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METHODOLOGY

A CRISPR/Cas9 assisted strategy for the conditional expression of human NF-kappaB c-Rel cDNA in mouse T cells: design, prospects, and challenges

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Abstract Nuclear factor-κB protein c-Rel is a critical regulator of autoimmune diabetes. We found that c-Rel O-GlcNAcylation at serine-350 increases with hyperglycemia, which results in increased transcription of proautoimmune Th1 cytokines, interleukin-2 (IL-2) and interferon-gamma (IFN-γ), and decreased transcription of the T regulatory cell transcription factor forkhead box 3 (FOXP3). To further study the translational relevance of c-Rel S350 O-GlcNAcylation in autoimmune diabetes, we sought to generate transgenic non-obese diabetic (NOD) mice conditionally expressing wildtype or mutant S350A human c-Rel cDNA in T cells downstream of the endogenous mouse *REL* promoter. We used CRISPR-Cas9 gene editing to insert a unique designer cassette containing floxed mouse c-Rel cDNA-STOP sequence to maintain whole body c-Rel expression, followed by a linker and human c-Rel cDNA-STOP sequence. Using comprehensive PCR analyses and high-throughput sequencing, we confirmed successful insertion of the cassette at the mouse *REL* locus and the expected deletion of the mouse c-Rel cDNA specifically in T cells following CD4-Cre mating. Additional characterization revealed that the knock-in transgenic mice lacked endogenous mouse c-Rel, further confirming desired interference with its natural start codon. Unexpectedly, these mice lacked mouse and human c-Rel protein expression from inserted cDNAs, which mechanistically correlated with increased CpG methylation of the c-Rel promoter

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region. Thus, our study presents a unique, universal molecular design and method for the generation of conditional knock-in transgenic mice expressing human genes at the endogenous mouse promoter. It also reveals a potential locus-specific challenge that may arise during the development of such novel transgenic mouse models.

Keywords NF-κB · Conditional knock-in · Type 1 diabetes · CRISPR-Cas9 gene editing · Epigenetic silencing

Introduction

A significant overlap exists between the human and mouse genomes (Emes et al. 2003), which facilitates the use of mice as a model organism in biomedical research. Transgenic mice that express specific human proteins can be created to increase the translational relevance of mouse studies. Importantly, human genes can maintain function when expressed in mice (Wallace et al. 2007). Conditional expression of human genes and their mutational variants in a tissue specific manner in mice is advantageous for studies on disease etiology and for the development of therapeutics. In this study, we sought out to generate a non-obese diabetic (NOD) mouse model expressing the human transcription factor, NF-κB c-Rel, as well as its variant c-Rel S350A, which is a point mutation that modulates its function. It has been shown previously that human NF-κB p100/p52 transgene retains function in mice (Connelly et al. 2007).

NOD mice spontaneously develop a human type 1 diabetes (T1D)-like disease and are a classical mouse model to study T1D based on significant similarities to T1D disease pathogenesis in humans (Van Belle et al. 2009). T1D is a chronic autoimmune disease characterized by destruction of pancreatic islet β-cells by autoreactive effector T lymphocytes (Katsarou et al. 2017). During homeostasis, T regulatory cells prevent autoimmune disease and maintain self-tolerance by suppressing autoreactive T cells (Rajendeeran and Tenbrock 2021). The transcription factor NF-κB c-Rel is a critical regulator of effector T lymphocyte differentiation and effector functions (Visekruna et al. 2012) and of T regulatory cell development and suppressor functions (Isomura et al. 2009; Ruan et al. 2009; Fulford et al. 2021).

We have previously shown that c-Rel deficiency results in accelerated development of autoimmune diabetes in NOD mice, which is associated with impaired expression of Th1 proinflammatory cytokines, such as IL-2 and IFN-γ, and significantly reduced FOXP3 expression and T regulatory cell numbers (Ramakrishnan et al. 2016). We have also shown that c-Rel is O-GlcNAcylated at serine 350 (Ramakrishnan et al. 2014) and that c-Rel O-GlcNAcylation is increased in diabetic mice (De Jesus et al. 2021). Mutation of serine 350 to alanine (S350A) prevents O-GlcNAcylation of c-Rel and results in reduced T cell receptor-induced expression of IL-2 and IFN-γ (Ramakrishnan et al. 2014) and increased FOXP3 expression in T cells (De Jesus et al. 2021). Thus, c-Rel S350 O-GlcNAcylation is expected to have a role in promoting autoimmune diabetes development by enhancing Th1 cytokine production by T cells and decreasing T regulatory cell development and function.

Here, to study the physiological role of c-Rel O-GlcNAcylation as a driver of autoimmunity in T1D, we sought out to generate two conditional knock-in mouse models in which human wildtype or S350A c-Rel cDNA was targeted to express at the endogenous *REL* locus of NOD mice. Specifically, we designed a cassette containing floxed mouse c-Rel cDNA, followed by a linker and human c-Rel cDNA. After mating with CD4-Cre mice, human c-Rel will be expressed in T cells, while mouse c-Rel will be expressed in all cell types except T cells in knock-in mice. Expression of human c-Rel in mice has high translational potential as it facilitates direct in vivo testing of therapeutic agents targeting human c-Rel, which may accelerate preclinical studies. We confirmed successful insertion of the cDNAs into the mouse *REL* locus with PCR and sequencing. Unexpectedly, we found no protein was expressed from the transgenic cDNAs, likely due to epigenetic gene silencing through DNA methylation of CpG islands. This study presents a unique, universally applicable conditional knock-in strategy to express human cDNAs in mice, along with a tale of potential challenges in the creation of such knock-in transgenic mice.

Methods

Mice

NOD mice (JAX:001976) and CD4-Cre NOD mice (JAX:013234) were purchased from Jackson Laboratories. All transgenic procedures were performed by the transgenics core at Case Western Reserve University. c-Rel knockout NOD mice (JAX:035009) were generated as previously described (Ramakrishnan et al. 2016). Mice were housed and handled in accordance with the National Institutes of Health (NIH) guidelines under the Institutional Animal Care and Use Committee protocol #2013–0134 approved by Case Western Reserve University.

Reagents and antibodies

Antibodies against the following proteins were used in this study: mouse c-Rel (D38BS, Cell Signaling Technology), human c-Rel (4727, Cell Signaling Technology), FLAG (M2, Sigma), c-Myc (9E10, Sigma), beta-actin (C4, Santa Cruz Biotechnology), and beta-tubulin (H300, Santa Cruz Biotechnology). For flow cytometric analysis, fluorescent antibodies against the following proteins were used in this study: CD4 (GK1.5, BioLegend), Zombie NIR Fixable Viability Kit (BioLegend), and FOXP3 (150D, BioLegend).

Design of targeting construct

To develop NOD mice conditionally expressing human c-Rel cDNA in T cells, we designed a unique construct with Myc tagged mouse c-Rel cDNA flanked by loxP sites and FLAG tagged wildtype or S350A mutant human c-Rel cDNA followed by BGH poly A sequences. Mouse c-Rel and human c-Rel cDNAs were separated with an intervening sequence. This cassette was flanked with a 2000 base pair (bp) 5’ homology arm and a 1000 bp 3’ homology arm corresponding to the genomic sequence around the ATG site of the endogenous mouse c-Rel (Fig. 1a-c).

CRISPR-Cas9 mediated gene editing

Specific targeting of the insert was achieved by using CRISPR-Cas9 gene editing. We designed six sgRNAs near the start codon of endogenous mouse c-Rel (Fig. 1d). The sgRNAs were generated and their efficiency was validated using Guide-it Complete sgRNA Screening System (Clontech). A DNA template with T7 promoter and sgRNA encoding sequence was generated by PCR using the Guide-it scaffold template. sgRNA was in vitro transcribed using Guide-it T7 Polymerase mix and purified by digestion using DNase I, followed by purification using MEGAclear Transcription Clean-Up Kit (Thermo Fisher Scientific) to minimize the risk of contamination with any off-target products. The cleavage template for screening the purified sgRNA was generated by PCR amplifying a 0.81 kb fragment of genomic DNA using the following primers (Forward: 5’ CACTAGTGA CCGGAGCGCGAAGATTCG 3’) and (Reverse: 5’ GGTTGAAGGGATCGAGGTCTCTACACGG 3’) surrounding the endogenous mouse c-Rel ATG site. Primers were selected with the target sequence located in an asymmetric position to produce two cleavage fragments of different sizes. Cleavage reaction was performed using the template, purified sgRNA, and Guide-it Recombinant Cas9 Nuclease. Efficiency of the cleavage reactions was validated by examining the digested DNA on an agarose gel (Fig. 1e).

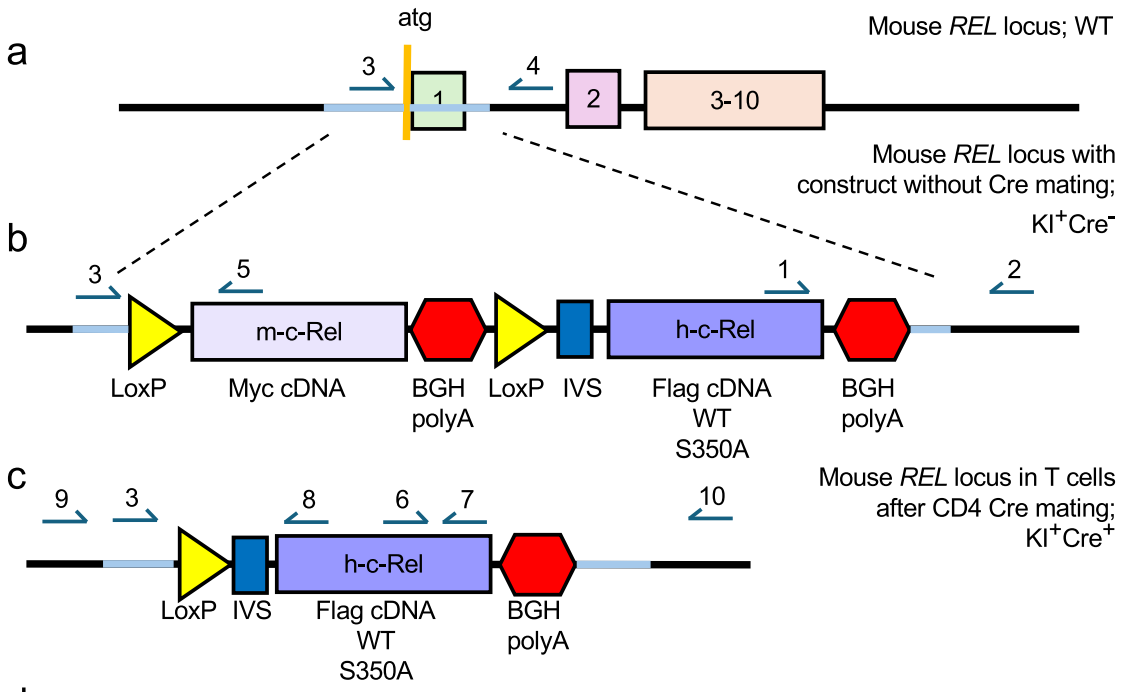
For in vivo targeting, we purchased synthetic sgRNA 6 and Cas9 protein (PNA Bio). Fertilized NOD mouse eggs were microinjected with the sgRNA, Cas9, and the wildtype or S350A mutant human c-Rel cDNA expressing constructs.

Primary cell isolation from mouse tissues

Single cell suspensions were prepared from mouse spleen and thymus by homogenizing the tissue using a rubber-capped plunger of a 3-cc syringe. Cells were then passed through a 40 µM cell strainer. Cells were centrifuged at 300 g for 5 min, and red blood cells were lysed using RBC lysis buffer.

Magnetic sorting

$CD4^+$ T cells were isolated from total splenocytes using a MojoSort™ Mouse CD4 T Cell Isolation Kit (BioLegend) following manufacturer’s instructions. Purity and viability of isolated cells were confirmed using flow cytometry.



d

CTAGTGACCGGAGCGCGAAGATTCGGGGGTGGGAAGGTGTGAGCCGCAAACCCAG

g1

g2

g3

AGGAGCCGGGAAAGAAGAGGAGGCCCGGGGGTGGTCCGAGGACGGCGTCCCGG

g4

CTGCGGAGGCTGCTCAGCCTCCTCACTGGTTGGCTGACTGACGGGCTAGCTGG

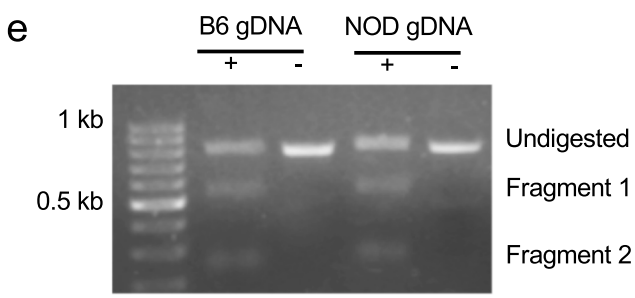
g5

CTGACTGCCTGCCTGACCGACTGCCTGGCTGACTGACTCACTGACTGACTGACTGG

CTGCCTTGCAGCCTCGGGCCGGACGCTGGAAGCTGCAGCGAGCGGCGCGCGGAG

g6

CCGAGCC**atg**GCCTCGA



◂**Fig. 1** Schematic for transgenic human c-Rel designer cassette and selection of guide RNA for CRISPR-Cas9 gene editing. Schematic diagrams of wildtype (WT) mouse *REL* locus with exons 1–10 (**a**), *REL* locus with transgenic cassette before CD4-Cre mating and in non-T cells after CD4-Cre mating (**b**), and *REL* locus with transgenic cassette after CD4-Cre mating in T cells (**c**). IVS: intervening sequence. Primers used for verification genotyping PCR are indicated as follows: (**a**), 3F/4R, (**b**), 3F/5R, 1F/2R, (**c**), 9F/10R, 3F/8R, 6F/7R. **(d)** The location of six sgRNAs (g1-g6) near the start codon of endogenous mouse c-Rel ATG (dotted underline, bold) are noted. **(e)** PCR with genomic DNA (gDNA) from C57BL/6 (B6) and NOD mice in cleavage reaction with purified sgRNA and Guide-it Recombinant Cas9 Nuclease (+). Undigested DNA (-) was used as a control

PCR genotyping

DNA was isolated from mouse ear snips using the HotSHOT method (Truett et al. 2000) and from mouse cells using DNeasy Blood and Tissue Kit (Qiagen). PCR was performed using DreamTaq PCR Master Mix (Thermo Fisher) following manufacturer's instructions. Primers used for PCR are listed in Supplemental Table 1. For the long-range PCR, LongAmp Taq Master Mix (NEB) was used following manufacturer's instructions. PCR products were then resolved in a 1% agarose gel and visualized following ethidium bromide staining.

Western blotting

Cells were lysed on ice for 30 min in lysis buffer (1.0% Triton-X100, 20 mM HEPES [pH 7.6], 150 mM NaCl, 1 mM EDTA, and complete protease inhibitor cocktail). Lysate was centrifuged at 12,000 g for 10 min, and supernatants were collected. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher). Normalized volumes of lysates were resolved in a 9% SDS-PAGE gels. Proteins were then transferred onto nitrocellulose membranes, probed with respective antibodies, and visualized using enhanced chemiluminescence assay.

Cell culture and transfection

HEK-293T cells were cultured in high glucose DMEM medium containing 10% super calf serum (SCS), 100 U/mL penicillin, streptomycin, and 1% L-glutamine in a 37 °C incubator with 5% CO_2. Cells were transfected using calcium phosphate precipitation method with plasmids expressing FLAG tagged human c-Rel and Myc tagged mouse c-Rel for use as positive controls in Western blotting.

Flow cytometry

Intracellular FOXP3 straining was performed using the True Nuclear Transcription Factor Buffer kit (BioLegend) following manufacturer's instructions. Briefly, cells were first stained for surface markers in FACS buffer, and then fixed, permeabilized, and stained with FOXP3 antibody overnight. Cells were then washed with permeabilization buffer and analyzed using Accuri or Cytoflex flow cytometer.

DNA library preparation and sequencing

DNA was quantified using the Qubit Fluorometer high sensitivity dsDNA kit (Invitrogen, US) and resolved on a 2% agarose gel. DNA was diluted to an input of 1 ng total in 5 µL and processed using the Nextera XT DNA Library Preparation Kit (Illumina, US). The Nextera tagmentation technology fragments and tags DNA with sequencing adapters in a single-tube enzymatic reaction. The tagmented DNA was amplified, which adds the unique indexes and adapters required for sequencing cluster generation. The final library was quantified using the Qubit Fluorometer high sensitivity dsDNA kit (Invitrogen, US) and the profile was verified on the Fragment Analyzer High Sensitivity NGS kit. Final library pool was quantified using the NEBNext Illumina Library Quant kit (NEB) and Life Technologies Quant Studio 7 Real Time PCR System. The pool was diluted and denatured for sequencing following Illumina standard protocols. High-throughput sequencing was carried out using an Illumina MiSeq v2 Nano flow cell, paired-end sequencing.

Targeted bisulfite methylation sequencing

DNA methylation analysis was performed by CD Genomics, NY, USA, as previously described (Xiu et al. 2022). Briefly, genomic DNA from splenic $CD4^+$ T cells was treated with bisulfite to convert all unmethylated cytosines to uracil. PCR was then used with specially designed primers (EpiDesigner) to obtain an amplification product with the T7 RNA

Table 1 Summary of protein expression data in wildtype (WT) and transgenic mice before (KI^+Cre^-) and after (KI^+Cre^+) CD4-Cre mating in various tissues

Tissue	Genotype	Expected	Observed
Spleen and thymus (Mix of $CD4^+$ and $CD8^+$ T cells and non-T cells)	WT	m c-Rel +	m c-Rel +
		Myc m c-Rel -	Myc m c-Rel -
		FLAG h c-Rel -	FLAG h c-Rel -
	KI^+Cre^-	m c-Rel -	m c-Rel -
		Myc m c-Rel +	*Myc m c-Rel* -
		FLAG h c-Rel -	FLAG h c-Rel -
	KI^+Cre^+	m c-Rel -	m c-Rel -
		Myc m c-Rel +	*Myc m c-Rel* -
		FLAG h c-Rel +	*FLAG h c-Rel* -
$CD4^+$ T cells	WT	m c-Rel +	m c-Rel +
		Myc m c-Rel -	Myc m c-Rel -
		FLAG h c-Rel -	FLAG h c-Rel -
	KI^+Cre^-	m c-Rel -	m c-Rel -
		Myc m c-Rel +	*Myc m c-Rel* -
		FLAG h c-Rel -	FLAG h c-Rel -
	KI^+Cre^+	m c-Rel -	m c-Rel -
		Myc m c-Rel -	Myc m c-Rel -
		FLAG h c-Rel +	*FLAG h c-Rel* -

Lines in italics represent instances where observed protein expression does not match expected protein expression

polymerase promoter sequence. Primer sequences used were: set 1 – 5'-aggaagagagAATTTAATTTGT TAGGTTTGGTAGTAAG-3' (forward) and 5'- cagtaatacgactcactatagggagaaggctCCTACCAAAATT TTTCAACTACACTA-3' (reverse); set 2 – 5'- aggaagagagGTTTTTAAGGATTTTGGAGGAGG (forward) and 5'-cagtaatacgactcactataggga gaaggctTCA ATCAATAAATCAATCAACCAAA-3' (reverse). With in vitro transcription, the amplified product was transcribed into RNA using T7 RNA polymerase. During this step, RNase A was used to create small RNA fragments that carry CpG sites. Time of flight mass spectrometry (MALDI-TOF MS) was used to detect methylated and unmethylated fragments based on molecular weights. The degree of methylation for each CpG site was then determined based on the relative quantitative ratio of methylated and unmethylated fragments for each CpG site. CpG sites that were not analyzed in one or more samples were excluded from further analysis.

Results

Design and insertion of designer human c-Rel cassette into mouse c-Rel ATG region using CRISPR-Cas9 gene editing

The main objective of our approach was to obtain T cell specific, conditional expression of wildtype (WT) and S350A mutant human c-Rel downstream of the mouse *REL* promoter in NOD mice. By inserting our transgene at the endogenous mouse *REL* locus, we can study expression of our transgene under the control of endogenous transcription factors and signaling pathways. For this, we targeted the ATG region in the first exon of mouse c-Rel (Fig. 1a). Because a standard insertion of the human cDNA will disrupt the start codon and result in whole body knockout of endogenous mouse c-Rel, we devised a novel strategy by adding Myc tagged mouse c-Rel cDNA with a polyA stop flanked by loxP sites into the construct (Fig. 1b). The addition of a polyA sequence after the mouse c-Rel cDNA will ensure that the downstream human c-Rel cDNA is not expressed before Cre mating (KI^+Cre^-). This approach allows for the expression of mouse c-Rel cDNA in all tissues of a knock-in mouse, until its specific deletion following mating with any tissue specific Cre mouse. This offers a versatile system to express heterologous but functionally redundant proteins in mouse tissues. Mating with a Cre mouse will result in the tissue specific expression of human c-Rel in KI^+Cre^+ mice (Fig. 1c).

To target the ATG region in the first exon of mouse c-Rel, six sgRNAs were bioinformatically identified in the region close to the start codon of endogenous mouse c-Rel (Fig. 1d). The sgRNAs were generated as described, and their efficiency was validated using Guide-it Complete sgRNA Screening System

(Clontech). A DNA template with T7 promoter and sgRNA encoding sequence was generated by PCR using the Guide-it scaffold template. sgRNA was in vitro transcribed using Guide-it T7 Polymerase mix and purified by digestion using DNase I. The cleavage template for screening the purified sgRNA was generated by PCR amplifying a 0.81 kb fragment of genomic DNA surrounding the endogenous mouse c-Rel ATG site. Cleavage reactions were performed using the template, purified sgRNA, and Guide-it Recombinant Cas9 Nuclease. Efficiency of the cleavage reactions was examined by separating the digested DNA on an agarose gel to visualize generation of cleaved fragments (Fig. 1e), and sgRNA 6 was selected as the most effective sgRNA in targeting.

Genotypic and sequencing validation of human c-Rel knock-in transgenic mice

Following CRISPR-Cas9 nuclease insertion of our wildtype and mutant c-Rel expressing constructs, we obtained 43 wildtype c-Rel founder mice and 58 mutant c-Rel founder mice. This approach is expected to yield both targeted and random insertions. To differentiate between the random and targeted insertions, we designed specific primers for PCR that are within the construct as well as its flanking regions (Fig. 1a-c, Supplemental Table 1). Successful insertion of our transgene construct was confirmed using standard genotyping using genomic DNA extracted from ear snips. To detect targeted insertions by PCR, we used one primer in the construct and the other primer in the endogenous chromosomal locus outside the arm of homology. We confirmed targeted insertion of the construct at the 3' end by PCR using primers 1 and 2, which are expected to yield a 1500 bp amplicon (Fig. 2a). Based on this reaction, out of the original founder mice, we obtained 5 mice with the wildtype human c-Rel construct and 20 mice with the mutant human c-Rel construct. This PCR validation of targeted insertion was performed using the founders as well as at least three progenies (F1–F3). The mating also included backcrossing of both male and female carriers with NOD background mice to ensure fixing of both Y and X chromosomes.

Once we confirmed the targeted presence and transmission of our construct to progeny, we followed a three primer genotyping strategy with primers 3, 4, and 5 to identify the mice that are homozygous for the inserted construct (Fig. 2b). Primer 3 is located in the 5' homology arm. Primer 4 is located in the intron between exon 1 and 2, which will produce a 272 bp product in wildtype mice. Primer 5 is located within mouse c-Rel cDNA and will produce a 375 bp product in knock-in mice with the construct. We also confirmed the presence of human c-Rel cDNA (Fig. 2c) in heterozygous and homozygous transgenic mice by PCR using primers 6 and 7, which bind internally within human c-Rel cDNA and will produce a 114 bp product. Based on the results of these PCRs (Fig. 2b-c), homozygous transgenic mice were used for further studies. Our comprehensive multiregional genotyping PCR showed that the entire cassette with Myc tagged mouse c-Rel, as well as wildtype or S350A mutant human c-Rel, were inserted at the intended endogenous *REL* locus (Fig. 2a-c).

To obtain the T cell specific expression of the human transgene, the mice were mated with CD4-Cre mice. We isolated DNA from splenocytes and splenic $CD4^+$ T cells of CD4-Cre mated mice and used primers 3 and 8 to further confirm using PCR that the mouse c-Rel cDNA in the insert had been removed while retaining human c-Rel cDNA (Fig. 2d, e).

To confirm the entirety of our cassette was inserted at the ATG region of the *REL* genomic locus without any mutations, we isolated $CD4^+$ T cells from KI^+Cre^+ transgenic mice, which should only express FLAG human c-Rel and not Myc mouse c-Rel. We then performed long-range PCR to amplify the entire transgenic insert and surrounding endogenous chromosomal regions using primers 9 and 10, which bind outside the 5' and 3' homology arms respectively (Fig. 2f). The ~ 10 kb PCR product was sequenced using high-throughput sequencing with Illumina MiSeq v2 Nano flow cell, paired-end sequencing (Supplemental Fig. 1). The sequencing identified that the complete cassette was inserted at the *REL* genomic locus with only 4 mutations compared to the reference sequence (Supplemental Table 2). The first two mutations were in the 5' arm of our transgene insert in a C-repeat region about 1.5 kb upstream of the transcription start site. The third mutation was the desired S350A in human c-Rel and represents the expected AG → GC mutation for the serine → alanine substitution. The fourth mutation is within the 3' arm of our transgene insert. This sequencing demonstrates that our entire transgene cassette was inserted into the correct genomic location and without any

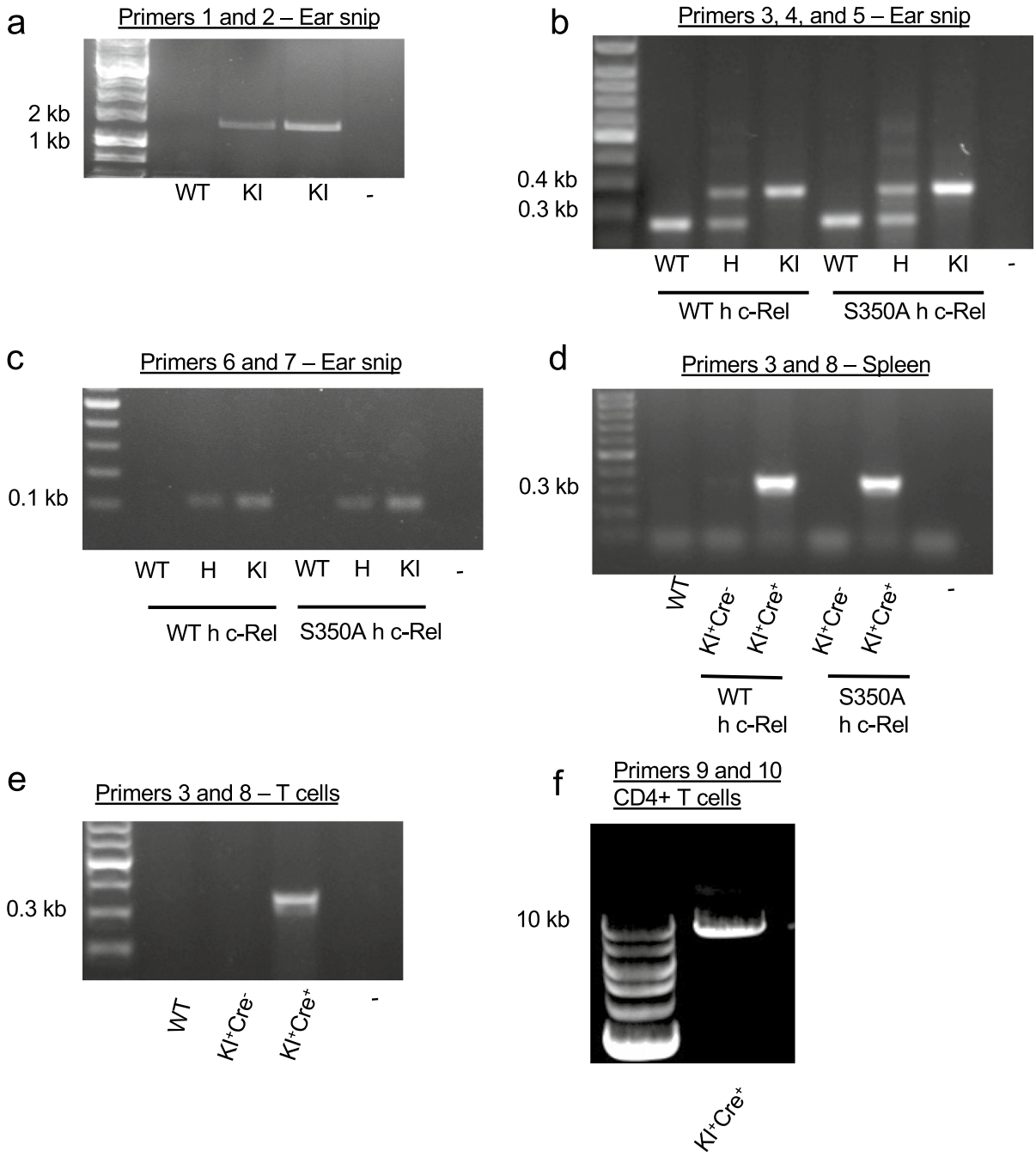


Fig. 2 Genotypic and sequencing validation of human c-Rel wildtype and S350A transgenic mice. (**a**) PCR genotyping to demonstrate targeted insertion of transgene with DNA from ear snips of wildtype (WT) and transgenic knock-in (KI) mice, (**b**, **c**) PCR genotyping with DNA from ear snips of WT, heterozygote (H) KI mice, and homozygous KI mice, (**d**, **e**) PCR genotyping with DNA from splenocytes or splenic $CD4^+$ T cells of WT and transgenic knock-in mice before (KI^+Cre^-) and after (KI^+Cre^+) CD4-Cre mating, (**f**) Genomic DNA was isolated from $CD4^+$ T cells of a KI^+Cre^+ S350A mouse. Long-range PCR was used to amplify the entire transgene insert. Data is representative of at least three independent experiments. Water (-) was used as a negative control

mutations that could affect expression or function (Supplemental Fig. 1).

Validation of protein translation of targeted mouse and human c-Rel cDNAs in cassette

Our next step was to examine the protein expression of mouse c-Rel cDNA in various tissues and human c-Rel cDNA in T cells after Cre mating in knock-in transgenic mice. We isolated the spleen, thymus, and splenic CD4$^+$ T cells from our knock-in transgenic mice before and after CD4-Cre mating and evaluated the protein expressions of endogenous mouse c-Rel, Myc tagged mouse c-Rel, and FLAG tagged human c-Rel. We expected that before CD4-Cre mating (KI$^+$Cre$^-$), Myc tagged mouse c-Rel will be expressed in all cell types in knock-in mice. After crossing knock-in mice with CD4-Cre mice (KI$^+$Cre$^+$), we expected that FLAG tagged human c-Rel will be expressed in T cells, while Myc tagged mouse c-Rel will be expressed in all other cell types (Table 1).

We examined splenocytes from KI$^+$Cre$^-$ and KI$^+$Cre$^+$ mice, which would contain a mixture of T cells and non-T cells and enable the detection of both mouse and human c-Rel protein expressed from their respective cDNAs. Against our expectations, we did not detect Myc tagged mouse c-Rel in KI$^+$Cre$^-$ and KI$^+$Cre$^+$ mice, nor FLAG tagged human c-Rel in KI$^+$Cre$^+$ mice (Fig. 3a). Additionally, as expected due to the disruption of the endogenous ATG start site, no endogenous mouse c-Rel was detected in splenocytes of KI$^+$Cre$^-$ and KI$^+$Cre$^+$ mice (Fig. 3b). This further confirms our genotyping data and the successful targeted insertion of the cassette at the *REL* genomic locus. These findings were consistent regardless of the wildtype or S350A human c-Rel genotype.

The knock-in transgenic mice also lacked protein expression of endogenous mouse c-Rel, Myc tagged mouse c-Rel, and FLAG tagged human c-Rel in thymocytes and in isolated splenic CD4$^+$ T cells with and without T cell receptor stimulation (Fig. 3c-e). A summary of Western blot findings regarding the presence or absence of protein expression is given in Table 1.

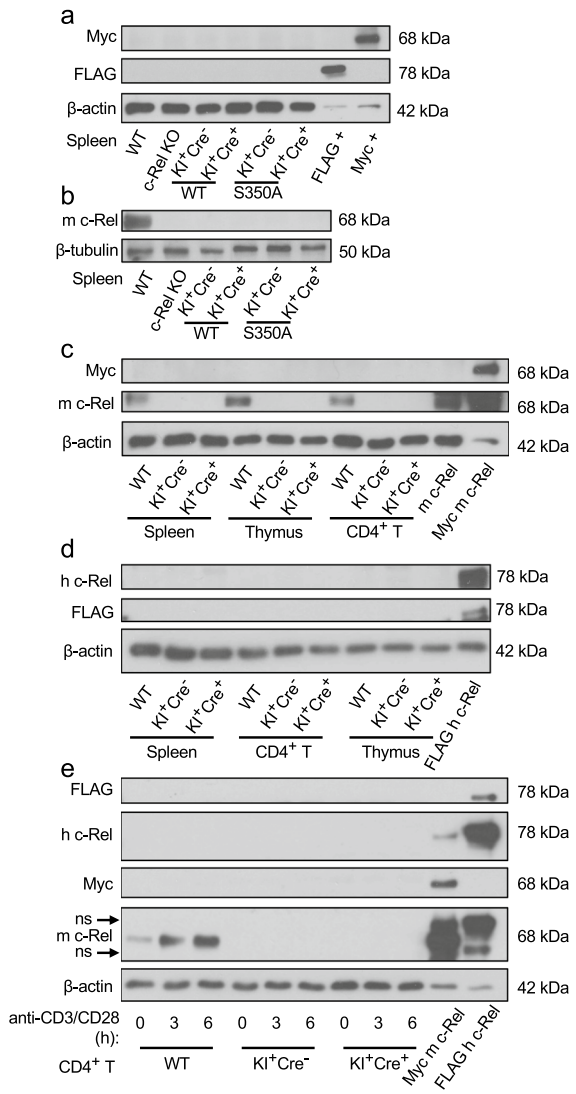


Fig. 3 c-Rel knock-in transgenic mice lack protein expression from both mouse and human c-Rel cDNAs. Expression of FLAG human c-Rel, Myc mouse c-Rel, and endogenous mouse c-Rel was assessed in the spleen (**a**–**d**), thymus (**c**, **d**), and splenic CD4$^+$ T cells (**c**–**e**) of wildtype (WT), c-Rel knockout (KO), transgenic mice before (KI$^+$Cre$^-$) and after (KI$^+$Cre$^+$) CD4-Cre mating. Additional positive controls include cell lysate from HEK-293T cells expressing FLAG human c-Rel or Myc mouse c-Rel (**a**, **c**, **e**) and FLAG human c-Rel expressed in mouse cells (**d**). Total cell lysates were prepared from the isolated tissues or cells and analyzed by Western blotting with indicated antibodies. Actin and tubulin were used as loading controls. Data is representative of two (**e**) or three (**a**–**d**) independent experiments with different mice

T regulatory cells were significantly reduced in c-Rel knock-in transgenic mice

We previously generated c-Rel knockout NOD mice, which have a significant decrease in FOXP3-positive T regulatory (Treg) cells in the spleen. This demonstrates the critical requirement of c-Rel for FOXP3 expression and Treg cell development (Ramakrishnan et al. 2016). We have also shown that c-Rel S350A mutation increases FOXP3 expression in T cells (De Jesus et al. 2021). Hence, the knock-in transgenic mice expressing wildtype human c-Rel were initially expected to maintain endogenous Treg cell numbers, while the S350A mutant human c-Rel expressing mice were expected to have increased Treg cell numbers. However, given our protein expression data where no c-Rel was detected, it was anticipated that our c-Rel knock-in transgenic mice would have decreased Treg cells compared to wildtype NOD mice.

We examined Treg cells in our knock-in transgenic mice and in accordance with the lack of c-Rel protein expression, both KI^+Cre^- and KI^+Cre^+ transgenic c-Rel mice had a significant decrease in Treg cells in the spleen compared to wildtype NOD mice (Fig. 4a, b). There was no significant difference in Treg cell numbers between KI^+Cre^- and KI^+Cre^+ mice and c-Rel knockout NOD mice (Fig. 4a, b). These results reemphasize the importance of c-Rel in Treg cell development and further suggest that our c-Rel knock-in transgenic mice lack all c-Rel protein expression and possess a c-Rel knockout phenotype.

CpG DNA methylation was increased upstream of *REL* transcription start site in c-Rel knock-in transgenic mice

Because the insertion of our cassette was at the desired locus and no unexpected mutations or stop codons were identified in the cDNAs inserted, we hypothesized that the lack of protein expression may be attributed to epigenetic silencing. It has previously been reported that human transgene DNA in rats shows heavy methylation that increases with successive generations (Li et al. 2015). We thus sought out to determine if DNA CpG methylation could be responsible for lack of expression of our transgene cassette. To address this, we isolated DNA from splenic $CD4^+$ T cells in wildtype, KI^+Cre^-, and KI^+Cre^+ mice and conducted targeted bisulfite methylation sequencing (CD Genomics). CpG methylation was determined in the promoter region of c-Rel with coverage approximately -1000 base pairs from the transcription start site. Compared to wildtype NOD mice, both KI^+Cre^- and KI^+Cre^+ knock-in transgenic mice had elevated CpG DNA methylation at several positions (Fig. 5), including CpG sites near known NF-κB binding sites in the promoter region (Grumont et al. 1993). This suggests that endogenous silencing of transcription by promoter CpG DNA methylation is a potential cause of lack of protein expression in our knock-in transgenic mice.

Discussion

Loss of self-tolerance is the main driver of autoimmunity in T1D and results in destruction of pancreatic islet β-cells. Therapeutic attempts to delay autoimmunity and slow progression of disease remain enigmatic. NF-κB c-Rel plays a critical role in regulating both effector and regulatory T cell functions. As such, delineating the function of c-Rel in T1D and targeting c-Rel appears to hold potential to control T1D progression. Our previous studies have shown that global deletion of c-Rel accelerates diabetes development in NOD mice (Ramakrishnan et al. 2016), and O-GlcNAcylation of c-Rel has a role in positive regulation of proautoimmune gene expression (Ramakrishnan et al. 2014) and in negative regulation of FOXP3 gene expression (De Jesus et al. 2021). Thus, it appears that posttranslational regulation of c-Rel is critical in dictating its autoimmune function in T1D. Based on our previous works, we hypothesized that lack of O-GlcNAcylation of c-Rel at S350 is expected to yield a phenotype that promotes immunosuppressive Treg cell function and prevents autoreactive T effector cell action, resulting in overall protection from T1D initiation and progression.

Because an in vivo disease model is necessary to validate this hypothesis, through this study, we sought out to generate a knock-in transgenic NOD mouse model expressing wildtype or mutant human c-Rel. We demonstrated successful insertion of the human c-Rel transgene at the endogenous mouse *REL* locus. Yet, further characterization of the knock-in transgenic mice revealed lack of protein expression from the inserted c-Rel cDNA plausibly associated with

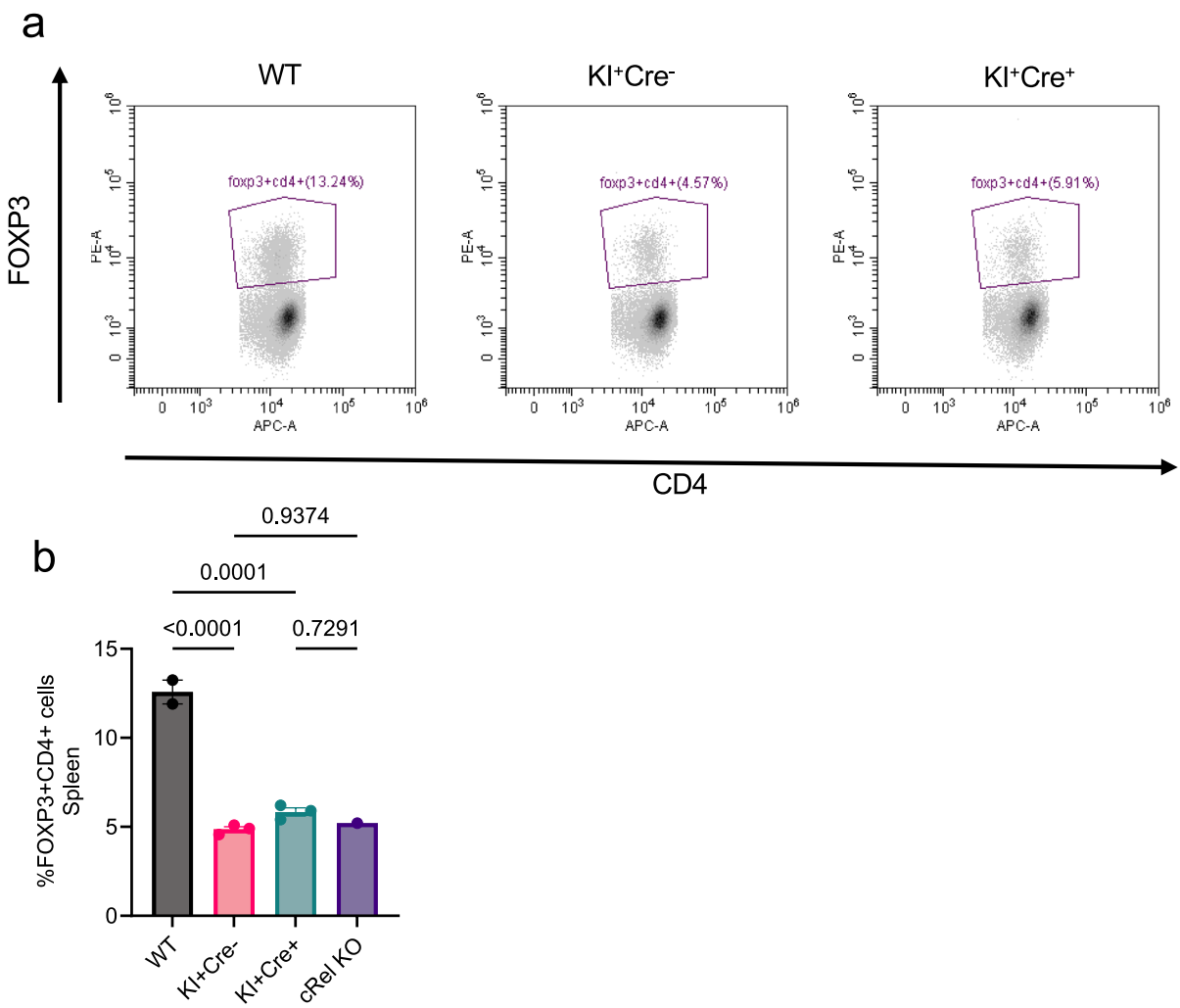


Fig. 4 c-Rel knock-in transgenic mice show significantly reduced FOXP3-positive T regulatory cells in the spleen. **(a)** Representative flow cytometry plots of surface CD4 expression and intracellular FOXP3 staining in splenocytes from wildtype (WT) NOD and transgenic mice before (KI^+Cre^-) and after (KI^+Cre^+) CD4-Cre mating, **(b)** Summary of FOXP3-positive T regulatory cells among $CD4^+$ T cells in spleen over multiple experiments. One-way ANOVA with multiple comparisons

increased methylation at several CpG sites in the mouse *REL* promoter region, including CpG sites near known NF-κB binding sites. The reason for increased CpG DNA methylation of the c-Rel promoter region in our c-Rel transgenic mice is unclear. Based on the design of the transgenic cassette, the *REL* promoter region in the transgenic mice is encompassed within the 2000 bp 5' homology arm inserted alongside our transgene. Hence, the mouse *REL* promoter sequence is expected to be the same between wildtype mice and c-Rel transgenic mice; however, the source of the DNA is technically different. It is hard to speculate whether the synthetic mouse *REL* promoter sequence encoded in the 5' homology arm of the cassette could be identified as "foreign DNA" in our transgenic mice and thus targeted for increased DNA methylation. Another point to consider is that we have significantly modified the native sequence near the original start codon by inserting the synthetic cassette with Myc-tagged mouse c-Rel cDNA, loxP sites, STOP signals, and FLAG-tagged human c-Rel cDNA, all devoid of natural intronic regions in the genomic DNA. It is not known whether these significant sequence manipulations will affect methylation in the adjacent locus or

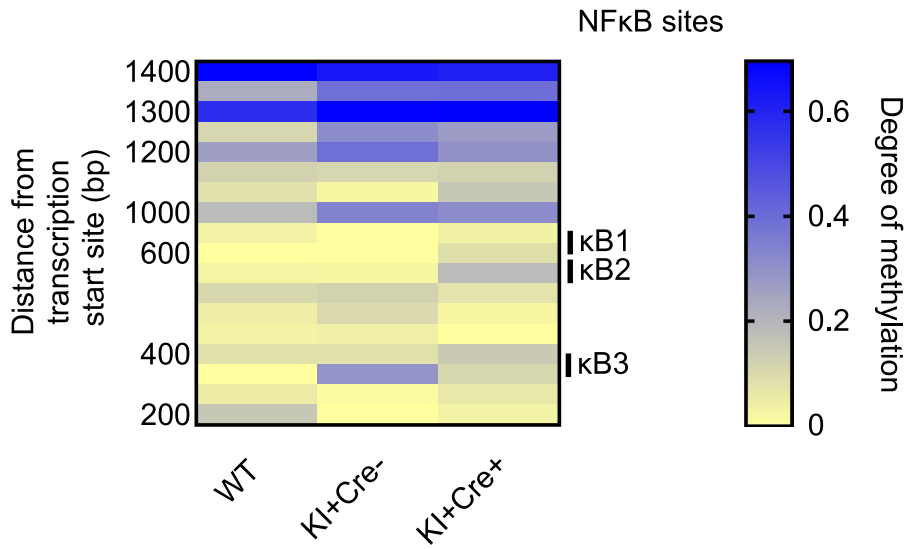


Fig. 5 c-Rel knock-in transgenic mice show increased CpG methylation in the *REL* promoter region. Genomic DNA was isolated from $CD4^+$ T cells in wildtype (WT) and transgenic mice before (KI^+Cre^-) and after (KI^+Cre^+) CD4-Cre mating. The degree of methylation at specific CpG sites was determined and was graphed relative to distance from the transcription start site and to NF-κB binding sites. Data is representative of n = 2 (WT), n = 2 (KI^+Cre^+), and n = 1 (KI^+Cre^-)

if there are other explanations for the increased DNA CpG methylation.

By inserting our transgene at the endogenous mouse *REL* locus, our model had the advantage of studying human c-Rel expression under the control of endogenous transcription factors and signaling pathways, offering increased translational relevance. This model contrasts with the insertion of the transgene at the commonly used *Rosa26* locus, which provides for more stable and constitutive expression of the transgene but lacks spatial and temporal control. Additionally, expression of transgenes at the *Rosa26* locus is not controlled by gene specific transcription factors and signaling pathways (Tratar et al. 2018). Nevertheless, the advantage of the endogenous locus was also possibly the downfall of our mouse model. Endogenous promoters with inserted transgenes can be more commonly affected by methylation and transcriptional repression compared to the *Rosa26* locus, which is not typically restricted by chromatin configuration (Chen et al. 2011).

Overall, our study demonstrates a novel design strategy for the development of knock-in transgenic mouse models where a particular human gene can be inserted at the endogenous mouse genomic locus of the same gene, in a cell-type specific manner. Our study also highlights potential challenges that may arise during this process and suggests that characterizing transgenic mouse models at the protein level during initial screening is of equal importance to genotyping for the desired DNA. It is possible that the observed increase in epigenetic silencing is a c-Rel locus specific response and gene insertions at other loci may not exhibit a similar negative regulation by methylation. The specific sequence of the inserted transgenic DNA downstream of the promoter may also influence and alter the methylation pattern at the upstream region. Since endogenous gene loci are under strict regulation to maintain homeostatic gene expression, these regions are expected to have minimal tolerance to accommodate foreign sequences. Hence, for genes where endogenous control of expression is not required, the insertion of the transgene may be considered at the commonly used *Rosa26* locus to achieve transgenic expression.

Directly studying human genes and their functions in a mouse model offers significant advantages for accelerating translational research. For example, transgenic mice expressing wildtype or mutated human *Npm1* at the *Rosa26* locus have been generated to study how *Npm1* mutations affect megakaryocyte differentiation and acute myeloid leukemia progression (Sportoletti et al. 2013). Similarly, 5xFAD mice, in which two human transgenes were inserted into the mouse genome under a neuron-specific promoter in a non-targeted manner (Oakley et al. 2006), are used to model amyloid plaque and neurofibrillary tangle formation, which aids in understanding how different genes may modulate neurodegeneration and in generating more translatable therapeutic options (Pádua et al. 2024). The therapeutic relevance of transgenic mouse models

expressing human genes was demonstrated with the FDA approval in 2023 of lecanemab, a monoclonal antibody that targets a soluble, neurotoxic form of amyloid-beta. The mouse monoclonal antibody form of this therapeutic was first validated and tested in transgenic ArcSwe APP (amyloid precursor protein) mice (Lannfelt 2023). These transgenic mice were generated with random integration of mutant human APP into the mouse genome under a neuron-specific promoter (Lord et al. 2006).

Transgenic mice that express human genes and that possess functional redundancy in the heterologous host will serve as relevant representative models of human disease. These mice will help to study disease etiology as well as the roles of any mutations involved in disease pathology. Moreover, having a mouse model expressing a human gene will allow direct functional testing of any drug's action against the human protein, avoiding concerns with potential differential effects on mouse and human proteins. Thus, our proposed strategy to develop a NOD mouse model conditionally expressing wildtype or mutant human c-Rel cDNA in a specific cell type to study their role in T1D, while retaining mouse c-Rel expression in the rest of the tissues, provides a universal concept to apply to study any human protein in relevant mouse disease models. Yet, the unexpected hindrance with lack of protein expression from the transgene highlights the need for in-depth molecular and functional analysis at early stages of any transgenic mouse model development to ensure the success of such an approach.

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Author contributions CNA, JTC, SS, RAC, and PR conceived and planned the experiments. CNA, JTC, SS, JH, and PR performed experiments and contributed to the interpretation of the results. CNA and PR wrote the original manuscript. All authors reviewed the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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