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ARTICLE

Dichotomous role of pancreatic HUWE1/MULE/ARF-BP1 in modulating beta cell apoptosis in mice under physiological and genotoxic conditions

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Abstract
Aims/hypothesis Diabetes mellitus represents a significant burden on the health of the global population. Both type 1 and type 2 diabetes share a common feature of a reduction in functional beta cell mass. A newly discovered ubiquitination molecule HECT, UBA and WWE domain containing 1, E3 ubiquitin protein ligase (HUWE1 [also known as MULE or ARF-BP1]) is a critical regulator of p53-dependent apoptosis. However, its role in islet homeostasis is not entirely clear.
Methods We generated mice with pancreas-specific deletion of *Huwe1* using a Cre-*loxP* recombination system driven by the *Pdx1* promoter (*Pdx1cre*$^{+}$ *Huwe1*$^{fl/fl}$) to assess the in vivo role of HUWE1 in the pancreas.
Results Targeted deletion of *Huwe1* in the pancreas preferentially activated p53-mediated beta cell apoptosis, leading to reduced beta cell mass and diminished insulin exocytosis. These defects were aggravated by ageing, with progressive further decline in insulin secretion and glucose homeostasis in older mice. Intriguingly, *Huwe1* deletion provided protection against genotoxicity, such that *Pdx1cre*$^{+}$ *Huwe1*$^{fl/fl}$ mice were resistant to multiple-low-dose-streptozotocin-induced beta cell apoptosis and diabetes.
Conclusion/interpretation HUWE1 expression in the pancreas is essential in determining beta cell mass. Furthermore, HUWE1 demonstrated divergent roles in regulating beta cell apoptosis depending on physiological or genotoxic conditions.

Keywords Apoptosis · ARF-BP1 · Beta cell · Diabetes · HUWE1 · MULE

Abbreviations

*C*m	Membrane capacitance
c-Myc	V-myc avian myelocytomatosis viral oncogene homologue
GSIS	Glucose-stimulated insulin secretion
HUWE1	HECT, UBA and WWE domain containing 1, E3 ubiquitin protein ligase
MCL1	Myeloid cell leukaemia sequence 1
MDM2	Mouse double minute 2 homologue
MLDS	Multiple low dose streptozotocin
PDX1	Pancreatic and duodenal homeobox 1
STZ	Streptozotocin

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Introduction

Type 1 diabetes is characterised by the autoimmune destruction of insulin-producing beta cells and typically has an early onset, while type 2 diabetes is associated with obesity and insulin resistance [1, 2]. Although caused by distinct underlying mechanisms, one common feature is an inadequate number of beta cells, which are required to maintain a sufficient plasma insulin level for glucose homeostasis.

Increased apoptotic cells have been observed in islets of patients with both type 1 and type 2 diabetes, in association with dramatic reduction in beta cell mass [1, 3]. The well-known tumour suppressor p53, when activated, leads to cell cycle arrest and apoptosis. It can be induced by a variety of insults, including DNA damage, oncogenic expression and ribosomal stress [4, 5]. The p53-dependent apoptotic pathway plays a critical role in beta cell demise during diabetes pathogenesis. The expression of p53 is increased in both human amylin- and nitric oxide-induced beta cell apoptosis in culture [6, 7]. In addition, beta-cell-specific p53 stabilisation and upregulation has been implicated in beta cell mass reduction in humans with diabetes [8, 9]. On the other hand, deletion of p53 in beta cells in culture provides resistance from NEFA-induced apoptosis [10]. Furthermore, p53-null mice are protected from streptozotocin (STZ)-induced beta cell death and type 1 diabetes [11], which suggests that p53 plays an important regulatory role in beta cell homeostasis under both physiological and pathological conditions. Thus, p53 and its regulators represent a viable target for diabetes.

For years, mouse double minute 2 homologue (MDM2) has been touted as the principle inhibitor of p53, via its specific E3 ubiquitin ligase activity leading to p53 degradation [12]. However, MDM2-null cells continue to undergo p53 stabilisation and subsequent apoptosis [13], suggesting an MDM2-independent regulation of p53. Indeed, multiple studies have shown molecules that directly regulate p53 function without MDM2 [14–16]. One such molecule is myeloid cell leukaemia sequence 1 (MCL1) ubiquitin ligase E3 (MULE) or HECT, UBA and WWE domain containing 1, E3 ubiquitin protein ligase (HUWE1 [also known as ARF-BP1 or LASU1]). The *Huwe1* gene is encoded on the X-chromosome and its protein product contains multiple domains, including BH3, which allows for polyubiquitination and degradation of p53 independent of MDM2 [17]. Thus, HUWE1 may have a regulatory role in p53-mediated apoptosis of beta cells that occurs during diabetogenesis.

Apart from p53, HUWE1 has multiple other polyubiquitination substrates [18–22]. Among them is MCL1, which belongs to the anti-apoptotic B cell CLL/lymphoma 2 (BCL-2) family of proteins [20]. MCL1 is rapidly induced after exposure to cell damaging stimuli, thus offering acute protection against apoptosis [23]. Therefore, from a functional point of view, the interaction of HUWE1 with MCL1 is pro-apoptotic in nature. This is in direct contrast to the anti-apoptotic role of HUWE1 in its polyubiquitination of p53. Therefore, it is very likely that the role of HUWE1 is cell-type- and context-specific [24], and the function of HUWE1 in beta cell homeostasis and diabetes pathogenesis remains unclear.

In this study, we examined the role of HUWE1 in beta cells by targeted deletion of *Huwe1* in mouse pancreas. Under basal conditions, we observed an overall anti-apoptotic role of HUWE1, predominantly through its inhibition of p53. As such, HUWE1-deficient mice showed a decline in beta cell mass with ageing without essential involvement of other known substrates of HUWE1 under physiological conditions. Furthermore, we showed that HUWE1 was critical for modulating exocytosis of insulin granules in beta cells. Intriguingly, *Huwe1* deletion demonstrated an opposite effect in response to genotoxic stress, whereby HUWE1-deficient beta cells were resistant to streptozotocin (STZ)-induced apoptosis and diabetes. Thus, HUWE1 is a critical modulator of beta cell integrity and function under both physiological and pathological conditions.

Methods

Mice Heterozygote *Huwe1*$^{fl/y}$ mice (Tak Mak, Toronto, ON, Canada; exons 76 and 77 of *Huwe1* flanked by *loxP* sites) were bred with *Pdx1cre* mice (Jackson Laboratories, Bar Harbor, ME, USA) [25, 26] to generate male *Pdx1cre*$^{+}$ *Huwe1*$^{+/y}$, male *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$, female *Pdx1cre*$^{+}$ *Huwe1*$^{+/+}$ and female *Pdx1cre*$^{+}$ *Huwe1*$^{fl/fl}$ mice. Male mice were used for experiments and littermates as controls. Genotypes were determined as described previously [27]. Mice were maintained on a mixed 129J-C57BL/6 background and housed in a pathogen-free facility on a 12 h light/dark cycle and fed ad libitum with standard irradiated rodent chow (5% fat; Harlan Tecklad, Indianapolis, IN, USA) without restriction of activity in accordance with University Health Network Animal Care Facility Protocol.

Metabolic studies Serum insulin, blood glucose, glucose tolerance tests, insulin tolerance tests and glucose-stimulated insulin secretion (GSIS) tests were performed as previously described [28]. Serum insulin was measured by enzyme-linked immunosorbent assay kit (Crystal Chem, Downers Grove, IL, USA).

STZ protocol Low doses of STZ were injected i.p. into mice (40 mg/kg body weight) for 5 days (consecutive) [29].

Immunohistochemistry and immunofluorescence The pancreas was fixed, stained and islet area quantified as previously

described [27]. Antibodies against insulin (Dako, Glostrup, Denmark), glucagon (Cell Signaling Technology, Beverly, MA, USA), HUWE1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TUNEL (Cell Signaling Technology), GLUT2 (Cell Signaling Technology), PDX1 (Santa Cruz Biotechnology), p53 (Cell Signaling Technology), p-p53 (Cell Signaling Technology) and MCL1 (Cell Signaling Technology) were used.

Western blotting Liver, muscle, visceral adipose tissue, islets and hypothalamus protein lysates were obtained as previously described [27]. Antibodies against actin (Santa Cruz Biotechnology), AMPK (Cell Signaling Technology), p-AMPK (Cell Signaling Technology), Akt (Cell Signaling Technology), p-Akt (Cell Signaling Technology), cleaved caspase 3 (Cell Signaling Technology), DNA polymerase beta (Abcam, Cambridge, MA, USA), GLUT2 (Chemicon, Temecula, CA, USA), MCL1 (Cell Signaling Technology), HUWE1 (Cell Signaling Technology), p53 (Cell Signaling Technology), PDX1 (Chemicon) were used.

Quantitative PCR RNA was extracted and quantitative (q)PCR performed as previously described [28, 30].

Electrophysiology Recording pipettes were from 1.5 mm borosilicate glass capillary tubes using a programmable micropipette puller. Pipettes were heat-polished and tip resistance ranged from 2 to 3 mΩ when filled with intracellular solution. For measurement of membrane capacitance, the intracellular solution contained: 125 mmol/l caesium glutamate, 10 mmol/l CsCl, 10 mmol/l NaCl, 1 mmol/l $MgCl_2$, 5 mmol/l HEPES, 0.05 mmol/l EGTA, 3 mmol/l MgATP, 0.1 mmol/l cAMP, pH to 7.2. The extracellular solution consisted of 118 mmol/l NaCl, 5.6 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 10 mmol/l $CaCl_2$, 20 mmol/l tetraethylammonium chloride, 5 mmol/l HEPES and 5 mmol/l D-glucose, pH 7.4. Cell membrane capacitance (Cm) was estimated by the Lindau–Neher technique, implementing the 'sine-DC' feature of the Lock-in module (40 mV peak-to-peak and a frequency of 500 Hz) in the whole-cell configuration. Recordings were conducted using an EPC10 patch clamp amplifier and the Pulse and X-Chart software programs (HEKA Electronik, Lambrecht, Germany).

Statistics Data are presented as means ± SEM and were analysed by one-sample t test.

Results

Generation of pancreas-specific Huwe1-knockout mice HUWE1 protein was ubiquitously expressed. It was detected in islets, hypothalamus, muscle, liver and visceral adipose tissue in both females and males, and expression was similar in both sexes (Fig. 1a and electronic supplementary material [ESM] Fig. 1a, b). The expression of *Huwe1* was significantly higher in pancreatic islets compared with surrounding exocrine tissue, indicating its potential important role in endocrine pancreatic function (Fig. 1b and ESM Fig. 1c).

The mouse *Huwe1* gene is located on the X-chromosome. To generate mice with *Huwe1* deletion specifically in pancreatic progenitor cells during early pancreatic development (E8.5-9), we employed the Cre-*loxP* system with *cre* expression regulated by the pancreas-specific promoter of pancreatic and duodenal homeobox 1. Both adult male *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice and female *Pdx1cre*$^{+}$ *Huwe1*$^{fl/fl}$ mice showed dramatic reduction in *Huwe1* mRNA (Fig. 1c and ESM Fig. 1d) in isolated islets. This translated into a significant reduction in HUWE1 protein (Fig. 1a and ESM Fig. 1a) and immunohistochemistry (Fig. 1b and ESM Fig. 1c). *Pdx1* promoter expression has also been reported in the central nervous system, especially the hypothalamus, the command centre for many endocrine functions such as glucose and energy homeostasis. However, in our model no observable reduction in HUWE1 protein level was detected in isolated hypothalami from *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ or *Pdx1cre*$^{+}$ *Huwe1*$^{fl/fl}$ mice compared with control littermates (Fig. 1a and ESM Fig. 1a). Accordingly, total body weight, a major hypothalamic regulatory target, remained similar to controls in both sexes (ESM Fig. 2a–d). The levels of HUWE1 protein was also comparable between genotypes in other peripheral tissues, including liver, muscle and visceral fat (Fig. 1a and ESM Fig. 1a).

Pdx1cre^{+} *Huwe1*$^{fl/y}$ *mice demonstrated age-dependent glucose intolerance and beta cell dysfunction without changes in insulin sensitivity* Male *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice showed similar fed blood glucose levels as controls (Fig. 2a) and by 2 months of age, exhibited higher fasting glucose levels (Fig. 2b). At this age, *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice also showed a trend towards increased glucose excursion during glucose tolerance test (Fig. 2c), while no difference in glucose lowering in response to peripheral insulin injection was seen (Fig. 2d). This metabolic defect worsened with age, with significantly impaired glucose tolerance at 6 months of age (Fig. 2e), while insulin sensitivity remained unchanged (Fig. 2f). This glucose intolerance following *Pdx1* promoter-mediated *Huwe1* deletion was observed only in male mice, while female *Pdx1cre*$^{+}$ *Huwe1*$^{fl/fl}$ mice showed comparable blood glucose, glucose tolerance and peripheral insulin sensitivity between genotypes (ESM Fig. 3a–d), which may have been affected by female hormones [31, 32]. Thus, only male *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ and control male *Pdx1cre*$^{+}$ *Huwe1*$^{+/y}$ littermates were used for further investigations.

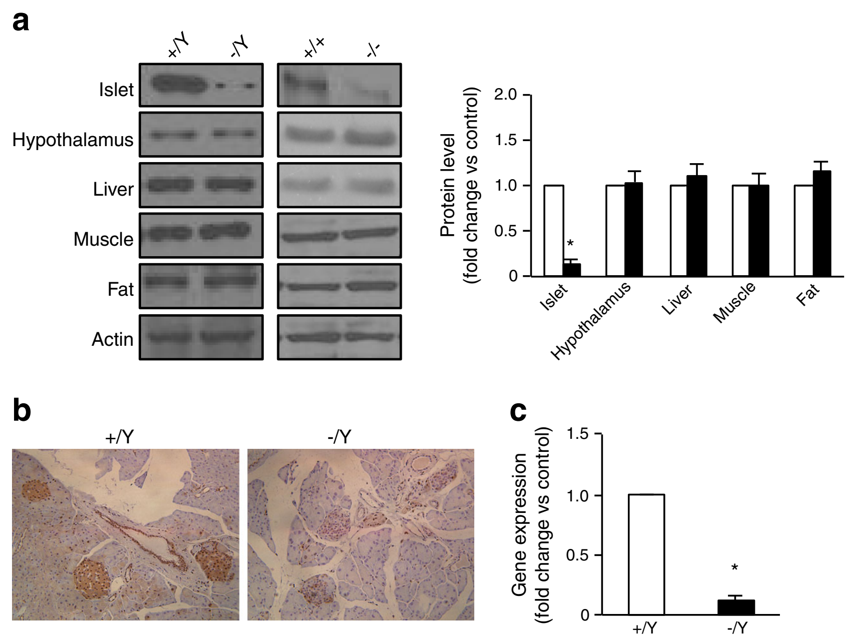

Fig. 1 Pancreas-specific deletion of *Huwe1*. (**a**) Western blot of HUWE1 and actin protein in tissues from male *Pdx1cre*$^{+}$ *Huwe1*$^{+/y}$ (+/Y) and *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ (−/Y), and female *Pdx1cre*$^{+}$ *Huwe1*$^{+/+}$ (+/+) and *Pdx1cre*$^{+}$ *Huwe1*$^{fl/fl}$ (−/−) mice with quantification for male mice (n=5). (**b**) Representative immunohistological staining of HUWE1 protein in pancreatic sections (n=5 mice). (**c**) *Huwe1* mRNA expression in islets (n=5 mice). $^{*}p<0.05$. White bars, +/Y; black bars, −/Y

Impaired glucose tolerance without a significant defect in peripheral insulin sensitivity in male *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice suggested an insulin secretory defect. In line with this hypothesis there was a reduction in circulating insulin levels at both 2 and 6 months of age in these mice (Fig. 3a, b). Furthermore, in vivo GSIS was diminished in these mice at 2 months of age (Fig. 3c), and this secretory defect was further aggravated at 6 months of age (Fig. 3d). Thus, PDX1-mediated *Huwe1* deletion impaired insulin secretion while sparing the peripheral metabolic actions of insulin.

Pdx1cre^{+} *Huwe1*$^{fl/y}$ *islets demonstrated age-dependent reduction in beta cell mass and increase in apoptosis* Total insulin secretion is the product of beta cell mass and secretory capacities of individual beta cells. Pancreatic sections from *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice showed a reduced total beta cell area at 2 months (ESM Fig. 4a, b) and further reduction at 6 months of age (Fig. 4a, b). In order to explore the mechanism of reduced beta cell mass in *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ pancreas, we measured their basal islet proliferation rate by Ki67 staining. Similar percentages of proliferating cells were observed in *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ and control islet tissue at both 2 and

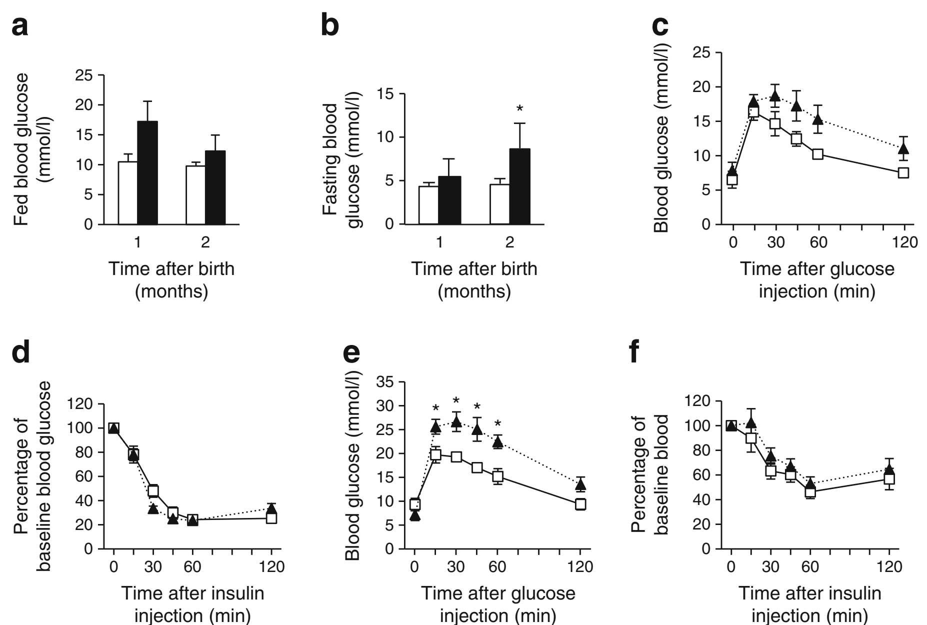

Fig. 2 Absence of HUWE1 leads to impaired glucose tolerance in male *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice. (**a**) Fed and (**b**) fasting blood glucose levels of *Pdx1cre*$^{+}$ *Huwe1*$^{+/y}$ (+/Y) and *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ (−/Y) mice at 1 and 2 months of age (n=9). (**c**) Glucose and (**d**) insulin tolerance test at 2 months of age (n=7). (**e**) Glucose and (**f**) insulin tolerance test at 6 months of age (n=7). $^{*}p<0.05$ vs +/Y mice at the same age. White bars/squares, +/Y; black bars/triangles, −/Y

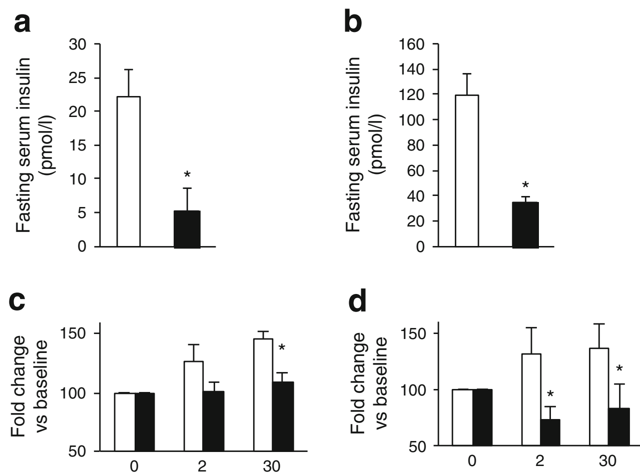

Fig. 3 Absence of HUWE1 leads to impaired insulin secretion in male *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice. (**a**, **b**) Fasting serum insulin and (**c**, **d**) GSIS of *Pdx1cre*$^{+}$ *Huwe1*$^{+/y}$ (+/Y) and *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ (−/Y) mice at 2 months (**a**, **c**) and 6 months (**b**, **d**) of age (n=5). $^{*}p$<0.05 vs +/Y mice at the same age. White bars, +/Y; black bars, −/Y

6 months of age (Fig. 4c and ESM Fig. 4c), suggesting that *Huwe1* deletion did not affect cellular proliferation. However, the number of TUNEL-positive cells was significantly elevated in *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ islets at 2 months of age (ESM Fig. 4d, e) and furthermore at 6 months of age (Fig. 4d, e), consistent with the worse insulin secretion and glucose intolerance with age. The numbers of alpha cells undergoing apoptosis were very small in both genotypes and total alpha cell area did not appear diminished (ESM Fig. 4f). Also, despite the increased apoptotic cells within their islets, gross architecture was intact (Fig. 4d and ESM Fig. 4d) and overall pancreas weight was not different between genotypes (ESM Fig. 2b, d). Thus, impairment in glucose homeostasis in *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice is most likely attributable to increased beta cell apoptosis resulting in reduced total insulin-secreting beta cell mass.

Individual beta cells from 6 month old male Pdx1cre^{+} *Huwe1*$^{fl/y}$ *mice demonstrated secretory dysfunction* To directly examine the insulin secretory function of individual beta cells in the male *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice, we employed patch–clamp *C*m measurements of single isolated beta cells. When compared with age-matched controls, the *C*m increase in beta cell isolated from 2-month-old *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice did not differ significantly with each depolarising pulse (Fig. 5a, b). The size of the readily releasable pool (RRP) of granules ($\Delta Cm_{1st\text{–}2nd}$ pulse) also showed no significant difference in *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ cell (28.5±6.8 fF/pF) when compared with controls (16.9±4 fF/pF) (Fig. 5c). In addition, the rate of granule refilling/mobilisation ($\Delta Cm_{3rd\text{–}10th}$ pulse) was comparable between genotypes at 2 months of age (Fig. 5c). However, when the experiments were performed with beta cells isolated from mice at 6 months of age, *C*m showed a stepwise decline with each pulsation in beta cells of *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice (Fig. 5d), which translated into a significant reduction in granule refilling/mobilisation rate (Fig. 5e). These results suggested that the mild glucose intolerance and reduction in circulating insulin

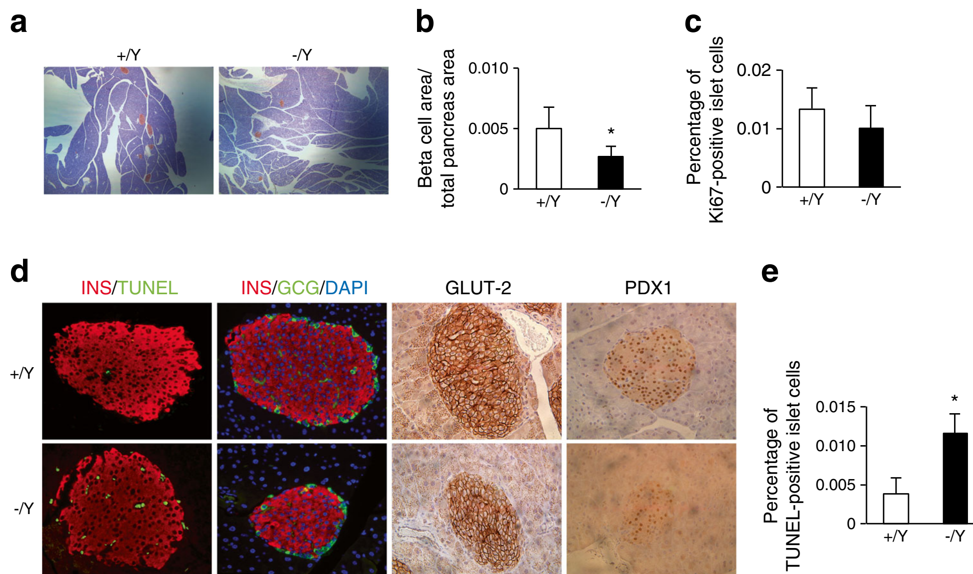

Fig. 4 Absence of HUWE1 leads to an increase in beta cell apoptosis and reduced beta cell area in 6-month-old *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice. (**a**) Representative pancreatic sections with immunohistochemical staining of insulin (×4 magnification) and (**b**) quantification of beta cell area of *Pdx1cre*$^{+}$ *Huwe1*$^{+/y}$ (+/Y) and *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ (−/Y) mice at 6 months of age (n=5). (**c**) Percentage Ki67-positive islet cells at 6 months of age (n=5). (**d**) Representative pancreatic sections with immunofluorescent staining of insulin/TUNEL, insulin/glucagon/DAPI and immunohistochemical staining of GLUT2 and PDX1 (×20 magnification) at 6 months of age (n=5). (**e**) Percentage of TUNEL-positive islet cells at 6 months of age (n=5). $^{*}p$<0.05. White bars, +/Y; black bars, −/Y. GCG, glucagon; INS, insulin

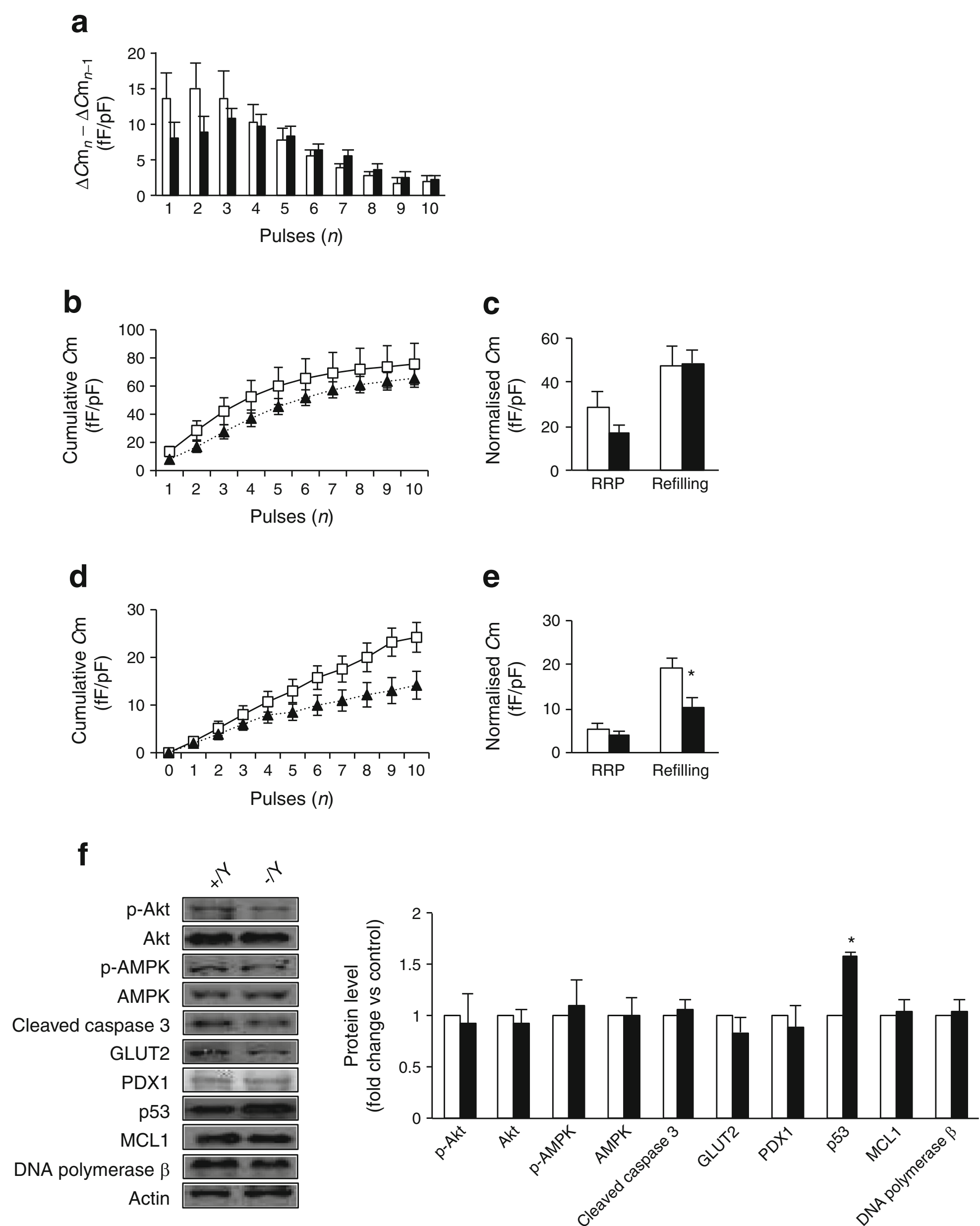

Fig. 5 *Huwe1* deletion in beta cells leads to age-dependent reduction in exocytosis. (**a**) Cell capacitance for each pulse during the train of ten 500 ms depolarisations in beta cells isolated from *Pdx1cre*$^+$ *Huwe1*$^{+/y}$ (+/Y) and *Pdx1cre*$^+$ *Huwe1*$^{fl/y}$ (−/Y) mice (n=12). (**b**) Cumulative changes in cell capacitance normalised to basal cell *C*m (fF/pF) in beta cells at 2 months of age (n=10). (**c**) The size of the RRP of insulin granules ($\Delta Cm_{1st\text{–}2nd\ pulse}$) and the rate of granule mobilisation ($\Delta Cm_{3rd\text{–}10th\ pulse}$) in beta cells isolated at 2 months of age (n=10). (**d**) Cumulative changes in cell capacitance normalised to basal cell *C*m (fF/pF) in beta cells at 6 months of age (n=12). (**e**) Size of the RRP of insulin granules ($\Delta Cm_{1st\text{–}2nd\ pulse}$) and the rate of granule mobilisation ($\Delta Cm_{3rd\text{–}10th\ pulse}$) in beta cells at 6 months of age (n=12). (**f**) Western blotting and quantification of phospho-Akt, Akt, phospho-AMPK, AMPK, cleaved caspase 3, GLUT2, PDX1, p53, MCL1, DNA polymerase beta and actin levels in isolated islets at 2 months of age (n=3). $^*p$<0.05 vs +/Y mice. White bars/squares, +/Y; black bars/triangles, −/Y

in young *Pdx1cre*$^+$ *Huwe1*$^{fl/y}$ mice was primarily due to reduced beta cell mass likely from increased apoptosis while secretory function of individual beta cells was not affected. Conversely, both beta cell mass and secretory function were diminished in islets of older *Pdx1cre*$^+$ *Huwe1*$^{fl/y}$ mice, and this contributed to impaired glucose homeostasis.

Huwe1 deletion preferentially activated p53-dependent apoptotic machinery Multiple polyubiquitination and degradation substrates of HUWE1 have been identified. Among them, p53, MCL1 and DNA polymerase beta are better characterised [16, 20, 33]. Intriguingly, p53 induces apoptosis while MCL-1 and DNA polymerase inhibit the apoptotic pathway. Thus, HUWE1 seemingly regulates two opposing pathways involved in cell survival. To identify the signalling alterations that may have resulted from *Huwe1* deletion in the pancreas in vivo, we measured the activation of p53 and MCL1 pathways. We found that *Huwe1* deletion in islets led to increased p53 in *Pdx1cre*$^+$ *Huwe1*$^{fl/y}$ mice at 2 months of age (Fig. 5f), with an even more significant difference in older mice at 6 months of age (ESM Fig. 5a). In keeping with the well-known pro-apoptotic role of p53, there was an associated increase in cleaved caspase 3 (ESM Fig. 5a). In comparison, levels of MCL1 and DNA polymerase beta were not significantly different between *Pdx1cre*$^+$ *Huwe1*$^{fl/y}$ and control islets (Fig. 5f and ESM Fig. 5a). Therefore, p53-dependent apoptosis in HUWE1-deficient islets likely contributed to reduced beta cell mass and glucose intolerance in *Pdx1cre*$^+$ *Huwe1*$^{fl/y}$

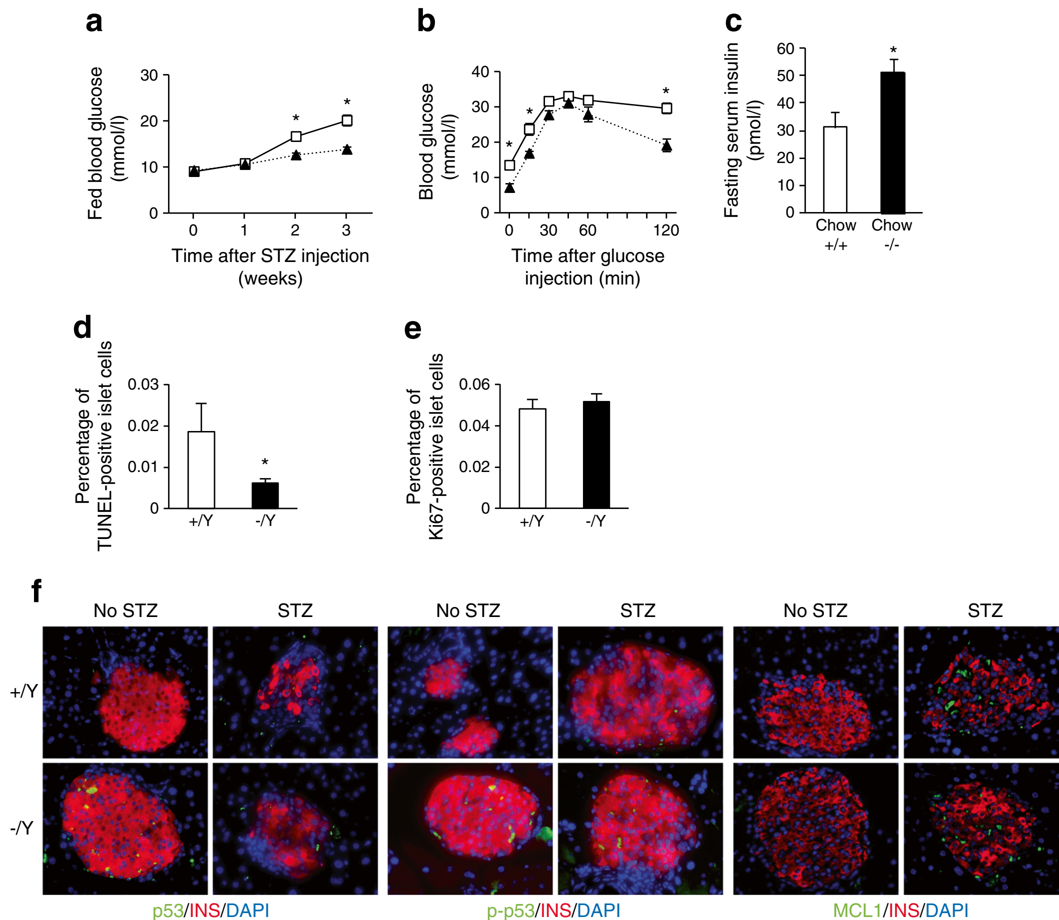

Fig. 6 Pancreas-specific deletion of *Huwe1* protects against MLDS-induced beta cell apoptosis. (**a**) Fed blood glucose in male *Pdx1cre*$^{+}$ *Huwe1*$^{+/y}$ (+/Y) and *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ (−/Y) mice at 2 months of age after MLDS injections (n=5). (**b**) Glucose tolerance test and (**c**) fasting serum insulin of 2-month-old mice 3 weeks after MLDS (n=5). (**d**) Percentage of TUNEL-positive and (**e**) Ki67-positive islet cells of 2-month-old mice 3 weeks after MLDS (n=5). (**c**) Representative pancreatic sections with immunofluorescent staining of p53/insulin (INS)/DAPI, phospho (p)-p53/INS/DAPI and MCL1/INS/DAPI (×20 magnification). $^{*}p$<0.05 vs +/Y mice. White squares, +/Y, black triangles, −/Y. INS, insulin

mice, while *Huwe1* deletion in islets had minimal effect on pro-survival pathways, such as p-Akt, p-AMPK, MCL1 and DNA polymerase beta (Fig. 5f and ESM Fig. 5a). Consistent with reduction in beta cell secretory function at 6 months of age, beta cell differentiation marker PDX1 also showed a reduction, while glucose-sensing receptor GLUT2 was similar in HUWE1-deficient islets compared with controls (Fig. 5f and ESM Fig. 5a). Coupled with reduced granule filling/mobilising rate we observed in individual beta cells, these findings suggest that glucose intolerance in HUWE1-deficient mice is due to both a defect in exocytosis and survival of pancreatic beta cells.

HUWE1 deletion paradoxically leads to protection against STZ-induced beta cell apoptosis The biological role of HUWE1 is highly specific for the cell type and is context-dependent. As such, HUWE1-deficient B lymphocytes demonstrated reduced viability under basal condition, while under genotoxic stress, they were resistant to apoptosis [17]. To test whether this context-dependent divergent biological role of HUWE1 occurred in pancreatic beta cells, we used an MLDS model to induce beta cell apoptosis in vivo. Consistent with the protective role of *Huwe1* deletion under cytotoxic conditions, *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ mice were protected against hyperglycaemia and glucose intolerance after STZ injection (Fig. 6a, b). Consistent with more viable beta cells and better glucose tolerance after STZ injection in HUWE1-deficient mice, insulin levels were higher compared with controls (Fig. 6c). There was also a significant reduction in the percentage of TUNEL-positive cells when compared with control mice (Fig. 6d), while islet proliferation remained comparable after STZ injection between genotypes (Fig. 6e). While higher levels of p53 and phosphorylated p53 were seen in islets under basal conditions, no significant difference in p53 or p-p53 levels were detected with STZ treatment between *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ and littermate control mice (Fig. 6f, ESM Fig. 5e). MCL1 also did not differ significantly between genotypes, suggesting that HUWE1 regulates these substrates differently

in distinct settings (Fig. 6f, ESM Fig. 5c). Thus, the biological role of HUWE1 is highly dependent on context in pancreatic beta cells. Under basal conditions, HUWE1 deletion leads to p53 stabilisation and an increase in apoptosis, whereas on DNA-damaging stress, HUWE1-deficient islets are protected against apoptosis in vivo.

Discussion

HUWE1 is a newly discovered apoptosis regulator that acts via its E3 ligase activity, which polyubiquitinates known players in apoptosis. Intriguingly, these HUWE1 targets consist of effectors of both pro-apoptotic and anti-apoptotic pathways, thus the precise function of HUWE1 in beta cells was unknown. This study showed that HUWE1 modulated beta cell homeostasis by exerting a tonic inhibition on p53-dependent apoptosis in beta cells, such that pancreas-specific *Huwe1* deletion led to increased beta cell death and glucose intolerance in mice under basal conditions. In contrast, HUWE1 appeared to play an opposite role when beta cells were under genotoxic stress. As such, the same HUWE1-deficient mice were paradoxically protected against MLDS-induced beta cell apoptosis and diabetes. This protection was not associated with a significant difference in expression of GLUT2, which is required for STZ action. Rather, these findings suggest that HUWE1 can regulate substrates such as p53 differently in specific settings, similar to the recent report on the dual role of HUWE1 in regulation of B lymphocyte survival and proliferation [17]. HUWE1 demonstrated similar dual function in pancreatic beta cells, depending on the experimental context in which the role of HUWE1 was examined.

In agreement with the physiological pro-survival role of HUWE1, Kon and colleagues reported that rat insulin promoter (RIP)-mediated *Huwe1* deletion in pancreatic beta cells led to similar beta cell apoptosis and diabetes starting at 6 months of age [34]. However, the possibility of RIP-mediated effects in the central nervous system shown by other groups [35–37] was not explored in their study. On the other hand, the hypothalamic expression pattern of PDX1 is much more restricted [38]. This was further supported in our mice where we used *Pdx1*-promoter-driven deletion of *Huwe1*, which resulted in no change in hypothalamic HUWE1 expression or peripheral insulin sensitivity. Furthermore, the region of the *Huwe1* gene that was targeted was different between the two groups (*loxP* sites flanked exon 11 in the report by Kon et al, whereas we used mice with *loxP* sites flanking exons 76 and 77) [34]. This difference is reflected in the early embryonic lethality (E12.5) of the whole-body knockout using the targeting construct used in our study, whereas perinatal lethality was observed by Kon et al [17, 34]. These differences suggest a more complete genetic ablation in mice that we used for our study in comparison to the report by Kon et al [17, 34].

Multiple HUWE1 substrates have been identified; among them is v-myc avian myelocytomatosis viral oncogene homologue (c-Myc) [18, 39]. *Huwe1* deletion leads to accumulation of c-Myc/zinc finger and BTB domain containing 17 (MIZ1) complex and subsequent downregulation of p21 in models of skin carcinogenesis [39]. Interestingly, in pancreatic beta cells, the absence of HUWE1 does not appear to have an impact on cell proliferation. It is possible that the intrinsic properties of low mitotic activity in these endocrine cells render them resistant against uncontrolled proliferation and tumorigenesis. Alternatively, this difference in HUWE1 function is a reflection of its context- and cell-type-dependent specificity in its biological role, which is highlighted by our findings in this report. Similarly, while HUWE1 can facilitate degradation of anti-apoptotic MCL1 [21, 24, 40], deletion of HUWE1 in the pancreas did not change MCL1 protein expression. This is consistent with the only slight differences in MCL1 that were seen at steady state in B lymphocytes with *Huwe1* deletion, suggesting MCL1 regulation is context-specific [18, 24]. In addition to the essential role of HUWE1 in determining beta cell survival, we also showed an age-dependent decline in secretory function. Thus, the role of HUWE1 may be more complex than previously thought. Indeed, HUWE1 has been shown to modulate histone ubiquitination and chromatin condensation in addition to its role in cellular survival [19]. Therefore, HUWE1 deletion may have a wide range of effects on protein expression important in other areas of cellular function, including potentially the machinery required for insulin granule secretion as suggested by our data showing a defect in exocytosis in *Pdx1cre*$^{+}$ *Huwe1*$^{fl/y}$ beta cells.

In summary, our study shows HUWE1 is an important physiological regulator of both beta cell function and survival, such that *Huwe1* deletion activates p53-dependent apoptosis and impairs beta cell secretory function with ageing under basal conditions. In contrast, HUWE1 has an opposite role under genotoxic conditions such that *Huwe1* deletion provides protection against further apoptosis. These complexities in the roles of molecules that determine beta cell fate illustrate the need for clearer understanding of their intricacies as we strive to find novel therapeutic targets that enhance beta cell survival and function for treatment of both type 1 and type 2 diabetes.

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