The KINGS Ins2\textsuperscript{+/G32S} mouse: a novel model of beta cell endoplasmic reticulum stress and human diabetes

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Running title: A new mouse model of beta cell ER stress

Tweet: The KINGS Ins2\textsuperscript{+/G32S} mouse: a novel model of beta cell endoplasmic reticulum stress and human diabetes. We hope the diabetes community will find this model of interest to study islet biology, beta cell ER stress and sex differences in diabetes.

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Representative image: Figure 2B
Abstract

Animal models are important tools in diabetes research as ethical and logistical constraints limit access to human tissue. Beta cell dysfunction is a common contributor to the pathogenesis of most types of diabetes. Spontaneous hyperglycaemia developed in a colony of C57BL/6J mice at King’s College London (KCL). Sequencing identified a mutation in the Ins2 gene, causing a glycine to serine substitution at position 32 on the B chain of the preproinsulin2 molecule. Mice with the Ins2\(^{+/G32S}\) mutation were named KCL Ins2 G32S (KINGS) mice. The same mutation in humans (rs80356664) causes dominantly inherited neonatal diabetes. Mice were characterised and beta cell function was investigated. Male mice became overtly diabetic at around 5 weeks of age whereas female mice had only slightly elevated non-fasting glycaemia. Islets showed decreased insulin content and impaired glucose-induced insulin secretion, which was more severe in males. Transmission electron microscopy and studies of gene and protein expression showed beta cell endoplasmic reticulum (ER) stress in both sexes. Despite this, beta cell numbers were only slightly reduced in older animals. In conclusion, the KINGS mouse is a novel model of a human form of diabetes that may be useful to study beta cell responses to ER stress.
Introduction

Diabetes is a collection of diseases characterised by hyperglycemia which is caused by an absolute or relative insulin deficiency. The cause varies from beta cell developmental defects, lack of beta cell adaptation to insulin demand, to beta cell destruction and functional decline. In addition to the most common types of diabetes (Type 1, Type 2, gestational), monogenic forms cause maturity onset of diabetes in the young (MODY) or neonatal diabetes. The common denominator in most forms of diabetes is the beta cell and thus understanding how beta cells can resist or adapt to damage or changing environments is important.

Animal models are a crucial tool in diabetes research as access to human cohorts and/or islets is limited, so animal models are therefore used extensively to understand pathogenic processes and test new therapies(1–3). It is important that a variety of well-defined models are available as the most robust therapeutic interventions will be successful in more than one model.

Spontaneous hyperglycaemia was discovered within some males of a C57BL/6J mice colony at King’s College London (KCL). Sequencing of the Ins2 gene revealed this to be due to a dominantly inherited single nucleotide polymorphism, resulting in a glycine to serine substitution at position 32 on the B chain of preproinsulin2 (Figure 1). Mice with the Ins2+/G32S mutation were named the KCL Ins2 G32S (KINGS) mice and a colony expanded through conventional breeding. The same mutation in the human insulin gene (rs80356664) causes dominantly inherited neonatal diabetes(4–6) and therefore the KINGS mouse provides an unique opportunity to undertake mechanistic studies. The aim of this study was to characterise the KINGS mouse, understand the mechanisms leading to hyperglycaemia and validate it as a new model of human diabetes.
Research Design and Methods:

Animals: All in vivo procedures were approved by our institution’s ethics committee and performed under licence in accordance with the U.K. Home Office Animals (Scientific Procedures) Act 1986 with 2012 amendments. The KINGS mice (C57BL/6J-Ins2<Kings>; MGI:6449740) were discovered in a colony with a C57BL/6J background and maintained on this background. Heterozygous males and females were studied from weaning until 20 weeks of age. In one study, KINGS mice were compared to Ins2<sup>+/Akita</sup> (Akita) mice which were obtained from The Jackson Laboratory (stock #003548, mouse genome informatics #1857572; Bar Harbor, ME) and maintained on the C57Bl/6J background by inhouse breeding. All mice were kept in standard laboratory conditions with a 12h light/dark cycle. They had access to water and standard chow ad libitum, unless otherwise stated. Nesting material, shelters and tunnels were provided in the cages as enrichment. According to our ethical guidelines, any animal losing 20% body weight was killed.

Genotyping: Ear clips were digested using lysis buffer (10% 10X Gitschier’s Buffer, 0.5% Triton X-100, 1% β-mercaptoethanol, 2% 50 µg/ml Proteinase K). The Kompetitive Allele Specific PCR (KASP, LGC, UK) was used to determine genotype. Forward primers with fluorescent tags corresponding to the wild-type Ins2 (GAAGGTGACCAAGTTCATGCTTTTGTCAACGACACCTTTGTG, FAM fluorophore) and KINGS mutant (GAAGGTCGGAGTCAACGGATTGCTTTTGTCAAGCAGCACCTTTGTA, HEX fluorophore), with a common reverse primer for Ins2 (AGAGCCTCCACCAGGTGGGA), were used. PCR was carried out using a LightCycler480 (Roche, Switzerland) to give different fluorescent signals corresponding to wild-type, heterozygous or homozygous genotypes.

Animal monitoring: Random morning (9am) blood glucose concentrations and body weights were measured between weaning (3-weeks) and 20-weeks and, for a subset of mice, measured daily between 3 and 6-weeks. Hyperglycaemia was defined as blood glucose concentrations >300 mg/dl and normoglycaemia as <200 mg/dl. Blood samples were taken using a 30G needle prick to the end of
the tail and glycaemia measured using a glucometer (Performa glucometer and Informa II test strips, Roche, UK). Samples exceeding the meter maximum (600 mg/dl) were re-assayed with a meter with a 900 mg/dl maximum capacity (Stat Xpress, Nova Biomedical, USA).

**Plasma insulin:** Mice were killed by overdose of anaesthetic (2g/kg Euthatal, Merial Animal Health, Essex, UK) and terminal blood samples were taken by cardiac puncture with heparinised needles. Blood was spun (1500 rpm, 10-mins, 4°C) and plasma removed and stored at -20°C until assayed (Ultrasensitive Insulin ELISA, Mercodia, Sweden).

**Glucose tolerance test:** Mice were fasted overnight for 16-hours prior to glucose tolerance test. Basal blood glucose concentrations were taken before administering glucose (2g/kg) by intraperitoneal injection. Blood glucose concentrations were measured at 15, 30, 60, 90 and 120-minutes post injection.

**Islet isolation:** Islets were isolated by collagenase digestion as previously described in detail (7,8). Collagenase (1mg/ml type XI; Sigma-Aldrich) was injected into the pancreas via the bile duct. The pancreas was excised and incubated at 37°C for 10-minutes. The resulting digest was centrifuged and washed using MEM media (Sigma-Aldrich) before histopaque-1077 (Sigma-Aldrich) was used to generate a density gradient from which the purified islets were collected. Islets were washed in MEM prior to use or culturing in RPMI medium with 10% fetal bovine serum.

**Transmission electron microscopy:** Islets were fixed with 2.5% glutaraldehyde (v/v) in 0.1 M sodium cacodylate buffer. Samples were rinsed with 0.1 M cacodylate buffer and post-fixed in 1% (v/v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4). Samples were then embedded with epoxy resin (TAAB, UK). Ultrathin sections (70-90 nm) were cut using a Leica-UC7 ultramicrotome mounted on 150 mesh copper grids and contrasted using Uranyless (TAAB, UK) and 3% Reynolds Lead citrate (TAAB,
Sections were examined at 120kV on a JEOL JEM-1400Plus TEM with a Ruby digital camera (2k x 2k).

Quantification of ER stress markers by PCR: Islets from 4, 10 and 20-week old mice were snap frozen and stored at -80°C until RNA extraction. Samples were lysed with lysis buffer (350µl RLT buffer (Qiagen, UK), 1% β-mercaptoethanol) and further homogenised using Qiashredder spin columns (Qiagen). RNA extraction (RNeasy Mini Kit, Qiagen) and cDNA conversion (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, UK) were completed as per manufacturer instructions. Real-time PCR was performed as previously described to investigate different branches of the unfolded protein response (UPR) using the following primers: CHOP (forward: CCAGCAGAGGTCAAGACGAC, reverse: CGCACTGACCACTCTGTTT), BiP (forward: CCACCAGATGCAGACATTG, reverse: AGGGCCTCACTTCCATAGA), XPB1 (forward: AAGAACACGCTTGGGAATGG, reverse: ACTCCCTTTGGCTCCAC), XBP1s (forward: GAGTCCGCAGCAGGTG, reverse: GTGTCAGAGATCCATGGGA). Ins2 (forward: CTCTCTACCTGGTGTGGG, reverse: TGGGTAGTGTTGGTGCTAGT) was measured in a subset of preparations. All genes were normalised against the geometric mean of three reference genes, GAPDH (forward: AACTTTGGCATTGTGGAAGG, reverse: GGATGCAGGGATGATGTTCT), beta-actin (forward: ACGGCCAGGTCATCACTATT, reverse: GTTGGCACAGGTCTTTACCG) and OAZ1 (forward: CACCATGCCGCTTCTTAGT, reverse: CCGGACCCAGGTACTACAG).

Quantification of ER stress markers by western blotting: Isolated islets were cultured for 1-day and as a positive control, one group of WT islets were then treated for 16h with 1µM Thapsigargin to induce ER stress (10). Islets pooled from 2-3 mice in each condition were lysed using RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail. 15µg protein per sample was subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Membranes were treated with antibodies towards BiP, XBP1s, p-eIF-2alpha (Cell Signalling technology) and beta actin.
(Santa Cruz technology). Densitometry analysis was performed on western blot images using ImageJ software.

**Immunofluorescence:** Whole pancreases from 4, 10 and 20-week old animals were formalin-fixed (4% formalin, 48 hours), wax embedded and cut into 5 µm sections. Sections from three different areas of the pancreas were de-waxed, underwent heat-mediated antigen retrieval (citrate buffer 10mM, pH 6) and stained using immunofluorescent techniques for insulin (guinea-pig anti-insulin 1:100, Dako, UK; anti-guinea pig Alexa Fluor® 647, 1:100, Jackson Laboratories, UK), glucagon (rabbit anti-glucagon 1:25, Abcam, UK; anti-rabbit Alexa Fluor® 488, 1:100, Jackson Laboratories), somatostatin (rat anti-somatostatin 1:25, Abcam; anti-rat Alexa Fluor® 597, 1:100, Jackson Laboratories) and DAPI (1:500, Invitrogen, UK). Pancreas sections were also co-stained for insulin, BiP (rabbit anti-BiP 1:100, Cell Signalling technology; anti-rabbit Alexa Fluor® 597 1:100, Jackson Laboratories) and DAPI (1:500, Invitrogen). Images were taken at 30x magnification using a Nikon Eclipse TE2000-U.

**Analysis of islet area and composition:** Due to the variable staining for insulin from KINGS mice, beta cell numbers were estimated by subtracting alpha and delta cell numbers from total islet cell count (DAPI staining). In a subset of sections, Nkx6.1 (rabbit anti-nkx6.1, 1:10, Thermo Fisher, UK; anti-rabbit Alexa Fluor® 488, 1:100, Jackson Laboratories) was stained for to identify beta cells in combination with insulin and DAPI to validate the use of counting beta cells by proxy of deducting alpha and delta cell count from total cell number. Islet area was measured by tracing around the islet edge. All images were analysed using ImageJ software.

**Insulin secretion and content:** Islets were cultured for 1-day. Insulin secretion and content were measured as previously described in detail(11). Briefly, islets were pre-incubated for 1-hour in RPMI-1640 containing 2mM glucose. Groups of 5-10 islets (3-5 replicates per animal) were picked and placed in Eppendorf tubes containing 500µl physiological salt buffer solution (Gey & Gey buffer), which was
either supplemented with 2 mM (basal) or 20 mM (stimulatory) glucose. Islets were incubated for 1-hour at 37°C after which supernatants were removed and stored at -18°C until analysis. Acidified alcohol was added to islet pellets before sonication and storage until analysis. Insulin secretion and content were measured by in-house radioimmunoassay (described in detail in (11)).

**Islet transplantation:** 500 islets from wild-type C57BL/6J mice were transplanted under the kidney capsule of 12-week KINGS males as previously described(7,8). Blood glucose and body weight were monitored daily for the first week and twice weekly thereafter. At 20-weeks of age, mice were killed and the endogenous islets isolated as described above.

**Statistical analysis:** Unpaired T-tests were used to compare two groups. ANOVAs with Holm-Sidak post-hoc tests were used to compare multiple groups. For repeated measurements over time, a two-way repeated measure ANOVA with Holm-Sidak post hoc test was used. All data are represented as mean±SD. P values <0.05 were considered significant. Statistical analyses were performed with GraphPad prism8.0 (GraphPad software Inc., San Diego, CA).

**Data and Resource Availability:** The datasets generated and/or analyzed during the current study and the KINGS mice (RRID:MGI:6449740) are available from the corresponding author upon reasonable request.

**Results**

**Random blood glucose concentrations are elevated in male mice but near normal in female mice:**
Both male and female homozygous mice were overtly diabetic at weaning (>540 mg/dl glucose; n=3-5), emaciated and had to be killed according to our ethical guidelines. Thereafter only heterozygous mutant mice were bred for this study.

Wild-type mice showed normal blood glucose concentrations (<200 mg/dl) from weaning to 20-weeks (Figure 2A,B). Male KINGS mice had higher blood glucose concentrations than at weaning by 28-days
which progressively increased to persistent and overt hyperglycaemia (>300 mg/dl) from 38-days onwards (Figure 2A,B). Female KINGS mice had an overall mildly elevated blood glucose compared to wild-type females (p<0.001, two-way repeated measure ANOVA). They had occasional hyperglycaemic spikes but maintained an average glycaemia within or just above the upper limit of normoglycaemia (<200 mg/dl) which did not worsen between weaning and 20-weeks. Bodyweight in the KINGS females was normal, but KINGS males had a reduced weight gain compared to wild-type males and were significantly lighter by 18 weeks (Figure 2C, p=0.027).

**Plasma insulin is detected in male and female KINGS mice:** Plasma insulin is detectable in KINGS mice (Figure 2D). In most male KINGS mice, the plasma insulin concentrations at each age-group is at the lower end of those seen in wild-type mice but this only reaches significance at 10-weeks. In female KINGS mice, there seems to be little difference in mean plasma insulin concentrations at 4 and 10-weeks but by 20-weeks the plasma insulin concentrations are significantly lower than in wild-type mice.

**Male and female mice are glucose intolerant:** At 4-weeks, random blood glucose concentrations were below hyperglycaemic levels in both male and female KINGS mice (Figure 2A). At this age both sexes showed impaired glucose tolerance compared to wild-type mice (Figure 2E). Impaired glucose tolerance persisted at 10 and 20-weeks compared to wild-type mice (Figure 2F, p<0.005 for all comparisons). Glucose intolerance progressively worsened in KINGS males between 4, 10 and 20-weeks whereas in KINGS female mice the glucose intolerance only worsened at 20-weeks (Figure 2F).

**Islet ultrastructure indicates ER stress:** At 10-weeks, male and female KINGS beta cells showed distended ER, indicative of ER stress, and swollen mitochondria with distorted cristae (Figure 3). Insulin granules were mostly depleted in KINGS males and to a lesser extent in females. However, there was heterogeneity between beta cells and the severity of ultrastructural changes was variable.

**Increased gene and protein expression of ER stress markers in the KINGS mice:**
CHOP mRNA levels were unchanged in female KINGS islets and either unchanged or significantly decreased in male KINGS islets at all ages compared to wild-type (Figure 4, A). Phosphorylation of eIF-2-alpha was slightly increased in KINGS male islets at 10-weeks (1.6-fold; Figure S1). The XBP1s/XBP1 mRNA ratio was increased in the KINGS males at all ages; in female KINGS islets the XBP1s/XBP1 ratio was only increased at 4-weeks (Figure 4, B). The XBP1s induction was confirmed at the protein level in 10-week KINGS males where XBP1s protein levels were 3.7-fold that of wild-type (Figure S1). BiP mRNA was upregulated in KINGS females at 4 and 10-weeks but this did not reach significance at 20-weeks (figure 4, C). In males, BiP induction at the mRNA level was not detected. However, immunofluorescent staining found BiP protein levels were increased at 4, 10 and 20-weeks in KINGS male islets (figure 4, D), which was also true for KINGS females. Western blotting also found increased BiP in 10-week male KINGS islets (Figure S1). Overall, this data suggests there is signalling in the IRE1 and ATF6 branches of the ER stress response (XBP1s and BiP), and mild or no activation of the PERK branch (eIF-2-alpha phosphorylation, CHOP). Ins2 mRNA expression was measured in 10-week-old mice and was found to be upregulated in KINGS male mice (2-fold increase compared to wild-type; 2170±332 vs 960±204, p=0.025, n=3-4). There was more variability in female KINGS mice, with no difference compared to wild-type (1500±530 vs 860±170, p=0.19, n=3-4).

Islet composition changes: Islet size range was similar in wild-type and KINGS mice (Figure 5A,B). Male KINGS mean islet area only differed from wild-type at 20-weeks. The scattergram shows that this is likely the result of the KINGS mice having more small islets rather than a loss of larger islets. In female mice, mean islet area in KINGS mice was similar to wild-type at all ages. In female mice, mean islet area in KINGS mice was similar to wild-type at 4, 10 and 20-weeks (Figure 5B). The composition of the islets was studied by expressing the number of beta cells as a percentage of total islet cells. KINGS islets had a moderate reduction in the percentage of beta cells at 10 and 20-weeks in male and female mice (Figure 5C,D). There was a corresponding increase of alpha and delta cell percentage
(Figure S2). KINGS islets show substantial cell disorganisation with alpha and delta cells located throughout the islet rather than at the periphery (Figure 5E).

**Islet glucose-induced insulin secretion and insulin content is reduced:** Insulin secretion and content were measured in islets from 4- and 10-week mice. At 4 weeks, both male and female (Figure 6A,B) KINGS mice showed the ability to respond to 20 mM glucose despite a significant reduction in insulin content (Figure 6C). By 10-weeks, when KINGS males were overtly hyperglycaemic, glucose-induced insulin secretion was almost completely abolished (Figure 6C,D) and islet insulin content was reduced 98% compared to wild-type (Figure 6F). In female mice, both glucose induced insulin secretion and islet insulin content was markedly reduced (Figure 6E and 6F), but not to the same extent as seen in male islets.

**Islet function is partially restored in a normoglycaemic environment:** To test whether impaired islet function in KINGS males was reversible, 12-week-old KINGS males were transplanted with wild-type islets under the kidney capsule for 8-weeks. Normoglycaemia after islet transplantation was achieved for between 2 and 7-weeks prior to endogenous islets being isolated (Figure 7A) and all mice gained weight (Figure 7B). Islets from “cured” KINGS males showed increased glucose-induced insulin secretion compared to age-matched non-grafted diabetic KINGS mice (Figure 7C). Insulin content within the islets was also higher in the “cured” KINGS islets compared to islets from diabetic KINGS mice (Figure 7D). However, it should be noted that the islet insulin content was still around 80% reduced compared to age-matched wildtype islets.

**KINGS mice have lower blood glucose than Akita mice:** A subset of KINGS mice were directly compared to Akita mice with regard to blood glucose concentrations. There were no differences between the two models at 4-weeks, however both male and female Akita mice had significantly higher blood glucose concentrations at 10-weeks than KINGS mice (Figure 8).
**Discussion**

This study introduces a novel model of diabetes, the KINGS mouse. The KINGS mutation causes a rare form of neonatal diabetes in humans (4–6) and therefore this model provides an unique opportunity to uncover the pathogenic mechanisms underlying this type of diabetes(12), as well as representing a model of how beta cells respond to ER stress.

Homozygous KINGS mice were hyperglycaemic at weaning and became emaciated, rapidly reaching our ethical endpoints for killing. Considering the ethical implications, together with the fact that only the heterozygous mutation has been described in humans(4–6), it was decided to breed the KINGS mice as heterozygotes, as typically done with mice carrying other insulin mutations (Akita and Munich mice)(13,14).

Heterozygous male and female KINGS mice maintain random non-fasted blood glucose concentrations around 200mg/dL at 4-weeks. Glucose tolerance tests carried out at this age show that both KINGS sexes are glucose intolerant. KINGS males consistently progress to severe hyperglycaemia which is maintained from 5-weeks of age onwards and their glucose tolerance deteriorates between 4 and 20-weeks. In stark contrast, the female mice do not develop overt hyperglycaemia. Their glucose intolerance initially remains stable but deteriorates between 10 and 20-weeks. There are clear signs of ER stress in both male and female KINGS islets. While there is no massive beta cell loss, islet function is impaired and severely so in the males.

Other animal models with Ins2 mutations, the Akita and Munich mice, have proven interesting models for preclinical diabetes research(14–16). These mutations cause cysteine residue substitutions on the A chain of preproinsulin2 and consequently disrupt the proinsulin A7-B7 disulphide bridge. This results in retention and aggregation of misfolded insulin in the ER, causing severe proteotoxic ER stress and activation of the UPR. Furthermore, aggregations can form between mutated and unmutated proinsulin, further increasing ER stress and depleting native insulin availability(17–19) These effects ultimately lead to beta cell loss through apoptotic cascades(20–25).
In contrast, the KINGS mouse mutation involves an amino acid which is not directly involved in disulphide bridge formation. However, the substitution of glycine with serine at this position introduces a side chain, which results in conformational change likely to indirectly impact the disulphide bond, subsequent proinsulin folding and structure(5,26). We have shown that the KINGS mutation drives beta cell ER stress. Electron micrographs reveal ultrastructural hallmarks of this with swollen ER and distorted mitochondria evident in KINGS beta cells. Furthermore, we found upregulation of the general ER stress marker, BiP, at the gene level in female mice and at the protein level in both female and male mice. BiP is regulated post-transcriptionally and this may explain BiP protein induction in the absence of detectable mRNA upregulation in the males (27). The UPR is activated in response to ER stress and, dependent upon the extent of the ER stress, this response can be adaptive or maladaptive and restore ER homeostasis or promote cellular dysfunction and death, respectively(28). Expression of UPR components were investigated in KINGS islets.

*XBP1s/XBP1* gene expression levels are increased at 4-weeks and 10-weeks in the male KINGS mice and increased XBP1s protein was also confirmed at 10-weeks. This suggests activation of the IRE1 UPR pathway which is associated with gene expression of ER associated degradation (ERAD) components to restore ER homeostasis. Interestingly, in the female KINGS mice *XBP1s/XBP1* gene expression is only increased at 4-weeks in the KINGS mice possibly implying differential UPR pathway activation between the sexes at later ages.

The adaptive PERK UPR pathway restores ER homeostasis by attenuating global protein translation and selectively promoting the translation of ATF4. The maladaptive PERK pathway is associated with the expression of the proapoptotic protein CHOP. Phosphorylation of eIF-2alpha was mildly increased at 10-weeks in KINGS male mice. This together with our finding that *CHOP* gene expression is not enhanced in KINGS mice at any age may suggest that ER stress is not robust enough to activate maladaptive PERK signalling. This contradicts findings
in the Akita mouse that eLF-2alpha phosphorylation is not enhanced(29) and CHOP expression is upregulated and thought to drive beta cell death(30).

Our study of islet composition indicates that the percentage of islet cells comprising of beta cells only drops by 10-15% in the KINGS mice. This contrasts the >50% reduction of beta cell volume in the Akita mouse(14) and indicates that ER stress in the KINGS mouse is not causing such extensive beta cell loss. Indeed, when we compared the KINGS mice to Akita mice, it was found that the KINGS mice had significantly lower blood glucose concentrations at 10-weeks. This is also consistent with our previous study using Akita mice, where female mice developed overt hyperglycaemia(16), which is in stark contradiction to the phenotype of KINGS females. Interestingly, whilst Ins2 mRNA is enhanced in the KINGS mice relative to the wild-type indicating a compensatory response of the beta cells, some studies have found Ins2 mRNA expression is reduced in Akita mice however other studies have suggested it is increased(29,30). Nevertheless, reduced plasma insulin levels and overt hyperglycaemia indicate that the attempt to compensate is not sufficient to prevent overt diabetes in male mice.

In vitro studies have suggested that the KINGS mutation may result in milder ER stress than the Akita mutation. When the KINGS (G32S) Ins2 gene was transfected into MIN6 cells, there was a modest retention of misfolded insulin in the ER compared to the more severe retention seen in the Akita (C96Y) Ins2 transfections(31). The same study showed G32S proinsulin, but not C96Y proinsulin, was partially recruited to granules. Another study showed limited insulin secretion from INS-1 cells transfected with either the Akita or KINGS-mutated insulin(32). However, since intracellular content of C-peptide was higher in cells with the KINGS mutation compared to the Akita mutation, it was concluded that the folding capacity of KINGS mutated insulin was superior. Together these
papers indicate that the misfolding, ER stress and UPR activation may be more modest in our model.

Glucotoxicity can drive beta cell dysfunction through various mechanisms including through increasing beta cell ER stress(33). To ascertain whether the hyperglycaemia seen in the males KINGS mice exacerbated the beta cell dysfunction, we corrected glycaemia by transplanting wild-type islets under the kidney capsule. Endogenous islets isolated from normoglycaemia-corrected KINGS mice showed a partial restoration of beta cell function and insulin content, indicating that glucotoxicity contributes to beta cell dysfunction. The functional data suggest that there is a ‘tipping point’ at which mice go from a reduced insulin content whilst maintaining the ability to secrete insulin (as in seen in 4-week male and female KINGS mice) to one where insulin content is reduced to such an extent that the islet can no longer meet the insulin demand (as seen in adult male KINGS mice). Male mice are significantly heavier than female mice and therefore a higher insulin demand may explain why male mice ‘tip’ into hyperglycaemia whilst females remain protected. In addition, circulating oestrogen acting upon islet oestrogen receptors can promote beta cell survival and proliferation which may contribute to the sex difference(43–45).

This study has validated the KINGS mouse as a novel model of monogenic diabetes caused by ER stress and glucotoxicity; pathogenic processes implicated in type 1 and type 2 diabetes. Previously published Ins2 mutant mice have shown severe islet ER stress and substantial beta cell loss. In contrast, the KINGS mouse does not show severe beta cell loss and may therefore provide key mechanistic insights into beta cell survival. Further work is required to understand the adaptive and protective mechanisms involved. In addition, the KINGS male mice have a reliable and predictable onset of overt hyperglycaemia, and thus could be used as a model of hyperglycaemia in which toxic agents such as streptozotocin can
be avoided. In conclusion, the KINGS mouse is not only a translational model of neonatal diabetes but also has great potential for investigations of hyperglycaemia, islet adaptation to stress and diabetic complications.

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Contributions
AA, LDG, MC, GS developed protocols for the experiments, conducted experiments, performed the statistical analysis, constricted figures and tables and actively participated in writing and reviewing the manuscript. DA, SS, CG and SB initially discovered hyperglycaemia in the mouse colony, sequenced Ins2, developed and carried out protocols for genotyping the mice and participated in protocol design and writing and reviewing of the manuscript. PJ and AK were lead supervisors, developed protocols for the experiments, participated in data analysis and writing the manuscript. AK is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Legends to the Figures

**Figure 1:**

Schematic of rodent preproinsulin2 molecule with KINGS (G32S), Akita (C96Y) and Munich (C95S) mouse amino acid substitutions shown.

**Figure 2:**

Random daily blood glucose concentrations from 22-42 days (A) and weekly blood glucose concentrations from 21-140 days (B) of WT male (open circles), KINGS male (filled circles), WT female (open triangles) and KINGS female (filled triangles) mice. KINGS male and KINGS female mice show significantly elevated blood glucose compared to sex matched wild-type littermates (p<0.001, Two-Way RM ANOVA, Holm-Sidak post-hoc, n=11-15). In male KINGS mice, blood glucose concentrations are elevated by five weeks, when compared to their starting levels measured at 3 weeks (p<0.001, Two-Way RM ANOVA, Holm-Sidak post-hoc, n=13). In all other groups blood glucose remained constant across the 20 weeks. (C) Weekly weight monitoring from 21-140 days. Male KINGS mice show lack of weight gain and are significantly different from wild-type mice by 126 days (p<0.05, Two-Way RM ANOVA, Holm-Sidak post-hoc; n=11-15). (D) Plasma insulin concentration in male and female WT and KINGS mice at 4, 10 and 20-weeks. Plasma insulin levels were significantly reduced in the KINGS males from 10-weeks and at 20-weeks in the KINGS females compared to WT littermates (p<0.05, unpaired T test, n=4-10). (E) Glucose tolerance test at 4 weeks of age for WT and KINGS male and female mice. (F) Area under the curve for glucose tolerance tests at 4, 10 and 20 weeks. Both male and female KINGS mice show significant glucose intolerance at all time points compared to sex matched wild-type mice. Male glucose intolerance worsens with age and females only show worsening at 20 weeks. (#= p<0.05 KINGS vs age and sex matched WT mice, *= p<0.05 vs 4-week age and genotype matched mice, **= p<0.05 vs 10 week age and genotype matched mice; Two-Way ANOVA, Holm-Sidak post-hoc test, n=5-7).
Figure 3:
Transmission Electron Microscopy images of representative beta cells from 10 week-old WT and KINGS male and female mice. WT beta cells show normal endoplasmic reticulum and mitochondria as well as dense core insulin granules (one representative image shown for each sex). KINGS beta cells (three representative images shown for each sex) have dilated endoplasmic reticulum (red asterisk), swollen mitochondria with distorted cristae (white hash) and a reduced density and number of insulin granules. The severity of the latter is heterogeneous between beta cells. 150000x magnification, scale bar= 500nm.

Figure 4: CHOP (A), BiP (B) and XBP1s/XBP1 (C) gene expression in islets from wildtype male (open circles), KINGS male (filled circles), wildtype female (open triangles) and KINGS female (filled triangles) mice at 4, 10 and 20-weeks of age. CHOP had reduced or similar expression in KINGS male and female islets compared to age and sex matched controls, whilst BiP had increased expression only in KINGS females at 4 and 10 weeks of age and XBP1s/XBP1 expression ratio was increased in both male and female KINGS mice at 4-weeks and male KINGS mice at 10 weeks (p=<0.05, unpaired T test, n=4-9). (D) Immunofluorescence analysis of BiP in islets from pancreases of wildtype male (open circles), KINGS male (filled circles), wildtype female (open triangles) and KINGS female (filled triangles) mice. BiP levels were significantly higher in both male and female KINGS mice from 4-weeks compared to age and sex matched wildtype littermates (p=<0.0001, unpaired T test, n=3, 8-49 islets per animal). (E) Representative immunofluorescent images of islets from male (upper panel) and female (lower panel) wildtype and KINGS mice stained for insulin (blue), DAPI (red) and BiP (green/white). Scale bar= 50μm.

Figure 5:
Individual islet areas measured in male (A) and female (B) WT and KINGS mice at 4, 10 and 20 weeks using immunofluorescence staining. Median area is indicated by the blue line, boxes represent the
interquartile range and whiskers indicate the range. Islet area was significantly reduced in 20-week male KINGS mice compared to age matched WT littermates (p<0.0005, unpaired T test, n=128-245). Beta cell percentage of total islet cells in WT male (open circles), KINGS male (filled circles) (C) and WT female (open triangles) and KINGS female (filled triangles) mice (D) at 4, 10 and 20 weeks of age. Beta cell percentage in KINGS males is slightly reduced compared to WT at 10-weeks and 20-weeks, and KINGS female beta cell percentage is reduced from 4-weeks (p=<0.05, unpaired T test, n=3, 128-258 islets per group). (E) Representative immunofluorescent images of islets from 10 week WT and KINGS male and female mice stained for insulin (white), glucagon (green), somatostatin (red). Scale bar= 50µm.

Figure 6:

Glucose stimulated insulin secretion at 4 week (A-B) and 10 week (D-E) for male and female KINGS (filled bars) and WT (unfilled bars) islets. The ability of KINGS islets to respond to 20mM glucose is impaired at 10 weeks (#=p<0.05 2mM vs 20mM, *= p<0.05 KINGS vs age and sex matched WT mice; Two-way ANOVA, Holm-Sidak post hoc, n=2-6). Insulin content of islets from 4-week (C) and 10-week (F) male and female KINGS (filled bars) and WT (unfilled bars) mice. Insulin content is reduced in 4- and 10-week KINGS mice compared to WT (p<0.0001, unpaired T test, n=9-37).

Figure 7:

(A) Random blood glucose concentrations after islet transplantation in KINGS male mice. All mice reach normoglycemia by 42 days post-transplantation and remain normoglycemic for at least three weeks. (B) Weight monitoring after islet transplantation in KINGS male mice. (C) Glucose stimulated insulin secretion and (D) islet insulin content for KINGS males (black bars) and cured KINGS males following 8 weeks of subcapsular islet transplantation (patterned bars). Cured KINGS males have significant recovery of 20mM glucose induced insulin secretion (P=<0.05, Two-way ANOVA, n=6-7) and insulin content (p<0.05, unpaired T test, n=5-14) compared to non-transplanted KINGS males.
Figure 8:

Non-fasted blood glucose concentrations in male (A) and female (B) KINGS (filled symbols) and Akita (open symbols) mice. Akita mice had significantly higher blood glucose concentrations at 10 weeks compared to age and sex matched KINGS mice (p<0.05, unpaired T test, n=5-7).
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338x170mm (300 x 300 DPI)
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Delta cell (A-B) and alpha cell (C-D) percentages in male and female WT (open symbols) and KINGS (filled symbols) islets at 4, 10 and 20 weeks determined through immunofluorescent staining. Delta cell percentage was increased in male and female KINGS mice at 20 weeks compared to sex and aged matched WT littermates (p<0.05, unpaired T test, n=3, 128-258 islets per group). Alpha cell percentages were significantly increased at 10 and 20 weeks in KINGS males and females (p<0.05, unpaired T test, n=3, 128-258 islets per group).

Supplementary figure 2:

Protein expression of ER stress markers BiP, XBP1s, p-eIF-2alpha measured by western blot in islets pooled from 10 week old WT and KINGS mice. In addition, WT islets treated with thapsigargin (TG) to induce ER stress were analysed.