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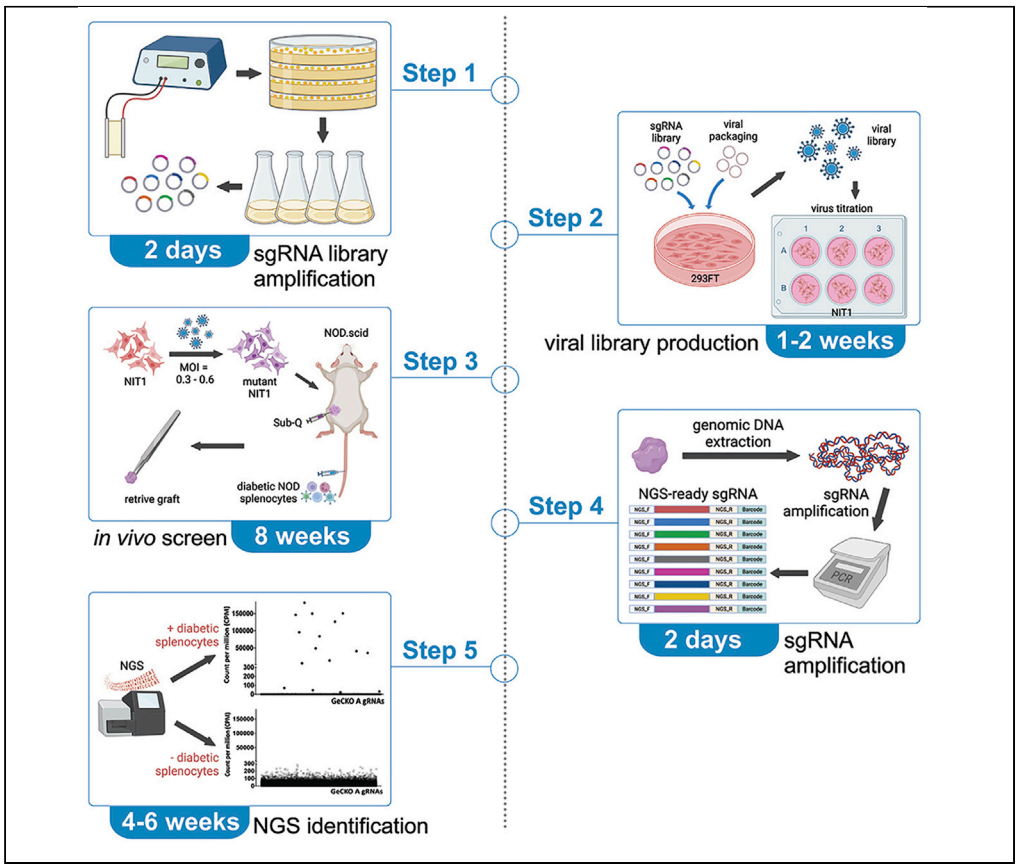
Protocol for genome-scale *in vivo* CRISPR screening to study protection of beta cells under autoimmunity in a type 1 diabetes mouse model



Autoimmunity-induced pancreatic beta cell failure is the main characteristic of type 1 diabetes (T1D). Here, we describe a protocol for genome-scale *in vivo* CRISPR-Cas9 screening for use in a mouse model of T1D. Using a non-obese-diabetic-derived mouse beta cell line, NIT-1, and a genome-wide CRISPR-Cas9 knockout library (GeCKO-v2), we describe how to identify genes that confer resistance to autoimmune killing. This protocol can be applied in other mouse models of autoimmunity.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for genome-scale CRISPR screens in an *in vivo* autoimmune diabetes mouse model

Conducting *in vivo* autoimmune beta cell killing assay using a reporter cell line

Detailed steps for generating CRISPR knockout library in NIT-1 beta cells

Li et al., STAR Protocols 4, 102155
June 16, 2023
https://doi.org/10.1016/j.xpro.2023.102155

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Protocol for genome-scale *in vivo* CRISPR screening to study protection of beta cells under autoimmunity in a type 1 diabetes mouse model

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https://doi.org/10.1016/j.xpro.2023.102155

SUMMARY

Autoimmunity-induced pancreatic beta cell failure is the main characteristic of type 1 diabetes (T1D). Here, we describe a protocol for genome-scale *in vivo* CRISPR-Cas9 screening for use in a mouse model of T1D. Using a non-obese-diabetic-derived mouse beta cell line, NIT-1, and a genome-wide CRISPR-Cas9 knockout library (GeCKO-v2), we describe how to identify genes that confer resistance to autoimmune killing. This protocol can be applied in other mouse models of autoimmunity.

For complete details on the use and execution of this protocol, please refer to Cai et al. (2020).[1]

BEFORE YOU BEGIN

Institutional permissions

The institutional permission on animal protocol is required for genome-scale *in vivo* CRISPR screens. NOD and NOD.scid (NOD.CB 17-*Prkdc*^{scid}/J) mice used in this protocol were purchased from The Jackoson Laboratory. Animals were housed in pathogen-free facilities at the Joslin Diabetes Center. All procedures were approved by the Joslin Diabetes Center Institutional Animal Care and Use Committee (protocol number 2013-03) and performed in accordance with institutional guidelines and regulations.

Choose a suitable sgRNA library

Timing: 1–2 days

1. Forward genetic screens, and genome-wide CRISPR screens in particular, are a powerful tool for the unbiased discovery of genes associated with a phenotype of interest. In recent years, many CRISPR/Cas9 systems have become available, and have been applied in different genome-scale screens.[2]
2. We choose the genome-scale CRISPR/Cas9 knockout library (GeCKO-v2) to screen genes that promote beta cell resistance to autoimmunity attacks.
   a. To achieve a better outcome, we have selected the single vector format of GeCKO-v2 library.[3]

   Note: The single vector lentiCRISPRv2 system has been recommended as a better suited model for *in vivo* or primary cell screening applications.[3] Current available CRISPR pooled

libraries can be found in Addgene (https://www.addgene.org/crispr/libraries/). You may choose a sgRNA library that is best suited for your experimental design.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Endura ElectroCompetent cells	Lucigen	Cat# 60242-1
One Shot™ Stble3™ Chemically Competent *E.coli*	Invitrogen	Cat# C7373-03
Chemicals, peptides, and recombinant proteins		
Gibco™ DMEM	Thermo Fisher	Cat# 10-313-039
Fetal bovine serum	Thermo Fisher	Cat#10437028
Penicillin/Streptomycin	Corning	Cat# 30-002-CI
Trypsin	Corning	Cat# 25-052-CI
DPBS	Sigma-Aldrich	Cat# D8537-6X500ML
GluaMAX	Gibco	Cat# 35-050-061
SOC medium	Thermo Fisher	Cat# BP9740-10X5
Ampicilin	Bio Basic	Cat# AB 0028
Puromycin dihydrochloride	Research Products International	Cat# P33020-0.025
Polyethylenimine (PEI)	Sigma-Aldrich	Cat# 764604-1G
Polybrene	EMD Millipore	Cat# TR-1003-G
Agarose	Thermo Fisher	Cat# BP1356-500
LB agar	Thermo Fisher	Cat# BP9724-500
Trypan blue solution, 0.4%	Thermo Fisher	Cat# 15250061
Deoxynucleotide (dNTP) solution mix	New England BioLabs	Cat# N0447L
TrackIt™ 1 kb Plus DNA Ladder	Thermo Fisher	Cat# 10-488-085
Blood glucose test strips	CONTOUR NEXT	Cat# B07CF8C4VX
NaCl	Thermo Fisher	Cat# BP358-212
NaOH	Thermo Fisher	Cat# SS255-1
Glycerol	Thermo Fisher	Cat# BP2291
D-Luciferin	Gold Biotechnology	Cat# LUCK-3G
Poly (ethylene glycol) PEG,10,000	Sigma-Aldrich	Cat# 81280-1KG
Red blood cell (RBC) lysis buffer	Sigma-Aldrich	Cat # R7757
Critical commercial assays		
Quick-DNA Midiprep Plus Kit	Zymo Research	Cat# D4075
ZymoPURE II Plasmid Maxiprep Kit	Zymo Research	Cat# D4202
ZymoPURE II Plasmid Midiprep Kit	Zymo Research	Cat# D4201
Experimental models: Cell lines		
NIT-1	ATCC	Cat# CR2055
HEK293FT cells	Thermo Fisher	Cat# R70007
Experimental models: Organisms/strains		
Mouse: adult (10–12 weeks) female NOD/ShiLtJ (NOD)	Jackson Laboratory	Cat# 001976
Mouse: adult (10–12 weeks) female NOD.Cg-Prkdcscid/J (NOD.scid)	Jackson Laboratory	Cat# 001303
Oligonucleotides		
NGS-Lib-KO-v2-Adapter-Fwd primer: GTAACTTGAAAGTATTTCGATTTCTT GGCTTTATATATCTTGTGGAAAGG ACGAAACACC	Invitrogen	N/A
NGS-Lib-KO- v2-Adapter-Rev primer: ACTTTTTCAAGTTGATAACGGACTAG CCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	Invitrogen	N/A
NGS-Lib-Fwd-1: AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTAC ACGACGCTCTTCCGATCTTAAGTAGA GGCTTTATATATCTTGTGGAAAGGACGAAACACC	Invitrogen	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NGS-Lib-Fwd-2: AATGATACGGCGACCACC GAGATCTACACTCTTTCCCTACACGACGCT CTTCCGATCTATCATGCTTAGCTTTATATAT CTTGTGGAAAGGACGAAACACC	Invitrogen	N/A
NGS-Lib-Fwd-3: AATGATACGGCGACCACCG AGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCTGATGCACATCTGCTTTATATATC TTGTGGAAAGGACGAAACACC	Invitrogen	N/A
NGS-Lib-Fwd-4: AATGATACGGCGACCACC GAGATCTACACTCTTTCCCTACACGACGCT CTTCCGATCTCGATTGCTCGACGCTTTATA TATCTTGTGGAAAGGACGAAACACC	Invitrogen	N/A
NGS-Lib-Fwd-5: AATGATACGGCGACCACC GAGATCTACACTCTTTCCCTACACGACGCT CTTCCGATCTTCGATAGCAATTCGCTTTATA TATCTTGTGGAAAGGACGAAACACC	Invitrogen	N/A
NGS-Lib-Fwd-6: AATGATACGGCGACCACC GAGATCTACACTCTTTCCCTACACGACGCT CTTCCGATCTATCGATAGTTGCTTGCTTTAT ATATCTTGTGGAAAGGACGAAACACC	Invitrogen	N/A
NGS-Lib-Fwd-7: AATGATACGGCGACCACCG AGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCTGATCGATCCAGTTAGGCTTTATA TATCTTGTGGAAAGGACGAAACACC	Invitrogen	N/A
NGS-Lib-Fwd-8: AATGATACGGCGACCACCGA GATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTCGATCGATTTGAGCCTGCTTTATATA TCTTGTGGAAAGGACGAAACACC	Invitrogen	N/A
NGS-Lib-Fwd-9: AATGATACGGCGACCACCG AGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCTACGATCGATACACGATCGCTTTAT ATATCTTGTGGAAAGGACGAAACACC	Invitrogen	N/A
NGS-Lib-Fwd-10: AATGATACGGCGACCACCG AGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCTTACGATCGATGGTCCAGAGCTTTAT ATATCTTGTGGAAAGGACGAAACACC	Invitrogen	N/A
NGS-Lib-KO-Rev-1: CAAGCAGAAGACGGCATA CGAGAT**TCGCCTTG**GTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTCCGACTCGGTGCCA CTTTTTCAA	Invitrogen	N/A
NGS-Lib-KO-Rev-2: CAAGCAGAAGACGGCATA CGAGAT**ATAGCGTC**GTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTCCGACTCGGTGCC ACTTTTTCAA	Invitrogen	N/A
NGS-Lib-KO-Rev-3: CAAGCAGAAGACGGC ATACGAGAT**GAAGAAGT**GTGACTGGAGT TCAGACGTGTGCTCTTCCGATCTCCGA CTCGGTGCCACTTTTTCAA	Invitrogen	N/A
NGS-Lib-KO-Rev-4: CAAGCAGAAGACGGCAT ACGAGAT**ATTCTAGG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCTCCGACTCGGTG CCACTTTTTCAA	Invitrogen	N/A
NGS-Lib-KO-Rev-5: CAAGCAGAAGACGGCAT ACGAGAT**CGTTACCA**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCTCCGACTCGGTG CCACTTTTTCAA	Invitrogen	N/A
NGS-Lib-KO-Rev-6: CAAGCAGAAGACGGCATA CGAGAT**GTCTGATG**GTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTCCGACTCGGTGCC ACTTTTTCAA	Invitrogen	N/A
NGS-Lib-KO-Rev-7: CAAGCAGAAGACGGCATA CGAGAT**TTACGCAC**GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTCAA	Invitrogen	N/A
NGS-Lib-KO-Rev-8: CAAGCAGAAGACGGCATAC GAGAT**TTGAATAG**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTCAA	Invitrogen	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
Mouse GeCKO-v2 (Genome-Scale CRISPR Knock-Out) Library A	Addgene	Cat# 1000000052
pMDLg/pRRE	Addgene	Cat# 12251
pRSV-Rev	Addgene	Cat# 12253
pMD2.G.	Addgene	Cat# 12259
Software and algorithms		
Benchling sgRNA designer tool (https://www.benchling.com)	Benchling	https://benchling.com/
BioRender illustration design tool	BioRender	https://biorender.com/
Other		
Nanodrop lite spectrophotometer	Thermo Fisher	Cat# ND-LITE
TC20 Automated Cell Counter	Bio-Rad	Cat# 1450102
IVIS Spectrum In Vivo Imaging System	PerkinElmer	Cat# 124262
0.22 μm vacuum filter	Millipore	Cat# SCGPT02R
0.45 μm filter	Celltreat	Cat# 229774
25 mL serological pipette	Celltreat	Cat# 229025B
245 mm square bioassay dish	Thermo Fisher	Cat# 240835
150 mm Petri dish	Corning	Cat# 430599

MATERIALS AND EQUIPMENT

Luciferase lentivirus production		
Reagent	Final concentration	Amount
pMDLg/RRE	N/A	2.5 μg
pRSV/Rev	N/A	2.5 μg
pMD2.G.	N/A	5 μg
Luciferase plasmid	N/A	10 μg
1 mg/mL PEI solution	N/A	60 μg
Total	**N/A**	**80 μg**

Store plasmids at −20°C for long-term. Filter PEI solution (pH = 7.0) using a 0.22 μm filter and store aliquots at 4°C for up to 3 months or at −20°C∼−80°C for long-term. Thaw and mix reagents at 20°C–25°C before use.

Pooled sgRNA lentiviral library production		
Reagent	Final concentration	Amount
pMDLg/RRE	N/A	5.06 μg
pRSV/Rev	N/A	5.06 μg
pMD2.G.	N/A	10.13 μg
Pooled sgRNA library	N/A	20.25 μg
1 mg/mL PEI solution	N/A	121.5 μg
Total	**N/A**	**162 μg**

Store plasmids and pooled sgRNA library at −20°C for long-term. Filter PEI solution (pH = 7.0) using a 0.22 μm filter and store aliquots at 4°C for up to 3 months or at −20°C∼−80°C for long-term. Thaw and mix reagents at 20°C–25°C before use.

STEP-BY-STEP METHOD DETAILS

Generation of a luciferase reporter NIT-1 cell line

Timing: 2–3 weeks

The following steps describe the production of luciferase lentivirus in HEK293FT cells (step 2) and luciferase lentiviral transduction in NIT-1 cells (step 3).

1. NIT-1 (#CRL-2055) and HEK293FT (#R7007) cell lines were obtained from ATCC and Thermo Fisher Scientific, respectively. Maintain cells in DMEM (Gibco, 10313039), supplemented with 10% fetal bovine serum (FBS, Gibco), GlutaMAX (Gibco), and penicillin/streptomycin (Corning), in a 37°C incubator with 5% CO_2.

   Note: NIT-1 insulinoma cell line was derived from female NOD mice, which is suitable for autologous transplantation to the NOD background mouse strains.

2. Preparing luciferase lentivirus.
   a. Choose Lenti-luciferase plasmids carrying a favorable drug selection marker from Addgene, such as Addgene: 21474 - pLenti-CMV V5-luciferase-Blast, or a homemade lenti-luciferase plasmid.
   b. Day 1: Seed 2×10^6 HEK293FT cells into a 100 mm dish with the maintenance media. The cells are expected to achieve 70%–80% confluency after 24 h incubation at 37°C with 5% CO_2.
   c. Day 2: Replace the culture media with 9 mL fresh maintenance media and add 1 mL (10% of total media volumn) DNA/PEI transfection mixture (See materials and equipment section).
      i. Prepare the luciferase plasmid and the lentiviral packaging at a 1:1 ratio.
      ii. Mix the plasmid DNA with polyethylenimine (PEI) at a ratio of 1:3 in 1 mL serum-free DMEM media without antibiotics.
      iii. Incubate the DNA/PEI mixture at RT in the biosafety cabinet for 20 min.
      iv. Add the DNA/PEI mixture dropwise to the 100 mm dish of HEK293FT cells.
      v. Gently rock the plate to mix the DNA/PEI mixture evenly. Return the plate to the cell incubator located in a biosafety level 2 area.
   d. Day 4: Collect the culture media containing lentiviruses to a 15 mL sterile tube and centrifuge the tube at 270 × *g* for 5 min at 4°C to pellet detached cells and debris.
   e. Remove the plunger from a 10 mL syringe and attach a 0.45 μm filter.
   f. After centrifugation, carefully transfer the lentiviral supernatant into the 10 mL syringe from step e.
   g. Put the plunger into the syringe and gently push it down to create 1 mL aliquots of filtrate in 1.5 mL microcentrifuge tubes. Store the luciferase lentivirus in the −80°C freezer.

   ⚠ CRITICAL: All lentiviral procedures must be perfomed in the biosafety level 2 desinated area and specific safety procedures must be followed. All materials including culture meida, cell culture consumables, and virus stocks that may have been in contact with viral materials must be bleached before disposal. We recommend that you consult your institutional safety office for the details of the safety protocols before initiating the lentiviral procedure.

   Pause point: The viral supernatant can be used immediately or stored at −80°C until needed.

3. Luciferase lentiviral transduction.
   a. Seed the NIT-1 cells onto a 6-well plate at a density of 2×10^6 cells/well and incubate at 37°C in 5% CO_2.

   Note: The cells are expected to achieve ~80% confluency after 24 h of incubation.

   b. Following aspiration of the cultured media, add 0 (control well), 0.1, 0.2, 0.5, 1, and 2 mL of the luciferase lentiviral supernatant to the ~80% confluent NIT-1 6-well plate. Fill each well to 3 mL volume with fresh DMEM media.

c. At 48 h post-transduction, replace the culture media with fresh DMEM media containing the selection marker, such as 4 μg/mL Blasticidin.
d. Once all control cells are dead, stop the drug selection process right away by replacing the drug-containing media with fresh DMEM media.

Note: You should be able to find that all cells in the control wells are dead by 3–5 days post-selection.

i. Pick the well with the lowest viral amount added.
ii. Grow these tranduced cells for transplantation and *in vivo* bioluminescence imaging.

Optimization of *in vivo* autoimmune killing of NOD-derived NIT-1 beta cells

Timing: 2–3 weeks

The following steps describe the setup and imaging procedure of *in vivo* autoimmune beta cell killing in a NOD mouse model.

⚠ CRITICAL: Induction of autoimmune diabetes in NOD mice may be affected by environmental factors, such as gut microbiota.[4] Therefore, we recommend to optimize the screening time frame for the efficiency of *in vivo* beta cell autoimmune killing in the experimental conducting sites.

4. *In vivo* autoimmune killing model setup. Troubleshooting 1.
   a. Use trypsin to detach the luciferase-expressing NIT-1 cells and resupend 10^7 luciferase-expressing NIT-1 reporter cells in 200 μL sterile DPBS.
   b. Prepare fresh purified diabetic NOD splenotyes.
      i. Remove the spleen from a diabetic NOD mouse and use a sterile surgical blade to cut it into 3–4 pieces.
      ii. Add the pieces to 5 mL sterile cold DPBS in a 15 mL falcon tube.
      iii. Take a 25 mm petri dish and place a 70 μm cell strainer inside the dish.
      iv. Pour out the spleen pieces and DPBS onto the cell strainer.
      v. Remove the excess DPBS from the dish.
      vi. Add 2–3 mL of RBC lysis buffer onto the strainer.
      vii. Use the plunger end of a 5 mL syringe to grind the spleen through the cell strainer.
      viii. Rinse the strainer with 1 mL sterile cold DPBS.
      ix. Transfer the suspended cells from the dish to a 15 mL falcon tube.
      x. Keep grinding and washing untill the liquid in the dish runs clear, transferring all rinses into the same tube.
      xi. Centrifuge the falcon tube at 505 × *g* for 5 min at 4°C.

Optional: If the cell pellet is still red and the supernatant is not clear (Figure 1A), resuspend it with 1 mL of RBC lysis buffer and add DPBS and centrifuge again. Repeat 2 times if needed to get a pink cell pellet with clear supernatant (Figure 1B).

      xii. Count the cells and resuspend 10^7 diabetic NOD splenocytes in 200 μL sterile DPBS.
      xiii. Store the isolated splenocytes on ice (up to 5 h) for the injection.

Note: In general, we recommend using the isolated splenocytes as soon as possible. Splenocyte isolation from one recent-onset diabetic NOD mouse may generate at least 8-10 × 10^7 splenocytes. However, the number and activity of splenocytes starts to decline after diabetic onset. We recommend not isolating splenocytes from NOD mice that have been diabetic for over 7–10 days.

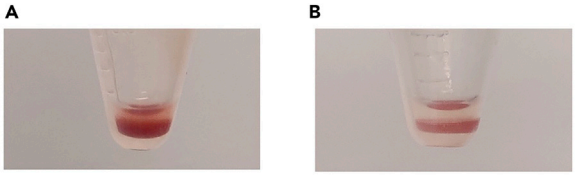

Figure 1. Lysis and removal of red blood cells during the splenocyte purification
(A) The cell pellet was not completely washed, as shown with a crimson or red color and cloudy supernatant.
(B) The cell pellet presented a pink color with clear supernatant was ready for the next step: resuspension and counting.

c. Cell injection.
   i. Subcutaneously transplant 10^7 luciferase-expressing NIT-1 reporter cells into each 8-week-old female NOD.scid recipient mouse.
   ii. Inject intravenously 10^7 diabetic NOD splenocytes in 200 μL sterile DPBS to induce autoimmunity. For a non-autoimmune control group, designate some NOD.scid mice to receive just the subcutaneously transplanted mutant NIT-1 cells, without intravaneously injected splenoctyes.
d. *in vivo* bioluminescence imaging.
   i. Dissolve D-luciferin (Gold Biotechnology, Cat#LUCK-3G) into sterile DPBS (without calcum or magnesium) to a final concentration of 15 mg/mL and filter sterilize (0.22 μm).
   ii. For bioluminescence imaging, inject D-luciferin intraperitoneally into mice transplanted with luciferase-expressing cells, at a dose of 150 mg/kg.
   iii. Measure ioluminescence using an IVIS Spectrum imaging system. The following example images were taken at day 1, 8 and 15 post-injection, as shown in Figure 2.

Note: To avoid missing the optimal autoimmune killing window, we recommend imaging the graft every other day for the initial experiment. More than 90% of the transplanted NIT-1 cells are expected to be immune-destructed by day 15 post-splenocyte injection.

Amplification of pooled sgRNA library

Timing: 2 days

The following steps describe the preparation and amplification of a pooled sgRNA library obtained from Addgene.

5. Pooled sgRNA library transformation.[2]
   a. Electroporate the library at 50 ng/μL using Endura ElectroCompetent cells following the manufacturer's directions.
   b. Pre-warm 1 large ampicillin$^+$ LB agar plate (245 mm square bioassay dish) per electroporation of the sgRNA library at 37°C (Repeat for a total of 1 electroporation per 10,000 sgRNAs in the library).
   c. Incubate the electroporated competent cells at 37°C for 1 h, mix well by inverting.
   d. Add same volume of LB medium to the electroporated competent cells, mix well by inverting for 5–10 times, plate 2 mL of electroporated competent cells on each large LB agar plate (245 mm square bioassay dish).
   e. Incubate the LB agar plates in an incubator at 37°C for 12–16 h.
6. Calculate electroporation efficiency.
   a. Dilute the electroporated competent cells for a 1000-fold dilution: add 5 μL of the electroporated competent cells to 495 μL of LB medium, mix well. Then add 100 μL of 100-fold dilution to 900 μL of LB medium (1000-fold dilution).

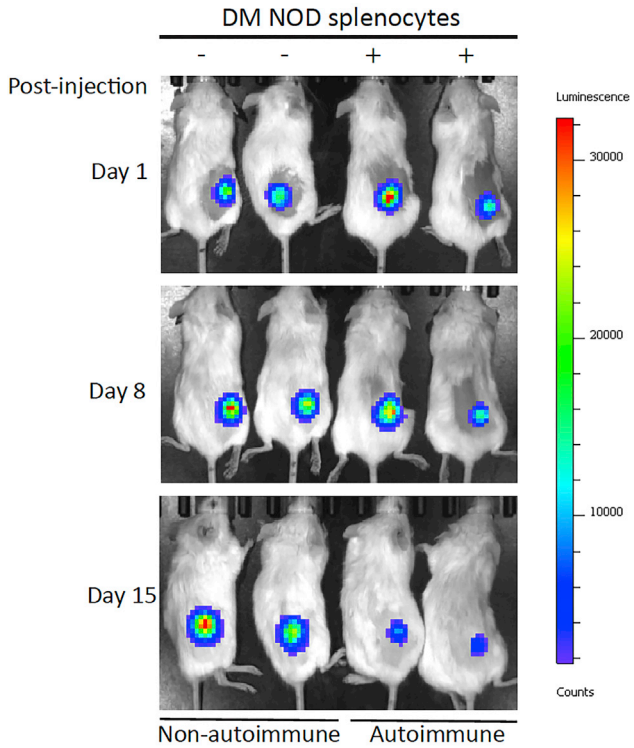

Figure 2. Autoimmune killing of NIT-1 cells in NOD mice can be visualized by bioluminescence imaging
Bioluminescence imaging of 10^7 NIT-1 cells transplanted subcutaneously into NOD.scid mice. Transplanted cells were engineered to carry a CMV-luciferase reporter. Some recipient mice were also injected intravenously with 10^7 splenocytes isolated from spontaneously diabetic (DM) NOD mice to cause beta cell killing. Images were taken at day 1, 8 and 15 post-injection. The Day 1 image was adapted from: 'Cai et al., Genome-scale *in vivo* CRISPR screen identifies RNLS as a target for beta cell protection in type 1 diabetes, 2020, Nat Metab.'[1]

b. Plate 100 μL of the 1000-fold dilution electroporated competent cells to a pre-warmed small ampicillin$^+$ LB agar plate (100 mm Petri dish). In total, plate 10 small ampicillin$^+$ LB agar plate.
c. Incubate the LB agar plates in an incubator at 37°C for 12–16 h.
d. On the next day, count the number of colonies on 10 small ampicillin$^+$ LB agar plate, then multiply the number by 1000.

Note: The ready-made GeCKO-A v2 library from Addgene contains 65,383 sgRNAs. It is recommended that each sgRNA be amplified at least 100 times. Thus, the A pool library should yield at least 6.54×10^6 colonies after transformation before the next step. If amplifying a custome library, it is recommended that at least 500 colonies per sgRNA in the library should be obtained.[2]

7. Harvest colonies from the LB agar plates.
   a. Pipette 7 mL of LB medium onto each large LB agar plate. Gently scrape the colonies off and transfer the liquid into a 50 mL tube.
   b. Repeat step 3a twice for a total of three LB medium washes.
   c. Prepare conical flasks with 500 mL LB medium with ampicillin, one for each large LB plate.
   d. Transfer the bacteria suspension to the flasks by dividing equally among them.
   e. Shake the conical flasks in a shaker incubator at 37°C at a speed of 200 RPM for 8–12 h.
   f. To collect, centrifuge the 8–12 h culture at 3,400 × *g* for 10 min. Discard the supernatant and proceed by following the ZymoPURE II Plasmid Maxiprep Kit's manufacturer's directions. Approximately 2 large LB agar plates' worth of bacteria can be condensed into 1 maxiprep.

g. Quantify the plasmid DNA by Nanodrop.

Pause point: The maxiprepped sgRNA library can be stored at −80°C for several years.

Note: Using an endotoxin-free plasmid kit is important for later virus production.

Optional: Next-generation sequencing of the sgRNA library for quality confirmation. The GeCKOv2 library was generated and quality confirmed by the Feng Zhang lab.[3] You may repeat the step of quality confirmation by following the published protocol.[2] In this protocol, the pre-screening controls are used in step 12 for quality control of the sgRNA copy number and coverage of the prepared sgRNA library.

CRISPR lentiviral library production and titration

Timing: 1–2 weeks

The following steps describe the selection of the optimized dosage of antibiotics in NIT-1 cells (step 8), the procedure to prepare a large-scale of concentrated CRISPR lentiviral library (step 9) and the calculation of the produced viral library titer (step 10).

8. Perform an antibiotic (puromycin) killing curve.
   a. Seed NIT-1 cells at 50% confluency in media containing a range of puromycin concentrations typically used for selection (0.5–10 μg/mL).
   b. Refresh media with puromycin every 3 days. After around 1 week, choose the lowest concentration of puromycin sufficient to kill all NIT-1 cells.

Note: It is important to use the lowest concentration of puromycin to kill all cells to avoid providing extra stress on the target cells.

9. Lentivirus production on HEK293FT cells.
   a. Expand HEK293FT cells to five 150 mm petri dishes at 70%–80% confluence.
   b. Refresh the culture media (10% FBS containing DMEM) with 17 mL media for each 150 mm dish.
   c. For each 150 mm dish: add the pooled sgRNA library, lentivirus packaging plasmids and PEI in 2 mL serum-free DMEM. Mix immediately by vortexing or pipetting (See materials and equipment section).
   d. Incubate the DNA/PEI mixture for 20 min at 20°C–25°C, then add the DNA/PEI mixture to the HEK293FT cells.
   e. Continue to incubate the HEK293FT cells for 48 h. Collect the supernatant containing viral particles in a 50 mL conical tube and centrifuge at 1,200 × *g* for 5 min to remove cell debris.
   f. Gently add another 17 mL media to the 150 mm dish and incubate for another 24 h. Collect the supernatant containing viral particles in a 50 mL conical tube and centrifuge at 1,200 × *g* for 5 min to remove cell debris. Combine the 48 h and 24 h supernatants and filter through a 0.45 μm syringe filter.
   g. After filtration, add 8.75 mL of 50% PEG 10000 solution, 3.72 mL of 4 M NaCl, and 4 mL of DPBS to 35 mL of media for a total volume around 51 mL.
   h. Mix the media and centrifuge solution by inverting, and put the 50 mL conical tubes into the 4°C cold room at least 12 h.
   i. On the second day, centrifuge the 50 mL conical tubes at 1,500 × *g* for 30 min at 4°C.
   j. Discard the supernatant and spin down again at 1,500 × *g* for 5 min at 4°C.
   k. Carefully remove the residual PEG solution and resuspend the lentiviral pellet in cold, sterile DPBS (1% of original vol., e.g., for 35 mL media, add 0.35 mL DPBS to resuspend the pellet).

Note: Lentivirus can be alternatively concentrated by ultracentrifugation.[5] However, the PEG precipitation protocol is more easy-to-handle and an efficient way to purify large volumes of lentivirus using a common centrifuge at speed of 1,500 × *g* (There are several protocols and kits are available, such as PEG-it™ Virus Precipitation Solution, Cat. # LV810A-1/ LV825A-1).

Pause point: The concentrated virus can be stored at −80°C for several years.

10. Calculation of lentiviral titer. Troubleshooting 2.
    a. Plate 6 wells of a 6-well plate at a density of 5×10^5 NIT-1 cells in DMEM medium per well.
        i. In each well add 20, 10, 5, 2.5, 0 and 0 μL (6th well as no-puromycin selection control) of lentivirus solution.
        ii. Fill to a total volume of 3 mL with DMEM medium, and supplement with 10 μg/mL polybrene.
    b. 2 days after virus infection, replace the medium with DMEM containing puromycin (2.0 μg/mL or your optimized concentration) into the virus-infected NIT-1 cells. Leave the 6th negative control well untreated (without puromycin).
    c. 3 days after starting the puromycin selection, when the 5th well (negative control) contains no live cells, and the 6th well (no-puromycin selection control) is 80%–90% confluent, rinse the cells with DPBS.
    d. Add 500 μL of trypsin to each well and incubate at 37°C for 2 min to dissociate the cells. Add 2 mL of DMEM and mix well.
    e. Mix 1 part cell suspension with 1 part trypan blue and incubate at 20°C–25°C for 3 min.
    f. Count and record the number of live cells in each well using the TC20 Automated Cell Counter.
    g. Calculate the lentiviral titer based on the cell viability as the number of cells in the puromycin treated well divided by the number of cells in the 6th negative control untreated well (without puromycin).

Note: The cell viability may be alternatively measured by a cell viability kit, such as Promega CellTiter-Glo 2.0 Cell Viability Assay (Cat# G9242).

In vivo screening of cells with the CRISPR GeCKO-A lentiviral library

Timing: 12–14 weeks

The following steps describe the generation of mutant NIT-1 cells using a genome-scale CRISPR knockout lentiviral library (step 11), the transplantation of mutant NIT-1 cells to NOD.scid mice and diabetes induction (step 12), and the process of graft genomic DNA extraction and preparation for next-generaiton sequencing (steps 13–18).

11. Transduction of cells with the sgRNA lentiviral library.[2,3,6] Troubleshooting 3.
    a. Prepare NIT-1 cells in five 150 mm petri dishes at 70%–80% confluency.
    b. Add the appropriate volume of lentivirus solution from step 10g to each 150 mm dish at MOI = 0.3, and supplement with 10 μg/mL polybrene.
    c. Incubate the NIT-1/lentivirus mixture at 37°C.
    d. 2 days after virus infection, replace the medium with DMEM containing puromycin (2.0 μg/mL) into the virus-infected NIT-1 cells.
    e. 3 days after puromycin selection, replace the medium with DMEM every 3 days until the cells recover to 80%–90% confluency.

CRITICAL: To ensure that most cells receive only one sgRNA, transduce the sgRNA library at MOI = 0.3. Scale up the sgRNA library coverage so that each sgRNA is expressed by

Figure 3. PCR amplication of pooled GeCKO gRNA
The PCR amplicons of the pooled GeCKO gRNA (~270 bp) are separated on a 2% agarose gel. The sizes of DNA ladder are shown as reference on the left.

more than 400 cells. For example, for a library size of 10000 unique sgRNAs, transduce 1.34×10^7 cells at an MOI of 0.3.

12. CRISPR GeCKO-A lentiviral library infected NIT-1 cells *in vivo* screen. A schematic of the screening procedure is shown in Figure 4.
    a. Pre-screening controls: save three of 10^7 mutant NIT-1 cell aliquots into three 1.5 mL Eppendorf tubes and store the cell pellets at −80°C to be used later in step 12f.
    b. Subcutaneously transplant 1×10^7 mutant NIT-1 cells into each 8-week-old female NOD.scid recipient mouse.
    c. Intravenously inject 1×10^7 of diabetic NOD splenocytes at the same time to induce autoimmunity.

Note: Designate some NOD.scid mice with subcutaneously transplanted mutant NIT-1 cells to serve as a non-autoimmune group, and do not inject them with splenocytes.

    i. Isolate diabetic NOD splenocytes from spontaneously diabetic female NOD mice as described in step 4.
    ii. Resuspend 1×10^7 of diabetic NOD splenocytes in 200 μL sterile DPBS.
    d. Monitor the blood glucose of mice for 8 weeks, at least 2 times per week.

Note: Mice in the non-autoimmune group may become hypoglycemic due to the transplanted graft expansion after ~3–6 weeks. The hypoglycemic control mice should be immediately taken out for graft retrieval. Please consult your institutional IACUC office for handling hypoglycemic animals. Mice in the autoimmune group usually develop diabetes in 6–8 weeks in our institute. You may adjust the screening timeframe in reponse to the diabetes onset time point in NOD mice housed in the experimental site.

    e. Terminate the screen at 8 weeks post-transplantation. Retrieve the remaining grafts from both the autoimmune group and the non-autoimmune group of mice.

Note: We recommend including at least three to five animals per group for screening.

    f. Extract the genomic DNA from the pre-screening control pellets and grafts using the Quick-gDNA midiprep kit (Zymo Research), following the manufacturer's protocol.

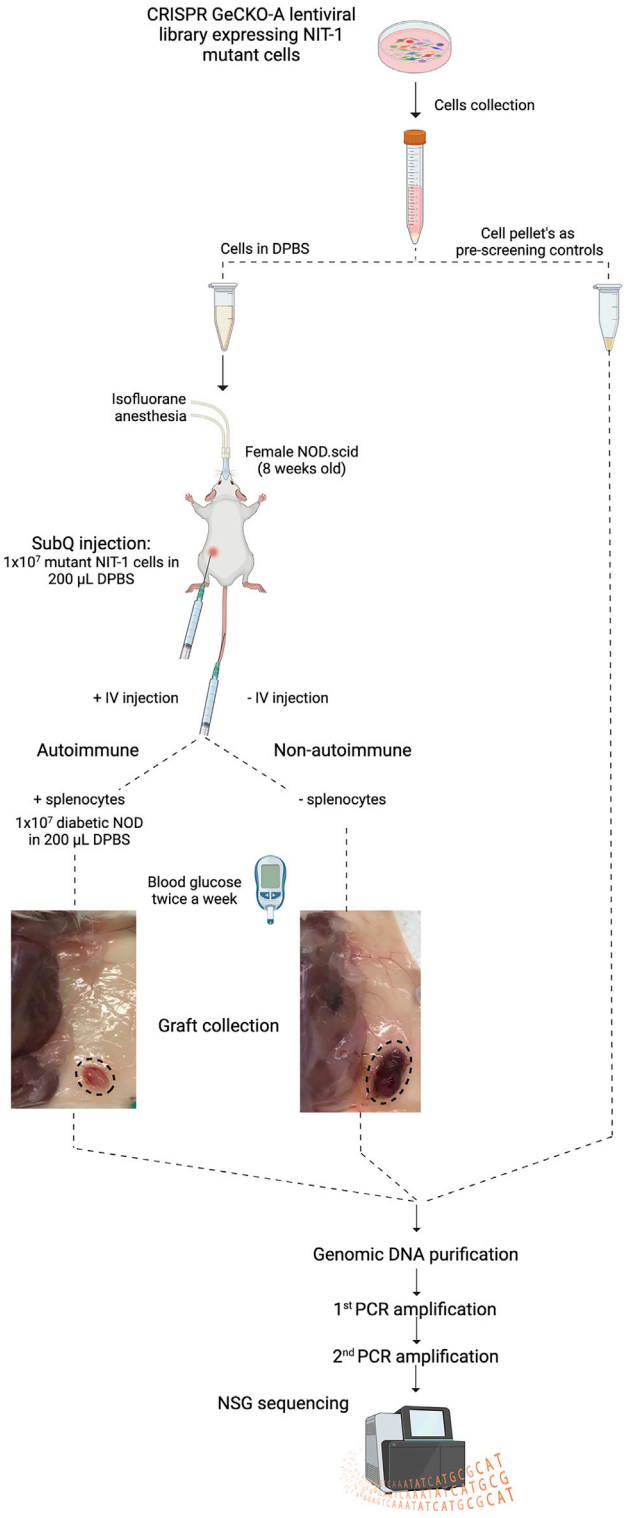

Figure 4. Genome-scale CRISPR/Cas9 screen of beta cell survival in the autoimmune NOD mouse model

NIT-1 cells transduced with the mouse GeCKO-A CRISPR lentiviral library (MOI = 0.3) and selected with puromycin. Three 10^7 NIT-1 mutant cell aliquots were collected as pre-screening controls. 10^7 NIT-1 mutant cells were

Figure 4. *Continued*
transplanted subcutaneously (SubQ) into each NOD.scid recipient mouse, with or without intravenous injection of 10^7 splenocytes from diabetic NOD mice. After 8 weeks, NIT-1 grafts were retrieved from recipients with (autoimmune) and without (non-autoimmune) splenocyte co-injection. Amplify the sgRNA library by PCR for next-generation sequencing.

⚠ CRITICAL: After screening, the surviving grafts may be variable in size, but in general they are very small grafts. Use sterile surgical tools for graft retrieval and avoid DNA contamination during the genomic DNA purification process.

Optional: To enhance the visibility of transplanted grafts, you may use luciferase-expressing NIT-1 cells for *in vivo* autoimmune screening. However, the reporter line has been pre-selected by an additional drug selection marker (puromycin is needed for the CRISPR library transduction), which may affect the cell sensitivity to stress and growth rate. Alternatively, you many generate a luciferase-expressing NIT-1 reporter line using a Rosa26 knock-in strategy to avoid double drug selections.

13. A two-step PCR method is used to amplify the sgRNA for next-generation sequencing (NGS). Troubleshooting 4.
    a. Perform the first PCR amplification of the pre-screening cell genomic DNA and the graft genomic DNA according to the following PCR program:

Reagent	Amount
Cell or graft genomic DNA from step 12f	5 μg in 10 μL
NEB Next High-Fidelity PCR Master Mix, 2×	50 μL
NGS-Lib-KO-v2-Adapter-Fwd primer (10 μM)	2.5 μL
NGS-Lib-KO- v2-Adapter-Rev primer (10 μM)	2.5 μL
ddH_2O	35 μL

Note: See the primer sequences in key resources table section.

⚠ CRITICAL: To reduce the PCR error, especially for low copy numbers of CRISPR-sgRNA sequences, prepare three PCR reactions for each genomic DNA sample if possible.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	10 s	20 cycles
Annealing	63°C	10 s	
Extension	72°C	25 s	
Final extension	72°C	2 min	1
Hold	4°C	forever	

    b. Combine and mix the three 100 μL PCR products in a 1.5 mL Eppendorf tube. Use the mixed 1st PCR products for the second PCR amplification.

Reagent	Amount
1st PCR DNA	10 μL
NEB Next High-Fidelity PCR Master Mix, 2×	50 μL
NGS-Lib-KO-Fwd primer (1–10 mix)	2.5 μL
NGS-Lib-KO-Rev primer (Barcode)	2.5 μL
ddH_2O	35 μL

Note: NGS-Lib-KO-Fwd primer (1–10 mix) and NGS-Lib-KO-Rev (Barcoded (bolded)) sequence can be found in Table 3 from a previous publication[2] and also listed in key resources table section. Equally mix NGS-Lib-KO-Fwd primer #1–10 for NGS and use different barcoded NGS-Lib-KO-Rev primer to label each sample.

c. Perform the second PCR amplification of the genomic DNA according to the following PCR program:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	10 s	25 cycles
Annealing	63°C	10 s	
Extension	72°C	25 s	
Final extension	72°C	2 min	1
Hold	4°C	forever	

14. Run the resulting PCR products on a 2% (wt/vol) agarose gel. Run the gel at 120 V for 30 min at 20°C–25°C.
15. Gel purification: visualize the gel using a Transilluminator and carefully cut out the ~270 bp product (Figure 3).

Note: The PCR may generate non-specific products. Gel purification is highly recommended.

16. Extract gel DNA using the Zymoclean Gel DNA Recovery Kit according to the manufacturer's directions.

Pause point: The gel-extracted DNA samples can be stored at −20°C for several months or at −80°C for long term.

17. Sequence the samples on the Illumia HiSeq/NovaSeq system.
18. Analyze sequencing data with MAGeCK to count sgRNA number[7] as described in step 14. Troubleshooting 5.

Note: We work with our institutional bioinformatics department to analyze the sequencing results.

EXPECTED OUTCOMES

Using this genome-scale *in vivo* CRISPR-Cas9 screen in a non-obese diabetic (NOD) derived mouse beta cell line, NIT-1, we successfully identified a group of genes, which potentially confer resistance to autoimmune killing. Further validation showed that deleting RNLS, a GWAS candidate gene for T1D, made beta cells resistant to autoimmune killing.[1]

LIMITATIONS

This protocol provides detailed materials and reagents for the loss-of-function screen on NIT-1 cells in one autoimmunity model (adoptive transfer), and can be applied for use with other mouse models of autoimmunity. The key to this *in vivo* screen is the selection of a suitable selection condition that will not be so harsh as to kill all the cells. Things like a luciferase containing reporter for *in vivo* imaging may help with this.

TROUBLESHOOTING

Problem 1

At step 4: autoimmune killing of cells in NOD mice. The condition is too harsh: all the cells were killed in seven days. The timeline expectation is to have ~90%–95% transplanted cells killed at day 15 post-splentocyte injection. Alternatively, the condition is too mild: more than 70% of the cells survived for more than one month.

Potential solution

Using the splenocytes purified from recent-onset diabetic NOD mice is highly recommended for reaching a better efficiency of autoimmune destruction. To avoid the unexpected high or low cell activities of purified splenocytes, we recommend select more than one spontaneously diabetic female NOD mice as a donor source for the splenocyte isolation. Re-Optimizing *in vivo* autoimmune killing (step 4) if necessary: If the condition is too harsh, decrease splenocyte number or increase the number of transplanted cells, and vice versa if the condition is too mild.

Problem 2

The viral titer is higher or lower than expected. It is difficult to calculate the titer of sgRNA library based on the the number of survival cells at step 10.

Potential solution

Infect the cells with the lower or higher concentration of virus or set up the titration of target cells again with a wider concentration gradient.

Problem 3

Cells were unhealthy after lentivirus infection and drug selection.

Potential solution

Avoid infecting the cells with high MOI and select the lowest concentration of antibiotic, which is sufficient to kill the negative control cells.

Problem 4

At steps 13–18: PCR bias led to false-negative results.

Potential solution

Adding Illumina adapter and barcode sequences in a one-step PCR might minimize the PCR bias.

Problem 5

Results of NGS sequencing were contamined. The common contamination is other genes-of-interest cloned in the same lentiviral vector in the laboratory, which present as the most dominant but implausible enriched sgRNAs in the screening results.

Potential solution

Using a DNA degradation solution, such as DNAZap (Thermo Fisher, Cat# AM9890), completely clean working area and lab tools before processing genomic DNA purification. Alternatively, use ultraviolet light to eliminate DNA cross contamination and process all DNA and PCR preparation under a PCR workstation/hood.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Erica P. Cai (ecai@indianabiosciences.org).

Materials availability

All reagents generated in this study are available from the lead contact with a Material Transfer Agreement.

Data and code availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request. For a complete example of next-generation sequencing read counts and analysis of screen results, please see.[1]

ACKNOWLEDGMENTS

This research was supported in part by funds from the Pittsburgh Foundation/Walton Fund to P.Y.; funds from the Myra Reinhard Family Foundation and grants from the Harvard Stem Cell Institute (DP-0167-17-00), JDRF (2SRA-2018-499-S-B), and NIDDK (1R01DK120445) to P.Y.; by postdoctoral fellowships from NIDDK (T32DK007260) to E.P.C.; and funds from the Mary K. Iacocca Foundation to E.P.C. We wish to acknowledge support from core facilities funded by the NIDDK Diabetes Research Center award P30DK036836 to the Joslin Diabetes Center and support from the Lilly Diabetes Center of Excellence at Indiana Biosciences Research Institute (IBRI) to E.P.C.

AUTHOR CONTRIBUTIONS

P.Y. and E.P.C. supervised the study. J.L. and E.P.C. performed both the experiments and analysis. J.L., Y.L., I.L.I., C.W., and E.P.C. wrote the manuscript. P.Y. and E.P.C. edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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