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ARTICLE

Human *Krüppel*-like factor 11 inhibits human proinsulin promoter activity in pancreatic beta cells

X. Niu · N. Perakakis · K. Laubner · C. Limbert · T. Stahl · M. D. Brendel · R. G. Bretzel · J. Seufert · G. Päth

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Abstract
Aims/hypothesis The *Krüppel*-like factor 11 (KLF11; TIEG2), a pancreas-enriched Sp1-like transcription factor, is a known negative regulator of pancreatic exocrine cell growth. A recent study indicated KLF11-induced activation of the human proinsulin promoter (hInsP).
Materials and methods We investigated the functional role of KLF11 in pancreatic beta cells.
Results Endogenous *KLF11* mRNA expression was found in whole rat pancreas, human pancreatic islets and INS-1E beta cells and was profoundly reduced by high glucose in INS-1E. Cotransfections of INS-1E and beta-TC3 beta cells with a human *(h)KLF11* expression plasmid and an hInsP-driven reporter plasmid resulted in a substantial dose-dependent and glucose-independent inhibition of proinsulin promoter activity. 5′-deletion of hInsP demonstrated that hKLF11 acts via DNA sequences upstream of −173 and requires the beta cell-specific transcription machinery, since hKLF11-mediated inhibition of promoter activity was abolished in HEK293 cells. Besides a previously described GC box, we further identified a CACCC box within the hInsP, both putative KLF11-binding motifs. Electrophoretic mobility shift analysis (EMSA) verified binding of in vitro translated hKLF11 to the GC box, but neither hKLF11-induced inhibition nor basal hInsP activity was altered by mutation or 5′-deletion of the GC box. In contrast, CACCC box mutation substantially reduced basal promoter activity and partially diminished hKLF11 inhibition, although binding of in vitro translated hKLF11 to the CACCC box could not be verified by EMSA.
Conclusions/interpretation In rodent beta cell lines, we demonstrate *hKLF11*-overexpression of human proinsulin gene expression and characterise a prominent role for the CACCC box in maintaining basal proinsulin promoter activity.

Keywords Beta-TC3 · CACCC box · GC box · Human proinsulin promoter · HEK293 · INS-1 · KLF11

Abbreviations

EMSA	electrophoretic mobility shift analysis
hInsP	human proinsulin promoter
HPRT	hypoxanthine phosphoribosyltransferase
KLF	*Krüppel*-like factor
MAOB	monoamine oxidase B
PDX-1	pancreatic duodenal homeobox protein-1
qPCR	quantitative real-time PCR
SEAP	secreted alkaline phosphatase
TIEG	TGF-β-inducible early response gene

This work is dedicated to X. Niu who died in December 2006 in China under tragic circumstances. An excellent research talent has died and left us in deep mourning.

X. Niu · N. Perakakis · K. Laubner · C. Limbert · T. Stahl · J. Seufert · G. Päth (✉)
Laboratory B9, Division of Endocrinology and Diabetology,
Department of Internal Medicine II,
University Hospital of Freiburg,
79106 Freiburg, Germany
e-mail: guenter.paeth@uniklinik-freiburg.de

X. Niu
Department of Hepatobiliary Surgery,
First Hospital of Xi'an Jiaotong University,
Xi'an, People's Republic of China

M. D. Brendel · R. G. Bretzel
Islet Transplantation Centre, Department of Internal Medicine III,
University of Giessen,
Giessen, Germany

Introduction

Krüppel-like transcription factor (KLF)11 is a member of the Sp1-like transcription factor family, which is defined by the presence of three conserved DNA-binding C-terminal zinc finger domains and variant N-terminal domains [1–3]. KLF/Sp1-like proteins bind with different selectivity to GC box or CACCC box promoter elements and take part as activators or repressors in virtually all aspects of cellular function, including cell proliferation, apoptosis, differentiation and neoplastic transformation.

Within the KLF/Sp1-like family KLF9, KLF10, KLF11, KLF13 and KLF16 are characterised by the existence of a repressor domain which interacts with the scaffold corepressor mammalian (m)Sin3A (SID or SID/R1 for KLF10 and KLF11) as a common structural feature [4]. SID/R1 mediates chromatin modification and other repressor mechanisms via recruitment of histone deacetylases and the nuclear receptor corepressor N-CoR, respectively [2]. Among the above-mentioned KLF proteins, KLF10 and KLF11 are further characterised by the existence of two additional repressor domains R2 and R3 [5] and TGF-β-induced expression [6, 7]. For this reason KLF10 and KLF11 form the TGF-β-inducible early response gene (TIEG) subfamily and were alternatively named TIEG1 and TIEG2, respectively. Due to parallel cloning, KLF11 was also named FKLF [8].

In the adult organism, KLF11 is ubiquitously produced, but enriched in muscle and pancreas [6]. Within the pancreas KLF11 has been described as a negative regulator of exocrine cell proliferation in transgenic mice with acinar cell-specific KLF11 overproduction in vivo and PANC1 epithelial cancer cells overexpressing *KLF11* in vitro [9]. As a result, *KLF11* transgenic mice develop a significantly smaller exocrine pancreas than controls due to reduced proliferation and enhanced apoptosis. Albeit a smaller exocrine pancreas, only mild changes in tissue architecture could be observed and levels of the acinar enzymes remained normal. In vivo and in vitro reduced proliferation is accompanied by enhanced apoptosis. This is, at least in part, explainable by KLF11-mediated downregulation of the scavengers superoxide dismutase 2 and catalase 1, resulting in increased susceptibility of cells to oxidative stress.

The role of KLF11 within the endocrine pancreas remained unestablished until recently Neve et al. [10] reported that, in beta cell lines, high glucose conditions stimulate *KLF11* mRNA expression and cotransfected human (h)KLF11 can activate the human proinsulin promoter (hInsP). In our own experiments, however, endogenous *KLF11* mRNA levels were reduced by high glucose in INS-1E beta cells. Furthermore, cotransfected hKLF11 dose-dependently and glucose-independently inhibits the activity of hInsP in INS-1E and beta-TC3 beta cells. Sequence analysis of hInsP not only retrieved the previously described GC box but also identified a CACCC box element, both putative binding sites of KLF11. Since our results are in contrast to those of Neve et al. [10] and to better understand how hKLF11 regulates human insulin gene expression, we here further studied the functional role of the GC and CACCC box by electrophoretic mobility shift analysis (EMSA), mutation and 5′-deletion constructs of the hInsP in INS-1E and beta-TC3 beta cells.

Materials and methods

Cell culture Cell lines were routinely cultured as follows. Rat INS-1E: RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μmol/l β-mercaptoethanol. Media contained 11.1 mmol/l glucose unless otherwise stated. Mouse beta-TC3 beta cells and human HEK293 cells: DMEM containing 10% FBS, 25 mmol/l glucose, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. INS-1E beta cells were kindly provided by C. B. Wollheim (University Medical Center, Geneva, Switzerland).

RT-PCR detection of KLF11 mRNA expression in pancreatic tissues and cells Total RNA extraction was performed using Trizol (Invitrogen, Karlsruhe, Germany) for tissue or RNeasy Mini Kit (Qiagen, Hilden, Germany) for cells. RNA samples were reverse transcribed into cDNA in the presence (RT+) or absence (RT−) of Superscript II Plus using oligo-(dT) primers (both Invitrogen). RT− samples served as controls for the absence of genomic DNA contamination. Primers: rat (r)KLF11, forward 5′-GAAGC GGCACGACAGCGAAAG-3′ and reverse 5′-AGCTC TGGGCTCTGAGGAGGAGTT-3′ [annealing temperature (Ta), 64°C; product length, 250 bp]; hKLF11, forward 5′-GGTGACCTGTTGCGGATAAG-3′ and reverse 5′-CACA GGGATCATCTGGCAAAGGA-3′ (Ta, 60°C; product length, 687 bp). PCR conditions: 94°C for 2 min; 35 cycles of 94°C for 30 s, primer-specific Ta for 30 s and 72°C for 30 s; 72°C for 2 min.

Plasmid construction The *hKLF11* coding sequence was amplified from cDNA derived from human islets of Langerhans by using high fidelity Pwo Master (Roche, Mannheim, Germany). Primers: forward 5′-CACGATGCAC ACGCCGGACTTC-3′ and reverse 5′-GCTAGCAAAAT CCCATGAGTGATGTCCTAATGG-3′. PCR conditions: 94°C for 2 min; 30 cycles, 94°C for 30 s−54°C for 30 s-72°C for 3 min. The resulting *hKLF11* coding sequence was subcloned into pcR2.1-TOPO by using a TOPO TA Cloning kit (both Invitrogen) and subcloned into the CMV promoter-driven pcDNA3.1+ (Invitrogen) to obtain the hKLF11-pcDNA3.1+

expression plasmid. The −881 to +54 hInsP fragment was amplified with a BD Advantage HF PCR Kit (BD Biosciences Clontech, Heidelberg, Germany) from human genomic DNA which was extracted from whole blood using a QIAamp DNA Blood Mini Kit (Qiagen). Primers: forward 5′-TCCCTCACTCCCACTCTCCCAC-3′ and reverse 5′-TTCGAATTGGAACAGACCTGCTTGATGGCC-3′. PCR conditions: 94°C for 3 min; 28 cycles of 94°C for 30 s, 55°C for 30 s and 68°C for 90 s; 68°C for 2 min. The resulting -881+54hInsP fragment was subcloned into pcR2.1-TOPO plasmid using a TOPO TA Cloning kit (both Invitrogen) and subcloned into pSEAP2-Basic (BD Biosciences Clontech) to obtain the −881hInsP-pSEAP reporter plasmid (SEAP, secreted alkaline phosphatase). −881hInsP-pSEAP was used for generation of 5′-deleted hInsP fragments (−387, −355, −323, −254, −173, −101 and −85) with an identical 3′-end (+54) by PCR, which were then subcloned via pcR2.1-TOPO into pSEAP2-Basic. GC box and CACCC box mutations were created using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany). The GC box was mutated from 5′-CCC GCCCT-3′ into 5′-CCCGAACT-3′ and the CACCC box from 5′-CCCACCCC-3′ into 5′-CCCGATTCC-3′. Accuracy of all sequences and mutations was checked by sequencing.

Protein extraction and in vitro translation For generation of whole cell extracts, freshly prepared RIPA buffer (Upstate/Biomol, Hamburg, Germany) containing one tablet per 10 ml Complete Mini EDTA-free protease inhibitor cocktail (Roche) was used. Nuclear extracts were acquired according to Schreiber et al. [11]. hKLF11 was in vitro translated from hKLF11-pcDNA3.1+ using a TNT Quick Coupled Transcription/Translation System (Promega, Mannheim, Germany). Control reactions were performed in the absence of hKLF11-pcDNA3.1+.

Western blot analysis Ten micrograms of protein from whole cell extract and nuclear extract or 1 μl in vitro translated reactions were separated on 10% PAGE-SDS gels. Proteins were transferred to polyvinylidene difluoride membranes by semi-dry blotting using Towbin transfer buffer. KLF11 was detected using a goat polyclonal antiserum (TIEG2 C-12, sc-23162; Santa Cruz, Heidelberg, Germany). Visualisation was achieved by a horseradish peroxidase-linked rabbit anti-goat secondary antibody (DAKO, Hamburg, Germany) and ECL Western Blotting Detection Reagent (Amersham, Freiburg, Germany).

SEAP reporter gene experiments INS-1E, beta-TC3 and HEK293 cells were seeded in six-well plates at a density of 3×10^5 cells per well. Next day, cells were transiently transfected using Metafectene (Biontex, Martinsried/Planegg, Germany). The proportion of DNA (μg) to Metafectene (μl) was 1:2. Each plasmid was transfected at a concentration of 0.5 μg unless otherwise stated. After 48 h, supernatants were collected for measurement of SEAP using a BD Great EscAPe SEAP Chemiluminescence Detection Kit (BD Biosciences Clontech).

Quantification of insulin gene expression in INS-1E beta cells in response to KLF11 overexpression INS-1E cells were seeded in six-well plates at a density of 3×10^5 cells per well in the presence of 11.1 mmol/l glucose. Next day, cells were transiently transfected with either rKLF11-pcDNA3.1+ or pcDNA3.1+ (mock control level) using Metafectene Pro (Biontex). The proportion of DNA (μg) to Metafectene Pro (μl) was 1:2. Each plasmid was transfected at a concentration of 1 μg/well. After 48 h, cells were harvested for analysis of monoamine oxidase B gene (*MAOB*), insulin-1 (*Ins1*) gene and insulin-2 (*Ins2*) gene mRNA expression levels using quantitative real-time PCR (qPCR) (see below).

Quantification of KLF11 mRNA levels in INS-1E beta cells in response to high glucose stimulation INS-1E cells were seeded in six-well plates at a density of 3×10^5 cells per well in the presence of 11.1 mmol/l glucose. Next day, medium was replaced by medium containing 2.8 mmol/l glucose. After 24 h, cells were washed with PBS and exposed to medium containing either 2.8 or 25 mmol/l glucose for 6–48 h. Finally, cells were harvested for analysis of *KLF11* mRNA expression levels using qPCR (see below).

qPCR Total RNA was isolated using the RNeasy Mini Kit (Qiagen), treated with Turbo DNA-free Kit (Ambion/Applied Biosystems, Darmstadt, Germany) and checked for contamination with genomic DNA by PCR with primers for rat hypoxanthine phosphoribosyltransferase gene (*HPRT*) (Quantitect Rn_HPRT1; Qiagen). RNA free from genomic DNA was reverse transcribed with Superscript III and oligo-(dT) primers (both Invitrogen). cDNA samples were investigated for mRNA levels by qPCR using Lightcycler 2.0 (Roche) and Faststart DNA Master+ CYBR Green I kit (Roche). Concentrations of mRNA were calculated from standard curves for each specific primer pair (dilution series: 1:1, 1:10, 1:100, 1:1,000 and 1:10,000) by Light Cycler Software 3.5 (Roche). All samples and standards were analysed in triplicates. Primer pairs for rat *HPRT* (Quantitect Rn_HPRT1), rat *Ins1* (Quantitect Rn_INS1), rat *Ins2* (Quantitect Rn_INS2) and rat *MAOB* (Quantitect Rn_MAOB) were obtained from a commercial supplier (Qiagen). Product length of all Quantitect primer pairs is 125 bp. See above for primers for *rKLF11*. qPCR conditions were: 95°C for 10 min; 40 cycles of 95°C for 15 s, 55°C (Quantitect primers) or 64°C (*rKLF11* primers) for 10 s and

72°C for 20 s; followed by melting curve analysis for specificity of qPCR products.

Glucose-induced insulin secretion from INS-1E beta cells INS-1E cells were seeded in six-well plates at a density of 3×10^5 cells per well in the presence of 11.1 mmol/l glucose. Next day, medium was replaced by medium containing 2.8 mmol/l glucose. After 24 h cells were washed twice with PBS and exposed to serum-free medium containing either 2.8 or 25 mmol/l glucose. After 1 h, medium samples were collected and centrifuged for 5 min at 16,000 *g*. Supernatant fractions were transferred to new tubes and stored at −20°C until measurement of secreted insulin using the High Range Rat Insulin ELISA kit (Mercodia, Uppsala, Sweden).

EMSA One microlitre of in vitro translated reactions was added to 5 × EMSA buffer (100 mmol/l KPO_4, pH 7.9, 5 mmol/l EDTA, 5 mmol/l dithiothreitol and 20% glycerol) and additionally supplemented with 50 mmol/l KCl, 1 μg poly-dIdC and 40,000 cpm/μl ^{32}P-labelled double-stranded oligonucleotide. The following DNA probes were generated by annealing two oligonucleotides, followed by a fill-in reaction with Klenow polymerase and dGTP, dCTP, dTTP and α-^{32}P-labelled dATP: GC box, 5′-GATCAAAGAGCCC CGCCCTGCAGCC-3′; GC box mutant, 5′-GATCAAAGA GCCCCGAACTGCAGCC-3′; CACCC box, 5′-GATCCG ACCCCCCCACCCCAGGCCC-3′; CACCC box mutant, 5′-GATCCGACCCCCCCGATTCCAGGCCC-3′. After incubation on ice for 15 min, samples were loaded onto a 5% polyacrylamide gel and run in 0.5 × Tris-borate-EDTA buffer for 2 h. For supershift assays, 2 μl of KLF11 antiserum (see above) were added and incubated on ice for 20 min before samples were loaded onto the gel.

Statistical analysis All results are presented as means±SD. Results were analysed by Bonferroni's multiple comparison test of selected groups (Figs. 3d, 5, 6 and 7) and a paired *t* test (Fig. 8) or ANOVA using GraphPad Prism 4.0.

Results

KLF11 *expression in pancreas, islets and beta cells, and by plasmid* RT-PCR demonstrated endogenous *KLF11* mRNA expression in whole rat pancreas, human islets of Langerhans and INS-1E beta cells (Fig. 1a). Western blotting detected endogenous KLF11 protein in nuclear extracts of INS-1E and beta-TC3 beta cells (Fig. 1b). An additional band around 90 kDa represents an artefact of unknown origin occurring with this antibody in some cell lines, i.e. Jurkat (see manufacturer's data sheet). Western blotting further confirmed functional hKLF11 protein production derived from hKLF11-pcDNA3.1+ in whole cell extracts of transiently transfected INS-1E beta cells (Fig. 1c) and in vitro translation (Fig. 1d).

hKLF11 *overexpression decreases hInsP activity* INS-1E cells were cotransfected with 1.5 μg −881hInsP-pSEAP or promoterless pSEAP2-Basic and increasing quantities (0–1.5 μg) of hKLF11-pcDNA3.1+. The amount of totally transfected DNA per well was adjusted to 3 μg DNA by addition of pcDNA3.1+. In this experimental setting *hKLF11* overexpression dose-dependently reduced −881hInsP activity, which is reflected by the magnitude of SEAP in the supernatants, up to 50% (Fig. 2). Marginal SEAP contents in pSEAP2-Basic transfected controls displayed background levels which remained unregulated by increasing *hKLF11* overexpression. The characteristic of *hKLF11* overexpression-induced inhibition of −881hInsP activity was not altered

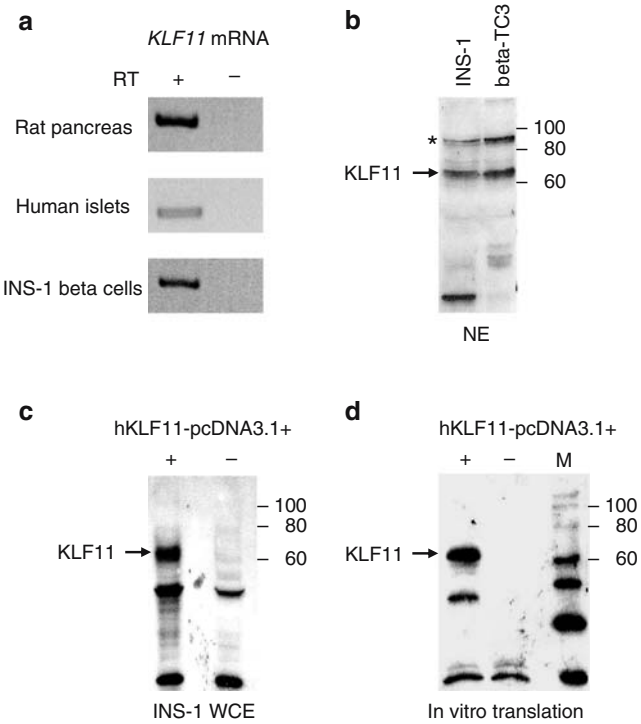

Fig. 1 *KLF11* mRNA and protein expression. **a** Endogenous *KLF11* mRNA is expressed in whole rat pancreas, human islets and INS-1E beta cells. *RT+*, reverse transcriptase added to reaction mix; *RT−*, reverse transcriptase not added. RT− samples serve as a control for contamination of cDNA with genomic DNA. **b** Endogenous KLF11 protein is produced in INS-1E and beta-TC3 beta cells. Western blots were performed using nuclear extracts (*NE*). *This band is a known artefact of the antibody used, which occurs in some cell lines, i.e. Jurkat (see manufacturer's data sheet). **c** INS-1E beta cells transiently transfected with the hKLF11-pcDNA3.1+ expression plasmids demonstrate functional *hKLF11* overexpression. Western blots were performed using whole cell extracts (*WCE*). **d** Western blotting detected successful in vitro translation of hKLF11 by using hKLF11-pcDNA3.1+ expression plasmids. Displayed are representative results; all experiments, $n=3$

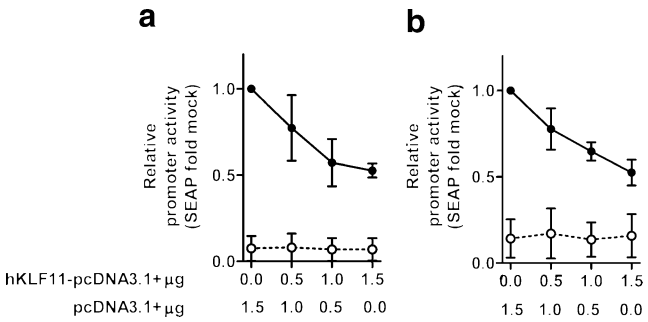

Fig. 2 *hKLF11* overexpression-mediated inhibition of cotransfected hInsP. INS-1E beta cells were transiently transfected with different amounts of the hKLF11-pcDNA3.1+ expression plasmids (*closed circles*) as indicated and cotransfected with either 1.5 µg of −881hInsP-SEAP reporter plasmids or 1.5 µg of promoterless pSEAP2-Basic empty plasmids as a background control (*open circles*). Each cotransfection was adjusted with pcDNA3.1+ mock plasmids to a total amount of 3 µg transfected DNA. The hKLF11-induced inhibition of hInsP is dose-dependent and stable in standard (**a**, 11.1 mmol/l) and high (**b**, 25 mmol/l) glucose conditions. Means±SD; $n=3$ for 11.1 mmol/l and $n=5$ for 25 mmol/l glucose

by high glucose conditions (25 mmol/l) as compared with standard culture conditions (11.1 mmol/l glucose).

Inhibition of insulin gene expression by KLF11 overexpression, glucose-induced insulin secretion and regulation of endogenous KLF11 mRNA expression by glucose Although a dose-dependent *KLF11* overexpression-induced increase in *MAOB* mRNA levels was described in both SH-SY5Y and HepG2 cells [12], we did not observe significant regulation of *MAOB* gene expression in INS-1E beta cells (not shown). However, qPCR analysis revealed that *hKLF11* overexpression significantly reduced *Ins2* gene expression while *Ins1* mRNA levels remained unaffected (Fig. 3a,b). Of note, the observed reduction of about 21% in the *Ins2* mRNA level is remarkable in the light of approximately one-third transfected cells; 100% transfection efficiency would result in a 63% reduction, a value similar to inhibition of hInsP activity by hKLF. The specific inhibition of *Ins2* gene expression is of interest since, in rat beta cells, only the *Ins2* and not the *Ins1* promoter sequence contains a CACCC box element (see below). Functional glucose-induced insulin secretion was tested at the protein level by increasing glucose concentrations in the medium from 2.8 to 25 mmol/l thereby enhancing 1-h insulin secretion about threefold (Fig. 3c). In contrast to insulin secretion, *KLF11* mRNA levels were significantly reduced by high glucose (Fig. 3d).

Binding of KLF11 to the GC and CACCC box Sequence analysis of the hInsP sequence identified the GC box from −340 to −348 and further a CACCC box from −88 to −96. Positions as well as wild-type and mutated sequences of both boxes are shown in Fig. 4a. EMSA analysis verified binding of in vitro translated hKLF11 to the GC box (Fig. 4b). Specificity of this binding was demonstrated by antibody-induced supershift. Under the same experimental conditions in vitro translated hKLF11 failed to bind the CACCC box (Fig. 4c).

Inhibition of hInsP 5′-deletion constructs by hKLF11 5′-deletion constructs of hInsP were investigated to verify the functional relevance of the GC box for hKLF11-mediated suppression of promoter activity. The position of important binding elements within hInsP and the length of 5′-deletion constructs are presented in Fig. 5a. In the experimental setting employed, either 0.5 µg pcDNA3.1+ (mock control) or 0.5 µg hKLF11-pcDNA3.1+ was cotransfected with 0.5 µg SEAP reporter plasmids driven by the −881hInsP or

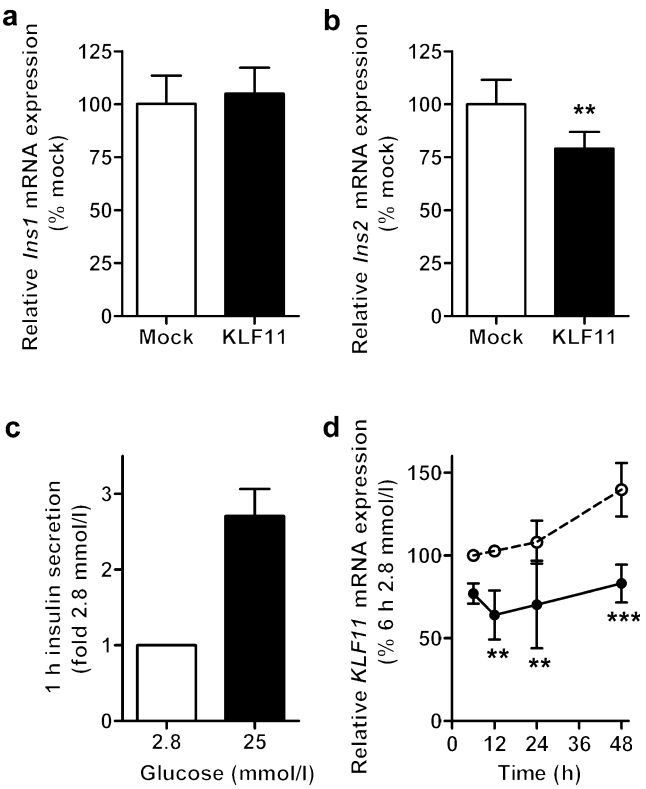

Fig. 3 Inhibition of insulin gene expression by *KLF11* overexpression, glucose-induced insulin secretion and regulation of endogenous *KLF11* mRNA expression by glucose. **a** INS-1E beta cells were transfected with rKLF11-pcDNA3.1+ expression plasmids or pcDNA3.1+ empty plasmids (mock control level). After 48 h, cells were harvested for qPCR analysis of *Ins1* mRNA levels. *rKLF11* overexpression did not affect *Ins1* gene expression. **b** In contrast, *rKLF11* overexpression significantly reduced *Ins2* gene expression. **c** INS-1E beta cells were precultured for 24 h in the presence of 2.8 mmol/l glucose and then incubated for 1 h with serum-free medium containing either 2.8 or 25 mmol/l glucose. High glucose conditions increase insulin secretion nearly threefold, demonstrating glucose responsiveness of the cells investigated. **d** INS-1E beta cells were precultured for 24 h in the presence of 2.8 mmol/l glucose and then exposed to 2.8 (*open circles*) or 25 mmol/l (*closed circles*) glucose. After 6–48 h cells were harvested for qPCR analysis. High glucose conditions significantly reduced endogenous *KLF11* mRNA expression. Means±SD; all experiments, $n=3$; $^{**}p<0.01$; $^{***}p<0.001$

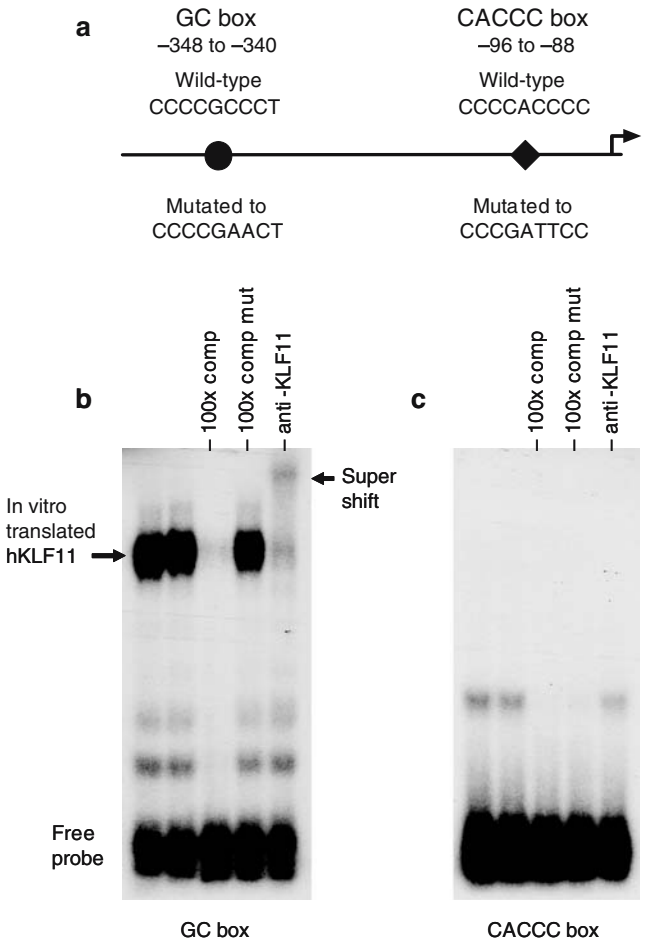

Fig. 4 Interactions of hKLF11 with GC and CACCC boxes. **a** Position of the GC and CACCC box sequences within hInsP. Wild-type and mutated sequences used in EMSA 25mer probes and reporter plasmids of this study are indicated. **b** In vitro translated hKLF11 specifically binds to the GC box sequence as demonstrated by a supershift in the presence of an antibody against KLF11. **c** In vitro translated hKLF11 did not bind to the CACCC box sequence. *Comp*, competitor; *comp mut*, competitor with mutated sequence. All experiments, $n=3$

its 5′-deletion constructs (Fig. 5b). Compared with the initial experiments (dose-dependency of KLF11-mediated inhibition of hInsP) the inhibition by 0.5 μg hKLF11-pcDNA3.1+ was more pronounced (50% instead of 30%). This may be explained by transfection of lower quantities of total DNA (1 μg instead of 3 μg), since it is known that high amounts of transfected DNA can alter cellular functions. Besides INS-1E, beta-TC3 beta cells were also investigated. It was confirmed that hKLF11-induced repression of −881hInsP activity did not significantly differ between both beta cell lines. In beta-TC3 beta cells, *hKLF11* overexpression-induced inhibition of hInsP activity was similar from −881 to −254 (about 60% inhibition compared with mock control) but was substantially reduced by further deletion to −173 (about 30% inhibition). Shorter 5′-deletion constructs were not affected by *KLF11* overexpression. These results demonstrate that complete hKLF11-mediated inhibition of hInsP depends on DNA sequences upstream of −173. Although expected, deletion of the GC box did not alter hKLF function. The functional relevance of the CACCC box could not be tested in this setting due to complete loss of KLF11-mediated inhibition of 5′-deletion constructs shorter than −173.

Effects of GC box and CACCC box mutation on hKLF11-inhibited and basal hInsP activity Beta-TC3 beta cells were cotransfected with −387hInsP-pSEAP (containing wild-type or mutated sequences) and either hKLF-pcDNA3.1+ or pcDNA3.1+ (mock) (Fig. 6). hKLF11 reduced the activity of −387hInsP-pSEAP to about 40% of mock control level. In accord with the results from 5′-deletion of hInsP this inhibition was not altered by mutation of the GC box. Although no binding of in vitro translated hKLF11 to the CACCC box was observed in EMSA experiments, CACCC box mutation significantly ($p<0.01$) reduced hKLF11-

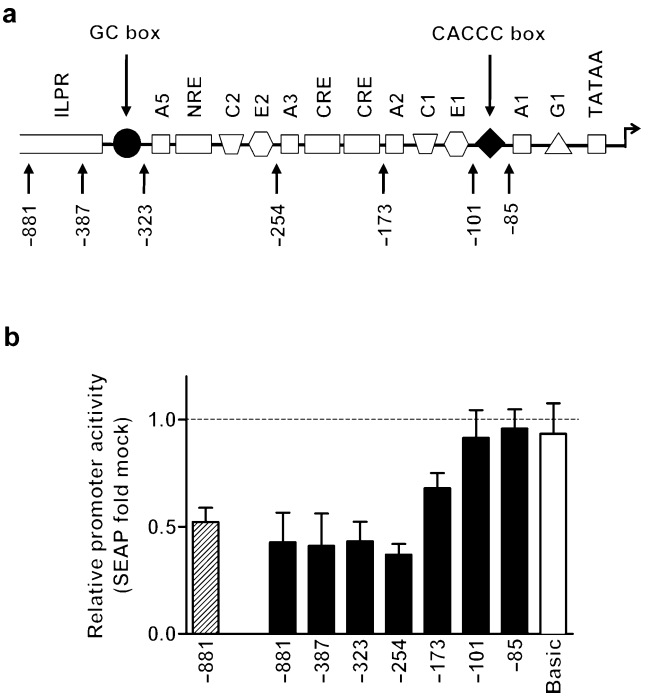

Fig. 5 KLF11-mediated inhibition of 5′-deletion fragments of hInsP. **a** Schematic delineation of the investigated hInsP full-length fragment and its 5′-deletion constructs subcloned into the promoterless pSEAP2-Basic reporter plasmid. *CRE*, cAMP response element; *ILPR*, insulin linked polymorphic region; *NRE*, negative regulatory element. **b** Cotransfection of hInsP-SEAP reporter plasmids with either the hKLF11-pcDNA3.1+ expression plasmid or pcDNA3.1+ empty plasmid (mock control level, *dotted line*). *hKLF11* overexpression-induced inhibition of hInsP activity was similar in INS-1E (*hatched column*) and beta-TC3 beta cells (*black columns*). Inhibition was stable between −254 to −881, diminished at −173 and abolished at −101. Note that inhibition was not altered by deletion of the GC box (−323 and −254). pSEAP2-Basic empty vector (*white column*) was used as a further control for the absence of non-specific regulation by KLF11. Note that plotted values from hInsP fragments and Basic represent relative changes in promoter activity and not differences in absolute SEAP concentrations. Means±SD; $n=5$

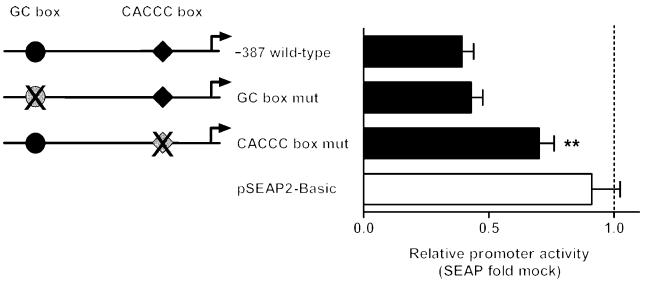

Fig. 6 Functional relevance of GC and CACCC box mutations (*box mut*) for KLF-mediated inhibition of 5′-deletion fragments of hInsP. Beta-TC3 beta cells were cotransfected with −387hInsP-pSEAP reporter plasmids containing wild-type or mutated (see Fig. 3a) GC and CACCC box sequences and either the hKLF11-pcDNA3.1+ expression plasmid or pcDNA3.1+ empty plasmid (mock control level, *dotted line*). GC box mutation did not alter hKLF11-induced inhibition of hInsP, while CACCC box mutation significantly diminished this inhibition. pSEAP2-Basic empty vector (*white column*) was used as a further control for absence of non-specific regulation by KLF11. Note that plotted values from hInsP fragments and Basic represent relative changes in promoter activity and not differences in absolute SEAP concentrations. Means±SD; $n=3$; $^{**}p<0.05$

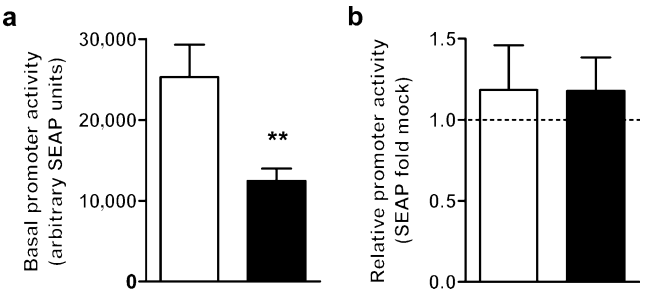

Fig. 8 Functional relevance of CACCC box mutation for basal hInsP activity and KLF11-mediated inhibition of hInsP in HEK293 cells. **a** Transfection with −323hInsP-pSEAP reporter plasmids containing wild-type (*white column*) or mutated (*black column*) (see Fig. 4a) CACCC box sequences. The −323hInsP fragment did not contain a GC box (see Fig. 5a). Also in non-beta cells the CACCC box mutation substantially reduced basal hInsP activity. **b** Cotransfection with −323hInsP-pSEAP reporter plasmids containing wild-type (*white column*) or mutated (*black column*) CACCC box sequences and either hKLF11-pcDNA3.1+ expression plasmids or pcDNA3.1+ empty plasmids (mock control level, *dotted line*). The hKLF11-induced inhibition of hInsP was completely abolished in non-beta cells. Means ± SD; all experiments, $n=3$; $^{**}p<0.01$

mediated inhibition of hInsP activity from about 40% to about 70% of the mock control level. Interestingly, while calculating relative promoter activities from SEAP data, we observed that absolute SEAP concentrations were substantially lower when the CACCC box was mutated. Therefore, we additionally investigated the functional relevance of the GC box and CACCC box for the basal activity of hInsP by transfection of beta-TC3 beta cells with −387hInsP-pSEAP containing wild-type or mutated sequences (Fig. 7). While mutation of the GC box had no significant influence, mutation of the CACCC box rigorously reduced basal hInsP activity to about 25% of wild-type control levels ($p<0.001$). We further tested this observation in HEK293 cells (Fig. 8a). The −323hInsP-pSEAP plasmid was used for experiments since its inhibition by *hKLF11* overexpression was identical to longer constructs and does not contain the GC box. Even in

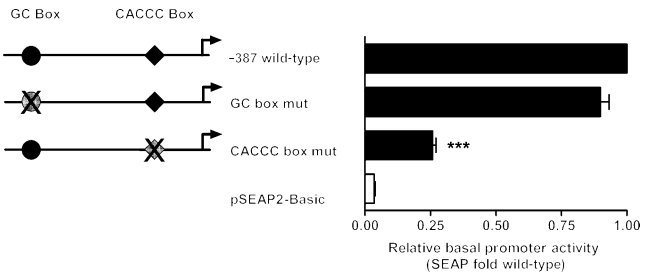

Fig. 7 Functional relevance of GC and CACCC box mutations (*box mut*) for basal hInsP activity. Beta-TC3 beta cells were transfected with −387hInsP-pSEAP2-Basic reporter plasmids containing wild-type or mutated (see Fig. 3a) GC and CACCC box sequences or the promoterless pSEAP2-Basic plasmid (background control). Basal activity of hInsP was substantially suppressed by CACCC box mutation, whereas GC box mutation had no effect. Means±SD; $n=3$; $^{***}p<0.001$

HEK293 cells, mutation of the CACCC box significantly reduced basal hInsP activity to about 50% of wild-type control levels. Finally, compared with mock control level (pcDNA3.1+), hKLF11 did not alter activity of either −323hInsP-pSEAP containing wild-type or mutated CACCC box (Fig. 8b).

Discussion

The TIEG subfamily, consisting of KLF10 and KLF11, is defined by the three N-terminal repressor domains SID/R1, R2 and R3. These domains have been demonstrated to repress transcription activity [4, 5]. In this sense, KLF11 acts as a dominant repressor of the caveolin-1 gene [13] and, besides its negative regulation of cell growth in the exocrine pancreas [9], *KLF11* overexpression also inhibits cell proliferation in Chinese hamster ovary cells [6]. KLF11 further suppressed oncogene-induced neoplastic transformation in mouse NIH-3T3 cells, and consequently *KLF11* mRNA expression was found to be significantly downregulated in a substantial amount (50%) of investigated pancreatic, breast and kidney tumours [9]. In this context, TGF-β inhibits growth of epithelial cells by activation of Smad signalling, which is potentiated through TGF-β-induced KLF11 by termination of the negative feedback loop imposed by Smad7 [14]. In pancreatic cancer cells with an oncogenic *Ras* mutation this function is inhibited by ERK/mitogen-activated protein kinase phosphorylation of KLF11, leading to disruption of KLF11-mSin3a interaction, and thereby ends silencing of the Smad7 promoter [14, 15].

In line with these transcription-repressing properties of KLF11, our results demonstrate a dose-dependent inhibition

of hInsP activity by cotransfected hKLF11 in INS-1E beta cells which is similar in standard and high glucose conditions. The observed hKLF11-mediated inhibition of hInsP activity is underlined by the reduction of *Ins2* gene expression by *rKLF11* overexpression. In line with this inhibitory function *KLF11* mRNA levels in INS-1E beta cells were repressed by high glucose conditions in which insulin production and secretion are enhanced. Interestingly, in a recent study Neve et al. [10] reported a high glucose-induced stimulation of *KLF11* mRNA expression in INS832/13 beta cells. This opposing observation may be explainable by possible differences between native INS-1E beta cells used in our study and the INS832/13 beta cell line, which is derived from a highly selected INS-1 subclone stably transfected with a plasmid containing the human proinsulin gene [16]. More importantly, Neve et al. [10] demonstrated activation of hInsP by cotransfected FLAG-tagged KLF11 in beta-TC3 beta cells. To be sure that this conflicting finding is not caused by the use of different beta cell lines, we confirmed the hKLF11-induced inhibition of cotransfected hInsP activity observed in INS-1E beta cells also in beta-TC3 beta cells. Noteworthy, the obtained inhibition was very similar in both beta cell lines demonstrating stable performance of our experimental approach in rodent beta cells independently of the species (rat and mouse). Since the hKLF11-induced inhibition of wild-type and mutated hInsP constructs was completely abolished in HEK293 cells, this function seems to be strictly dependent on the beta cell-specific transcription machinery.

Sp1-like proteins are known to bind with different selectivity to CGCCC or CACCC core sequences in GC-rich sites [2]. The requirement of GC box sequences for functional KLF11-induced repression of promoter activity has been evaluated by several studies [6, 13, 14]. Based on this established concept Neve et al. [10] predicted, although they demonstrate activation and not repression, that KLF11 influenced hInsP via an identified GC box sequence, which they have tested by EMSA. We also verified binding of hKLF11 to the GC box and further investigated the functional relevance of this interaction, but unexpectedly, neither 5′-deletion nor mutation of the GC box altered *hKLF11* overexpression-induced inhibition of hInsP activity. This demonstrates that, at least in the context of hInsP, the inhibitory function of hKLF11 is GC box-independent. Moreover, the GC box is also dispensable for the maintenance of basal hInsP activity.

Searching for alternative KLF11-binding sites within hInsP we identified a CACCC box located from −88 to −96. The CACCC sequence was initially reported to be required for KLF11-mediated activation of γ-globin gene promoter [8], whereas a later study failed to induce significant alterations of γ-globin gene promoter activity by cotransfected KLF11 [17]. However, KLF11 seems to be expendable for globin gene expression since $KLF11^{-/-}$ mice display normal haematopoiesis at all stages of development [18]. Interestingly, Ou et al. [12] confirmed binding of KLF11 to both Sp1/GC and CACCC sites, thereby influencing the human *MAOB* promoter as an activator via its Sp1/GC site or as a repressor via its CACCC site. In contrast, we could not detect binding of in vitro translated hKLF11 to the CACCC box sequence within hInsP. This indicates that the observed decrease in hKLF11 inhibitory function due to CACCC box mutation may be caused by indirect interactions. Nevertheless, an involvement of the CACCC box in KLF11 action is supported by the specific KLF11-mediated inhibition of *Ins2* gene expression while *Ins1* gene expression remains unaffected. Of note, only the *Ins2* promoter sequence contains a CACCC box.

Interestingly, mutation of the CACCC box substantially downregulated basal activity of hInsP in beta-TC3 beta cells and also HEK293 cells to about 25 and 50% of wild-type control levels, respectively. These results suggest that the CACCC box is mainly a target for general transcription factors independently of the beta cell-specific transcription machinery and demonstrates the requirement of a functional CACCC box for the maintenance of normal human proinsulin gene expression. From our results we conclude that this function is independent of the involvement in hKLF-mediated inhibition of hInsP.

Considering all findings we presume that hKLF11 may mainly act via another yet unknown site of hInsP or, more likely, indirectly by interfering with beta cell-specific transcription factors. This reasoning was initiated by the described interaction of KLF1 [19, 20], KLF2 [21], KLF4 [22, 23] and KLF13 [24, 25] with the transcriptional cofactors CBP and p300, which also interact with the pancreatic duodenal homeobox protein (PDX)-1, a major transactivator of proinsulin gene expression in pancreatic beta cells [26, 27]. Of note, KLF13 and KLF11 belong to the same KLF subfamily that is functionally characterised by the SID motif. The speculation of interactions between KLF11 and PDX-1 is supported by the results from 5′-deletion of hInsP demonstrating stepwise decrease of KLF11-induced inhibition. PDX-1 is known to bind to A elements present in the proinsulin promoter region. Although KLF11 function is unaffected by deletion of A5 (−254hInsp fragment), further deletion of A3 (-173hInsp fragment) reduced and deletion of both A3 and A2 (−101hInsP fragment) completely abolished KLF11-mediated inhibition.

In summary, high glucose conditions stimulating insulin production and secretion repress endogenous *KLF11* expression in INS-1E beta cells. This is in line with our results characterising hKLF11 as a glucose-independent negative regulator of hInsP in INS-1E and beta-TC3 beta cells. Interestingly, KLF11 specifically reduced *Ins2* but not *Ins1* gene expression in INS-1E beta cells and only the *Ins2* promoter contains a CACCC box. Although we could not

verify KLF11 interaction with the CACCC box, KLF11-mediated inhibition of human proinsulin promoter activity depends on a functional CACCC box, thereby indicating an indirect mechanism. In contrast, and regardless of observed specific binding, the GC box was dispensable for this KLF11 function. Moreover, we demonstrate a new and substantial role for the CACCC box in maintaining basal hInsP activity. In conclusion, these findings may contribute to a better understanding of the complex regulation of proinsulin gene expression and may suggest that dysregulation of KLF11 function and CACCC box mutation have an impact on the development and clinical manifestation of diabetes mellitus.

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Duality of interest There is no duality of interest to report.

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