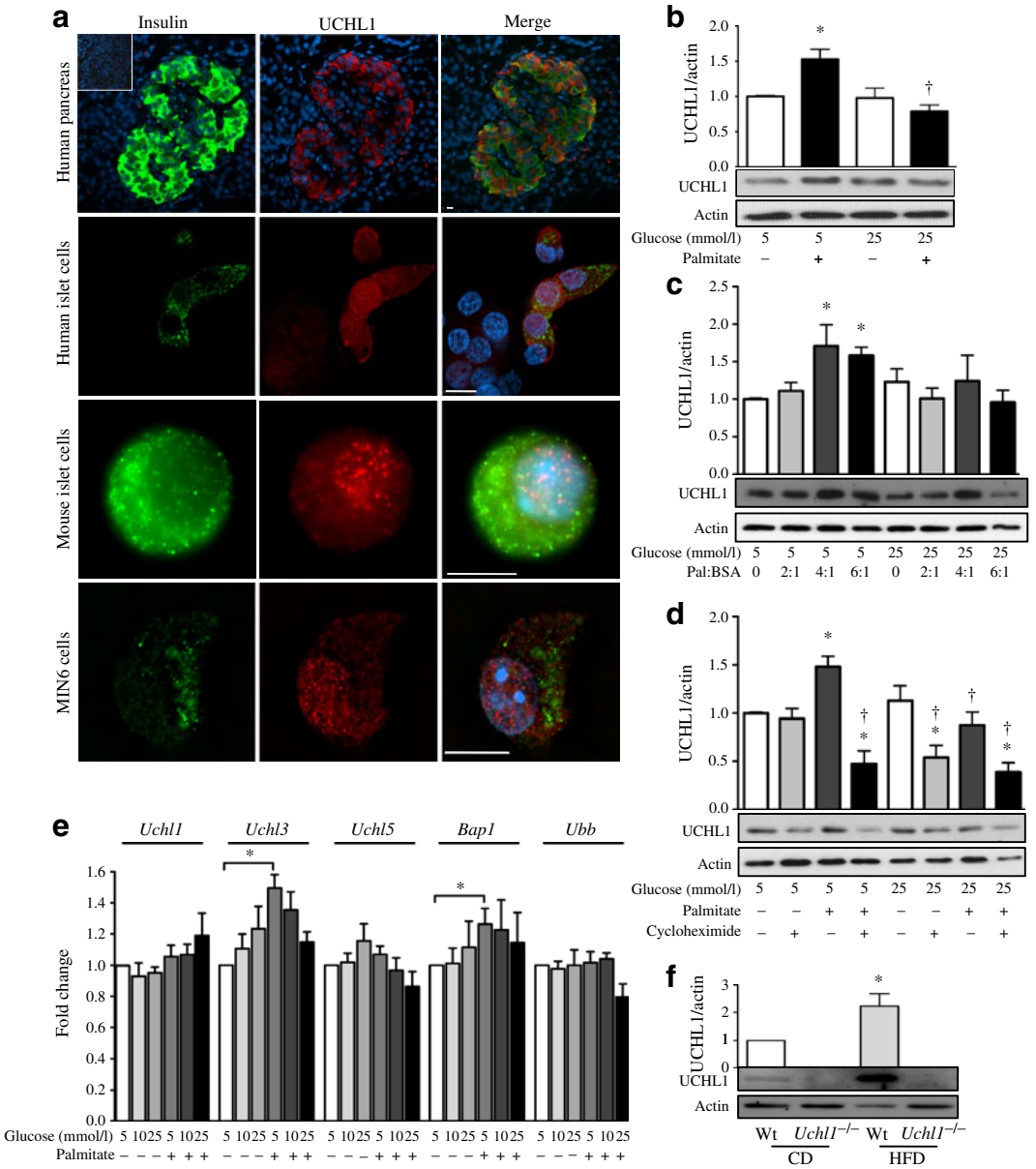


Fig. 1 Localisation of UCHL1 in human and mouse islet cells. **a** Immunofluorescent staining of UCHL1 (red), insulin (green) and DAPI (nuclei, blue) in normal human pancreas, dispersed human islet cells, mouse islet cells and MIN6 cells. Insert is the negative staining control. Scale bar, 10 μm. UCHL1 protein levels in MIN6 cells after 24 h treatment with (**b**) 1.5 mmol/l palmitate and (**c**) 0.5, 1 or 1.5 mmol/l (2:1, 4:1 or 6:1 palmitate [Pal] to BSA, respectively) palmitate, and (**d**) 20 mg/l cycloheximide with or without 1.5 mmol/l palmitate under 5.6 mmol/l (low) or 25 mmol/l (high) glucose (n=4). **e** mRNA levels of ubiquitin C-terminal hydrolases and ubiquitin precursor genes in MIN6 cells under 5, 10 or 25 mmol/l glucose with or without 1.5 mmol/l palmitate were detected by quantitative real-time RT-PCR (n=4). **f** UCHL1 protein levels in isolated wild-type (Wt) or *Uchl1*$^{-/-}$ mouse islets after a 4-week high-fat diet (HFD) were examined (n=5). CD, control diet. Values that differ significantly from the control of the low glucose concentration or wild-type with control diet, *p<0.05. Protein levels in Western blots were normalised to actin. Significant difference from the palmitate group of the low glucose, †p<0.05

ubiquitination. UCHL1 is reported to play a role in preventing degradation of free mono-ubiquitin in neurons [12]. In the present study, palmitate at basal glucose did not alter free ubiquitin levels in the mouse beta cells, even as increased UCHL1 levels were detected (Fig. 2a), reflecting the relatively short treatment period and/or the known redundancy in ubiquitin-processing enzymes. On the other hand, high glucose reduced free mono-ubiquitin levels in a palmitate-independent manner (Fig. 2a).

We also investigated ubiquitination of cellular proteins under several conditions. Palmitate increased general ubiquitination, while elevated glucose induced an even

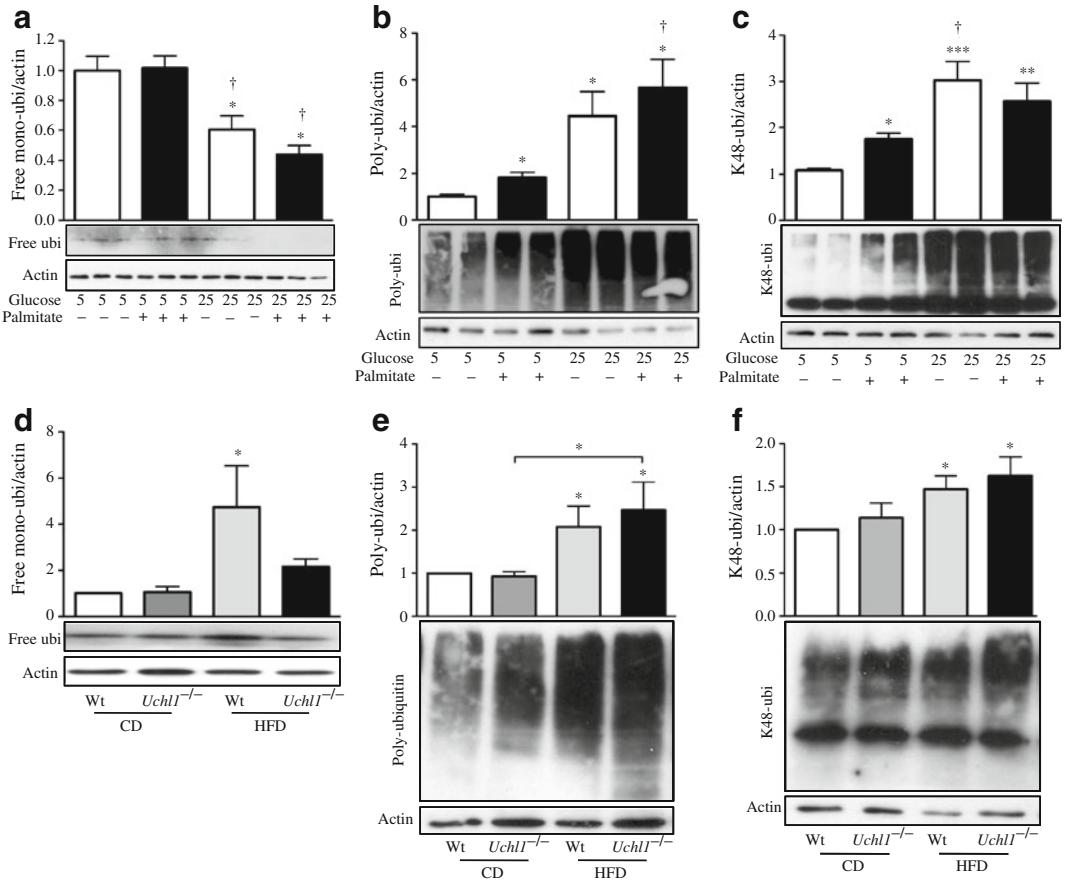


Fig. 2 Regulation of free mono-ubiquitin (ubi) levels and ubiquitination by palmitate and high-fat feeding. Free mono-ubiquitin levels in MIN6 cells after (**a**) 24 h treatment with or without 1.5 mmol/l palmitate in low or high glucose (n=4–8). **b** Total poly-ubiquitin and (**c**) K48-linked poly-ubiquitin levels in MIN6 cells after 24 h incubation with or without 1.5 mmol/l palmitate in low or high glucose (n=4–8). Glucose concentrations in mmol/l. **d** Free mono-ubiquitin, (**e**) total poly-ubiquitin and (**f**) K48-linked poly-ubiquitin levels of islets isolated from *Uchl1*$^{-/-}$ or wild-type (Wt) mice after 4 weeks of high-fat (HFD) or control diet (CD) (n=4–7). Protein levels were normalised to actin. Values that differ significantly from the low glucose control or the wild-type under control diet or the group indicated by the line, $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Significant difference from the palmitate group of the low glucose, $^{\dagger}p<0.05$

higher degree of ubiquitination (Fig. 2b). Simultaneously, ubiquitination at the lysine 48 linkage (K48), which targets proteins to the 26S proteasome for degradation [37, 38], was increased in the palmitate-treated MIN6 cells (Fig. 2c). Glucose also increased K48-linked ubiquitination, but this effect was not additive to that of palmitate. Together with our analysis of multiple ubiquitin C-terminal hydrolases (Fig. 1e), these data point to complex roles for palmitate and glucose on the beta cell ubiquitin landscape.

Short-term high-fat diet impairs glucose homeostasis in Uchl1 null mice Diet-induced obesity is characterised by hyperlipidaemia and is known to predispose both humans and rodents to type 2 diabetes. A short-term high-fat diet induced significant increases in free ubiquitin and ubiquitinated proteins, including K48-linked chains (Fig. 2d–f), reflecting a general upregulation of the ubiquitin system. However, high fat feeding failed to increase free ubiquitin levels in the *Uchl1*$^{-/-}$ mice (Fig. 2d), suggesting that the majority of increase in the free ubiquitin pool required the activity of UCHL1. The implication that UCHL1 was essential for the increase in the free ubiquitin levels observed under high-fat-fed conditions prompted us to analyse the glucose tolerance of these mice after a 4-week high-fat diet. Indeed, *Uchl1*$^{-/-}$ mice were glucose intolerant relative to their wild-type littermates after a brief course of high fat feeding, while they showed normal glucose homeostasis with a control diet (ESM Table 2 and ESM Fig. 2). Glucose tolerance of *Uchl1*$^{-/-}$ mice was mildly impaired after only 1 week of high-fat diet (Fig. 3a) before any weight gain (ESM Table 3) and further deteriorated after a 4-week high-fat diet (Fig. 3b). Insulin sensitivity was similar in the wild-type and mutant mice regardless of diet (Fig. 3c), pointing to a defect at the level of the beta cell. Indeed, high fat-fed *Uchl1*$^{-/-}$ mice exhibited reduced glucose-stimulated insulin secretion compared with wild-

type littermates (Fig. 3d). Fasting glucagon levels were similar between the control diet-fed wild-type (128.22±0.15 pmol/l) and *Uchl1*$^{-/-}$ mice (128.56±12.38 pmol/l), as well as the high fat-fed wild-type (119.23±16.20 pmol/l) and *Uchl1*$^{-/-}$ mice (118.86±15.78 pmol/l). Therefore, it is likely that glucose intolerance in high-fat-fed *Uchl1*$^{-/-}$ mice is due to a defect in insulin secretion from the endocrine pancreas.

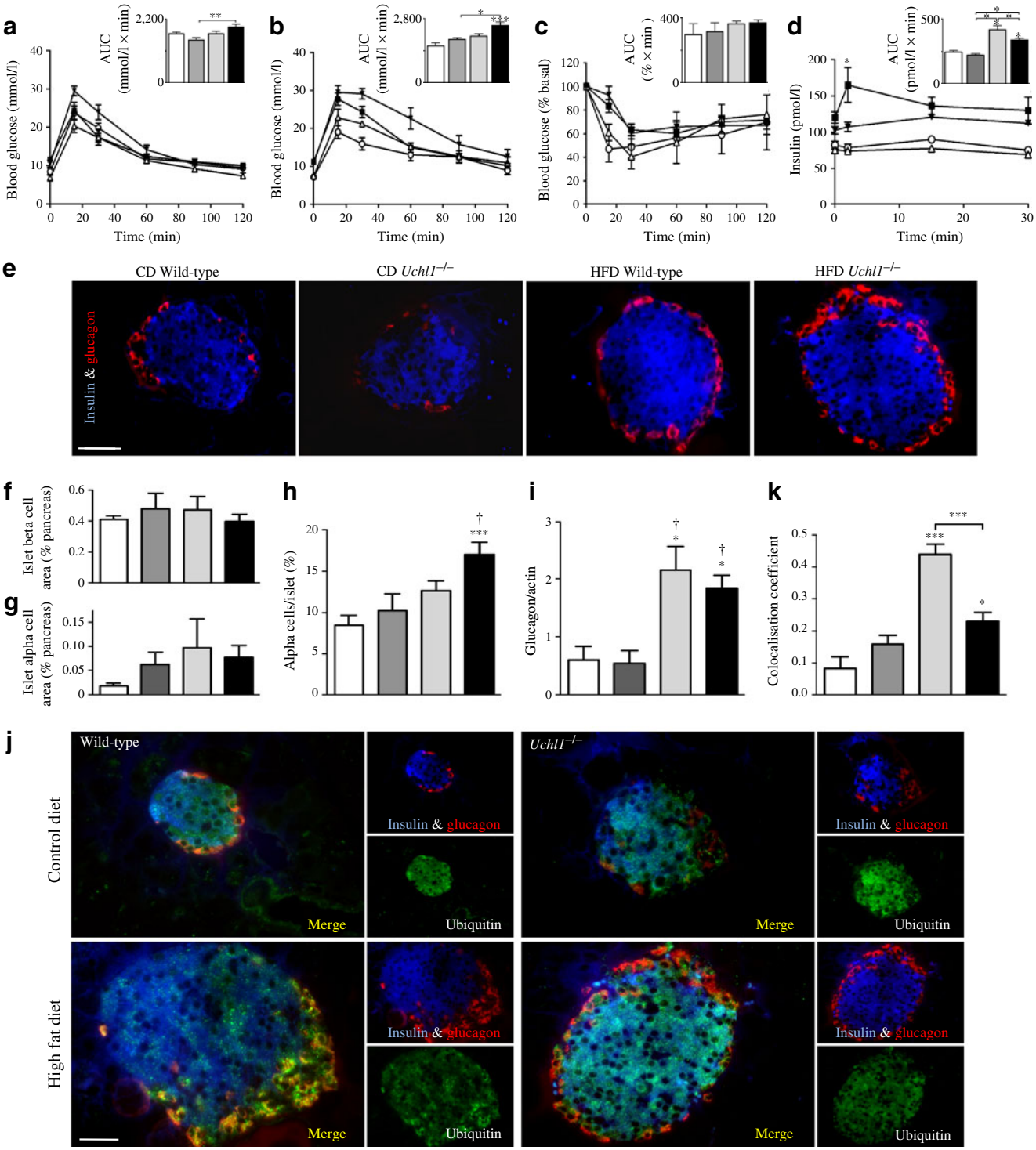
Uchl1 deficiency accelerates alpha cell hyperplasia UCHL1 has been proposed as a pancreatic progenitor marker [20, 39], but its role in pancreatic development has not been examined using a rigorous loss-of-function genetic approach. Total beta cell and alpha cell mass were not significantly different in this short-term study (Fig. 3e–g). Interestingly, while *Uchl1*$^{-/-}$ mice displayed grossly normal islet architecture, we did observe an increase in the alpha cell area per islet in high-fat-fed *Uchl1*$^{-/-}$ mice (Fig. 3h). This phenomenon is in line with observations of increased alpha cell proliferation in wild-type mice fed a longer-term (18 weeks) high-fat diet [40], suggesting the acceleration of a normal process. Glucagon content per islet from the *Uchl1*$^{-/-}$ mice was not different from the wild-type, although this variable was increased by the high-fat diet (Fig. 3i). Ubiquitin levels increased in alpha cells after the high-fat diet, an effect that was blunted in the *Uchl1*$^{-/-}$ mouse pancreas (Fig. 3j, k).

UCHL1 has roles in apoptosis and proliferation of beta cells We and others have previously shown that impaired in vivo insulin secretion can result from a decrease in beta cell survival or function, or a combination of both [36]. On a control diet, *Uchl1*$^{-/-}$ mice tended to have more apoptotic cells per islet, although this difference was not statistically significant. However, after a high-fat diet, beta cell apoptosis was dramatically and significantly increased in *Uchl1*$^{-/-}$ mice (Fig. 4a, b). To determine whether this increase in apoptosis was intrinsic to the islets, rather than the result of systemic effects, we examined isolated islets. Indeed, under basal conditions *Uchl1*$^{-/-}$ islets displayed elevated ER stress markers, CHOP and BiP [27, 33]. Importantly, *Uchl1*$^{-/-}$ islets were more susceptible to ER stress caused by in vitro lipotoxicity, but not glucolipotoxicity (Fig. 4c, d). Caspase-3-dependent apoptosis was enhanced in *Uchl1*$^{-/-}$ islets in all culture conditions (Fig. 4e). We tested the hypothesis that acutely inhibiting UCHL1 activity would be sufficient to increase ER stress-induced apoptosis using a chemical inhibitor. Indeed, the UCHL1 inhibitor dose-dependently increased the protein levels of CHOP and cleaved caspase-3 (Fig. 4f, g). Despite the caveat that high doses of this drug may inhibit UCHL3, these data together suggest that UCHL1 normally suppresses ER stress and programmed cell death in beta cells.

Fig. 3 Glucose tolerance, insulin secretion, beta cell and alpha cell area in *Uchl1*$^{-/-}$ mice after high-fat diet. IPGTT of male *Uchl1*$^{-/-}$ and wild-type mice after a (**a**) 1-week (n=7) and (**b**) 4-week (n=6–12) control (CD) or high-fat (HFD) diet. Inserts are AUC. **c** Insulin resistance measured by insulin tolerance test (n=8–10) and (**d**) in vivo insulin secretion (n=5) of mice after 4-week high-fat diet. Values that differ significantly from the wild-type (under control diet in [**b**]) or the group indicated by the line, *p<0.05. White circles, control diet, wild-type; black squares, high-fat diet, wild-type; white triangles, control diet, *Uchl1*$^{-/-}$; black inverted triangles, high-fat diet, *Uchl1*$^{-/-}$. **e** Immunofluorescent stain for insulin (blue) and glucagon (red) on pancreatic sections from wild-type or *Uchl1*$^{-/-}$ mice with control or high-fat diet (n=6). Beta cell (**f**) and alpha cell (**g**) area (as a percentage of pancreatic section area; n=5–6). **h** Alpha cell area per islet area. **i** Glucagon protein normalised to actin in the isolated islets from *Uchl1*$^{-/-}$ or wild-type mice with control or high-fat diet (n=4). **j** Immunofluorescent ubiquitin (green) staining in the islet cells represented by insulin (beta cell, blue) and glucagon (alpha cell, red) on pancreatic sections from *Uchl1*$^{-/-}$ or wild-type mice with control or high-fat diet and (**k**) the Pearson's correlation coefficient between ubiquitin and glucagon staining (n=4). White bar, control diet, wild-type; dark grey bar, control diet, *Uch*$^{-/-}$; light grey bar, high-fat diet, wild-type; black bar, high-fat diet, *Uch*$^{-/-}$. For immunofluorescent staining, five sections from each mouse and four mice per group were examined. Values that differ significantly from the wild-type with control diet or the group indicated by the line, *p<0.05, **p<0.01, ***p<0.001. Significant difference from the *Uchl1*$^{-/-}$ group with control diet, $^{\dagger}p$<0.05. Scale bar, 10 μm

Next, we asked whether UCHL1 in the beta cell might play a role in proliferation, a cellular function that is known to be regulated by ubiquitination [41]. Interestingly, *Uchl1*$^{-/-}$ mice showed a higher percentage of proliferating cells than wild-type, while the 4-week high-fat diet abolished this effect (Fig. 5a, b). Similarly, chemical inhibition of UCHL1 increased the proliferation of cultured beta cells under control conditions, but not in the presence of elevated palmitate (Fig. 5c). Together, these data suggest that UCHL1 normally acts as a brake against in vivo and in vitro beta cell proliferation.

UCHL1-dependent effects of lipotoxicity on exocytosis proteins While the above findings pointed to a critical role for UCHL1 in beta cell survival, we also sought to address the possibility that insulin secretion might be affected directly. Perifusion of isolated islets from high-fat-fed *Uchl1*$^{-/-}$ mice revealed a significant decrease in KCl-stimulated insulin release relative to the wild-type (Fig. 6a). The magnitude of this secretory defect (~40%) could not be accounted for by changes in intracellular Ca^{2+} evoked by KCl (Fig. 6b), suggesting a modest but physiologically important defect in insulin exocytosis. To examine the molecular mechanisms by which this defect occurs, we measured protein levels of the major components of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex including syntaxin 1 (STX1), STX4, SNAP25 and VAMP2 (Fig. 6c–f), which are critical in controlling the exocytosis of secretory granules. In wild-type mouse islets, STX1 and SNAP25 protein levels were augmented by in vivo lipotoxicity,



while VAMP2 was decreased. STX4 and SNAP25 levels were significantly reduced in islets from high-fat-fed *Uchl1*$^{-/-}$ mice compared with the high-fat-fed wild-type littermates (Fig. 6d, e). VAMP2 protein was 50% lower in high-fat-fed *Uchl1*$^{-/-}$ mouse islets than in both control diet-fed *Uchl1*$^{-/-}$ and wild-type mouse islets (Fig. 6f). Together, these experiments demonstrate that a short-term high-fat diet results in robust changes in total protein levels of multiple SNARE proteins, and that some of these changes require the presence of UCHL1.

In order to study the role of ubiquitination in the alteration of SNARE levels, we performed an immunoprecipitation specific for ubiquitinated proteins and examined the extent to which SNARE proteins were pulled down.

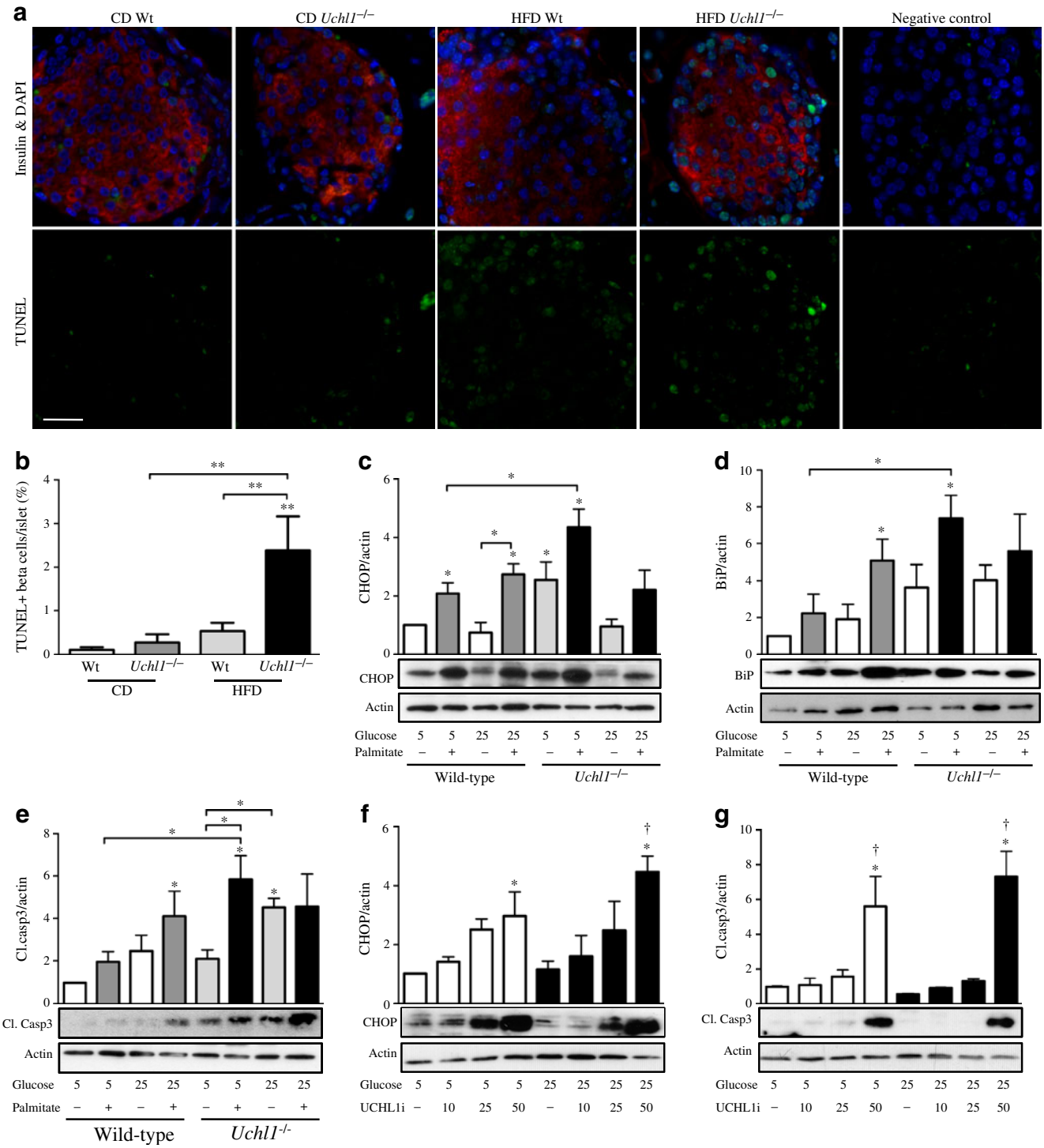


Fig. 4 Increased apoptosis and ER stress in beta cells of *Uchl1*$^{-/-}$ mice islets after high-fat diet. **a** TUNEL (green) on pancreatic sections with beta cells labelled with insulin (red) and nuclei with DAPI (blue) from wild-type (Wt) or *Uchl1*$^{-/-}$ mice with control (CD) or high-fat (HFD) diet, where (**b**) the percentage was plotted (n=5–6). Scale bar= 10 μm. ER stress assessed by (**c**) CHOP and (**d**) BiP levels, and (**e**) apoptosis detected by the cleaved caspase-3 (Cl. Casp3) levels from isolated *Uchl1*$^{-/-}$ or wild-type mouse islets followed by 48 h 1.5 mmol/l palmitate treatment in low or high glucose (n=4). **f** CHOP and (**g**) cleaved caspase-3 levels of MIN6 cells treated with indicated doses of UCHL1 inhibitor (UCHL1i, μmol/l) under 5 mmol/l or 25 mmol/l glucose for 24 h (n=4). Significantly different values from wild-type mouse islets with control diet or treated with 5 mmol/l glucose control or the 5 mmol/l glucose group without UCHL1 inhibitor or the group indicated by the line, *p< 0.05, **p<0.01. Significant difference from the group of high glucose only, $^{\dagger}p$<0.05

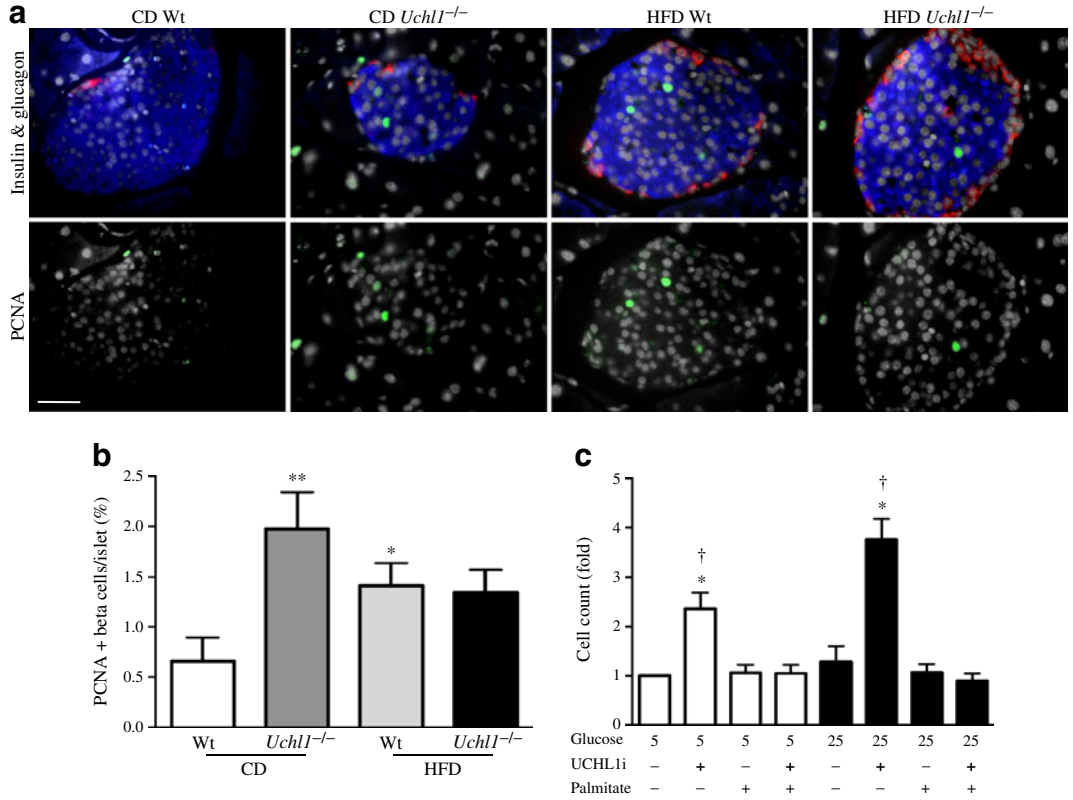


Fig. 5 Increased proliferation in *Uchl1*$^{-/-}$ mouse islets is lost with high-fat diet (HFD). **a** PCNA (green) staining of proliferating cells on pancreatic sections. Insulin-positive beta cells (blue), glucagon-positive alpha cells (red) and nuclei labelled with DRAQ5 (grey). Scale bar, 10 μm. **b** Percentage of PCNA labelled beta cells (n=5–6). **c** Cell count of MIN6 cells after 10 μmol/l UCHL1 inhibitor (UCHL1i) treatment with 5 or 25 mmol/l glucose for 24 h (n=4). *Values that differ significantly from the wild-type with control diet or the 5 mmol/l glucose group with neither UCHL1 inhibitor nor palmitate. *p<0.05, **p<0.01. Significant difference from the group of high glucose only, $^{\dagger}p$<0.05

Surprisingly, STX1 and SNAP25, which were previously reported to be ubiquitin targets [42, 43], were not conjugated to ubiquitin in our hands (not shown). However, we did observe conjugation of VAMP2 to ubiquitin (Fig. 6g). Moreover, palmitate increased ubiquitination of VAMP2 proteins as demonstrated by their shift in apparent molecular mass. Inhibition of UCHL1 activity further increased the degree of ubiquitination (Fig. 6h). It should be noted that UCHL1 inhibition alone augmented VAMP2 ubiquitination. Collectively, these studies demonstrate that UCHL1 has important roles in the beta cell ubiquitin landscape and regulates diverse cellular processes including apoptosis, proliferation and exocytosis.

Discussion

The experiments in the present study tested the hypothesis that UCHL1 has important in vivo roles in glucose homeostasis, as well as beta cell fate and function, under lipotoxic conditions. Emerging evidence points to the importance of UCHL1 in pancreatic beta cells [6, 7, 44], and UCHL1 was suggested to be a marker for newly formed pancreatic beta cells [20, 39]. Notwithstanding previous correlative studies, our genetic loss-of-function data clearly show that UCHL1 is not required for normal mouse islet development and is dispensable under non-stressed conditions. However, our studies point to complex roles for UCHL1 in beta cell survival, proliferation and function in the control of adult glucose homeostasis in the context of lipotoxic stress. In our hands, palmitate treatment in low-glucose cultures or a 4-week high-fat diet that was too brief to result in significant fasting hyperglycaemia (ESM Table 3) significantly increased beta cell UCHL1 protein levels, probably via augmented protein translation. Mice lacking UCHL1 had significantly impaired glucose tolerance after this brief high-fat diet due to a combination of defects in beta cell survival and function (i.e. functional beta cell mass). Had we been able to study *Uchl1*$^{-/-}$ mice after a longer high-fat diet, we expect that the accumulated apoptosis would have resulted in an eventual decline in physical beta cell mass. In many

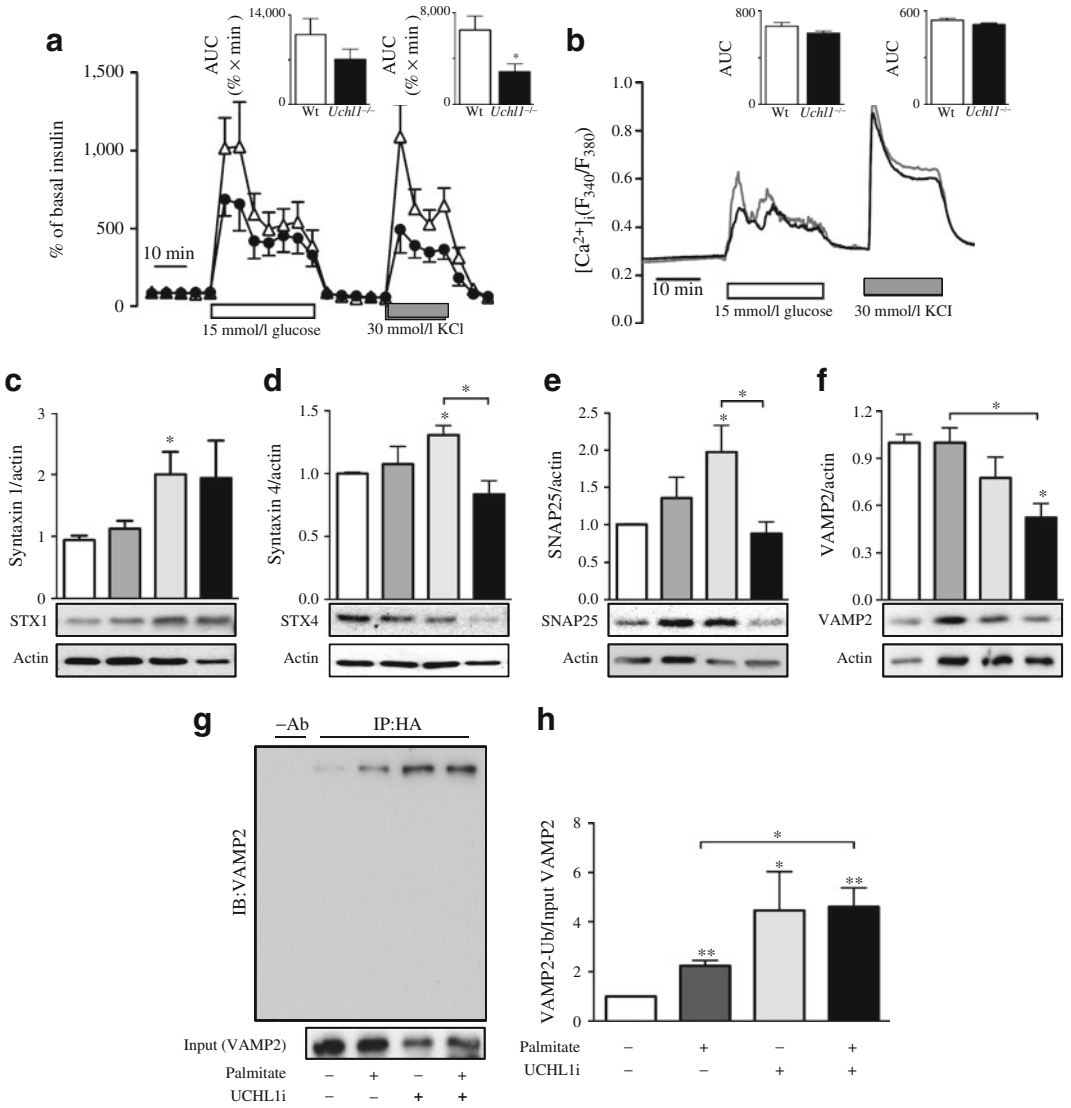


Fig. 6 Dysregulated SNARE proteins in *Uchl1*$^{-/-}$ mouse islets after a high-fat diet (HFD). **a** In vitro insulin secretion of isolated islets from *Uchl1*$^{-/-}$ and wild-type (Wt) mice fed a high-fat diet for 4 weeks. One hundred isolated islets were incubated with 3 mmol/l glucose Krebs buffer, otherwise treated as indicated. Sample perifusates were collected every 5 min (n=4–5). White triangles, high-fat diet, wild-type; black circles, high-fat diet, *Uchl1*$^{-/-}$. **b** Intracellular Ca^{2+} signal from dispersed islet cells of *Uchl1*$^{-/-}$ and wild-type mice after 4-week high-fat diet. The dispersed islet cells were incubated in Krebs buffer with 3 mmol/l and then treated as indicated. Mean of the traces are shown. Inserts are the plots of AUC. Protein levels of SNARE proteins (**c**) STX 1, (**d**) STX4, (**e**) SNAP25 and (**f**) VAMP2 of isolated islets from *Uchl1*$^{-/-}$ and wild-type mice after 4-week control (CD) or high-fat diet (normalised to actin). White bar, control diet, wild-type; dark grey bar, control diet, *Uchl1*$^{-/-}$; grey bar, high-fat diet, wild-type; black bar, high-fat diet, *Uchl1*$^{-/-}$. Values that differ significantly from the wild-type with control diet or the group indicated by the line, *p< 0.05. **g** Immunoprecipitation (IP) of HA-ubiquitin tagged proteins with HA antibody (Ab) from MIN6 cells treated with palmitate and/or 10 μmol/l UCHL1 inhibitor (UCHL1i) under 5 mmol/l glucose for 24 h and then immunoblotted (IB) with VAMP2 antibody. **h** Quantification of ubiquitinated VAMP2 against total VAMP2. Values that differ significantly from the control group or the group indicated by the line, *p<0.05, **p<0.01

regards, the phenotype of *Uchl1*$^{-/-}$ mice is similar to that of *Pdx1*$^{+/-}$ mice [36], which require many months to manifest the effects of beta cell apoptosis on physical beta cell mass.

UCHL1 plays a modulatory suppressive role in proliferation in other systems. For example, silencing of UCHL1 via promoter methylation triggered proliferation in cancer cells via p53 [45, 46]. However, we did not observe significant changes in p53 levels in *Uchl1*$^{-/-}$ mouse islets with or without a high-fat diet (not shown). Some cancer models, including pancreatic ductal carcinomas, show increased UCHL1 levels [47, 48] and augmented UCHL1/JAB1-mediated p27 degradation [49]. We did not observe differences in islet p27 levels (not shown). Thus, the targets of UCHL1 hydrolase activity that mediate the anti-proliferative and anti-apoptotic effects in islet cells remain

to be identified. UCHL1 may also dampen alpha cell hyperplasia in the context of lipotoxicity. The increased number of alpha cells seen in control diet-fed *Uchl1*$^{-/-}$ mice may represent an acceleration of the previously reported IL-6-dependent alpha cell hyperplasia [40]. In the absence of UCHL1, degradation of ubiquitin may be increased. Increased ubiquitin levels in alpha cells after high-fat feeding may suppress their proliferation, which may otherwise disrupt normal glucose homeostasis. However, fasting plasma glucagon levels were not different in *Uchl1*$^{-/-}$ mice, consistent with a lack of extrinsic stimulation. Elucidating how UCHL1-associated ubiquitination regulates alpha cell mass and/or function awaits the conditional knockout of this gene in alpha cells.

Apart from the increased UCHL1 protein levels, short-term high-fat feeding also induced increases in both free mono-ubiquitin levels and the degree of ubiquitination in wild-type mouse islets. Recent data from obese humans also demonstrated a global increase in islet ubiquitination [44]. UCHL1 is known to have complex effects on the amount of free ubiquitin and ubiquitination, including: (1) processing of the ubiquitin precursor into free mono-ubiquitin destined for use in ubiquitination [41]; (2) inhibiting free mono-ubiquitin degradation [12]; and (3) liberating free ubiquitin monomers from poly-ubiquitin chains on proteins destined for degradation at proteasomes. Thus, UCHL1 activity can result in increased ubiquitin flux through this pathway and support the degradation of target proteins. The fact that normal levels of free mono-ubiquitin could be found in islets of unstressed *Uchl1*$^{-/-}$ mice indicates that additional enzymes are important in basal conditions, demonstrating the redundancy of this enzyme in beta cell development and function. Our results suggest that UCHL1 is selectively involved in the control of free ubiquitin levels in the context of lipotoxicity. Given the role of UCHL1 in the removal of poly-ubiquitin chains on the regulatory subunit of proteasomes as well as deubiquitination of proteins before their degradation, we suggest that deletion of UCHL1 would result in the accumulation of poly-ubiquitinated proteins and the subsequent depletion of the free ubiquitin pool. In the context of increased ubiquitination, depletion of free mono-ubiquitin and accumulation of poly-ubiquitinated proteins would be expected to lead to ER stress and apoptosis [50]. Since the loss of UCHL1-dependent free mono-ubiquitin liberation prevents adaptation to the high-fat diet, we propose that these events are part of a programme of functional compensation, proliferation and survival signalling. We propose that UCHL1 is upregulated by lipotoxicity in the early stages of obesity as a protective measure, but later falls with chronic hyperglycaemia.

Our new data, together with previous reports, demonstrate opposing actions of lipids and glucose on UCHL1 levels [7]. Other ubiquitin C-terminal hydrolase members and the ubiquitin precursor were also differentially regulated by palmitate and glucose, and this is consistent with our previous evidence for distinct lipotoxic and glucolipotoxic mechanisms [27, 33]. High glucose alone had a significant effect on the ubiquitin landscape, generating a large increase in protein ubiquitination and a decrease in free ubiquitin. These data agree with reports of increased poly-ubiquitination in the islets of patients with type 2 diabetes and the HIP rat model of type 2 diabetes [44]. In our studies, high glucose appeared to over-ride the effects of palmitate on UCHL1 and free ubiquitin levels, perhaps resulting in the accumulation of poly-ubiquitin chains and overloaded proteasomes.

The present study also revealed novel roles for UCHL1 and ubiquitination in insulin exocytosis. The SNARE proteins, SNAP25 and VAMP2, were altered in the *Uchl1*$^{-/-}$ islets. Interestingly, although STX1 and SNAP25 have previously been reported to be targets for ubiquitin-mediated proteasomal degradation in neurons [42, 43], we did not observe ubiquitination of these two proteins in our immunoprecipitation experiments. Instead, we provide evidence that VAMP2 is reduced under lipotoxic conditions via ubiquitination. Interestingly, VAMP2 is reduced by synuclein knockout [51], and alpha-synuclein is stabilised by UCHL1 in mouse brain [13]. Additional analysis, including unbiased proteomics, is required to more thoroughly reveal underlying mechanisms and identify ubiquitinated proteins in beta cells under a variety of stress conditions.

In conclusion, this study demonstrates that an increase in UCHL1 is essential for beta cell function and survival during lipotoxic stress. The absence of UCHL1 results in increased ER stress and apoptosis, as well as altered turnover of key SNARE proteins such as VAMP2. Together with other recent studies, this work highlights an emerging understanding of the importance of UCHL1 and the ubiquitin proteasome system in the pathobiology of lipotoxicity and type 2 diabetes. Better understanding of the common roles and mechanisms of lipotoxicity, ER stress, apoptosis and secretory pathway dysfunction in metabolic and neurological diseases is likely to open new avenues for therapy.

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