Gcg^{CreERT2} knockin mice as a tool for genetic manipulation in pancreatic alpha cells

Chiyo Shiota^{1,2} · Krishna Prasad¹ · Ping Guo¹ · Joseph Fusco¹ · Xiangwei Xiao¹ · George K. Gittes¹

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Abstract

Aims/hypothesis The Cre/loxP system, which enables tissue-specific manipulation of genes, is widely used in mice for diabetes research. Our aim was to develop a new Cre-driver mouse line for the specific and efficient manipulation of genes in pancreatic alpha cells.

Methods A *Gcg*^{CreERT2} knockin mouse, which expresses a tamoxifen-inducible form of Cre from the endogenous preproglucagon (*Gcg*) gene locus, was generated by homologous recombination. The new *Gcg*^{CreERT2} mouse line was crossed to the *Rosa26*^{tdTomato} (*R26*^{tdTomato}) Cre reporter mouse line in order to evaluate the tissue specificity, efficiency and tamoxifen dependency of *Gcg*^{CreERT2}-mediated recombination. Cell types of pancreatic islets were identified using immunohistochemistry. Biochemical and physiological data, including blood glucose levels, plasma glucagon and glucagon-like peptide (GLP)-1 levels, and pancreatic glucagon content, were collected and used to assess the overall effect of *Gcg* gene targeting on *Gcg*^{CreERT2/w} heterozygous mice.

Results Tamoxifen-treated *Gcg*^{CreERT2/w}; *R26*^{tdTomato/w} mice displayed Cre reporter activity, i.e. expression of tdTomato

red fluorescent protein (RFP) in all known cells that produce proglucagon-derived peptides. In the adult pancreas, RFP was detected in 94–97% of alpha cells, whereas it was detected in a negligible (~0.2%) proportion of beta cells. While more than 98% of cells labelled with tamoxifen-induced RFP were glucagon-positive cells, 14–25% of pancreatic polypeptide (PP)-positive cells were also positive for RFP, indicating the presence of glucagon/PP bihormonal cell population. Tamoxifen-independent expression of RFP occurred in approximately 6% of alpha cells. In contrast to alpha cells and GLP-1-producing neurons, in which RFP expression persisted for at least 5 months after tamoxifen administration (presumably due to rare neogenesis in these cell types in adulthood), nearly half of RFP-positive intestinal L cells were replaced with RFP-negative L cells over the first 2 weeks after tamoxifen administration. Heterozygous *Gcg*^{CreERT2/w} mice showed reduced *Gcg* mRNA levels in islets, but maintained normal levels of pancreatic and plasma glucagon. The mice did not exhibit any detectable baseline physiological abnormalities, at least in young adulthood.

Conclusions/interpretation The newly developed *Gcg*^{CreERT2} knockin mouse shows faithful expression of CreER^{T2} in pancreatic alpha cells, intestinal L cells and GLP-1-producing neurons. This mouse line will be particularly useful for manipulating genes in alpha cells, due to highly specific and efficient CreER^{T2}-mediated recombination in this cell type in the pancreas.

Keywords Cre/loxP · CreER^{T2} · Pancreatic alpha cells · Proglucagon

Abbreviations

BAC Bacterial artificial chromosome
E Embryonic day

✉ Chiyo Shiota
chiyo.shiota@vanderbilt.edu

✉ George K. Gittes
gittesgk@upmc.edu

¹ Division of Pediatric Surgery, Department of Surgery, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, 4401 Penn Ave, Pittsburgh, PA 15224, USA

² Present address: Department of Molecular Physiology and Biophysics, Vanderbilt University, 2215 Garland Ave, Nashville, TN 37232, USA

ES	Embryonic stem
FRT	Flippase recognition target
GLP	Glucagon-like peptide
iCre	Improved Cre
NST	Nucleus of the solitary tract
PP	Pancreatic polypeptide
PPG neuron	Preproglucagon neuron
qRT-PCR	Quantitative RT-PCR
RFP	Red fluorescent protein

Introduction

Pancreatic islet alpha cells are specialised to produce the glucagon that counteracts insulin for glucose homeostasis. Defects in insulin production and release by islet beta cells, insulin signalling to target organs or both are primary causes of diabetes mellitus. However, it has become evident that dysregulation of glucagon secretion by alpha cells also contributes to diabetes development and severity, indicating that alpha cells could also be a therapeutic target for better management of the disease [1, 2]. In addition, recent studies have suggested that alpha cells might be a potential source for the generation of new beta cells to cure diabetes [3–5].

The *Gcg* gene encodes preproglucagon, which consists of an N-terminal signal peptide and proglucagon. In the pancreas, the *Gcg* gene is only expressed in alpha cells. Outside the pancreas, the *Gcg* gene is expressed in intestinal L cells [6] and in a subset of neurons in the lower brain stem [7], most of which are in the nucleus of the solitary tract (NST) and some in the intermediate reticular nucleus [8, 9]. Tissue-specific differential processing of proglucagon yields glucagon in alpha cells, but yields glucagon-like peptide (GLP)-1 and GLP-2 in L cells and neurons. Intestinal GLP-1 is one of the incretins that are released after food intake and augment insulin secretion from beta cells, thereby lowering the blood glucose level [10]. GLP-1-producing NST neurons, so-called preproglucagon (PPG) neurons, project to multiple brain regions where GLP-1 receptors are expressed. This central GLP-1 controls neurological and cognitive functions, including appetite regulation and glucose homeostasis [11], and activation of PPG neurons reduces food intake and body weight in mice [9].

Mouse models have been extensively used in islet studies. The use of Cre/lox site-specific recombination systems, which allow cell-type-specific deletion or activation of genes by expressing Cre recombinase in distinct cell populations, has greatly enhanced our knowledge of islet biology, both in normal conditions as well as in the pathogenesis of diabetes. For genetic manipulation of alpha cells, the transgenic mouse line in which the Cre gene is expressed under the control of the 1.6 kb fragment of the rat *Gcg* gene promoter has been widely used over the years [12]. Previously, we have also generated *Gcg*-Cre transgenic mice using an 8 kb mouse *Gcg* promoter

and codon-optimised Cre (improved Cre; iCre) [13], and other groups have developed *Gcg*-Cre mice using a construct based on the bacterial artificial chromosome (BAC) [9, 14]. The transgenic approach is unable to control the insertion site and copy number of transgenes, leading to some unpredictability. A relatively low recombination rate in alpha cells has been reported in some studies with the rat *Gcg*-Cre mouse [15, 16], perhaps due to silencing of the transgene [17], while we noted off-target recombination in a large percentage of beta cells in our mouse *Gcg*-Cre mouse despite the use of a large 8 kb promoter [13]. Although not conclusive, it has been suggested that the *Gcg* gene is expressed at low levels in beta cells or their progenitors, and amplified *Gcg* promoter activity due to multiple copies of transgene produced enough Cre to cause recombination, even though endogenous *Gcg* promoter activity was low. In fact, gene expression analysis of single mouse beta cells has consistently demonstrated that beta cells express genes for other islet hormones at very low levels [18, 19].

Given the need for more precise manipulations of alpha cells, we developed an alternative Cre-driver mouse line that enables specific and efficient Cre-mediated recombination in alpha cells. To this end, we designed a new Cre-driver mouse with the following features: (1) use of the *Gcg* promoter to drive Cre expression in alpha cells to take advantage of its strong and specific activity in alpha cells within the pancreas, even though there will also be activity in GLP-1-producing cells; (2) use of a knockin strategy to express Cre under the control of endogenous regulatory elements for *Gcg* gene transcription; and (3) use of CreER^{T2}, a tamoxifen-inducible form of Cre, to reduce off-target recombination, which would more likely occur during embryogenesis and early postnatal development. Although we did not incorporate a bicistronic expression system into our knockin strategy due to concerns about CreER^{T2} expression levels, Ackermann et al recently developed *Gcg*^{IRE5-CreERT2} knockin mice that express both glucagon and CreER^{T2} from a targeted *Gcg* allele and demonstrated specific and efficient Cre-mediated recombination in alpha cells [20]. Here, we describe our new *Gcg*^{CreERT2} knockin mouse, with additional information regarding a *Gcg*^{iCre} knockin mouse that we also generated. Given the similarity of our *Gcg*^{CreERT2} knockin mice to those reported by Ackermann et al [20], the results from the extended characterisation of CreER^{T2} expression in our mice may be applicable to their mice, and thus would supplement their report.

Methods

Vector construct and gene targeting The BAC clone RP24-439G8, which harbours the *Gcg* gene locus of C57BL/6J mouse genomic DNA, was obtained from the BACPAC Resources Center at the Children's Hospital Oakland Research Institute (Oakland, CA, USA) and directly modified

to create a targeting vector using a *galK*-based BAC recombineering technique [21]. Briefly, a DNA sequence encoding the first 22 amino acids of preproglucagon in exon 2 was replaced with a CreERT² coding sequence followed by an SV40 polyA (pA) signal. A neomycin resistance gene (*neo*) expression cassette flanked by flippase recognition target (FRT) sites was inserted immediately after the CreERT²-pA sequence. The modified exon 2, along with 2 kb upstream and 7 kb downstream sequences, was retrieved into the PGKdtabpA plasmid (a gift from P. Soriano, Icahn School of Medicine at Mount Sinai, New York, NY, USA; Addgene plasmid no. 13440) [22] to add a diphtheria toxin A expression cassette to the end of 5' homology arm (Fig. 1a). The resulting targeting construct was electroporated into F1 hybrid (129S6/SvEvTac × C57BL/6Ncr)-derived G4 embryonic stem (ES) cells [23] to obtain gene-targeted ES cells. Correctly targeted ES cells were screened by long-range PCR using LA Taq DNA polymerase (Takara Bio, Mountain View, CA, USA) and primers specific to the targeted allele and to the outside of targeting sequence (Fig. 1b). Chimeric mice were generated using a morula aggregation method.

Plasmid and *E. coli* necessary for the BAC recombineering were a gift from Neal G. Copeland (National Cancer Institute, Frederick, MD, USA). CreERT² fragment was obtained from pCAG-CreERT2 (a gift from C. Cepko, Harvard Medical School, Boston, MA, USA; Addgene plasmid no. 14797) [24].

Mice Chimeric mice were bred with *Rosa26*^{FLPo} knockin mice (The Jackson Laboratory, Bar Harbor, ME, USA, stock no. 012930) to remove the neomycin resistance gene cassette

(Fig. 1c), and resulting *Gcg*^{CreERT2/w} mice were backcrossed to C57BL/6J mice (The Jackson Laboratory, stock no. 000664) for seven generations while collecting data for this study. The *Rosa26*^{tdTomato} Cre reporter (*R26*^{tdTomato}) strain was obtained from The Jackson Laboratory (stock no. 007909) and bred with *Gcg*^{CreERT2} mice to obtain double-mutant mice. Mouse genotypes were determined by PCR using DNA from tail biopsies. To induce Cre-mediated recombination, *Gcg*^{CreERT2} mice were treated with s.c. injection of tamoxifen dissolved in corn oil (10 mg/ml), using regimens indicated in the Results section.

Mouse colonies were maintained in a specific pathogen-free barrier facility in the Children's Hospital of Pittsburgh of UPMC (Pittsburgh, PA, USA). All mouse handling and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh, and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunohistochemistry Tissues were fixed by transcardiac perfusion of 4% paraformaldehyde in PBS for 10–15 min, followed by 6 h immersion in the same fixative. Overnight fixation was avoided to preserve fluorescence of dtTomato protein. Cryosections (6 μm) were used for all histological analyses. The primary antibodies used are listed in Table 1. These antibodies were validated using mouse tissues known to express and not to express the target protein. Secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). For staining with Cre antibody, an antigen retrieve was performed using a citrate-microwave method according to the manufacture's protocol.

Fig. 1 Gene targeting for *Gcg*^{CreERT2} knockin mice. **(a)** Schematic representation of the *Gcg* locus, targeting vector and targeted alleles before and after FLPo-mediated removal of the neomycin resistance gene cassette (*neo*). Primers (P1–4), used for screening of targeted ES cells, are shown under the *Gcg*^{CreERT2-neo} allele. Black boxes, *Gcg* coding regions; triangles, FRT sites. **(b)** PCR screening of targeted ES cells. Representative results of 5' and 3' PCR reactions in wild-type (–) or correctly targeted (+) ES cell clones are shown. M, size marker. **(c)** PCR genotyping of *Gcg*^{CreERT2-neo} and *Gcg*^{CreERT2} mice using primers specific to CreERT² and neomycin resistance gene

Table 1 Primary antibodies used for immunohistochemistry

Antibody	Dilution	Vendor	Catalogue no.
Rabbit anti-human E-cadherin	1:500	Cell Signaling, Danvers, MA, USA	3195
Rat anti-mouse E-cadherin	1:500	Life Technologies/Invitrogen, Grand Island, NY, USA	13-1900
Rabbit anti-Cre	1:1000	Millipore/Novagen, Billerica, MA, USA	69050-3
Rabbit anti-GLP-1	1:200	Phoenix, Burlingame, CA, USA	H-028-13
Rabbit anti-glucagon	1:2000	Millipore/Linco, Billerica, MA, USA	4030-01
Guinea pig anti-human insulin	1:1000	Millipore/Linco, Billerica, MA, USA	4011-01
Rabbit anti-mouse MafB	1:250	Bethyl, Montgomery, TX, USA	IHC-00351
Guinea pig anti-rat PP	1:2000	Millipore/Linco, Billerica, MA, USA	4041-01
Rabbit anti-human somatostatin	1:500	Agilent/Dako, Santa Clara, CA, USA	A0566

Blood glucose measurement and hormone assay Blood glucose levels were measured using an Accu-Chek glucose meter (Roche, Indianapolis, IN, USA). Plasma glucagon and total GLP-1 levels were determined with glucagon RIA and a total GLP-1 ELISA kit (Millipore, Billerica, MA, USA), respectively. Pancreatic glucagon was measured as previously described [13]. All assays were performed in duplicate.

Islet isolation, cell sorting and gene expression analyses

For islet isolation, pancreas was digested by intraductal injection of Clzyme RI enzyme solution (VitaCyte, Indianapolis, IN, USA), followed by 15 min incubation at 37°C [25]. Islets were hand-picked after a density-gradient purification using Histopaque (Sigma-Aldrich, St Louis, MO, USA) [26] and dispersed into single cells in Accumax solution (Stemcell, Cambridge, MA, USA). Resulting single-cell populations were sorted on a FACS Aria cell sorter (BD Biosciences, San Jose, CA, USA) to isolate tdTomato red fluorescent protein (RFP)-positive and -negative cells. RNA was extracted from the isolated islets or the sorted cells using an RNeasy Mini kit (Qiagen, Hilden, Germany) or an Arcturus PicoPure RNA isolation kit (Applied Biosystems, Waltham, MA, USA), and cDNA was synthesised using a QuantiTect Reverse Transcription Kit (Qiagen). Gene expression was measured by real-time PCR using QuantiTect primers (Qiagen) and iTaq SYBR Green mix (Bio-Rad, Hercules, CA, USA). cDNA samples were run in duplicate.

Statistical analysis Data are expressed as means $\pm$ SD. Statistical significance was assessed with an unpaired two-tailed Student *t* test or two-way ANOVA, and $p < 0.05$ was considered statistically significant. The experimenters were not blind to group assignment and no data were omitted.

Results

Alpha cell-specific expression of CreER^{T2} in *Gcg*^{CreERT2} mouse islets We first examined CreER^{T2} expression in

Gcg^{CreERT2/w} mouse pancreas by immunohistochemistry using anti-Cre antibody. Since it is difficult to detect CreER^{T2} protein when the protein is distributed diffusely throughout the cytoplasm, *Gcg*^{CreERT2/w} mice were treated with daily injection of 1 mg tamoxifen for 4 days at 2–3 weeks of age to accumulate CreER^{T2} proteins in the nucleus. Pancreases were fixed 1 day after the last tamoxifen injection and processed for immunohistochemistry. Cre staining was observed in glucagon-positive cells, but was hardly detected in insulin-positive cells in these mice (Fig. 2a). Because the gene targeting was designed to substitute CreER^{T2} expression for preproglucagon expression, homozygous *Gcg*^{CreERT2/CreERT2} mice were expected to be deficient in proglucagon-derived peptides. To confirm this, we generated *Gcg*^{CreERT2/CreERT2} mice and examined their islet morphology. Indeed, there was no glucagon staining observed. In addition, *Gcg*^{CreERT2/CreERT2} mouse islets displayed the expected hyperplasia of Cre-positive cells that were identified as alpha cells by positive staining for MafB (Fig. 2b). Alpha cell hyperplasia is the typical phenomenon in glucagon or glucagon receptor deficiency. Thus, we verified that the *Gcg*^{CreERT2} allele functions as expected.

Tamoxifen-induced recombination in *Gcg*^{CreERT2} mouse alpha cells To assess tissue specificity and efficiency as well as the tamoxifen dependency of recombination mediated by CreER^{T2} in *Gcg*^{CreERT2} mice, we bred *Gcg*^{CreERT2} mice with *R26*^{tdTomato} Cre reporter mice. The *R26*^{tdTomato} mouse expresses tdTomato RFP when Cre excises the transcription stop cassette. Because of robust expression of the tdTomato gene through the CAG promoter and exceptional brightness of tdTomato fluorescence, RFP-positive cells were easily identified on sections subjected to immunostaining. To examine CreER^{T2} activity in embryonic stages, pregnant female mice were injected with 1 mg tamoxifen at 11.5 days postcoitum for embryonic day (E)13.5 pancreases or at 14.5 days postcoitum for E16.5 pancreases. Double staining for glucagon and insulin on *Gcg*^{CreERT2/w}; *R26*^{tdTomato/w} pancreatic sections revealed that more than 60% of glucagon-positive cells expressed RFP on E13.5 (Fig. 3a), and that RFP expression expanded to more

Fig. 2 Cre expression in *Gcg^{CreERT2}* mice. **(a)** Triple immunostaining for Cre, insulin and glucagon on *Gcg^{w/w}* and *Gcg^{CreERT2/w}* mouse pancreatic sections. Cre staining is merged with glucagon staining or insulin staining. DAPI nuclear staining is shown in blue. Scale bars, 50 µm. **(b)** Immunostaining for Cre and MafB on *Gcg^{CreERT2/CreERT2}* mouse pancreatic sections. Merged images with co-stained insulin (green) and DAPI (blue) are shown to the right. Scale bars, 50 µm

than 80% of glucagon-positive cells on E16.5 (Table 2). At E13.5, about 10% of insulin-positive cells were RFP-positive, and most were also positive for glucagon staining. It is known that co-expression of glucagon and insulin occurs in pancreatic endocrine cells at early developmental stages, but not in late stages [12, 27]. Consistently, we observed that fewer than 1% of insulin-positive cells were labelled with RFP in E16.5 pancreases (Table 2).

We next examined CreER^{T2}-mediated recombination in adult mice. At 5 weeks of age, *Gcg^{CreERT2/w};R26^{tdTomato/w}* mice were randomised into groups receiving either three doses of 1 mg tamoxifen or vehicle over a week, and pancreases were fixed at 2 or 5–6 months of age. Pancreatic sections were double-stained for glucagon and insulin or for pancreatic polypeptide (PP) and somatostatin. Although some RFP-positive cells were seen in the islets of vehicle-injected mice at both ages examined, a dramatic increase in RFP expression was observed among alpha cells in tamoxifen-injected mice at 2 months of age, and the pattern of RFP expression was maintained to 5–6 months of age (Fig. 3b). Quantitative analysis further demonstrated an efficient induction of RFP expression by tamoxifen in alpha cells (> 90%), with low frequency of

tamoxifen-independent ('leaky') RFP expression (< 6%) in this cell type (Table 2). Importantly, more than 98% of RFP-positive cells were alpha cells, indicating that CreER^{T2}-mediated recombination occurred almost exclusively in alpha cells. Consistently, RFP expression in beta and delta cells was negligible. However, a substantial proportion of PP cells (10–25%) were labelled with RFP (Table 2). Since only a few RFP-positive cells were negative for glucagon staining ($1.2 \pm 0.7\%$ and $1.1 \pm 0.5\%$ of total RFP-positive cells at 2 and 5–6 months of age, respectively), this indicates that some alpha cells stained positively with anti-PP antibody. Indeed, double staining for glucagon and PP demonstrated the co-existence of both hormones in some cells (Fig. 3c), which is consistent with previous immunohistochemical studies on rat [28] or mouse [19] islets.

In addition to morphological assessments, expression of *Gcg* and *Ins2* genes was measured by quantitative RT-PCR (qRT-PCR) in flow-sorted RFP-positive and -negative islet cells (Fig. 3d). *Gcg* gene expression was detected nearly exclusively in RFP-positive cells (Fig. 3e), whereas *Ins2* gene expression was detected solely in RFP-negative cells, as expected (Fig. 3f). Collectively, the data confirmed that recombination of the floxed target sequence is tamoxifen-dependent and highly specific to alpha cells in *Gcg^{CreERT2}* mouse pancreases.

Recombination by *Gcg^{CreERT2}* in extrapancreatic proglucagon-producing cells Besides pancreatic alpha cells, the *Gcg* gene is expressed in intestinal L cells and some neurons in the brain stem in which proglucagon is processed to GLP-1. In contrast to pancreatic alpha cells, the population of which is normally maintained by replication of existing alpha cells, L cells are constantly replenished by neogenesis from intestinal progenitor cells, and thus the population of labelled cells by an inducible Cre is assumed to diminish as time passes after tamoxifen exposure. For this reason, we examined intestine obtained from 3- to 4-month-old *Gcg^{CreERT2/w};R26^{tdTomato/w}* mice that were treated with tamoxifen at 2–5 days, 2 weeks or 3 months before harvesting tissue. RFP-positive cells were occasionally observed in the epithelium of small intestine 2–5 days after tamoxifen treatment (Fig. 4a), and these cells stained positively with anti-GLP-1 antibody, indicating that CreER^{T2}-mediated recombination occurred in L cells (Fig. 4b). Quantitative analysis revealed that $80.7 \pm 3.2\%$ of GLP-1-positive cells were RFP positive at 2–5 days after tamoxifen treatment. The proportion of RFP-expressing cells in the GLP-1-positive cell population decreased to $42.9 \pm 12.2\%$ and to only $2.6 \pm 2.4\%$ at 2 weeks and 3 months after tamoxifen administration, respectively. The analysis also showed that $11.1 \pm 8.8\%$ and $21.1 \pm 7.8\%$ of RFP-positive cells were negative for GLP-1 staining at 2–5 days and 2 weeks after tamoxifen treatment, respectively. It remains to be determined if these cells were L cells that had ceased GLP-1 production, or were perhaps other types of enteroendocrine cells.

Fig. 3 CreERT²-mediated recombination in *Gcg*^{CreERT2/w};*R26*^{tdTomato/w} mice. **(a)** Detection of tdTomato epifluorescence (red) and co-immunostaining for glucagon (green) and insulin (blue) in *Gcg*^{CreERT2/w};*R26*^{tdTomato/w} embryonic pancreas at E13.5. Scale bars, 50 μ m. **(b)** Detection of tdTomato epifluorescence (red) and immunostaining for glucagon (green) on 2-month-old (2M) and 5-month-old (5M) non-treated (–) or tamoxifen (Tam)-treated (+) *Gcg*^{CreERT2/w};*R26*^{tdTomato/w} mouse pancreatic sections. DAPI nuclear staining is shown in blue. Scale bars, 50 μ m. **(c)** Detection of tdTomato epifluorescence (red) and co-immunostaining for glucagon (green) and PP (blue) on a 5-month-old tamoxifen-treated *Gcg*^{CreERT2/w};*R26*^{tdTomato/w} mouse pancreatic section. Arrows indicate an RFP-positive cell stained with both glucagon and PP antibodies. Scale bars, 20 μ m. **(d)** Representative flow plot showing clear separation of tdTomato RFP-positive cells (red) from RFP-negative cells (purple) by FACS. **(e, f)** qRT-PCR analysis of gene expression in tdTomato RFP-positive and -negative cell populations. Relative expression levels of *Gcg* gene **(e)** and *Ins2* gene **(f)** to *Ppia* gene are shown. $n = 3$. Data are means $\pm$ SD

2–3 months of age. Measurements of gene expression in islets by qRT-PCR demonstrated a 60% reduction in *Gcg* mRNA levels but no change in *Ins2* mRNA levels in *Gcg*^{CreERT2/w} mice compared with wild-type mice (Fig. 5a, b). However, pancreatic glucagon content and plasma glucagon levels were normal in *Gcg*^{CreERT2/w} mice, indicating that glucagon net content is maintained at normal levels by post-transcriptional mechanisms in the presence of *Gcg* gene heterozygosity (Fig. 5c, d). In addition, plasma total GLP-1 levels were not affected by disruption of the *Gcg* gene in the *Gcg*^{CreERT2} allele (Fig. 5e). Consistently, there were no differences in body weight or blood glucose levels in either the fed or fasted conditions between *Gcg*^{w/w} and *Gcg*^{CreERT2/w} mice (Fig. 5f, g).

***Gcg*^{iCre} knockin mice with an unexpected duplication mutation of the *Gcg* locus** We generated *Gcg*^{iCre} knockin mice using the same strategy as the *Gcg*^{CreERT2} mice, with the only difference being insertion of an iCre coding sequence instead of a CreERT² sequence. While the iCre sequence was knocked into the *Gcg* gene as we designed, subsequent analysis of *Gcg*^{iCre/CreERT2} hemizygous mice, in which we observed glucagon expression, indicated that the targeted allele still carried an intact *Gcg* gene, and that a duplication mutation had occurred in the *Gcg*^{iCre} mouse line, most likely during homologous recombination for gene targeting in ES cells. To determine the structure of the duplication, we performed a long-range PCR analysis of *Gcg*^{iCre/CreERT2} mouse genomic DNA using primers specific to iCre or the 3' portion of exon 2, which does not exist in the *Gcg*^{iCre} or *Gcg*^{CreERT2} gene, in combination with various primers for upstream and downstream of the *Gcg* gene. The results revealed that the duplicated region included all exons of the *Gcg* gene, which spans 9 kb, and extended upstream for at least 17 kb and downstream for at least 7.8 kb. The 3' portion of the adjacent *Fap* gene, which is located upstream of *Gcg* and encodes fibroblast activation protein, was involved in the duplication. Due to such a large size of the duplication, we could not determine

In the brain, RFP expression was detected in neurons of the NST, but not in other areas (Fig. 4c). As expected, RFP-positive neurons were still seen 5 months after tamoxifen injection.

Normal physiology in *Gcg*^{CreERT2/w} mice An effect of haploinsufficiency on phenotype has not been reported in global *Gcg* null mutant mice. To confirm that our *Gcg*^{CreERT2} mice also do not have any phenotypical changes due to heterozygosity, we collected biochemical and physiological data from wild-type *Gcg*^{w/w} and *Gcg*^{CreERT2/w} mice at

Table 2 RFP labelling in *Gcg^{CreERT2/w};R26^{tdTomato/w}* mouse islet cells

Age	E13.5	E16.5	2–3 months		5–6 months	
Tamoxifen	+	+	–	+	–	+
Alpha cells (%)	64.9 ± 5.4	81.8 ± 6.8	6.1 ± 0.2	93.7 ± 6.0	5.8 ± 1.5	96.6 ± 1.5
Beta cells (%)	10.1 ± 0.8	0.9 ± 0.4	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.2	0.2 ± 0.1
PP cells (%)	ND	ND	2.6 ± 0.2	13.8 ± 6.3	2.2 ± 1.5	24.9 ± 4.4*
Delta cells (%)	ND	ND	0.4 ± 0.3	2.2 ± 1.5	1.6 ± 0.2	2.3 ± 2.2

Data are means ± SD for four mice

RFP expression was assessed by counting 100 glucagon-positive (alpha) cells and 50 insulin-positive (beta) cells in E13.5 embryos, and 400 alpha cells and 400 beta cells in E16.5 embryos. For adult mice, 300 alpha cells, 1000 beta cells, 200 PP-positive cells and 200 somatostatin-positive (delta) cells were counted in each animal

* $p < 0.05$ compared with 2- to 3-month-old mice

ND, not determined

duplication junction points or the relative position of the *Gcg^{iCre}* gene to the *Gcg* gene. Despite the lack of full information, we thought it was worthwhile to characterise this mouse line because the *Gcg^{iCre}* gene has at least 17 kb upstream sequence of the *Gcg* gene.

Immunohistochemical examination of *Gcg^{iCre/w};R26^{tdTomato/w}* mice at 3 weeks and 2 months of age demonstrated that most alpha cells were labelled with RFP (Fig. 6a, Table 3). While quantitative analysis showed no difference in the labelling efficiency in alpha cells between *Gcg^{iCre/w}* and tamoxifen-treated *Gcg^{CreERT2/w}* mice, slight but significant increases in RFP expression were observed in beta and PP cells in *Gcg^{iCre/w}* mice compared with *Gcg^{CreERT2/w}* mice ($p < 0.0001$ in beta cells and $p = 0.0006$ in PP cells, two-way ANOVA). Consistently, 3–6% of RFP-positive cells were negative for glucagon staining in *Gcg^{iCre/w}* mouse pancreases.

In the intestine, RFP expression was observed in L cells that were marked with GLP-1 staining (Fig. 6b). RFP-negative L cells were often seen in crypts, indicating a time

lag between GLP-1 expression and RFP expression in newly formed L cells, with the latter requiring iCre expression and recombination of the *R26^{tdTomato}* gene prior to RFP expression. In contrast to *Gcg^{CreERT2}* mice, non-specific RFP expression was widely seen in the brain in *Gcg^{iCre/w};R26^{tdTomato/w}* mice (Fig. 6c, d). RFP-labelled cells often formed large clusters, suggesting that the recombination of the *R26^{tdTomato}* gene occurred in early developmental stages with subsequent clonal expansion (Fig. 6d).

Discussion

While we and Ackermann et al [20] used a knockin strategy to generate new *Gcg^{CreERT2}* or *Gcg^{iCre}* mouse lines, two other groups have established new *Gcg*-Cre mouse lines by means of BAC transgenesis and successfully applied them to gene manipulation in L cells [14] or PGP neurons [9]. BAC transgenes include long flanking sequences on both sides of

Fig. 4 CreER^{T2}-mediated recombination in 2-month-old *Gcg^{CreERT2/w};R26^{tdTomato/w}* mouse intestine and brain. **(a)** Jejunum section showing tdTomato epifluorescence (red) and immunostaining for E-cadherin (green). DAPI nuclear staining is shown in blue. Scale bar, 50 µm. **(b)** Jejunum section showing tdTomato epifluorescence (red) and

co-immunostaining for GLP-1 (green) and E-cadherin (blue). Scale bar, 50 µm. **(c)** Brain section showing tdTomato-positive neurons in the NST. The area in the small square is shown at high magnification in the inset. Scale bar, 200 µm

Fig. 5 Biochemical and physiological variables in 2- to 3-month-old *Gcg^{CreERT2/w}* mice. (a, b) qRT-PCR analysis of gene expression in isolated islets. Relative expression levels of *Gcg* gene (a) and *Ins2* gene (b) to *Ppia* gene are shown. $n = 3$. * $p < 0.05$, unpaired two-tailed Student *t* test. (c) Pancreatic glucagon content. (d) Plasma glucagon levels. (e) Plasma total GLP-1 levels. (f) Body weight. (g) Blood glucose levels. White circles, *Gcg^{w/w}*; black squares, *Gcg^{CreERT2/w}*. Data represent individual values and means $\pm$ SD

the Cre inserted into the *Gcg* gene, and thereby confer better control of *Gcg*-Cre gene expression by *cis* elements similar to

the endogenous *Gcg* gene. Nevertheless, it was reported that approximately 20% of beta cells were labelled with Cre reporter in one of the BAC transgenic lines [29]. This is in contrast to our two new mouse models, in which activation of Cre reporter in beta cells was negligible ($< 1\%$), even in the *Gcg^{iCre}* mice in which the targeted *Gcg* gene locus was disarranged by a duplication mutation.

Upon tamoxifen injection, the *Gcg^{CreERT2}* mice displayed Cre reporter activation specifically in cells that express preproglucagon in adult mice. However, labelling NST neurons by the reporter seemed less effective compared with a recent study using BAC *Gcg*-Cre mice [9]. A further study is

Fig. 6 Cre-mediated recombination in 2-month-old *Gcg^{iCre/w};R26^{tdTomato/w}* mice. (a) Detection of tdTomato epifluorescence (red) and co-immunostaining for glucagon (green) and insulin (blue) on a pancreatic section. Scale bar, 50 μ m. (b) Jejunum section showing tdTomato epifluorescence (red) and co-immunostaining for GLP-1 (green) and E-cadherin (blue). Cells positive for RFP and GLP-1 (arrowheads) and positive for GLP-1 but not for RFP (arrows) are seen. Scale bar, 50 μ m. (c, d) Detection of tdTomato epifluorescence (red) and staining for myelin (green) on brain sections, including the region of the hypothalamus (c) and the brain stem (d)

Table 3 RFP labelling in *Gcg^{iCre/w};R26^{tdTomato/w}* mouse islet cells

Cell type (%)	Age	
	3 weeks	2 months
Alpha cells	95.2 $\pm$ 3.2	93.9 $\pm$ 1.2
Beta cells	0.6 $\pm$ 0.1	0.92 $\pm$ 0.3
PP cells	37.9 $\pm$ 5.8	24.5 $\pm$ 2.3*
Delta cells	3.3 $\pm$ 1.1	4.5 $\pm$ 4.2

Data are means $\pm$ SD for four mice

RFP expression was assessed by counting 500 glucagon-positive (alpha) cells, 1000 insulin-positive (beta) cells, 200 PP-positive cells and 200 somatostatin-positive (delta) cells in each animal

* $p < 0.05$ compared with 3-week-old mice

required to determine the efficiency of Cre-mediated recombination in the *Gcg*^{CreERT2} mouse brain, but it is possible that induction of recombination by tamoxifen is weaker in the brain than in other organs since the penetration of 4-hydroxytamoxifen, the primary active tamoxifen metabolite, into the brain is partially restricted by the P-glycoprotein (ABCB1) transporter [30]. In contrast, the efficiency of Cre reporter activation in *Gcg*^{CreERT2} mouse L cells was similar to that in the BAC *Gcg*-iCre mice [14]. Consistent with previous estimates of the turnover time of enteroendocrine cells using ³H-thymidine labelling [31], half of the Cre reporter-positive L cells disappeared during the first 2 weeks after tamoxifen treatment, indicating that the effect of genetic manipulation on L cells does not last longer in this inducible system. Taken together, our *Gcg*^{CreERT2} mouse favours studies on alpha cells, although each study must give careful consideration to the possible effects of Cre-mediated gene modifications in extrapancreatic sites.

The *Gcg*^{IREs-CreERT2} mice reported by Ackermann et al express both preproglucagon and CreER^{T2} as two independent proteins from a single *Gcg*-IREs-CreERT2 mRNA [20]. The *Gcg*^{iCre} mice described here express preproglucagon in addition to Cre from the targeted allele due to a duplication mutation. In contrast, our *Gcg*^{CreERT2} mice are heterozygous null mutants for the *Gcg* gene. Although we did not detect any physiological abnormalities in the current analysis, *Gcg* heterozygosity may affect the functionality of preproglucagon-producing cells in extreme conditions or during ageing. Therefore, including *Gcg*^{CreERT2} mice as a control is crucial.

The lineage labelling analysis of the *Gcg*^{CreERT2} and *Gcg*^{iCre} mouse islets showed that 14–25% of PP-positive cells co-expressed glucagon in adult animals. This result is comparable with findings from a recent study that identified glucagon/PP double-positive cells (16% of PP-positive cells) by RNA fluorescence in situ hybridisation analysis of mouse islet cells [19]. Our data showed that the size of this bihormonal cell population was relatively stable over the experimental period, suggesting the presence of a subpopulation of alpha cells. Lineage analysis using PP cell-specific Cre might determine if this subpopulation is an independent lineage from the majority of alpha cells that are negative for PP.

In conclusion, we describe a basic characterisation of a new *Gcg*^{CreERT2} knockin mouse. The data demonstrate that this mouse line is a useful tool, particularly for studies on pancreatic alpha cells. In addition to *Gcg*^{CreERT2} knockin mice, we report a new *Gcg*^{iCre} knockin mouse that also displayed specific and efficient recombination in alpha cells. However, it should be noted that the *Gcg*^{iCre} knockin mouse line is not adequate for some studies due to widespread Cre lineage labelling in the brain.

The mouse strains reported here are available at The Jackson Laboratory as stock no. 030681 for *Gcg*^{CreERT2} mice and no. 030663 for *Gcg*^{iCre} mice.

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