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## Generation of mice encoding a conditional allele of *Nkx2.2*

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### Abstract

*Nkx2.2* is a homeobox transcription factor that is expressed in the pancreas, intestine and central nervous system (CNS) during embryogenesis and in the adult. In mice, global deletion of *Nkx2.2* results in cell mis-specification in each of the tissues where it is expressed, and the null mice die as neonates with severe hyperglycemia. Although, a whole body knockout demonstrates the importance of *Nkx2.2* in cell specification and postnatal viability, it precludes assessment of the cell-autonomous and postnatal functions of *Nkx2.2*. In this study we report the generation and functional characterization of mice encoding a conditional allele of *Nkx2.2*. We demonstrate the functional integrity of the conditional *Nkx2.2* allele and report successful *in vivo* deletion using a pancreas-specific Cre recombinase. The pancreas-specific deletion of *Nkx2.2* results in similar defects found in the *Nkx2.2* null pancreas and the mice die shortly after birth, demonstrating that the neonatal lethality associated with the null allele is caused by pancreatic islet dysfunction. The generation of a conditional *Nkx2.2* allele provides an important tool for identifying the cell-autonomous and postnatal functions of *Nkx2.2* in establishing and maintaining cell type identities and functions in the pancreas, intestine and CNS.

### Keywords

*Nkx2.2*; Cre-lox; pancreas; cell-specific knock-out

### Introduction

*Nkx2.2* is a homeodomain-containing transcription factor expressed in the pancreas, intestine and central nervous system (CNS) (Arnes et al. 2012). In the pancreas, *Nkx2.2* is expressed throughout the epithelium of the developing pancreatic buds, beginning at embryonic day (E) 8.75 (Jorgensen et al. 2007). As pancreatic development proceeds, *Nkx2.2* remains expressed in a population of ductal cells, as well as specific endocrine cell subtypes. In the postnatal pancreas, *Nkx2.2* becomes restricted to insulin-producing beta cells, glucagon-producing alpha cells, ghrelin-producing epsilon cells, and pancreatic polypeptide-producing PP cells (Arnes et al. 2012; Prado et al. 2004; Sussel et al. 1998).

Global deletion of *Nkx2.2* in the mouse results in dramatic alterations to the complement of hormone-producing islet cells in the pancreas. Specifically, beta cells are absent, alpha and PP cells are reduced, and epsilon cells are concomitantly increased (Prado et al. 2004; Sussel et al. 1998). As a result of these defects, the mice are hyperglycemic at birth and die within the first week of postnatal life. In addition to the pancreatic defects, cell specification in the intestine and CNS are also affected by deletion of *Nkx2.2*. Similar to the pancreas, intestinal

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enteroendocrine cells are formed in altered ratios; ghrelin cells are dramatically increased with a corresponding decrease in many of the other enteroendocrine populations, including those expressing serotonin, somatostatin and GIP (Desai et al. 2008). Moreover, several neuronal cell populations are re-specified in the developing CNS (Briscoe et al. 1999; Qi et al. 2001). Due to the early postnatal lethality associated with the *Nkx2.2* null allele, we were unable to determine the physiological consequences of the intestinal and CNS phenotypes. Furthermore, the early lethality associated with these mice precluded analysis of any postnatal functions of *Nkx2.2* in each of the tissues where it is expressed.

To determine the tissue-specific and postnatal functions of *Nkx2.2*, we have used a gene targeting approach to generate *Nkx2.2* conditional knockout mice. We have determined that the inducible allele does not disrupt normal *Nkx2.2* function. Moreover, when the *Nkx2.2<sup>flox</sup>* allele is homozygous and combined with *Pdx1-cre* deleter mice (Hingorani et al., 2003), *Nkx2.2* is efficiently deleted in the pancreas and the *Nkx2.2* null islet phenotype is recapitulated. Furthermore, these mice die with hyperglycemia shortly after birth, confirming that the early postnatal lethality is primarily caused by the pancreatic defects.

## Materials and Methods

### Generation of mice encoding a conditional allele of *Nkx2.2*

A loxP site (L83) and a FNFL cassette (FRT-Neo-FRT-LoxP) were engineered to flank exon 2 (E2) of the *Nkx2.2* gene to generate the *Nkx2.2* conditional targeting construct on a Bacterial Artificial Chromosome (BAC). The gene targeting vector was generated by retrieving a 10kb long homology arm (5' end to L83), floxed sequence containing exon 2, FNFL cassette, and 2kb short homology arm (end of FNFL to 3' end) into a vector carrying the Diphtheria toxin alpha chain (DTA) negative selection marker. The FNFL cassette conferred G418 resistance during gene targeting in PTL1 ES cells (129/B6 hybrid; Precision Targeting Lab, LLC), and the DTA cassette provided an autonomous negative selection to reduce random integration events during gene targeting. Five targeted ES cells were identified by PCR screening and clone 9G was injected into C57BL/6 blastocysts to generate chimeric mice. Two chimeric males conferred germline transmission. Male chimeras were bred to *B6;SJL-Tg(CTFLPe)9205Dym/J (ACTB<sup>FLPe/FLPe</sup>*; (Rodriguez et al. 2000)) female mice on a C57BL/6 background to both transmit the floxed *Nkx2.2* allele through the germline and to simultaneously remove the Neomycin cassette. Two founder lines of mice carrying the *Nkx2.2* conditional allele (*Nkx2.2<sup>flox</sup>*) were bred to determine the affect of homozygosity of the conditional allele.

Mice carrying the conditional allele were homozygous by mating *Nkx2.2<sup>flox/+</sup>* animals, producing *Nkx2.2<sup>flox/flox</sup>* mice. Both male and female heterozygous animals were followed from birth through 12 weeks of age and subjected to metabolic study. *Nkx2.2<sup>flox/+</sup>* mice were also mated with *Tg(Ipfl-cre)1Tuv (Pdx1-cre*; (Hingorani et al. 2003), and subsequently maintained as *Nkx2.2<sup>flox/+</sup>;Pdx1-cre* on an outbred Black Swiss background (NTac:NIHBS, Taconic). To generate mutant animals, *Nkx2.2<sup>flox/+</sup>;Pdx1-cre* animals were mated with either *Nkx2.2<sup>flox/+</sup>;Pdx1-cre* or *Nkx2.2<sup>flox/flox</sup>* animals. *Nkx2.2<sup>flox/flox</sup>;Pdx1-cre* mutants and littermate controls were assessed at E15.5 and postnatal day (P) 1. All experiments involving mice were approved by the Columbia University Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health guidelines for the care and use of animals.

For genotyping, a portion of embryonic or postnatal tail was removed, digested with proteinase K, and DNA extracted. Previously described conditions and primers were used to genotype for *Pdx1-cre* (Hingorani et al. 2003). Genotyping for the *Nkx2.2<sup>flox</sup>* allele was carried out using two sets of primers: L83-forward: 5 -

TCCTTTTAAAAATCTGCCACGTCT-3 , L83-reverse: 5 -  
 GGATTTGGAGCTCGAGTCTTGG-3 ; FL146-forward 5 -  
 GGGTTATCCAGACAGTGGAGGAGTG-3 , FL146-reverse 5 -  
 GAGGTCAACTAGGCCTCAACTTGGT-3 . An initial denaturation at 94C for 5min was used, followed by 35 cycles of 94C for 30sec, 60C for 30sec, 72C for 1min, and a final extension of 72C for 5 . The L83 primer set produced two possible amplification products of 397bp (wildtype) and 480bp (*Nkx2.2<sup>flox</sup>*); the FL146 primer set produced two possible amplification products of 420bp (wildtype) and 566bp (*Nkx2.2<sup>flox</sup>*).

### Metabolic analyses

Animals were maintained on a normal chow diet, and body weight was measured weekly using a standard scale. Blood glucose measurements were obtained with an Accu-Check Compact Plus glucose monitor (Model GT; Roche). Glucose tolerance tests (GTT) were performed after an overnight fast, as previously described (Banks et al. 2011).

### Immunofluorescence

Immunofluorescence was performed according to previously published protocols, on pancreas sections from E15.5 embryos and postnatal pancreas tissue (P0 and P14) (Mastracci et al.; Mastracci et al. 2011). In all cases, tissue was embedded in OCT, after fixation with 4% PFA and cryopreservation in 30% sucrose. Frozen sections (8µm) were cut and mounted on glass slides. Sections were stained with goat -ghrelin (1:800; Santa Cruz), guinea pig -glucagon (1:1000; Linco/Millipore, MA), guinea pig -insulin (1:1000; Millipore), rabbit -insulin (1:1000; Cell Signaling Technology), rabbit -somatostatin (1:200; Phoenix Pharmaceuticals), rabbit -pancreatic polypeptide (1:200; Zymed) and rabbit -amylase (1:1000; Sigma). Donkey -guinea pig-Cy2, -Cy3 or -Cy5, -rabbit-Cy2 or -Cy3, and -goat Cy2 or -Cy5 secondary antibodies were used (1:400, Jackson ImmunoResearch). DAPI (1:1000; Invitrogen) was applied for 30 minutes following secondary antibody incubation. Images were acquired on a Leica DM5500.

### RNA in situ hybridization

RNA *in situ* hybridization was performed on 8µm sections mounted on glass slides as previously described (Mastracci et al.) using an antisense riboprobe transcribed from a linearized plasmid containing the full-length *Nkx2.2* cDNA (Hartigan and Rubenstein 1996). RNA *in situ* hybridization was performed on pancreas tissue sections from *Nkx2.2<sup>flox/+</sup>*, *Nkx2.2<sup>flox/flox</sup>* and *Nkx2.2<sup>flox/flox</sup>;Pdx1-cre* at E15.5.

### Real time PCR

Pancreas was dissected from each embryo and stored in RNAlater (Ambion) until RNA was extracted using the NucleoSpin RNAII Kit (Clontech). Subsequently, cDNA was made with equal amounts of RNA for each sample (Superscript III Kit, Invitrogen, CA). Real time PCR was performed using TaqMan gene expression assays-on-demand (Applied Biosystems) for *glucagon* (Mm00801712\_m1), *ghrelin* (Mm00445450\_m1), *insulin1* (Mm01950294\_s1), *insulin2* (Mm00731595\_gH), *pancreatic polypeptide* (Mm00435889\_m1) and *somatostatin* (Mm00436671\_m1). *CyclophilinB* was used as a control housekeeping gene, and was assayed using a probe and primer set previously described (Anderson et al. 2009). *Nkx2.2* was also assayed with a probe and primer set previously described (Anderson et al. 2009). Statistical analyses were conducted with Prism Software (GraphPad Software, La Jolla, CA) using a standard t-test.

## Results and Discussion

To generate a conditional allele of *Nkx2.2*, loxP sites were introduced into the *Nkx2.2* genomic locus, flanking exon 2. The 5' loxP site was positioned within intron 1 and the 3' loxP site and Neomycin cassette were inserted into a non-conserved region of the 3' UTR (Figure 1a). The construct was electroporated into 129/B6 hybrid PTL1 ES cells. 192 colonies were screened by PCR, using primers from within the construct sequence to a genomic region located outside the sequences included in the construct (Figure 1b). Five positive *Nkx2.2<sup>lox</sup>*-targeted ES clones were identified and clone 9G was used to generate mice. Mating of chimeras for germline transmission and removal of the Neomycin cassette occurred simultaneously using mice expressing FLP recombinase on a B6 background (*B6;Sjl-Tg(ACTFLPe)9205Dym/J(ACTB<sup>FLPe</sup>/FLPe)*). Two founder lines were identified (CKO line A and CKO line B). Both lines of *Nkx2.2<sup>lox/+</sup>* animals were viable and fertile, and when the allele was homozygosed (Figure 1c) no alteration to breeding/fertility was observed. CKO line A was used for all subsequent analysis.

To ensure that the presence of flanking lox sites within the *Nkx2.2* genomic locus did not adversely affect *Nkx2.2* function, mice carrying the *Nkx2.2<sup>lox</sup>* allele were mated for homozygosity and analyzed for pancreatic/metabolic function. Males and females were followed from weaning (3 weeks) to adult (12 weeks), and no change in body weight was observed in *Nkx2.2<sup>lox/lox</sup>* compared with both *Nkx2.2<sup>lox/+</sup>* and wildtype mice (Figure 2a,b). Moreover, males and females at weaning age (3 weeks) were fasted overnight and then subjected to an intraperitoneal glucose tolerance test (GTT). Following glucose injection, blood glucose was measured at timed intervals over 120 minutes. Glucose clearance was unaltered in *Nkx2.2<sup>lox/lox</sup>* mice compared with controls (Figure 2c,d); area under the curve shows no significant difference (Figure 2e). Mice subjected to GTT were of similar weight (Figure 2f), and blood glucose before fasting was not different between genotypes (Figure 2g).

Pancreas tissue was analyzed during development and postnatally to determine that all hormone-expressing cells were present and islet morphology was unaltered. At E15.5, P0 and 2 weeks of age, the complement of hormone-expressing cells appeared unaltered between wildtype and *Nkx2.2<sup>lox/lox</sup>* animals (Figure 3). In addition, islet morphology displayed the expected structure of a core of insulin-producing beta cells surrounded by glucagon-, ghrelin-, somatostatin, and PP-expressing cells (Figure 3m-r).

To test that *Nkx2.2* could be deleted efficiently, *Nkx2.2<sup>lox</sup>* mice were bred to *Pdx1-cre* transgenic mice to produce mice with a deletion of *Nkx2.2* in all *Pdx1*-expressing cells, which includes the entire pancreatic domain. Embryos were harvested at E15.5 and the pancreas was analyzed using RNA *in situ* hybridization for the presence of *Nkx2.2* transcript. Whereas the expected expression pattern of *Nkx2.2* was observed in wildtype and *Nkx2.2<sup>lox/lox</sup>* pancreas, pancreatic tissue from *Nkx2.2<sup>lox/lox</sup>;Pdx1-cre* embryos was devoid of *Nkx2.2* (Figure 4a-e). Moreover, *Nkx2.2<sup>lox/lox</sup>;Pdx1-cre* animals were born but died within the first week of life; pups analyzed at P1 displayed elevated blood glucose levels (Figure 4g), as reported for the *Nkx2.2* null animals, which are devoid of insulin-producing beta cells (Sussel et al. 1998). Given this similarity, *Nkx2.2<sup>lox/lox</sup>;Pdx1-cre* mutant embryos were further analyzed to determine if the islet cell phenotype of the *Nkx2.2* null was recapitulated in the *Nkx2.2<sup>lox/lox</sup>;Pdx1-cre* mutants. As expected, *ghrelin* (*Ghr*) was dramatically increased in the *Nkx2.2<sup>lox/lox</sup>;Pdx1-cre* compared with wildtype (Figure 5a). *Nkx2.2* was expectedly decreased, along with *insulin1* (*Ins1*), *insulin2* (*Ins2*), *glucagon* (*Gcg*), and *pancreatic polypeptide* (*Ppy*) transcript; *somatostatin* (*Sst*) expression was unchanged (Figure 5a). Moreover, pancreas tissue from E15.5 embryos was analyzed by

immunofluorescence and the observed cellular phenotype recapitulated the expression data and phenocopied the *Nkx2.2* null embryos (Figure 5b–e).

Altogether these data demonstrate that the *Nkx2.2<sup>fllox</sup>* allele does not disrupt normal *Nkx2.2* function and successfully allows for the efficient deletion of *Nkx2.2* in the presence of a Cre recombinase. In addition, *Nkx2.2<sup>fllox/fllox</sup>;Pdx1-cre* mutant animals recapitulate the pancreatic phenotype associated with the whole body deletion of *Nkx2.2* (Prado et al. 2004; Sussel et al. 1998). These studies also confirm that the neonatal lethality associated with the *Nkx2.2* null mice is due to loss of *Nkx2.2* activity in the pancreas. The generation of this conditional allele of *Nkx2.2* facilitates further analysis of the cell autonomous and stage-specific functions of *Nkx2.2* in the pancreas, intestine and CNS.

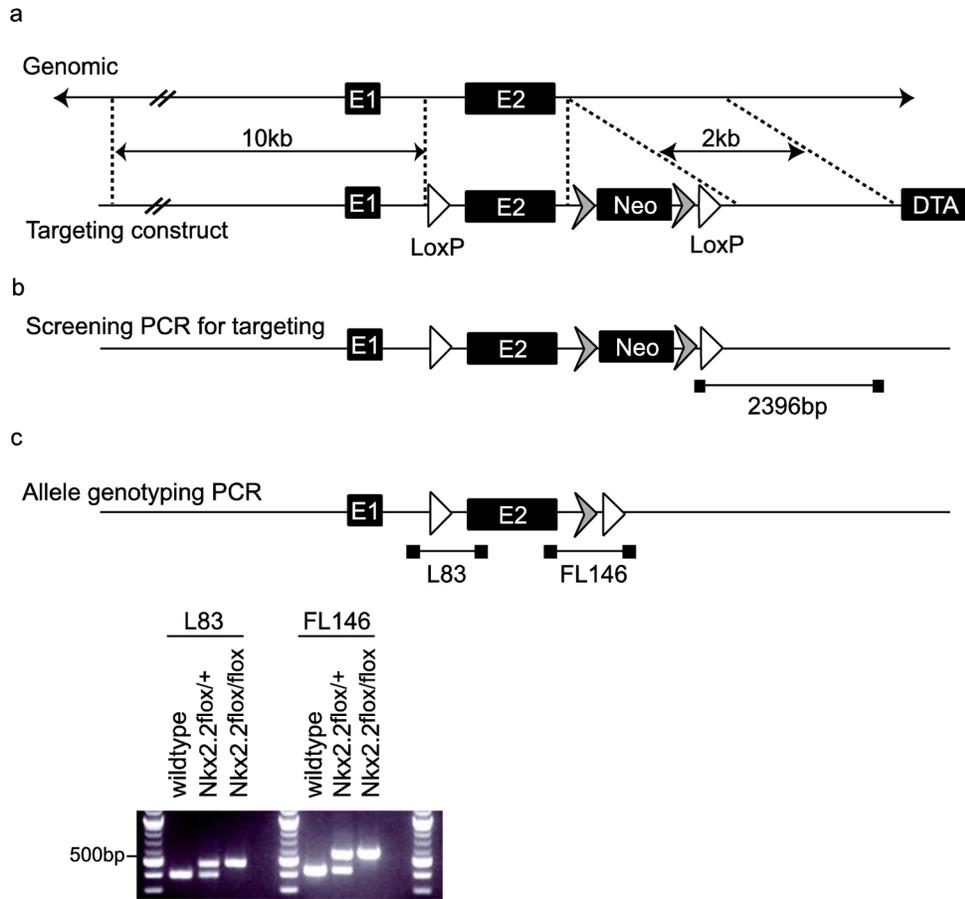
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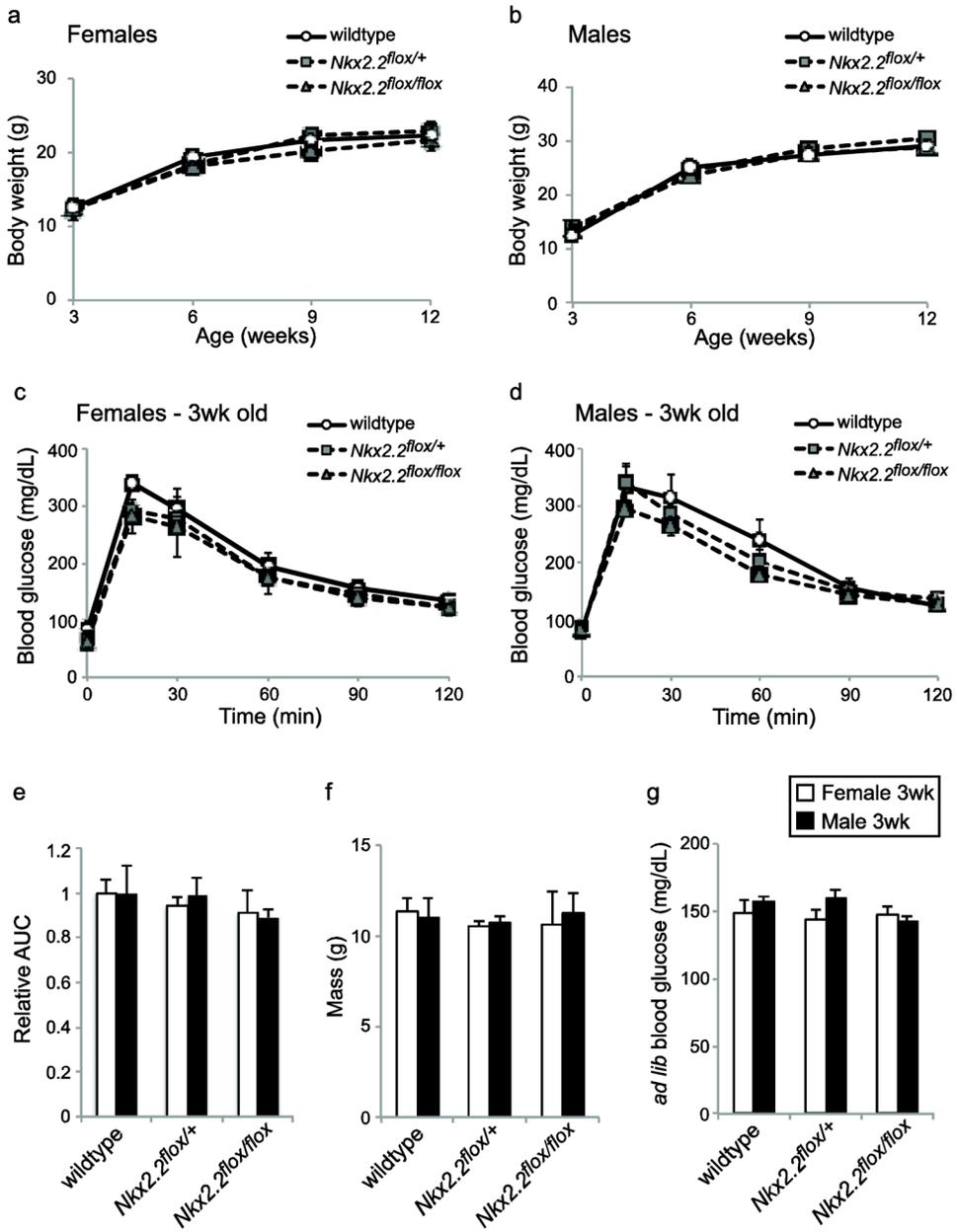
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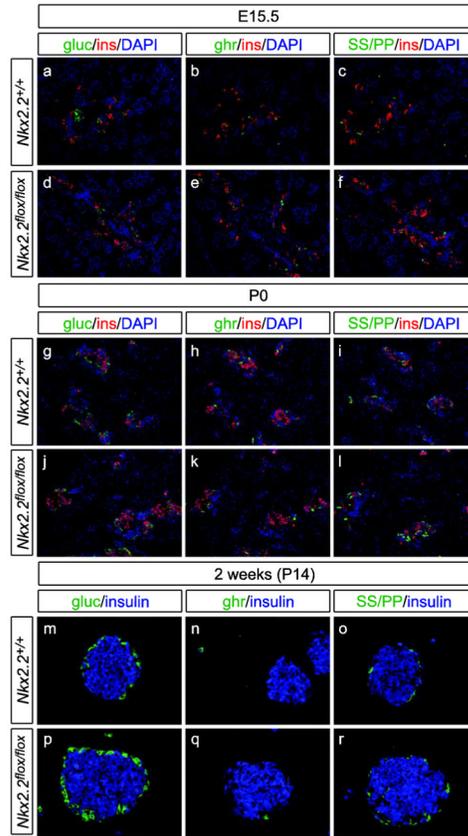


**Fig. 1. *Nkx2.2* conditional allele construct**

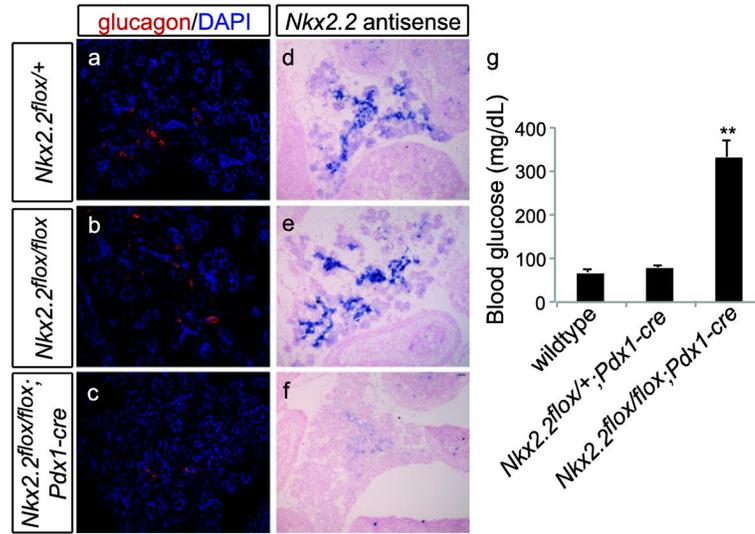
(a) A loxP site (L83) and a FNFL cassette (FRT-Neo-FRT-LoxP) were engineered to flank exon 2 (E2) of the *Nkx2.2* gene to generate the *Nkx2.2* conditional targeting construct on a Bacterial Artificial Chromosome (BAC). The targeting construct was retrieved onto a vector carrying the diphtheria toxin alpha chain (DTA) negative selection marker. (b) Targeted ES cells were identified by PCR screening, producing a 2396bp product if the construct had incorporated. Male chimeras were bred to *B6;SJL-Tg(ACTFLPe)9205Dym/J* (*ACTB<sup>FLPe/FLPe</sup>*; (Rodriguez et al. 2000)) female mice on a C57BL/6 background to both transmit the *Nkx2.2<sup>flox</sup>* allele through the germline and to simultaneously remove the Neomycin cassette. (c) Mice carrying the *Nkx2.2* conditional allele (*Nkx2.2<sup>flox</sup>*) were genotyped for the presence of both loxP sites; the additional sequence added by the presence of the loxP site or the loxP and fragment of the FNFL cassette provided a sequence size difference in PCR product between the *Nkx2.2<sup>flox</sup>* allele and the wildtype locus. Representative genotyping PCR amplification of wildtype, *Nkx2.2<sup>flox/+</sup>* and *Nkx2.2<sup>flox/flox</sup>* mice, using both the L83 and FL146 primer sets.



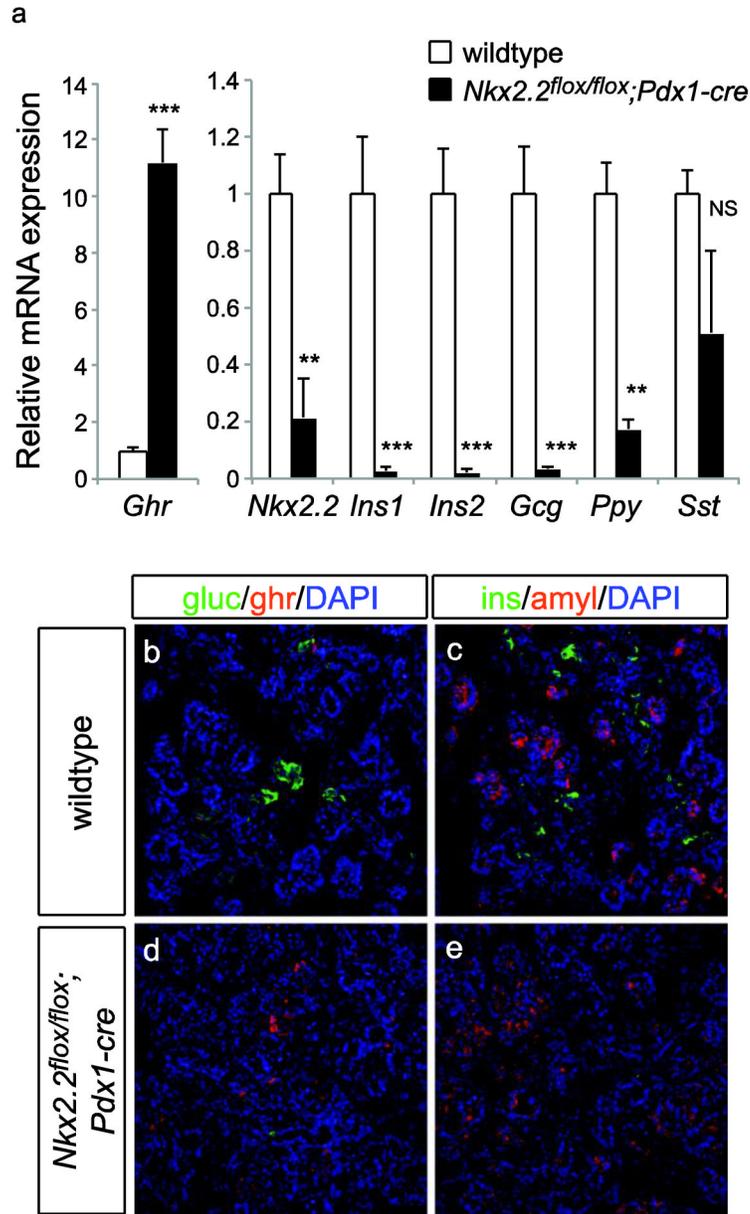
**Fig. 2. *Nkx2.2<sup>flox</sup>* mice have normal growth and glucose tolerance**  
 Body weight was measured in female (a) and male (b) mice from 3 weeks (weaning) until 12 weeks of age, and the presence of the *Nkx2.2<sup>flox</sup>* allele did not affect mass. Glucose tolerance tests were performed on female (c) and male (d) mice at 3 weeks of age, and the *Nkx2.2<sup>flox</sup>* allele did not alter glucose tolerance. For these mice there was no significant difference in area under the curve (e), body weight (f) and *ad lib* blood glucose (g).



**Fig. 3. Islet cell hormone expression and morphology in the *Nkx2.2<sup>lox</sup>* pancreas**  
 The presence of hormone-expressing endocrine cells was assessed by immunofluorescence staining on pancreas tissue sections from *Nkx2.2<sup>lox/lox</sup>* mice and wildtype control littermates. At E15.5 and P0, the presence of cells expressing glucagon (a, d, g, j), insulin (a–l), ghrelin (b, e, h, k), somatostatin or pancreatic polypeptide (c, f, i, l) appeared unchanged between genotypes. By 2 weeks of age (P14) there continued to be no difference in islet hormone expression and islet morphology appeared identical between wildtype (m – o) and *Nkx2.2<sup>lox/lox</sup>* (p – r) mice.



**Fig. 4. *Nkx2.2*<sup>flox/flox</sup>;*Pdx1-cre* mutants recapitulate the *Nkx2.2* null pancreatic phenotype**  
Using immunofluorescent staining for the expression of glucagon, the presence of alpha cells was observed in the pancreas from *Nkx2.2*<sup>flox/+</sup> (a) and *Nkx2.2*<sup>flox/flox</sup> (b) embryos at E15.5, whereas a significant decrease of alpha cells was observed in the *Nkx2.2*<sup>flox/flox</sup>;*Pdx1-cre* pancreas (c). The known pattern of expression for *Nkx2.2* was observed in the control pancreas sections (d, e) compared with the efficient loss of *Nkx2.2* in the mutant (f) due to *Pdx1-cre* mediated recombination of the *Nkx2.2*<sup>flox</sup> allele. Similar to the phenotype observed in the *Nkx2.2* null mice, pups were born and had severely elevated blood glucose (g). DAPI marks nuclei (a–c). Images are 20X



**Fig. 5. Pancreatic hormone expression in the *Nkx2.2<sup>flox/flox</sup>;Pdx1-cre* mutants is similar to *Nkx2.2* null mice**

(a) The relative expression of islet hormones was assessed in the *Nkx2.2<sup>flox/flox</sup>;Pdx1-cre* mice and wildtype control littermates at P0. The expression of *ghrelin (Ghr)*, *insulin1 (Ins1)*, *insulin2 (Ins2)*, *glucagon (Gcg)*, *pancreatic polypeptide (Ppy)* and *somatostatin (Sst)* appeared similar to the expected pancreas phenotype of the *Nkx2.2* null mice. *Pdx1-cre* mediated recombination of the *Nkx2.2<sup>flox</sup>* allele was efficient in reducing *Nkx2.2* transcript. E15.5 pancreas tissue from wildtype (b, c) and *Nkx2.2<sup>flox/flox</sup>;Pdx1-cre* (d, e) embryos was subject to immunofluorescence for glucagon, ghrelin, insulin and amylase. DAPI marks nuclei. Images are 40X.