METHOD ARTICLE

A CRISPR/Cas9 genome editing pipeline in the EndoC-βH1 cell line to study genes implicated in beta cell function [version 1; peer review: 3 approved, 1 approved with reservations]

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Abstract

Type 2 diabetes (T2D) is a global pandemic with a strong genetic component, but most causal genes influencing the disease risk remain unknown. It is clear, however, that the pancreatic beta cell is central to T2D pathogenesis. In vitro gene-knockout (KO) models to study T2D risk genes have so far focused on rodent beta cells. However, there are important structural and functional differences between rodent and human beta cell lines. With that in mind, we have developed a robust pipeline to create a stable CRISPR/Cas9 KO in an authentic human beta cell line (EndoC-βH1). The KO pipeline consists of a dual lentiviral sgRNA strategy and we targeted three genes (INS, IDE, PAM) as a proof of concept. We achieved a significant reduction in mRNA levels and complete protein depletion of all target genes. Using this dual sgRNA strategy, up to 94 kb DNA were cut out of the target genes and the editing efficiency of each sgRNA exceeded >87.5%. Sequencing of off-targets showed no unspecific editing. Most importantly, the pipeline did not affect the glucose-responsive insulin secretion of the cells. Interestingly, comparison of KO cell lines for NEUROD1 and SLC30A8 with siRNA-mediated knockdown (KD) approaches demonstrate phenotypic differences. NEUROD1-KO cells were not viable and displayed elevated markers for ER stress and apoptosis. NEUROD1-KD, however, only had a modest elevation, by 34%, in the pro-apoptotic transcription factor CHOP and a gene expression profile indicative of chronic ER stress without evidence of elevated cell death. On the other hand, SLC30A8-KO cells demonstrated no reduction in K<sub>ATP</sub> channel gene expression in contrast to siRNA silencing. Overall, this strategy to efficiently create stable KO in the human beta cell line EndoC-βH1 will allow for a better understanding of genes involved in beta cell dysfunction, their underlying functional mechanisms and T2D pathogenesis.
Keywords
EndoC-βH1, human beta cells, CRISPR/Cas9, gene knockout, Type 2 Diabetes, insulin secretion, NEUROD1

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Introduction

Type 2 diabetes (T2D) affects around 400 million people worldwide and is a complex disease with genetic and non-genetic risk factors. Genome-wide association studies (GWAS) have so far identified more than 240 loci which are robustly associated with disease risk. The vast majority of these exert their impact on T2D-risk through the pancreatic beta cell and therefore authentic human beta cell models are essential for functional follow-up studies.

A lack of a stable and functional human beta cell line, restricted access to human cadaveric pancreatic islets and functional limitations of induced pluripotent stem cells (iPSC)-derived beta-like cells have long been a challenge in understanding beta cell biology. Meanwhile, rodent beta cell lines have provided valuable insights into beta cell function and pathophysiology. Although they share many similarities with human beta cells, there are also fundamental structural, transcriptional and functional differences, aside from having a distinctive genetic background. Human pancreatic islets have a substantially different architecture than rodent islets as they have fewer beta cells, a mixed cell distribution throughout the whole islet, with alpha, beta and delta cells being adjacent to each other and alpha cells clustering around blood vessels. Rodent islets on the other hand have a higher vascular density and are made up of a distinct beta cell core and non-beta cell mantle. Transcriptional analysis in purified beta cells from mice and human demonstrated a set of common core beta cell genes. However, the studies also highlighted a substantial number of uniquely expressed genes in either species and significantly differentially expressed genes such as GAD2, IAPP, MAFF and PPARg, some of which are involved in Type 1 (T1D) and T2D pathology. Differences in key components of the glucose-stimulated insulin secretion (GSIS) pathway emphasise unique functional signatures. The principal glucose transporter in rodent beta cells is SLC2A2, whereas human beta cells mainly utilise SLC2A1 and SLC2A3, leading to distinct glucose uptake dynamics. Furthermore, rodent beta cells express two insulin genes, INS1 and INS2, which is in contrast to human beta cells, which only have one insulin gene. Other distinguishing factors are the proliferative capacity and ion channel composition of rodent cells compared to human beta cells.

In 2011, Scharffmann and colleagues released the EndoC-βH1 cell line, a human beta cell line which opened up the possibility of studying human beta cell physiology and pathology in vitro and provided a valuable alternative to rodent beta cell lines. To generate this cell line, fetal pancreatic buds were transduced with oncogene simian virus 40 large tumour antigen (SV40LT) and human telomerase reverse transcriptase (hTERT). Between each transduction, the cells were transplanted into SCID mice to expand and form insulinomas. The isolated and passaged cells were able to secrete insulin in response to different glucose and secretagogues stimulation, expressed key beta cell markers and were negative for other pancreatic cell markers like glucagon. Insulin content is a magnitude lower than in primary human beta cells, but secreted insulin as percentage of content and the stimulation index are in the same range as for pancreatic islets. Along with their similar electrophysiological properties, EndoC-βH1 are therefore a representative model of human beta cells and physiological insulin secretion. Further independent investigations have also demonstrated their suitability for both high-throughput screening and individual gene function studies.

Robust protocols for generating gene KO using CRISPR/Cas9 in EndoC-βH1 studies have not yet been described. This genome editing tool has revolutionised genetic manipulations by being an easily programmable RNA-guided endonuclease. The application in EndoC-βH1, however, is not straightforward, as their proliferation rate is low and they are very sensitive to seeding densities. It is thus not possible to expand a culture from a single cell, which precludes the generation of a modified clonal cell line. Furthermore, the cells have a very low transfection efficiency, batch-to-batch variation and have to be closely monitored across passages to ensure their beta cells characteristics. A recent study created an HNF1A KO cell line in EndoC-βH1 using CRISPR/Cas9, this cell line, however, does not demonstrate complete HNF1A depletion and has not been fully characterised.

Despite the technical challenges of this cell line, we have successfully developed a lentiviral-based pipeline to create stable non-clonal CRISPR/Cas9 KO cell lines in EndoC-βH1 and have performed genomic and functional characterisation for several proof of concept genes. These KO cell lines could be a valuable tool in understanding human beta cell function and genes underlying the pathology for both T2D and T1D.

Methods

Cloning of individual sgRNA into plentiCRISPRv2 plentiCRISPRv2 was purchased from Addgene (#52961) and sgRNA sequences were retrieved from the TKO Library v3. Two sgRNAs per gene were chosen based on highest specificity and lowest off-target score which were evaluated on CRISPOR.org (Table 1). BsmBI compatible tails, 5’CACCG3’ and 5’AAACYC3’, with X and Y being complementary sequences to the sgRNA, were added to each oligonucleotide. plentiCRISPRv2 vector was digested with FastDigest BsmBI (Fermentas) for 30 min at 37°C and gel-purified using a 0.8% agarose gel. sgRNA oligos were annealed (1 μl of each 100 μM stock) and phosphorylated using T4 PNK (NEB) for 30 min at 37°C, 5 min at 95°C, then the heating block was shut off to let the samples could cool down to room temperature. 20 ng BsmBI digested plentiCRISPRv2 and 2 μl of 1:100 diluted annealed sgRNA oligonucleotides were ligated using Quick Ligase (NEB) for 1 h at RT. Next, 5 μl of the ligation reaction were transformed into Stbl3 competent cells and successful sgRNA insertion was confirmed using Sanger sequencing.

Cell culture

EndoC-βH1 cells were cultured as previously described and passaged every 7 days. They were grown in culture vessels coated with 2 μg/ml Fibronectin and 1% extracellular matrix (ECM) (Sigma-Aldrich) and cultured in DMEM containing...
5.5 mM glucose (Gibco), 2% bovine serum albumin (BSA), 2 mM glutamine, 10 mM nicotinamide, 100 international units (U)/ml penicillin, 100 μg/ml streptomycin (P/S), 50 μM β-2-mercaptoethanol, 5.5 μg/ml transferrin and 6.6 ng/ml sodium selenite (all Sigma-Aldrich).

Lenti-X HEK293T cells (Clontech) were cultured in DMEM 6429 (Sigma-Aldrich) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were tested negative for mycoplasma and grown at 37°C and 5% CO₂.

### Functional lentiviral titer

At 48 h before transduction, EndoC-βH1 were plated at 20,000 cells per well in a 96-well plate. A viral dilution curve ranging from 1:50 to 1:6400 was prepared in 100 μl P/S free media and the cells were infected for 6 h. After 48 h, media was changed on half of the wells per dilution into 4 μg/μl puromycin and the cells were incubated for 7 days. Cell viability was analysed using the CyQUANT Direct Cell Proliferation assay (Invitrogen). The puromycin selected cell counts were normalised to their respective non-selected controls to determine the percentage of survival, which represents transduced cells. The functional titer in transducing units (TU)/μl can be calculated based on:

$$\text{TU/μl} = \frac{\text{#Cells} \times m}{\text{Virus (μl) used in transduction}}$$

The probability that a cell is infected by a certain number of viral particles at a given multiplicity of infection (MOI) ($m$) can be modelled using the Poisson distribution (PD). Simplifying the original PD equation, gives the following:

$$P(n > 0) = 1 - e^{-m}$$

with $P(n>0)$ being the probability that a cell gets infected by at least one viral particle. A MOI of 0.3 would lead to ~26% transduced cells, most of them being infected by a single viral particle, therefore this MOI is a good constant in determining the functional titer. To determine the MOI relative to the virus (μl) used in transduction in Equation 1, a linear regression for the percentage of alive cells against the amount of infected virus was performed in the linear, unsaturated range of the puromycin selection curve. The amount of virus needed for a MOI of 0.3 was then calculated by inserting 26% as the percentage of alive cells and solving the linear equation for the amount of virus (μl) needed in the transduction. Along with the number of plated cells, the TU/μl could then be determined.

### Generation of EndoC-βH1 KO cell lines

To generate stable CRISPR KO lines, cells were transduced at a MOI of 8 which was calculated based on the functional titer. They were selected in 4 μg/μl puromycin for 7 days, with media changes if necessary, to remove dead cells and add fresh puromycin. After selection, cells were grown in normal EndoC-βH1 culture medium and passaged weekly.

| sgRNA | Target gene | Target region | Sequence (5’ → 3’) |
|-------|-------------|---------------|--------------------|
| sgRNA 1 | PAM | Exon 1 | GAACTAGCAGGCTAGGGACG |
| sgRNA 2 | PAM | Exon 12 | GTTCAGAACCCTACCCACAG |
| sgRNA 3 | IDE | Exon 1 | TACCCACAGGGCGCTCCGG |
| sgRNA 4 | IDE | Exon 8 | CATTAATGTGGACTTGACC |
| sgRNA 5 | INS | Exon 2 | CACATGCGCCGCTTCTGCA |
| sgRNA 6 | INS | Exon 2 | CATCTGCTCCATCCAGC |
| sgRNA 7 | NEUROD1 | Exon 2 | CTGCAAAACGTCTGAACGA |
| sgRNA 8 | NEUROD1 | Exon 2 | GCTGCCTAGGGCGTGGC |
| sgRNA 9 | SLC30A8 | Exon 2 | GTGCCGAGGAGAGGACCAG |
| sgRNA 10 | SLC30A8 | Exon 8 | GCACACCTCACCATTGAG |
Insulin secretion assay
Cells were starved overnight in 2.8 mM glucose followed by 30 min starvation in 0 mM glucose and stimulation for 1 h in one of the following conditions: 2.8 mM, 5.6 mM, 11 mM, 15 mM, 20 mM, 25 mM glucose, 15 mM glucose + 100 μM tolbutamide or diazoxide. All conditions were prepared in glucose-free EndoC-BH1 culture medium. Supernatant was collected and cells were lysed in acid ethanol to collect insulin content. Secreted and intracellular insulin were measured using the Insulin (human) AlphaLISA Detection Kit and the EnSpire Alpha Plate Reader (both Perkin Elmer), diluted 1:10 and 1:200, respectively. Secreted insulin was displayed as percentage of insulin content and insulin content was normalised to cell count, which was measured using the CyQUANT Direct Cell Proliferation assay (Invitrogen).

siRNA silencing
Cells were transfected in 6-well plates at 24 h after plating with siRNAs at a final concentration of 15 nM (SMARTpool ON-TARGETplus human NeuroD1 #L-008667-00 and SLC30A8 #L-007529-01, non-targeted control pool #D-001810-10, Dharmacon). The required amount of siRNA (μM) (Gibco) was prepared in Opti-MEM reduced serum-free medium to constitute 0.1% of the volume. A Lipofectamine RNAiMAX (Invitrogen) mix was prepared to account for 0.4% of the same total volume and both transfection mixes were incubated for 5 min at RT. The RNAiMAX and siRNA mix were pooled and further incubated for 20 min before they were added dropwise to the cultured cells. Cells were harvested 72 h past transfection for RNA and protein extraction.

Gene expression analyses
RNA was extracted using the TRIzol reagent (Invitrogen) following manufacturer’s instructions. First-strand cDNA was synthesised using the Super Script III First-Strand Synthesis System (Invitrogen), oligo(dT) primer and 50-500 ng total RNA as input. Quantitative PCR (qPCR) to measure gene expression levels were performed with TaqMan Gene Expression Assays and TaqMan Gene Expression Master Mix on a 7900HT (all Applied Biosystems) using the following thermocycling conditions: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 60°C (Table 2). TaqMan probes with binding sites outside of the regions targeted by sgRNAs were used. Ct values were analysed using the ΔΔCt method and target genes were normalised to three housekeeping genes (TBP, PPIA and GAPDH).

Western blot analyses
Cell pellets for protein analyses were lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing 1X protease inhibitor cocktail (Roche). Protein concentration was quantified by DC protein assay (Bio-Rad) and 10 μg of protein per lane were prepared. Lysates were denatured at 80°C for 10 min and run on a Mini-PROTEAN TGX 4-20% precast gel (Bio-Rad) at 300 V for 15 min. The gel was activated on a ChemiDoc MP Imaging System and transferred to a Trans-Blot Turbo polyvinylidene fluoride (PVDF) membrane using the Trans-Blot Turbo Transfer System (all Bio-Rad). Membranes were blocked in 3% BSA for 1 h at RT, incubated with primary antibodies overnight at 4°C followed by 1 h incubation at RT with secondary antibodies (antibodies are given in Table 3). The membranes were subsequently incubated for 4 min at RT with Clarity Western enhanced chemiluminescence (ECL) reagent and imaged on the ChemiDoc MP Imaging System (Bio-Rad). To normalise for protein loading, the membrane was further incubated with a loading control antibody of appropriate size (tubulin or GAPDH) (Table 3). Western Blot images were quantified using Image Lab 6.0 software (Bio-Rad). Protein bands of interest were normalised to a loading control on the same blot and displayed relative to a control sample.

PCR and sequencing analyses
Genomic DNA for PCR amplification was extracted using the NucleoSpin Tissue extraction kit (Macherey-Nagel). PCR reactions were prepared containing the following components per sample: 2 μl Immobuffer (10x), 0.6 μl MgCl₂ (50 mM), 1 μl each of forward and reverse primer (10 μM) (Table 4), 0.4 μl dNTPs (10 mM), 0.2 μl Immolase DNA Polymerase (5 U/μl) (all Bioline), 4 μl Q-Solution (5x) (Qiagen) and 10.8 μl of the DNA sample (100 ng). The PCR amplification was performed for 10 min at 94°C, 32 cycles of 1 min at 94°C and 1 min at 64°C and 30 sec at 72°C, followed by 10 min at 72°C. DNA samples were run on a 2% agarose gel for 1 h at 120 V and PCR bands were visualised on a GelDoc transilluminator system (Bio-rad). Ahead of sending samples for sequencing, excess primers and nucleotides were removed from PCR reactions using an ExoSAP enzymatic clean up. The reaction was performed with 10 μl PCR product, 0.05 μl ExoI, 0.5 μl SAP, 1 μl SAP buffer (all Affymetrix) and 0.45 μl nuclelease-free water and incubated for 30 min at 37°C and 5 min at 95°C. The sequencing reaction was premixed using 13.5 μl nuclelease-free water, 1.5 μl ExoSAP treated PCR sample and 2 μl sequencing

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Table 2. TaqMan gene expression assays.

| TaqMan Probe | Assay details | Target region |
|--------------|---------------|---------------|
| INS          | Hs00355773_m1 | Exon 1–2      |
| IDE          | Hs00610452_m1 | Exon 24–25    |
| PAM          | Hs01804504_m1 | Exon 22–23    |
| NEUROD1      | Hs01922995_s1 | Exon 2        |
| GAPDH        | Hs02786624_g1 | Exon 8        |
| TBP          | Hs00427620_m1 | Exon 3–4      |
| PPIA         | Hs01634221_s1 | Exon 1        |
| SLC30A8      | Hs00545182_m1 | Exon 2–3      |
| DIT3         | Hs99999172_m1 | Exon 1–2      |
| XBP1s        | Hs03929085_g1 | Exon 5        |
| HSPA5        | Hs00607129_gH | Exon 1–2      |
| ATF6         | Hs00232586_m1 | Exon 6–7      |
| ATF4         | Hs00909569_g1 | Exon 1–2      |
# Table 3. Antibody specifications.

| Antibody | Company, catalogue number | Dilution | Species           | RRID/Ref   |
|----------|---------------------------|----------|-------------------|------------|
| INS      | Santa Cruz, sc-377071     | 1:1000   | Mouse monoclonal | AB_2800506 |
| IDE      | Santa Cruz, sc-393887     | 1:1000   | Mouse monoclonal | AB_2800507 |
| PAM      | Santa Cruz, sc-514110     | 1:1000   | Mouse monoclonal | AB_2800508 |
| NEUROD1  | Santa Cruz, sc-46684      | 1:1000   | Mouse monoclonal | AB_671759  |
| Cas9     | Santa Cruz, sc-517386     | 1:1000   | Mouse monoclonal | AB_2800509 |
| CHOP     | Abcam, ab179823           | 1:1000   | Rabbit monoclonal| AB_10703186|
| pPERK    | Cell Signaling, #3179     | 1:1000   | Rabbit monoclonal| AB_2095853 |
| pIRE1    | Abcam, ab48187            | 1:1000   | Rabbit monoclonal| AB_873899  |
| Cleaved Caspase 3 | Cell Signaling, #9661 | 1:500 | Rabbit polyclonal | AB_2341188 |
| ZnT8     | /                         | 1:1000   | Mouse monoclonal  | 44         |
| β-Tubulin| Santa Cruz, sc-365791     | 1:2000   | Mouse monoclonal | AB_10841919|
| GAPDH    | Abcam, ab181602           | 1:10 000 | Rabbit polyclonal | AB_2630358 |
| α-mouse IgG HRP | Thermo Scientific, 31450 | 1:2500   | Rabbit polyclonal | AB_228427  |
| α-rabbit IgG HRP | Thermo Scientific, 31460 | 1:2500   | Goat polyclonal   | AB_228341  |

# Table 4. Primer specifications.

| Primer name | Target region | Experiment | Sequence (5’ → 3’) |
|-------------|---------------|------------|---------------------|
| LKO1.5R     | lentiCRISPRv2  | sgRNA sequence | GTTGATAACGGGACTAGCT |
| lentiCRv2_Cas9 | Cas9       | sgRNA integration | CAGGCGAGATGTACCTT |
| PAM_sgRNA1  | sgRNA1        | sgRNA integration | ACACGGAATCAGGCAAG |
| PAM_sgRNA2  | sgRNA2        | sgRNA integration | GACGAAACACCGGTGCAAC |
| IDE_sgRNA3  | sgRNA3        | sgRNA integration | AAACACGCACACACAGAG |
| IDE_sgRNA4  | sgRNA4        | sgRNA integration | CTCGTTAGCCTGGCTT |
| INS_sgRNA5  | sgRNA5        | sgRNA integration | CAATGCCACGCTTGCAG |
| INS_sgRNA6  | sgRNA6        | sgRNA integration | CATCTGCTTCCCTACCAG |
| NEUROD1_sgRNA7 | sgRNA7    | sgRNA integration | GACGAAACACCGGTGCAAA |
| NEUROD1_sgRNA8 | sgRNA8    | sgRNA integration | CTGAAATGCCTGGGGTGGTTT |
| PAM1_F      | sgRNA1 target site | Editing efficiency | GCTGAGGGAGGAAAGCTTC |
| PAM1_R      | sgRNA1 target site | Editing efficiency | TTTTTCACGACGGGAGT |
| PAM2_F      | sgRNA2 target site | Editing efficiency | TTGCTGAGATCTAGGGCC |
| PAM2_R      | sgRNA2 target site | Editing efficiency | TCCCTGAGGTGATTTCCC |
| IDE3_F      | sgRNA3 target site | Editing efficiency | AGTGGCGATTCCTTACC |
| IDE3_R      | sgRNA3 target site | Editing efficiency | CTAATGCAGTACCGATGC |
| IDE4_F      | sgRNA4 target site | Editing efficiency | TCCATGAAACAAAGCCAGT |
| IDE4_R      | sgRNA4 target site | Editing efficiency | CACAGCTTCTGACCATTC |
| INS5_F      | sgRNA5 target site | Editing efficiency | CATCTTCCGCTGGAGAGAG |
| INS5_R      | sgRNA5 target site | Editing efficiency | TCCCTAATCTGGAGTCCAG |
| INS6_F      | sgRNA6 target site | Editing efficiency | CTCAGGTCCACACCCAGT |
| INS6_R      | sgRNA6 target site | Editing efficiency | AAGACACAGAGACGACAGGACAG |
| sgRNA1_MED15_F | sgRNA1 off-target | Off-targets | GGGGACACAGAGACGACAGGAGT |
| sgRNA1_MED15_R | sgRNA1 off-target | Off-targets | TGGACACGAGGAGACGAGGAGT |
| sgRNA2_GPM6B_F | sgRNA2 off-target | Off-targets | ATCATGAGGGAGGAGAGAGAGT |
| sgRNA2_GPM6B_R | sgRNA2 off-target | Off-targets | CAGGACCACACTCCAGAGT |

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primer (10 μM) and sent to Eurofins Genomics. Sequence traces were visualised using SnapGene Viewer 4.3 and analysed using TIDE 2.0 and ICE 1.1

Statistical analysis
Statistical analyses were performed in Prism 8.0 (GraphPad Software) and data are shown as mean with standard error of the mean (SEM). Values displayed as fold changes were analysed as log-transformed values and statistical tests were performed as indicated in the figure legends. In general, values normalised to a control group such as western blot data were analysed using one-sample Student’s t-test and two or more groups were compared using two-sample Student’s t-test or one-way analysis of variance (ANOVA) followed by Sidak’s multiple comparison test.

Results
A CRISPR/Cas9 pipeline to create EndoC-βH1 KO cells
To demonstrate that this lentiviral CRISPR/Cas9 pipeline can robustly generate KOs in EndoC-βH1, we created KO cell lines for three proof-of-concept genes. We chose genes with known relevance in beta cell function, namely peptidyl-glycine alpha-amidating monoxygenase (PAM)32, insulin-degrading enzyme (IDE)47,48 and insulin (INS)49,50. In brief, we transduced EndoC-βH1 with lentivirus containing Cas9 and two sgRNAs, selected for successfully transduced cells and characterised the generated heterogeneous KO cell lines (Figure 1A). As a vector system, we chose lentiCRISPRv2 which is a one-plasmid system containing Cas9, a puromycin resistance cassette and the cloned sgRNA (Figure 1B). To increase the KO efficiency in our editing approach, we...

Figure 1. A lentiviral CRISPR/Cas9 pipeline to genome edit EndoC-βH1. (A) Strategy to genome edit EndoC-βH1 using a lentiviral dual sgRNA-based approach resulting in a population with cells containing individual or both sgRNA edits. (B) CRISPR plasmid, lentiCRISPRv2 containing Cas9 and a single sgRNA. (C-E) Gene targeting strategy using two sgRNA per gene for PAM (C), IDE (D) and INS (E). Numbers indicate exons of the respective gene. (F) Puromycin kill curve in EndoC-βH1. Relative fluorescence units (RFU) are representative for cell viability. Results are representative of the optimization experiment performed right before selection of the KO cell lines. The graph displays the mean of six technical replicates with standard error of the mean (SEM).
utilised a dual sgRNA strategy using two sgRNAs in separate lentivirus targeting different parts or exons of each gene (Figure 1C-E). The sgRNA sequences were retrieved from the genome-wide CRISPR KO library Toronto KnockOut version 3.0 (TKOv3) which are optimized for high on-target efficiency and minimal off-target cutting. In the case of IDE, only one of the two protein-coding isoforms was targeted. We packaged lentivCRISPRv2 into lentivirus and transduced EndoC-βH1 at a high MOI of 8, ensuring that each cell is infected by several lentivirus and increasing the likelihood to achieve KOs. The cells were selected in 4 μg/ml puromycin to remove untransduced cells. The ideal concentration of puromycin was determined right before selection as EndoC-βH1 cells have different susceptibilities to antibiotics depending on their passage (Figure 1F). After antibiotic selection, the transduced cells are a heterogeneous population having either no edit, an insertion or deletion (indel) from one sgRNA, a large deletion from simultaneous cutting of both sgRNA or two indels from both sgRNA cutting individually (Figure 1A). These stable CRISPR cell lines were routinely cultured like regular EndoC-βH1 cells and their genomic and functional characteristics investigated.

Genomic modifications of EndoC-βH1 KO cells

To characterise the genomic modifications resulting from stable lentiCRISPRv2 integration and CRISPR editing, we analysed sgRNA and Cas9 integration, sgRNA efficiency and potential off-target cutting. A control cell line created with the same lentiviral backbone but without sgRNA was included as empty vector (EV) or Cas9 only control in all experiments. A PCR-based approach (PCR 1) using a sgRNA specific primer and a primer targeting the lentivCRISPRv2 backbone was used to detect sgRNA integration (Figure 2A). Both sgRNAs were detectable in each KO cell line and not in EV control cells indicating successful transduction with both sgRNA lentivirus. Stable Cas9 expression was demonstrated in all cells lines transduced with lentiCRISPRv2 (KO cell lines and EV) but not in untransduced wild-type cells (WT) (Figure 2B). Individual sgRNA editing efficiency was assessed by amplifying sgRNA target sides (PCR 2) and measuring indel frequency using TIDE (Figure 2C). All sgRNA target sides demonstrate an editing efficiency greater than 87.5%, leaving only around 1% of cells without indels. The sgRNA target sides in INS-KO cells (sgRNA 5 and 6) are within range of a single PCR and Sanger sequencing reaction, which means it is possible to assess the frequency of large deletions from simultaneous cutting of both sgRNA using the Inference of CRISPR Edits (ICE) tool. In 33.4% of INS-KO cells, the approximately 55 bp region between both sgRNA target sides has been deleted through concurrent sgRNA cutting (Figure 2C). In PAM-KO and IDE-KO cells, the presence of large deletions between both sgRNAs was demonstrated by performing a PCR with primers on either side of the sgRNA target side (PCR 3) (Figure 2D). If the region between the two sgRNAs is still present, the fragment is too large for PCR amplification but if both sgRNAs cut simultaneously and the region between the sgRNA target sides has been deleted, a PCR product can be amplified. Such PCR product is present in both cell lines, PAM-KO and IDE-KO, indicating a large deletion of 94 kbp and 66 kbp, respectively, that has not occurred in the WT and EV control cells. In INS-KO cells and confirming the results of the ICE analysis, the presence of two bands in contrast to controls indicate the presence of both fragments, a shorter PCR product containing the 55 bp deletion and the normal PCR product which results from cells containing small indels or no edits. Further, we tested if the stable integration of sgRNA and Cas9 in our CRISPR pipeline increased potential off-target activity. We determined the off-target potential by assessing the cutting frequency determination (CFD) score of sgRNA 1 and 2 off-targets in PAM-KO cells. The regions with the highest CFD score, introns in MED15 (0.43) and GPM6B (0.54) were sequenced and no off-target activity could be detected (Figure 2E and F). In summary, this CRISPR pipeline in EndoC-βH1 is highly efficient in creating edited populations containing individual indels or large deletions from two double-strand breaks.

Functional characterisation of EndoC-βH1 KO cells

Having established efficient editing of the cells, we next sought to determine if this translated into functional KO cells. We therefore investigated the insulin secretion characteristics of Cas9 expressing cells and determined both mRNA expression and protein levels of the targeted genes in the KO cells. To investigate if the transduction and selection pipeline or general expression of Cas9 affects the functionality of the cells, we compared EV to WT cells and assessed their insulin secretion and content characteristics. The secretory capacities of EV and WT cell lines in response to physiological glucose concentrations stimulation are similar (Figure 3A). Both cell lines were susceptible to the ATP-sensitive potassium (KATP) channel activator and blocker, diazoxide and tolbutamide. Diazoxide reduced insulin secretion by 66.2% and 59.8% (p=0.81), whereas tolbutamide further potentiated insulin secretion 3.5- and 2.8-fold (p=0.94) in WT and EV cells, respectively (Figure 3A). Insulin secretion increased 2.48- vs 2.64-fold (p=0.94) in EV compared to WT cells after stimulation with 15 mM glucose (Figure 3B). Intracellular insulin was equally similar between EV and WT cells, averaging at 33.44 ng and 34.83 ng/2×10⁶ cells (p=0.83) (Figure 3C). mRNA levels of targeted genes were measured by RT-qPCR and were significantly decreased in PAM-KO and IDE-KO by 77.8% (p=0.035) and 66% (p=0.034), respectively. INS-KO cells also demonstrate a strong reduction of INS transcript by 54.2% (p=0.056) compared to EV control cells (Figure 3D). These reductions of mRNA levels indicate the successful introduction of frameshift mutation and the generation of a premature stop codon (PSC) followed by degradation through nonsense-mediated mRNA decay (NMD). The detection of residual transcript is in line with previous studies demonstrating incomplete NMD in KO genes after induction of frameshift mutations in coding regions of the gene. Due to the heterogeneity of the KO cell population however, it cannot be ruled out that the detected transcript also includes mRNA from...
**Figure 2. Genomic modifications of EndoC-βH1 KO cell lines.** (A) Stable genomic integration of lentiCRISPRv2 in transduced EndoC-βH1. **Upper panel:** PCR strategy (PCR 1) using primer specific for each sgRNA together with generic Cas9 primer. PCR product is only present upon successful integration of the specific sgRNA. **Lower panel:** PCR products for individual sgRNAs in control EV cell line or KO cell lines. (B) Western blot analysis of Cas9 protein and β-Tubulin in KO cell lines and control cells (WT and EV). (C) Editing efficiency of individual sgRNAs. **Upper panel:** PCR strategy (PCR 2) using primer specific for each sgRNA target site. **Lower panel:** PCR products for individual sgRNA target sites were Sanger sequenced and analysed for editing efficiency using TIDE and ICE. (D) Editing efficiency of dual sgRNA cutting. **Upper panel:** PCR strategy (PCR 3) using primers flanking the region encompassed by both sgRNAs. PCR product (or shorter fragment) is only present upon deletion of the full region between both sgRNA target sites. **Lower panel:** PCR products indicating large deletion in WT, EV or KO cell lines. (E, F) Sequencing of top off-target hits in PAM-KO cells. Sequencing trace for sgRNA1 off-target MED15 (E) and sgRNA2 off-target GPM6B (F) are displayed for EV and PAM-KO cells.
Figure 3. Functional characterisation of EndoC-βH1 KO cell lines. (A-C) Insulin secretion as percentage of content in different glucose conditions (A), fold change between 2.8 mM and 15 mM glucose stimulation (B) and insulin content (C) in WT cells compared to EV cells. (D) qPCR expression data for PAM, IDE and INS genes in EV and respective KO cell lines. Values for KO cell lines were normalised to EV within each experiment and fold changes are displayed as percentage of EV, which is indicated as a dotted line at 100%. (E) Western blot analysis of PAM, IDE and INS in EV and respective KO cells with β-Tubulin as loading control. All data are mean ± SEM from three independent experiments and for insulin content as 21 replicates from seven conditions in three independent experiments. Fold changes were log-transformed for statistical analysis. P values * < 0.05, ** < 0.01 and *** < 0.001 using two-way ANOVA Sidak's multiple comparison test (A), two-sample t-test with Holm-Sidak correction for Diazoxide and Tolbutamide induction and glucose stimulation (A, B), one-way ANOVA with Sidak's multiple comparison test for insulin content (C) and one-sample t-test for expression data (D).
unedited cells or cells that do not contain a frameshift mutation or PSC. In IDE-KO cells, only one protein-coding isoform was targeted for CRISPR editing. However, the detected IDE transcript is representative for both isoforms, suggesting that part of the residual transcript originates from the non-targeted expressed isoform. In fact, when protein levels were assessed by western blot in IDE-KO, the targeted isoform (Isoform 1) was not detectable, whereas the non-targeted isoform demonstrates unchanged protein expression. In contrast, we observed complete depletion of PAM in PAM-KO and insulin precursor and mature insulin in INS-KO cells (Figure 3E). In addition, when insulin content was assessed in INS-KO cells using a sensitive AlphaLISA-based method, insulin was not detectable within the dynamic range of the assay (p<0.0001 vs EV) (Figure 3C). The complete absence of protein indicates that the created KO cell lines are all indeed loss-of-function (LoF) cell lines. We can conclude that this lentiviral CRISPR pipeline in EndoC-βH1 creates functional KO cells with complete protein depletion and without any adverse effects on insulin secretion due to stable expression of Cas9 or antibiotic selection.

EndoC-βH1 KO cells represent a distinct loss-of-function model
To assess EndoC-βH1 KO cells as a LoF model, we compared KO cells to siRNA silencing strategies. siRNA mediated effects are based on mRNA degradation and unlike stable KO cells, they represent a transient LoF system. To investigate the different strategies, we generated SLC30A8-KO and NEUROD1-KO cell lines and compared them with their respective siRNA models. NEUROD1 is a key transcription factor for beta cell function and pancreas development and is implicated in both T2D-risk and monogenic diabetes.

The NEUROD1-KO cell line creation was successful as assessed by sgRNA integration (Figure 4A). However, it was not possible to generate a stable KO cell line as NEUROD1-KO cells were not able to survive. Within a few passages and after an initial reduction in NEUROD1, the protein level returned to baseline indicating that NEUROD1-KO cells were depleted, and only unedited cells survived and expanded (Figure 4B). Protein samples taken during the brief period of NEUROD1 reduction demonstrated an increased level of markers for endoplasmic reticulum (ER) stress and apoptosis.

![Figure 4. NEUROD1-KO cells demonstrate elevated ER stress and apoptosis.](image-url)
Unfolded protein response (UPR) signal activators, PERK and IRE1, which are activated through phosphorylation (pPERK and pIRE1) to initiate downstream signalling aimed at restoring ER homeostasis were increased 10.6- and 8.7-fold respectively (Figure 4C and D). However, a 2.2-fold upregulation of the pro-apoptotic transcription factor CHOP and a 1.6-fold increase in the activated death protease cleaved caspase 3 indicated persistent and severe ER stress resulting in apoptosis in NEUROD1-KO cells (Figure 4E and F). To compare these severe consequences of NEUROD1 depletion to a transient model, we performed siRNA KD achieving a mean protein reduction of 93.7% (p=0.007) (Figure 5A and B). CHOP, a downstream transcription factor mediating apoptosis was significantly upregulated by 34% (p=0.016) compared to non-targeting control siRNA (Figure 5C and D). Other markers of UPR activation and apoptosis like pPERK (112.3%, p=0.422) and cleaved caspase 3 (98.0%, p=0.801) were not significantly increased (Figure 5E–H and Extended data). A combined readout for cell death and proliferation does not show a difference between control and NEUROD1 silenced cells (100% vs 97.78%, p=0.894), illustrating no apoptotic effects in cells treated with siNEUROD1 (Figure 5I).

Figure 5. NEUROD1 siRNA silencing results in increased CHOP and chronic ER stress. (A-H) Western Blot analysis in cells treated with siRNA targeting NEUROD1 (siNEUROD1) compared to control siRNA (siNT). Western blots and quantification for NEUROD1 (A, B), CHOP (C, D), pPERK (E, F) and cleaved caspase 3 (G, H), β-Tubulin and GAPDH are displayed as loading controls. Protein values were normalised to their respective loading controls and siNT within each experiment and fold changes are displayed as percentage of siNT, which is indicated as a dotted line at 100%. (I) Cell count data for siNEUROD1 cells is normalised to siNT. (J) Expression data analysis for genes involved in ER stress in siNT and siNEUROD1 cells. All data are mean ± SEM from three independent experiments for western blots and cell count and six independent experiments for expression data. Fold changes were log-transformed for statistical analysis. P values * < 0.05, ** < 0.01 and *** < 0.001 using one-sample t-test for western blot data (B, D, F, H) and two-sample t-test for cell count (I) and expression data (J).
of expression levels in siRNA treated cells confirm efficient silencing of \textit{NEUROD1} mRNA levels by 79.72\% (p<0.0001) (Figure 5J). \textit{DDIT3}, which encodes for CHOP was in contrast to its protein level, not significantly increased (113.93\%, p=0.506). The active form of \textit{XBP1}, spliced \textit{XBP1} (\textit{XBP1s}) is a downstream transcription factor inducing the expression of UPR target genes, which was significantly downregulated by 30.82\% (p=0.023). \textit{HSPA5}, encoding the ER chaperone BiP was also significantly decreased to 79.73\% (p=0.005). There was also a trend towards \textit{ATF6} reduction to 90.80\% (p=0.143). \textit{ATF4} and \textit{INS} were not significantly changed (p=0.453 and p=0.596). This observed expression phenotype is consistent with an adaptive UPR response to chronic ER stress\cite{59}. The siRNA silencing approach confirms an effect of \textit{NEUROD1} on ER stress; however, the phenotype is more pronounced and severe in the KO model. Whereas the silencing of \textit{NEUROD1} does not have an apoptotic effect, complete loss of \textit{NEUROD1} protein in \textit{NEUROD1}-KO cells leads to cell death.

As a second comparison between the KO and siRNA model, we chose the \textit{SLC30A8} gene, which encodes the zinc transporter ZnT8. LoF variants in \textit{SLC30A8} have shown to be protective in T2D and siRNA KD of \textit{SLC30A8} in EndoC-\beta\textsubscript{H1} has been associated with improved glucose sensitivity and reduced expression of \textit{K\textsubscript{ATP}} channel subunits, amongst other effects\cite{33}. To assess differences between the siRNA and KO model, we focused on comparing the expression of the \textit{K\textsubscript{ATP}} channel subunits, \textit{KCNJ11} and \textit{ABCC8}. siRNA silencing of \textit{SLC30A8} reduced ZnT8 protein by 76.39\% (p=0.001) and mRNA expression was decreased by 84.54\% (p=0.002) (Figure 6A and C). \textit{SLC30A8}-KO cells demonstrate complete ZnT8 protein depletion and a reduced \textit{SLC30A8} gene expression by 57.01\% (p=0.041) (Figure 6B and C). \textit{KCNJ11} and \textit{ABCC8} expression in silenced cells was as previously described, reduced by 28.23\% (p=0.007) and 28.46\% (p=0.011), respectively (Figure 6C)\cite{33}. In \textit{SLC30A8}-KO cells, however, \textit{KCNJ11} and \textit{ABCC8} mRNA levels were unchanged compared to EV control cells (104.20\%, p=0.948 and 94.66\%, p=0.529) (Figure 6C).

This comparison between siRNA and KO models in EndoC-\beta\textsubscript{H1} demonstrate that KO LoF models can potentiate the phenotype due to complete and permanent loss of protein, as seen in \textit{NEUROD1}-KO cells. On the other hand, the phenotype in stable cell lines can also be diminished or not detectable at all as described in \textit{SLC30A8}-KO cells. These additional KO cell lines show the importance of using multiple approaches to study the role of genes of interest and highlight how our KO pipeline in EndoC-\beta\textsubscript{H1} cells has added an extra dimension.

![Figure 6. SLC30A8-KO cells do not replicate siRNA mediated effects on \textit{K\textsubscript{ATP}} channels. (A, B) Western blot analysis for Znt8 in cells treated with si\textit{SLC30A8} compared to control si\textit{NT} (A) and \textit{SLC30A8}-KO cells compared to EV cells (B). \textit{β}-Tubulin is indicated as loading control. C, Gene expression analysis of \textit{SLC30A8}, \textit{KCNJ11} and \textit{ABCC8}. \textit{SLC30A8}-KO cells and si\textit{SLC30A8} were normalised within each experiment to their respective control (EV or si\textit{NT}) which are displayed as a dotted line at 100\%. All data are mean ± SEM from three independent experiments and fold changes were log-transformed for statistical analysis. P values * < 0.05, ** < 0.01 and *** < 0.001 using one-sample t-test comparing treated cells to their control at 100\%.](image-url)
Discussion

The EndoC-βH1 cell line is an authentic human beta cell line, which is arguably the best current model to study human beta cell function. Due to its challenging growth and culture characteristics, robust protocols for CRISPR/Cas9 disease modelling to study genes implicated in human beta cell function have not been described yet in EndoC-βH1. Here, we have developed a pipeline to create stable EndoC-βH1 KO cell lines and have characterised the modifications in several proof-of-concept KO cell lines. Overall, we have successfully created five independent EndoC-βH1 KO cell lines with different gene structures, isoform expression, protein localisation and function, demonstrating that this pipeline is not restricted to certain subset of genes. The proof-of-concept cell lines, PAM-KO, IDE-KO and INS-KO cell lines showed an editing efficiency greater than 87.5% for each sgRNA and complete protein depletion, indicating high KO efficiency.

Editing strategies using only one sgRNA rely on a single cleavage event followed by non-homologous end-joining-mediated introduction of a frameshift mutation leading to a PSC. However, only two-thirds of frameshift mutations introduce a PSC and low sgRNA efficiencies, alternative splicing to avoid the introduced PSC and mutations escaping NMD can reduce the KO frequency\(^66\). To increase the likelihood of achieving a functional KO, we implemented a strategy using two separate sgRNAs.\(^74\) Performing CRISPR/Cas9 editing with two sgRNA does not only increase the probability of creating a PSC at an individual sgRNA target site, it might also result in a large deletion by creating a pair of double-strand breaks (DSB) and thus rendering the resulting protein non-functional. Using several sgRNAs, however, also increases the chance of off-target cleavage. Sequencing of selected high chance off-target sides in PAM-KO has not demonstrated any significant off-target effects. As off-target effects are sgRNA-specific, it cannot be excluded that DSBs have occurred at other sites, for any of the other sgRNAs or large deletions or rearrangements are present which exceed the range of the performed targeted PCR and sequencing reaction\(^62\). Further, it might be possible that off-target activity increases during long-term culturing due to stable integration of Cas9 and the sgRNAs. We applied this dual sgRNA strategy by transducing EndoC-βH1 with individual lentivirus for each sgRNA. To obtain a more homogenous population, avoid transduction variabilities and achieve efficient dual sgRNA-based deletions, using a single expression vector containing both sgRNAs would further advance this pipeline. This can be achieved through dual-sgRNA cloning into a Cas9 containing backbone. A cost-effective and versatile protocol has recently been described and successfully been used to delete transcriptional enhancers in EndoC-βH1.\(^33\) EndoC-βH3 is a drug inducible conditionally immortalized human beta cell line with similar characteristics as EndoC-βH1 albeit not glucose-responsive when left untreated and only demonstrating a stable phenotype for a limited time in culture after transgene excision\(^45\).

A recent study describing an HNF1A KO cell line in EndoC-βH1 has utilised a similar approach based on lentiviral transduction with a single sgRNA in a modified lentCRISPRv2 vector.\(^94\) However, the study has not investigated if the transduction and selection process had any impact on the functionality of the cells and the resulting cell line does not demonstrate a complete protein depletion, as 10% of the cells still contain HNF1A protein. When only HNF1A negative sorted cells were studied, expression analysis could validate their findings from embryonic stem cell models and thus illustrates how KO models in EndoC-βH1 can provide relevant insights into beta cell function. Our KO models in comparison showed a complete protein depletion, which could be due to our dual sgRNA strategy, which results in a higher editing efficiency. Such a cell line with complete protein depletion makes it possible to study the KO consequences without a low level of background expression, which could mask some effects or increase the complexity of the pipeline by having to sort for complete KO cells.

Even though complete protein depletion is present in KO cells, phenotypic consequences can vary greatly compared to temporary protein reduction through siRNA-based approaches as demonstrated in assessing KO and KD strategies for SLC30A8 and NEUROD1. NEUROD1-KO and SLC30A8-KO cells exhibit opposite directions of effect compared to siRNA KD. NEUROD1-KO show a potentiated impact on ER stress and apoptosis whereas effects on target genes in SLC30A8-KO are diminished. These differences are in line with previous studies demonstrating contrasting effects between KO and KD approaches\(^46\)–\(^48\). The genetic compensation response, which might be masking KO mediated effects, has recently been attributed to nonsense-induced transcriptional compensation through degradation of PSC containing mRNA and should be taken into consideration when designing future KO sgRNA strategies\(^69\)–\(^70\).

Interestingly, EndoC-βH1 NEUROD1-KO cells were not viable, which is consistent with a detected increase of ER stress and apoptosis markers. In line with this, pancreases from NeuroD1 null mice have 14-fold more apoptotic cells.\(^71\) Mice with conditional NeuroD1-KO in insulin expressing cells on the other hand do not demonstrate increased apoptosis as measured by activated caspase 3.\(^37\) Overexpression of NeurO1 in rodent beta cell lines prevents ethanol induced expression of Ddit3 (CHOP), reduces apoptosis and highlights Ddit3 as a downstream target of NeuroD1.\(^72\) This is in accordance with the results of both, our NEUROD1-KO and siRNA model which show an increase in CHOP protein, confirming the observed relation between NEUROD1 and Ddit3 in a human beta cell model and without extrinsic stress stimuli. Whereas temporary silencing of NEUROD1 induces a phenotype similar to chronic ER stress but without any effects on cell viability, NEUROD1-KO cells are not able to compensate for the permanent and complete loss of NEUROD1 protein and demonstrate elevated ER stress and apoptosis. However, further studies...
are needed to investigate the potential regulatory role of NEUROD1 in ER stress and apoptosis, and its implications for diabetes.

This genome editing pipeline in EndoC-βH1 is an efficient strategy to robustly create KO cell lines in a human beta cell line. The generated KO cell lines could be used to study the function of genes in human beta cells, investigate their role in diabetes pathology and as a protein free cellular system to overexpress and study genetic variants implicated in disease. As every other LoF model, observed phenotypes might be specific to this strategy and should be validated with complementary approaches such as transient siRNA transfection. The successful generation of these KO cell lines demonstrate the feasibility of CRISPR/Cas9 genome editing in EndoC-βH1 and open up further possibilities for CRISPR/Cas9 based strategies such as CRISPR interference (CRISPRi), CRISPR activation (CRISPRa), genome-wide CRISPR screening, epigenome and base editing.

**Data availability**

**Underlying data**

European Nucleotide Archive: A CRISPR/Cas9 genome editing pipeline in the EndoC-βH1 cell line to study genes implicated in beta cell function. Accession number PRJEB34547; http://identifiers.org/ena.embl:PRJEB34547.

Open Science Framework: A CRISPR/Cas9 genome editing pipeline in the EndoC-βH1 cell line to study genes implicated in beta cell function. [https://doi.org/10.17605/OSF.IO/2KYAN](https://doi.org/10.17605/OSF.IO/2KYAN).

This project contains the following underlying data:
- Figure 1: 1F Puromycin kill curve (Raw data in an Excel file)
- Figure 2:
  - 2A IDE sgRNA, 2A INS sgRNA_1D IDE KO, 2A INS sgRNA_1D INS KO, 2A PAM sgRNA, 2B Cas9, 2B Cas9_INSKO, 2B Tubulin, 2B Tubulin_INSKO and 2D PAM KO (Uncropped images in Image Lab and TIFF files)
  - 2E_MED15 EV (Raw sequencing file)
  - 2E_MED15 PAMKO (Raw sequencing file)
  - 2F_GPM6B EV (Raw sequencing file)
  - 2F_GPM6B PAMKO (Raw sequencing file)
  - Figure 2C (Raw sequencing files)
- Figure 3:
  - 3A Secretion data, 3B Fold change, 3C Insulin content and 3D mRNA expression (Raw data in Excel files)
  - 3E IDE, 3E INS, 3E PAM, 3E Tubulin_IDE, 3E Tubulin_INS and (Uncropped images in an Image Lab and TIFF file)
- Figure 4 (All uncropped images in Image Lab and TIFF files)
- Figure 5:
  - 5A GAPDH, 5A NEUROD1, 5C CHOP, 5CE TUBULIN, 5E pERK, 5G Cleaved Caspase3 and 5G TUBULIN (Uncropped images in Image Lab and TIFF files)
  - 5BDFH Quantification (Raw data in an Excel file)
  - 5I Cell Count (Raw data in an Excel file)
  - 5J Gene expression (Raw data in an Excel file)
- Figure 6:
  - 6A SLC30A8, 6A TUBULIN, 6B SLC30A8 and 6B TUBULIN (Uncropped images in Image Lab and TIFF files)
  - 6C Gene expression (Raw data in an Excel file)
- Extended data:
  - A GAPDH_Traf2, A Traf2, C pIRE1, E Ki67 and CE Tubulin_pIRE1 and Ki67 (Uncropped images in Image Lab and TIFF files)
  - BDF Quantification (Raw data in an Excel file)

**Extended data**

Open Science Framework: A CRISPR/Cas9 genome editing pipeline in the EndoC-βH1 cell line to study genes implicated in beta cell function. [https://doi.org/10.17605/OSF.IO/2KYAN](https://doi.org/10.17605/OSF.IO/2KYAN).

This project contains the following extended data:
- Extended data.tif: (A–F), Western Blot analysis in cells treated with siRNA targeting NEUROD1 (siNEUROD1) compared to control siRNA (siNT). Western blots and quantification for TRAF2 (A, B), pIRE1 (C, D) and Ki67 (E, F), β-Tubulin and GAPDH are displayed as loading controls. Protein values were normalised to their respective loading controls and siNT within each experiment and fold changes are displayed as percentage of siNT, which is indicated as a dotted line at 100%. Fold changes were log-transformed for statistical analysis. P values * < 0.05, ** < 0.01 and *** < 0.001 using one-sample t-test.

Data held by Open Science Framework are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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Bridget K. Wagner
Chemical Biology and Therapeutics Science Program, Broad Institute of MIT and Harvard, Cambridge, MA, USA

In this study, Grotz et al. have developed a method for CRISPR/Cas9-based genome editing in a human beta cell line, EndoC-bH1. The authors used three genes known to be important for beta-cell function, INS, IDE, and PAM, to validate the method. Further, they employ a dual-sgRNA strategy and high MOI, to increase the likelihood of transducing cells with both guides and to increase the likelihood of knocking out the gene of interest.

They first demonstrate that introduction of Cas9 to the cells does not interfere with glucose-stimulated insulin secretion. The genes of interest were also verified to be knocked out. The KO cells were then compared to siRNA-mediated silencing, and the authors found differences in gene expression and sensitivity to apoptosis, suggesting that KO brings additional information to the study of genes of interest, and that multiple approaches should probably be taken to understand the biology.

Overall, this study is solidly performed, and the authors have been careful in their use of the EndoC cells and in the design of sgRNAs. I had a few questions regarding the comparison of KO with KD. First, the EV cells showed quite a high level of CHOP (Fig. 4E), even though the KO increases that somewhat. Does Cas9 expression induce UPR as well? On the other hand, this was true of the siNT cells as well (Fig. 5C), so is it possible the EndoC cells themselves were under stress, even in basal conditions?

Second, it would be nice to see a gene-expression analysis comparing NEUROD1-KO with KD, similar to that done for SLC30A8 (Fig. 6C).

Finally, could the differences between KO and KD cells be explained by seed effects in the siRNA treatment, especially since a pool of siRNAs was used?

Most of these questions can be addressed by editing some of the text.

Is the rationale for developing the new method (or application) clearly explained? Yes
Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Beta cell biology, chemical biology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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**Author Response 21 Apr 2020**

**Antje Grotz, University of Oxford, Oxford, UK**

In this study, Grotz et al. have developed a method for CRISPR/Cas9-based genome editing in a human beta cell line, EndoC-bH1. The authors used three genes known to be important for beta-cell function, INS, IDE, and PAM, to validate the method. Further, they employ a dual-sgRNA strategy and high MOI, to increase the likelihood of transducing cells with both guides and to increase the likelihood of knocking out the gene of interest. They first demonstrate that introduction of Cas9 to the cells does not interfere with glucose-stimulated insulin secretion. The genes of interest were also verified to be knocked out. The KO cells were then compared to siRNA-mediated silencing, and the authors found differences in gene expression and sensitivity to apoptosis, suggesting that KO brings additional information to the study of genes of interest, and that multiple approaches should probably be taken to understand the biology.

1. Overall, this study is solidly performed, and the authors have been careful in their use of the EndoC cells and in the design of sgRNAs. I had a few questions regarding the comparison of KO with KD. First, the EV cells showed quite a high level of CHOP (Fig. 4E), even though the KO increases that somewhat. Does Cas9 expression induce UPR as well? On the other hand, this was true of the siNT cells as well (Fig. 5C), so is it possible the EndoC cells themselves were under stress, even in basal conditions?

First of all, we would like to thank you for taking the time to review this manuscript and for the constructive and helpful feedback. Cas9 expression does not induce UPR which we assessed by comparing basal levels of CHOP in WT (no Cas9) and EV (Cas9 only) cells. We clarified this in the main text by adding: ‘There was no difference in ER stress or apoptotic markers between WT and
EV cells (not shown)’. EndoC-βH1 cells have high basal levels of ER stress which could be due to high baseline ER stress which is observed in beta-cells as a result of their function as secretory cells and the associated insulin production. However, we have not compared their ER stress levels to other models of beta-cells and therefore have no indication if the levels are even further increased. In line with our results about basal levels of high ER stress in EndoC-βH1, Oleson et al demonstrated the same observation for markers such as cleaved caspase 3 or HSP70 1. As the high levels of CHOP have been consistent across experiments and researchers and can be induced even further, we can assume that the cells were not stressed beyond their baseline.

2. Second, it would be nice to see a gene-expression analysis comparing NEUROD1-KO with KD, similar to that done for SLC30A8 (Fig. 6C).

Thank you for this suggestion, we agree that it would have been interesting to explore this aspect. However, due to the ongoing cell death and resulting very low cell numbers of NEUROD1-KO cells, we were unable to collect sufficient cells for both, protein and RNA analysis. We therefore decided to prioritise analysing the differences in protein levels between NEUROD1-KO and EV cells for validated markers of UPR activation and ER stress.

3. Finally, could the differences between KO and KD cells be explained by seed effects in the siRNA treatment, especially since a pool of siRNAs was used?

We agree that this is an important aspect to be considered which we have not discussed sufficiently. We have now addressed this in the discussion section of the revised manuscript: ‘Another consideration for comparing KO and KD experiments are potential seed-based, microRNA-like off-target effects from siRNA due to partial sequence complementarity to 3’ UTRs regions of other mRNA transcripts 2. To increase the confidence that the observed phenotype results from silencing of the intended target gene and not due to off-target effects, it is crucial to confirm the pooled siRNA approach using multiple individual siRNAs, lentiviral delivered shRNA or rescue experiments 3,4.’

Most of these questions can be addressed by editing some of the text.

1. Oleson, B. J. et al. Distinct differences in the responses of the human pancreatic β-cell line endoc-βh1 and human islets to proinflammatory cytokines. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 309, R525–R534 (2015).
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4. Jackson, A. L. & Linsley, P. S. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. Nat. Rev. Drug Discov. 9, 57–67 (2010).

**Competing Interests:** No competing interests were disclosed.
In this work, Grotz et al. develop a robust method to generate human beta cell lines harbouring gene KOs with high efficiency. A fact that perhaps will be elusive to those from outside the pancreatic β cell field is that cells from the EndoC βH family grow slowly and very sensitive to culture conditions. Consequently, it would be extremely challenging to generate clonal populations with these cells as it is normally done in the genome editing field, particularly by those working with stem cells or cancer cell lines. Even so, EndoC βH cells are currently a gold standard human beta cell model, when studies on human tissue are impractical, and therefore efforts to develop new methodologies around them are welcomed by the community.

The work scientifically sound and I praise the authors for the detailed description of the methods and results. Below I offer minor suggestions for improvement, which the authors may want to address to improve the manuscript further:

• The authors set the tone very well in the introduction. In addition to the functional features that the authors already describe, I would only suggest adding a short reference to previous work demonstrating the utility of EndoC βH1 cells to model transcriptional and epigenomic features, given their resemblance with primary human beta cells (Lawlor et al. Cell Reports 2019).

• The western blot data showing complete gene KO is very convincing. It would be interesting to know how stable these KO lines are, especially after the observations made by the authors in Figure 4B, comparing NEUROD1 KO at weeks 1 and 2. How long did the authors maintain the other KO lines in culture? This information could be added in the main text and/or in the figure legends.

• Regarding the design, a few additional clarifications could have been provided to add even more value to the pipeline. In particular, the authors mention that two sgRNAs were selected per gene, based on highest specificity and lowest off-target score. Did the authors also require that the expected excised fragment yields a gene KO (i.e. that the length of the excised coding sequence is not a multiple of three)? If this was the case, as it seems from the INS example, it should be stated in the manuscript.

• While for the INS gene, the authors produced KO cells carrying a 55 bp deletion, for the other examples, the authors designed KOs of several kbs. The reasoning to choose specific sgRNAs for the targeting is sound (highest predicted specificity and lowest off-targeting). Nevertheless, deleting large fragments may produce undesired results such as the KO of intronic enhancers and other noncoding regulatory elements, which may be involved in the regulation of other genes.
Could this somehow affect the results observed with these KO lines, especially in the comparisons between KO and KD? The authors should at least discuss this possibility.

- The comparison between KO and KD cells is very interesting conceptually. It is debatable however whether the results are directly comparable given the fact that factors such as time after induction of loss-of-function, delivery method (lentiviral vs. transfection), molecular machinery deployed and associated cytotoxicity are distinct between the two approaches. Could these experimental setup factors contribute to differential susceptibility to cell death (NEUROD1) or transcriptional dysregulation (SLC30A8)? The authors could explore these possibilities further in the discussion. Still, what these results point out is that multiple approaches are necessary to drill into the molecular mechanism of a particular locus and that there is no “one-fits-all” solution. This is an important point that the authors transmit well.

**Other considerations:**

These two types of experiment (siRNA and CRISPR KO) are often applied and interpreted as if they were the same, when in fact the authors observed different results between KO and KD, where residual protein could still be present. It seems logical that a KO will produce a more severe phenotype than a transient KD of the same gene, as the authors observe with NEUROD1 (Figure 5). In Figure 6, however, the authors observe the opposite trend, with the gene KD affecting the expression of the two genes encoding the subunits of a pancreatic β cell potassium channel, but not the KO. Previous work by the Gloyn group established that not only SLC30A8 KD, but also presence of specific variants in SLC30A8 associated with less transcription of the gene, were linked with decreased KCNJ11 and ABCC8 expression. Moreover, human islet data revealed correlation between mRNA levels of SLC30A8 and the other two genes. The observations with the lentiCRISPR-mediated KO are thus very puzzling. They are however in line with previous observations of genetic compensation, as explained by the authors. Given the clinical importance of ABCC8, the results of this study invite further work to identify the genes that may be compensatory for ABCC8 loss. In my opinion however, such studies would be outside the scope of this work.

In summary, this work provides a straightforward approach to generate LoF human pancreatic β cell models within a reasonable timeframe for mechanistic investigations. As with any method, its applicability is limited and additional methods should be applied to fully characterise genetic loci of interest.

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1. Lawlor N, Márquez E, Orchard P, Narisu N, et al.: Multiomic Profiling Identifies cis-Regulatory Networks Underlying Human Pancreatic β Cell Identity and Function. *Cell Reports*. 2019; **26** (3): 788-801.e6 Publisher Full Text
If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Epigenomics, Gene regulation, CRISPR-Cas9 tools, Genetics of metabolic disorders

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 21 Apr 2020

**Antje Grotz,** University of Oxford, Oxford, UK

In this work, Grotz et al. develop a robust method to generate human beta cell lines harbouring gene KOs with high efficiency. A fact that perhaps will be elusive to those from outside the pancreatic β cell field is that cells from the EndoC βH family grow slowly and very sensitive to culture conditions. Consequently, it would be extremely challenging to generate clonal populations with these cells as it is normally done in the genome editing field, particularly by those working with stem cells or cancer cell lines. Even so, EndoC βH cells are currently a gold standard human beta cell model, when studies on human tissue are impractical, and therefore efforts to develop new methodologies around them are welcomed by the community.

The work scientifically sound and I praise the authors for the detailed description of the methods and results. Below I offer minor suggestions for improvement, which the authors may want to address to improve the manuscript further:

1. **The authors set the tone very well in the introduction. In addition to the functional features that the authors already describe, I would only suggest adding a short reference to previous work demonstrating the utility of EndoC βH1 cells to model transcriptional and epigenomic features, given their resemblance with primary human beta cells (Lawlor et al. Cell Reports 2019)¹.**

We would like to thank you first for taking the time to review this manuscript and for the thoughtful and constructive feedback. We agree that this is an important and relevant publication which readers will find useful and we have therefore added the following paragraph in the introduction: ‘Multiomic profiling in EndoC-βH1 cells including epigenomic and transcriptomic maps largely recapitulate primary human islets signatures and along with their similar electrophysiological properties, EndoC-βH1 are therefore a representative model of human beta cells and physiological insulin secretion²⁸, ²⁹, ³⁰.’

2. **The western blot data showing complete gene KO is very convincing. It would be interesting to know how stable these KO lines are, especially after the observations made by the authors in Figure 4B, comparing NEUROD1 KO at weeks 1 and 2. How long did the authors maintain the other KO lines in culture? This information could be added in the main text and/or in the figure**
Thank you for pointing this out, we agree that this important information had been missing and we added the following sentence ‘The KO cell lines were stably cultured for more than six months without losing their KO phenotype.’

3. Regarding the design, a few additional clarifications could have been provided to add even more value to the pipeline. In particular, the authors mention that two sgRNAs were selected per gene, based on highest specificity and lowest off-target score. Did the authors also require that the expected excised fragment yields a gene KO (i.e. that the length of the excised coding sequence is not a multiple of three)? If this was the case, as it seems from the INS example, it should be stated in the manuscript.

We agree that this would be an additional criterion to ensure gene KO, however we did not specifically consider this in our current sgRNA design but we would recommend to incorporate it in future KO pipelines.

4. While for the INS gene, the authors produced KO cells carrying a 55 bp deletion, for the other examples, the authors designed KOs of several kbs. The reasoning to choose specific sgRNAs for the targeting is sound (highest predicted specificity and lowest off-targeting). Nevertheless, deleting large fragments may produce undesired results such as the KO of intronic enhancers and other noncoding regulatory elements, which may be involved in the regulation of other genes. Could this somehow affect the results observed with these KO lines, especially in the comparisons between KO and KD? The authors should at least discuss this possibility.

Thank you for this excellent suggestion, we have added the following paragraph to address this: ‘In addition to the non-functional proteins, large deletions however might also result in the excision of intronic enhancers and non-coding regulatory elements. This might affect the regulation and expression of other genes and potentially introduce unintended phenotypes which could explain some of the differences between KD and KO phenotypes.’

5. The comparison between KO and KD cells is very interesting conceptually. It is debatable however whether the results are directly comparable given the fact that factors such as time after induction of loss-of-function, delivery method (lentiviral vs. transfection), molecular machinery deployed and associated cytotoxicity are distinct between the two approaches. Could these experimental setup factors contribute to differential susceptibility to cell death (NEUROD1) or transcriptional dysregulation (SLC30A8)? The authors could explore these possibilities further in the discussion. Still, what these results point out is that multiple approaches are necessary to drill into the molecular mechanism of a particular locus and that there is no “one-fits-all” solution. This is an important point that the authors transmit well.

You raise an important point and we agree that many factors could play a role in the observed differences, we have therefore added the following sentence in the discussion to emphasise this point: ‘In addition, it cannot be excluded that the experimental setups including distinct reagents have contributed to the observed differences between KO and KD cells for example by inducing different levels of susceptibility to cell death.’

**Competing Interests:** No competing interests were disclosed.
In this manuscript, Grotz et al. reported using stable CRISPR/Cas9 KO in EndoC-βH1 cells to study the role of beta cell key regulators in beta cell survival. First, sequencing and western blotting were used to validate the KO efficiency. Then, the authors compared the KO cell lines siRNA-mediated knockdown (KD) approaches and found different phenotypes. NEUROD1-KO cells were not viable and displayed elevated markers for ER stress and apoptosis. NEUROD1-KD, however, only had a modest elevation of CHOP without evidence of elevated cell death. In addition, SLC30A8-KO cells demonstrated no reduction in K channel gene expression in contrast to siRNA silencing. The manuscript reported a useful platform to study the role of beta cell regulators in beta cell function and survival. However, additional work need to be done to be considered for indexing.

Major issues:
a. Several genes KO in this study are key beta cell regulators, such as INS, IDE, PAM, NEUROD1, and SLC30A8. However, the characterization of the KO cells are very limited. Please systematically examine:
   1. The key markers of beta cells.
   2. Total insulin content.
   3. Beta cell function.
b. Additional experiments are needed to explain the difference between KO and KD lines. It is due to the remaining dose of the protein in KD lines. If yes, an inducible shRNA system might be required to study the dose-dependent activity of NEUROD1 and SLC30A8.

Is the rationale for developing the new method (or application) clearly explained?
Partly

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Partly
Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Beta cell biology and gene knockout.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

---

**Author Response 21 Apr 2020**

**Antje Grotz,** University of Oxford, Oxford, UK

1. In this manuscript, Grotz et al. reported using stable CRISPR/Cas9 KO in EndoC-βH1 cells to study the role of beta cell key regulators in beta cell survival. First, sequencing and western blotting were used to validate the KO efficiency. Then, the authors compared the KO cell lines siRNA-mediated knockdown (KD) approaches and found different phenotypes. NEUROD1-KO cells were not viable and displayed elevated markers for ER stress and apoptosis. NEUROD1-KD, however, only had a modest elevation of CHOP without evidence of elevated cell death. In addition, SLC30A8-KO cells demonstrated no reduction in K channel gene expression in contrast to siRNA silencing. The manuscript reported a useful platform to study the role of beta cell regulators in beta cell function and survival. However, additional work need to be done to be considered for indexing.

First, we would like to thank you for the thoughtful feedback and for taking the time to review this manuscript. We have addressed your concerns with detailed comments below and hope this will clarify the intended aims of our paper. We would like to stress that our objective is to provide a methodological paper detailing a protocol which we feel is a valuable resource for the community in as timely a manner as possible without performing additional in-depth functional follow-up work. We now make this point clearer in our revised manuscript so that readers can understand that the characterisation of the cell lines is not the main objective of the study and consequently our characterisation is not comprehensive. We have added the following sentence in the introduction 'This CRISPR/Cas9 pipeline and the resulting KO cell lines could be a valuable tool in understanding human beta cell function and genes underlying the pathology for both T2D and T1D.'

2. **Major issues:**
   a. Several genes KO in this study are key beta cell regulators, such as INS, IDE, PAM, NEUROD1, and SLC30A8. However, the characterization of the KO cells are very limited. Please systematically examine:
      1. **The key markers of beta cells.**
      2. **Total insulin content.**
      3. **Beta cell function.**

   It would certainly be interesting and valuable to systematically characterise the INS, IDE, PAM, NEUROD1, and SLC30A8 KO cell lines. However, the aim of our manuscript was to provide details of our pipeline to generate CRISPR KOs in this human beta cell line and not to characterise the role of these genes in human beta-cell function. Our goal was to provide details of our pipeline for
those wishing to generate their own KO-cell lines and not to characterise these genes. We feel that these additional studies are beyond the scope of this manuscript.

3. Additional experiments are needed to explain the difference between KO and KD lines. It is due to the remaining dose of the protein in KD lines. If yes, an inducible shRNA system might be required to study the dose-dependent activity of NEUROD1 and SLC30A8.

Again, we agree that the difference in cellular phenotype between these two experimental approaches is fascinating and future studies should indeed explore the relationship between transcript expression levels and phenotype. There are many potential explanations for this phenomenon (such as transcriptional adaptation as discussed in Ref 69 and 70) and addressing this comprehensively would require a substantial body of additional work which is in our opinion, beyond the scope of this methodological report. To reiterate our aim with this methodological report was to provide details of how to create KO cell models in this line and not to determine the differences between KD and KO loss-of-function models or dose-dependent activities of NEUROD1 and SLC30A8.

4. If any results are presented, are all the source data underlying the results available to ensure full reproducibility? Partly

We would also like to note that all underlying source data has been made available at Open Science Framework in cooperation with the editors as explained in the ‘Data availability’ section.

**Competing Interests:** No competing interest
in humans, which would be more difficult in rodent-derived cells.

The authors have applied their approach to five target genes. For two of them, the results are compared with cells where the same gene has been silenced by RNA interference. The essential aspect of their approach is that two instead of the customary one guide RNAs are used. It is argued, and largely shown, that this will result in a more “controlled” and effective knock out of the targeted genes: it is intended that sequence between the sites where the guide RNAs bind will be excised. All the components of the system are cloned into a lentiviral vector, since it is known to be reasonably effective in EndoC-betaH1 cells. A potential caveat of this approach is that the lentivirus will integrate into the host genome, which in itself may have an impact on the cells. On the other hand, a stable transduction will be achieved. This can be checked by sequencing. Indeed, the authors have looked for off-target effects, also from Cas9-mediated nuclease activity, but find none. An alternative approach would have been to include a Piggybac sequence in the construct, which could be excised, and hence prevent scarring of the host DNA, or use adenoviruses, which do not integrate.

As the authors describe, no clonal selection of the transduced EndoC-betaH1 cells was made. Hence, the transduced and edited cells will be made out of a heterogenous population of cells with long and short excisions. Nevertheless, a PCR-based analysis of the INS-KO cells show that a major proportion of transduced cells have a major part excised of the sequence between the sites where the guide RNAs have cut. This notwithstanding, the somewhat inconsistent functional and expression results that were found may be due to this heterogeneity of editing.

The analyses of the cells are mostly derived from expression analyses (QPCR and western blots). The only analysis of secretion is a comparison of cells transduced with empty vector and wild type cells. Although there is quite high variability in secretion, which is not strongly stimulated other than by tolbutamide, no differences between the manipulations are seen, suggesting that the molecular manipulations leave the secretion machinery largely intact.

A very interesting and thought-provoking aspect of the work is the comparisons of knock out and silencing approaches to target the same gene; the latter has become a well-established approach in cell biology. It is thus surprising that the results, using these two approaches are so distinct and variable. Knock out of NEUROD1 resulted in cell death, inferred from the fact that knock out cells are largely absent after a few days, and instead proliferation of cells in which gene targeting has not occurred. In contrast, efficient silencing of NEUROD1 does not induce apoptosis. For manipulation of SLC30A8, expression of KCNJ11 and ABCC8 mRNA was used as read outs. Equally puzzling, levels of these important genes were lowered in silenced cells but unaffected in knock out cells. While the present work points out the problem it makes no attempts to explain it. Although it seems that deletion and silencing of the target genes and their encoded proteins have been comparable, the impact on phenotype has been variable. Clearly, there are many aspects of silencing and knock out (CRISPR) which are different: onset, extent, vector, molecular mode of action, toxicity, off-target effects, durability and more. An important lesson from this paper is that all approaches, as wonderful and effective they may seem, have limitations, and this should always be borne in mind when drawing conclusions from research.

Another reflection from my part is that the methodological development of the genome editing techniques is astounding. Just the other week, prime-editing was published. Here addition of reverse transcript and an RNA template to the nickase affords unprecedented accuracy in single base substitutions as well as deletion of sequences of choice. Thus, the approach described here has already been surpassed by a, seemingly, superior approach. Again, this underscores that addressing research questions may be more durable than publishing methodology. Moreover, the single base approach is really what is needed in
functional cellular genomics, where the impact of SNPs in risk alleles for complex diseases is analysed. The pipeline described here does not address this issue.

In sum, the work from Anna Gloyn’s group has introduced a novel and robust way of manipulating gene expression in highly differentiated insulin-secreting cells. It is also a cautionary tale – understand the limitations of your methods when interpreting your results.

**Is the rationale for developing the new method (or application) clearly explained?**
Yes

**Is the description of the method technically sound?**
Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**
Yes

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Islet and diabetes research; metabolism

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.