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Human *Krüppel*-like factor 11 differentially regulates human insulin promoter activity in β-cells and non-β-cells via p300 and PDX1 through the regulatory sites A3 and CACCC box

Nikolaos Perakakis [a], Despoina Danassi [a], Marcus Alt [a], Eleni Tsaroucha [a], Amir E. Mehana [a,b], Natalie Rimmer [a], Katharina Laubner [a], Haiyan Wang [c], Claes B. Wollheim [d], Jochen Seufert [a], Günter Päth [a,*]

[a] *Division of Endocrinology and Diabetology, Department of Internal Medicine II, University Hospital of Freiburg, Hugstetter Strasse 55, 79106 Freiburg, Germany*
[b] *Institute of Biology II, Faculty of Biology, University of Freiburg, Schaenzlestrasse 1, 79104 Freiburg, Germany*
[c] *F. Hoffmann-La Roche AG, Metabolic and Vascular Diseases, Basel, Switzerland*
[d] *Department of Cell Physiology and Metabolism, University Medical Center, University of Geneva, 1 Rue Michel-Servet, 1211 Geneva 4, Switzerland*

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ABSTRACT

Human *Krüppel*-like factor 11 (hKLF11) has been characterised to both activate and inhibit human insulin promoter (hInsP) activity. Since KLF11 is capable to differentially regulate genes dependent on recruited cofactors, we investigated the effects of hKLF11 on cotransfected hInsP in both β-cells and non-β-cells. hKLF11 protein interacts with hp300 but not with hPDX1. Overexpressed hKLF11 stimulates PDX1-transactivation of hInsP in HEK293 non-β-cells, but confers inhibition in INS-1E β-cells. Both hKLF11 functions can be neutralised by the p300 inhibitor E1A, increased hp300 levels (INS-1E), dominant negative (DN)-PDX1 and by mutation of the PDX1 binding site A3 or the CACCC box. In summary, hKLF11 differentially regulates hInsP activity depending on the molecular context via modulation of p300:PDX1 interactions with the A3 element and CACCC box. We postulate that KLF11 has a role in fine-tuning insulin transcription in certain cellular situations rather than representing a major transcriptional activator or repressor of the insulin gene.

1. Introduction

Krüppel-like factor (KLF)11 is a member of the Sp1/KLF transcription factor family which shares binding to GC-rich or CACCC sequences via three conserved C-terminal zinc-finger domains (McConnell and Yang, 2010). KLF proteins are ubiquitously expressed throughout mammalian tissues and participate in virtually all aspects of cellular function as transcriptional repressors or activators depending on the molecular context (Kaczynski et al., 2003; Ou et al., 2004). Among KLFs, KLF10 and KLF11 form a subgroup defined by TGF-β-inducible expression and were therefore alternatively named TGF-β-inducible early response genes (TIEG)1 and 2, respectively (Cook et al., 1998; Subramaniam et al., 1995).

KLF11 is enriched in muscle and pancreas (Cook et al., 1998) and published results characterised KLF11 as a negative regulator of exocrine pancreatic cell proliferation in transgenic mice with acinar cell-specific KLF11 overproduction (Fernandez-Zapico et al., 2003). Within the endocrine pancreas, KLF11 was initially characterised as an activator of the human insulin gene promoter (hInsP) (Neve et al., 2005). This study further reported an association between KLF11 gene variants and early-onset diabetes as well as common type 2 diabetes in French and Northern European populations, respectively. Based on these associations, KLF11 gene variants which failed to activate the insulin promoter were proposed to represent a new MODY (MODY7) (Fernandez-Zapico et al., 2009). However, associations between KLF11 gene/promoter variants and MODY or type 2 diabetes could not be confirmed in other populations including full-heritage Pima Indians (Ma et al., 2008), Danish (Florez et al., 2006; Gutierrez-Aguilar et al., 2008) and Japanese individuals (Kuroda et al., 2009; Tanahashi et al., 2008).

Abbreviations: CBP, CREB-binding protein; h, human; InsP, insulin promoter; KLF, *Krüppel*-like factor; MAO B, monoamine oxidase B; mut, mutated; PDX1, pancreatic duodenal homeobox protein 1; SEAP, secreted alkaline phosphatase; Sp1, specificity protein 1; TIEG, TGF-β-inducible early response genes; wt, wild type.

* Corresponding author. Address: Research Lab B9, Division of Endocrinology and Diabetology, Department of Internal Medicine II, University Hospital of Freiburg, 79106 Freiburg, Germany. Tel.: +49 761 270 73 270; fax: +49 761 270 33 720.
E-mail address: guenter.paeth@uniklinik-freiburg.de (G. Päth).

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Nevertheless, a significant association between KLF11 gene/promoter variants and reduced insulin sensitivity in glucose tolerant Danish subjects was verified (Gutierrez-Aguilar et al., 2008). Variability of these results may be at least in part explainable by the close structural and functional relationship of KLF11 to KLF10 (Subramaniam et al., 2007). As an example for shared function among KLFs, coexpressed KLF10 as well as KLF13, KLF14 and KLF16 have recently been demonstrated to activate the hInsP (Bonnefond et al., 2011).

The initial report by Neve et al. (2005) and the recent study of Bonnefond et al. (2011) characterise KLF11 as a stimulator of cotransfected hInsP reporter in β-cell lines. Our own research, however, could not confirm these findings. We observed that hKLF11 inhibits hInsP in rat INS-1E as well as mouse β-TC3 β-cells (Niu et al., 2007). A negative regulation of hInsP by cotransfected hKLF11 was also observed by others in mouse MIN6-m9 β-cells (Kuroda et al., 2009). These opposite findings raise the question whether the employed experimental settings reflect differential actions of KLF11 that depend on the exact molecular context as it has been reported earlier for the human monoamine oxidase gene (MAO) B gene (Ou et al., 2004). Differential regulation of gene expression is a general feature in several other KLFs because of their ability to recruit transcriptional corepressors as well as coactivators in distinct cellular situations (Kaczynski et al., 2003; McConnell and Yang, 2010). Alternatively, KLF11 may influence hInsP indirectly since it can bind the conserved area II region within the pancreatic duodenal homeobox protein (PDX)1 gene promoter and is potentially able to stimulate expression of PDX1 which is a major transactivator of insulin gene expression (Fernandez-Zapico et al., 2009). However, PDX1 function strictly depends on the transcriptional cofactor p300, and KLF11 has been demonstrated to interact with p300 (Fernandez-Zapico et al., 2009). The importance of p300 is underscored by a reduction of KLF11-mediated hInsP activation if a mutant dominant negative p300 is coexpressed (Bonnefond et al., 2011).

Taken together, there is consensus that KLF11 can regulate hInsP. The described conflicting findings on stimulation versus inhibition indicate that the exact molecular mechanism is not yet fully understood. To characterise context-dependent regulation of the hInsP by KLF11, we analysed the contributing roles of the p300:PDX1 transcriptional complex and relevant promoter response elements in both β-cells and non-β-cells.

2. Methods

2.1. Cell culture

Rat INS-1E β-cells and INS-1-derived transgenic INSrβ β-cell lines were routinely cultured in RPMI 1640 (11.1 mM glucose) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol. INSrβ β-cell lines enable doxycycline (Dox)-inducible overexpression of either mouse Pdx1 or its mutated dominant negative (DN)-Pdx1 variant lacking the complete N-terminal transactivation domain (the first 79 amino acids). INSrβ β-cell lines were generated and characterised as described earlier (Wang et al., 2001). Human embryonic kidney 293 cells (HEK293) were cultured in DMEM (25 mM glucose) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/mL streptomycin.

2.2. Plasmid construction

Construction of human KLF11 expression plasmid (hKLF11-pcDNA 3.1) and human insulin promoter-driven secreted alkaline phosphatase (SEAP) reporter plasmids (5′-deletion series −881 + 54 to −101 + 54hInsP-pSEAP) has been described previously (Niu et al., 2007). The human p300 coding sequence was subcloned from CMVß-p300 (kindly provided by Timothy J. Kieffer, University of British Columbia Vancouver, BC Canada) into the CMV promoter-driven pcDNA3.1 (Invitrogen/Life Technologies GmbH, Darmstadt, Germany) to obtain the hp300-pcDNA3.1 expression plasmid. The human PDX1 coding sequence was subcloned from hPDX1-pCMV5 (kindly provided by Doris A. Stoffers, University of Pennsylvania, School of Medicine, Philadelphia, PA, USA) into pcDNA3.1 to generate the hPDX1-pcDNA3.1 expression plasmid. The pBJ9Q-E1A plasmid (Bannister and Kouzarides, 1995) expresses E1A under the control of the Rous sarcoma virus (RSV) promoter (kindly provided by Bernd Groner, Georg-Speyer-Haus, Institute for Biomedical Research, Frankfurt am Main, Germany). A1, A3 and GG2 box of −881 + 54hInsP promoter sequence were mutated as described earlier (Le Lay and Stein, 2006). The CACCC box within a −387 + 54hInsP promoter fragment was mutated from CCCCACCCC to CCCGATTCC as described earlier (Niu et al., 2007). All mutations were performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany). Potential protein binding between hKLF11, hPDX1 and hp300 was anlysed by using the CheckMate™ Mammalian Two-Hybrid System (Promega GmbH, Mannheim, Germany). hKLF11, hPDX1 and hp300 coding sequences were subcloned from above described plasmids into multiple cloning regions of each of the assay-provided expression plasmids pACT (expresses VP16 fusion proteins) and pBIND (expresses GAL4 fusion protein). Binding of pACT and pBIND expressed fusion proteins activates luciferase expression by the pG5*luc* reporter plasmid. Accuracy of all sequences and mutations was checked by sequencing.

2.3. Protein extraction and Western blot analysis

HEK293, ß-TC3 and INS1E cells were seeded in 100 mm dishes and grown to optical confluency. Whole cell extracts were generated by using RIPA buffer (Upstate/Biomol, Hamburg, Germany) containing 1 tablet/10 ml Complete Mini EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). Nuclear extracts were generated as described earlier (Schreiber et al., 1989). Three million INS-rβ β-cells were seeded in 60 or 100 mm dishes. After two days day ectopic PDX1 or DN-PDX1 expression was induced by addition of Dox. Whole cell extracts were generated 24 h post addition of Dox as explained above. In general, 7 μg extracted proteins per sample were separated on 10% polyacrylamide SDS gels and transferred to PVDF membranes by semi-dry blotting using Towbin transfer buffer. KLF11, actin and PDX1 were detected by their respective goat polyclonal antisera (TIEG2 C-12, sc23162; actin I-19, 2c-1616; PDX-1 A17, sc-14664; all Santa Cruz Biotechnology, Heidelberg, Germany). Visualization of signals was achieved by using a horse radish peroxidase-linked donkey anti-goat secondary antibody (donkey anti-goat IgG-HRP, sc-2033, Santa Cruz Biotechnology) and ECL + Western Blotting Detection Reagent (Amersham, Freiburg, Germany).

2.4. SEAP reporter gene experiments

INS-1E β-cells or HEK293 cells were seeded in 6-well culture plates at a density of 300,000 cells/well in their respective medium. Next day cells were transiently transfected using Metafectene Pro (Biontex, Martinsried/Planegg, Germany) using a ratio of 2 μl Metafectene Pro per 1 μg DNA. After 48 h supernatants were collected for measurement of SEAP using the BD Great EscAPe SEAP Chemiluminescence Detection Kit (BD Biosciences Clontech, Heidelberg, Germany). INSrβ-PDX1 and INSrβ-DN-PDX1 β-cells were seeded 300,000 cells/well each in 6-well plates. Next day ectopic gene expression was induced by addition of 500 ng/ml Dox

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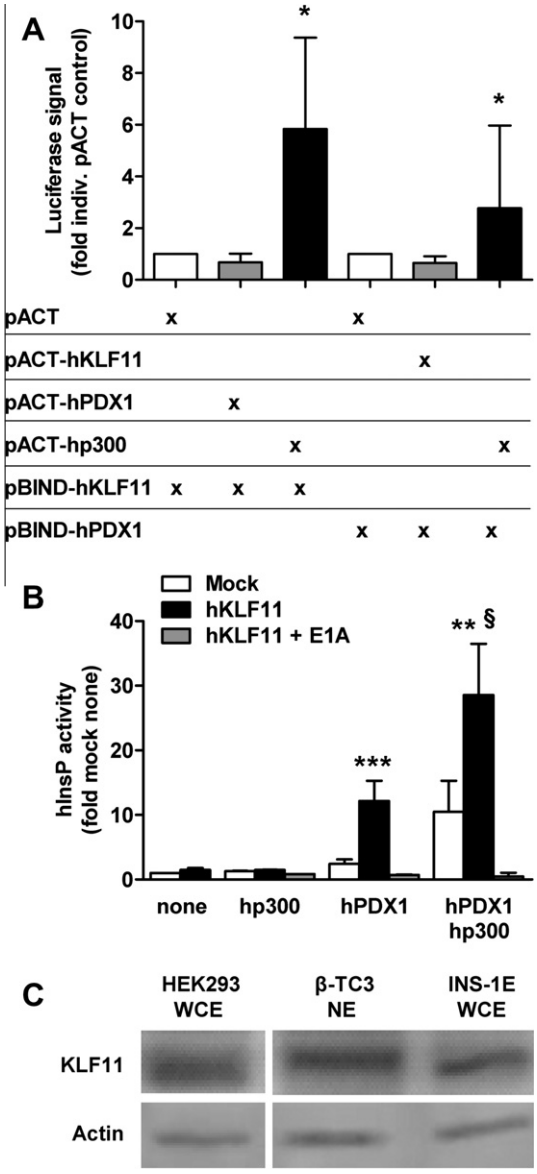

Fig. 1. hKLF11 interacts with hp300 and synergistically stimulates p300/PDX1-induced hInsP activity in non-β-cells. **(A)** Protein interactions were evaluated by a mammalian two-hybrid system. HEK293 cells were transiently cotransfected with pG5*luc* reporter plasmid and pACT and pBIND expression plasmids containing coding sequences of proteins as indicated in the figure. pACT/pBIND-hKLF11 and pACT/pBIND-hPDX1 signals define unspecific signal background and the known interaction between hp300 and hPDX1 served as positive control. Protein interaction was observed between hKLF11 and hp300 but not between hKLF11 and hPDX1. Means ± SD, $n = 4$; $^{*}p < 0.05$ vs. individual control. **(B)** HEK293 cells were transiently cotransfected with -884 + 54hInsP reporter plasmid, hp300, hPDX1 and hKLF11 or pcDNA3.1 empty vector (mock). Compared to mock, overexpression of hKLF11 alone or in combination with hp300 did not affect hInsP but hKLF11 significantly enhanced hPDX1- and hp300/hPDX1-induced promoter activation. Additional cotransfection of the p300/PDX1 inhibitor E1A completely abolished hInsP activation. Means ± SD, $n = 3$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. individual mock; §, $p < 0.05$ vs. hKLF11 and hPDX1. **(C)** Detection of KLF11 protein in whole cell extracts (WCE) and nuclear extracts (NE) of HEK293, β-TC3 and INS-1E cells. Representative blot, $n = 3$.

according to Wang et al. (2001). Cells were transiently transfected at day 3 and supernatants were collected at day 7 after seeding. Generally, total amount of transfected DNA was adjusted to 1 μg by addition of pcDNA3.1 empty vector. In exception, total transfected DNA was 1.25 μg in experiments shown in Fig. 1B and 1.5 μg in those for Fig. 3A.

2.5. Analysis of protein interaction

We employed the Check Mate Mammalian Two-Hybrid assay consisting of three plasmids as described above in *Plasmid construction*. HEK293 cells were seeded in 24-well plates at a density of 50,000 and directly transfected in parallel with pACT-(X), pBIND-(Y) and pG5*luc* (0.33 μg each) by using Metafectene Pro as described above. Transfected DNA amount was 1 μg in all experimental groups. X and Y refer to coding sequences of two respective proteins tested for interaction as indicated in Fig. 6. Forty eight hours after transfection cells were harvested for analysis of luciferase signals by using the Dual-Luciferase Reporter Assay System (Promega GmbH) according to the manufacturer's protocol.

2.6. Statistical analysis

Results were analysed for statistical differences by *t* test (comparison between two groups) or ANOVA followed by Bonferroni post hoc test (three and more groups) using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

3. Results

3.1. hKLF11 interacts with hp300 and synergistically stimulates p300/PDX1-induced hInsP activity in non-β-cells

In our initial report, we postulated that KLF11 may interact with p300 or PDX1. Therefore, we analysed potential protein interactions by a mammalian-two hybrid system. HEK293 cells were transiently cotransfected with a pG5*luc* reporter plasmid as well as pACT and pBIND expression plasmids containing coding sequences of proteins as indicated (Fig. 1A). Combinations of pACT empty vector with pBIND-hKLF11 or pBIND-hPDX1 were used for defining nonspecific background level. The known interaction between p300 and PDX1 (pACT-hp300 and pBIND-hPDX1) served to define the level of positive interaction signals. No signals over background were detected if hKLF11 was paired with hPDX1 (pACT-hPDX1 and pBIND-hKLF11 or pACT-hKLF11 and pBIND-hPDX1) while coexpression of pACT-hp300 and pBIND-KLF11 resulted in positive signals above the level of the p300/PDX1 positive control. The mammalian-two hybrid system does not finally prove that observed interactions are direct. However, the observed direct or indirect interactions between hKLF11 and hp300 confirm results of immunoprecipitation experiments by Fernandez-Zapico et al. (2009).

To further explore the underlying molecular mechanism, we evaluated hKLF11 effects on hInsP in HEK293 cells (Fig. 1B). Cells were transiently cotransfected with −884 + 54hInsP reporter plasmid, hp300, hPDX1 and hKLF11 and E1A (0.25 μg each). hKLF11 was absent in individual mock controls. Activity of hInsP in the absence of the β-cell-specific transcription machinery was very low and defines the background which was not altered by further coexpression of hKLF11. Overexpression of hp300 alone or in combination with hKLF11 did not affect hInsP activity in HEK293 cells. Substantial hInsP activation could be induced by coexpression of hPDX1 alone (endogenous p300 is present in HEK293 cells) and was much more pronounced by the combination of hPDX1 and hp300. This was an expected result since β-cell-specific PDX1 is absent in non-β-cells and the concentration of p300 is known to be the limiting factor of PDX1 action (Stanojevic et al., 2004). Interestingly, hKLF11 synergistically stimulated hPDX1- and hp300/PDX1-induced hInsP activity in HEK293 cells. This finding contrasts with our earlier observed KLF11-mediated inhibition of hInsP activity in β-cell lines (Niu et al., 2007). In some experiments, we further

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coexpressed the established p300/PDX1 inhibitor E1A (Qiu et al., 2002) which completely abrogated hInsP activity. This observation confirms that p300 and (ectopic) PDX1 are strictly required for the stimulatory impact of hKLF11 on hInsP in HEK293 non-β-cells.

In addition, we have verified endogenous KLF11 protein expression in HEK293 whole cell extracts and for control also in β-TC3 nuclear extracts and INS-1E whole cell extracts (Fig. 1C). Endogenous KLF11 expression in both β-cell lines confirms our previous findings (Niu et al., 2007).

3.2. Inhibition of hInsP by KLF11 in β-cells depends on p300 and PDX1

To investigate the role of p300 for the previously reported inhibition of hInsP in INS-1E and β-TC3 β-cells (Niu et al., 2007), we transiently transfected INS-1E with the −884 + 54hInsP reporter plasmid, hKLF11 (both 0.25 μg) and different amounts of hp300. Mock controls lack hKLF11. Significant reduction of hInsP activity by coexpressed hKLF11 was observed as reported earlier (Fig. 2A). Interestingly, additional coexpression of 0.1 or 0.5 μg hp300 completely abolished the inhibitory impact of hKLF11 indicating that its inhibitory function can be blocked by excessive concentrations of hp300 protein.

To evaluate the specific role of PDX1 in this context, we employed two INSrβ β-cell lines with Dox-inducible overexpression of either mouse Pdx1 (rβ-PDX1) or its dominant negative variant (DN)-PDX1 that lacks the complete transactivation domain (rβ-DN-Pdx1). Dox-induced ectopic protein production was analysed by Western blotting and found maximal at 500 ng/ml Dox (Fig. 2B). In whole cell extracts, we observed in both INSrβ cell lines a signal at 31 kDA. These signals indicated by asterisks likely represent the cytosolic 31 kDa variant of PDX1 described by Macfarlane et al. (1999) and served as loading control. Note that ectopic DN-PDX1 expression is slightly leaky (band at 0 mM Dox). Consequently, expansion of INSrβ-DN-Pdx1 β-cells is slower than in INSrβ-Pdx1.

INSrβ-PDX1 and INSrβ-DN-PDX1 β-cells with induced ectopic protein expression were transiently cotransfected with −884 + 54hInsP reporter plasmid and hKLF11 (0.5 μg each). Mock controls lack hKLF11. As observed before in INS-1E β-cells (Fig. 2A), overexpression of hKLF11 substantially inhibited hInsP activity in both INSrβ-PDX1 and INSrβ-DN-PDX1 β-cells (Fig. 2C). The inhibitory effect of hKLF11 was not altered by induced ectopic PDX1 but was almost completely diminished by induced ectopic DN-PDX1 expression. Note that Fig. 2C does not reflect DN-PDX-mediated general reduction of hInsP transactivation since values were normalised to individual mock level.

These results demonstrate that hKLF11-mediated inhibition of hInsP in β-cells is mediated via PDX1. Promoter repression by hKLF11 can be blocked by excessive free hp300 indicating a situation where hp300 molecules neutralise hKLF11 and remaining free hp300 molecules still support the formation of functional PDX1 transcriptional complexes.

3.3. Effects of hInsP 5′-deletion and mutation of PDX1 binding sites or the CACCC box on hPDX1 and hKLF11-induced promoter activity in non-β-cells

To locate the promoter region responsible for synergistic enhancement of PDX1-mediated hInsP activation by hKLF11, we investigated a series of hInsP 5′-deletion constructs in HEK293 cells (Fig. 3A). The −881 + 54hInsP full length fragment contains the PDX1 binding elements A5 (−319 to −307), A3 (−216 to −207), GG2 (−145 to −140) and A1 (−83 to −75) which were deleted from 5′ in a stepwise approach. HEK293 cells were transiently transfected with −881 + 54hInsP or its 5′-deletion constructs, hPDX1 and hKLF11 (0.5 μg each plasmid). Individual mock controls

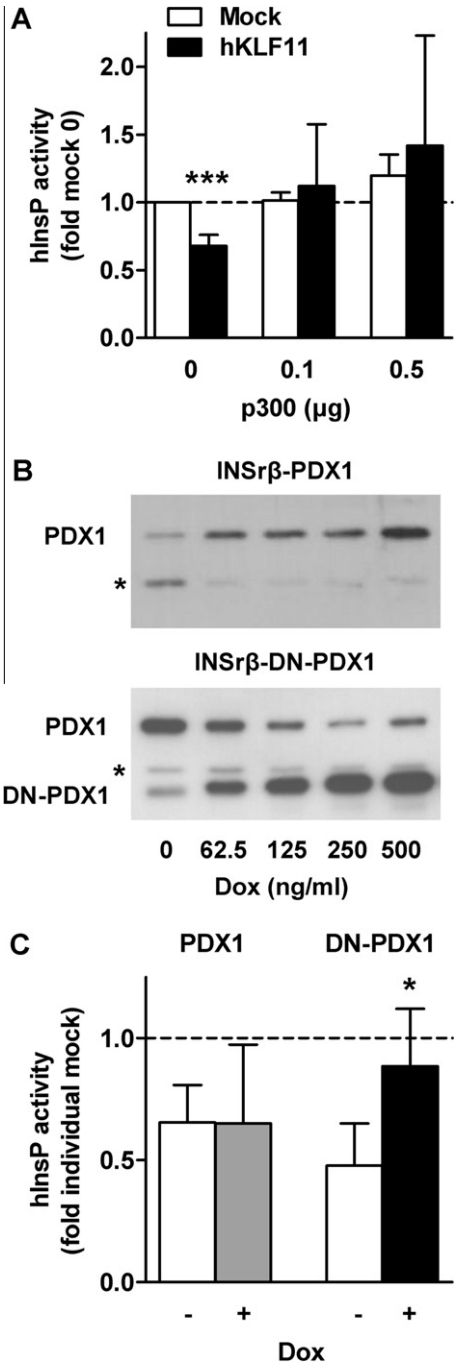

Fig. 2. KLF11-mediated inhibition of hInsP in β-cells depends on p300 and PDX1. **(A)** INS-1E β-cells were transiently cotransfected with -884 + 54hInsP reporter plasmid, different amounts of hp300 and hKLF11 or pcDNA3.1 (mock; mock 0 level, dashed line). Compared to individual mock, inhibition of hInsP by hKLF11 in the presence of endogenous p300 is completely abolished by hp300 overexpression. Means ± SD, $n = 5$; $^{***}p < 0.001$ vs. mock 0. **(B)** Doxycycline (Dox)-inducible overexpression of mouse PDX1 and its mutated dominant negative variant DN-PDX1 (lacking the complete transactivation domain) in whole cell extracts of INS-1-derived rβ-PDX1 and rβ-DN-PDX1 β-cells, respectively. PDX1 signals in the absence of Dox represent endogenous PDX1. Signals indicated by asterisks likely represent the cytosolic 31 kDa variant of PDX1 described by Macfarlane et al. (Macfarlane et al., 1999) and served as loading control. Representative blot, $n = 3$. **(C)** INSrβ-PDX1 (PDX1) and INSrβ-DN-PDX1 (DN-PDX1) β-cells were transiently cotransfected with -884 + 54hInsP reporter plasmid and hKLF11 or pcDNA3.1 (mock, dashed line). Ectopic protein production was induced by 500 ng/ml Dox. Overexpression of PDX1 had no effect while overexpression of DN-PDX1 nearly completely reduced hKLF11-mediated hInsP inhibition. Note, the plot does not reflect DN-PDX suppression of hInsP activity since values were normalised to individual mock. Means ± SD, $n = 4$; $^{*}p < 0.05$ vs. individual control w/o Dox.

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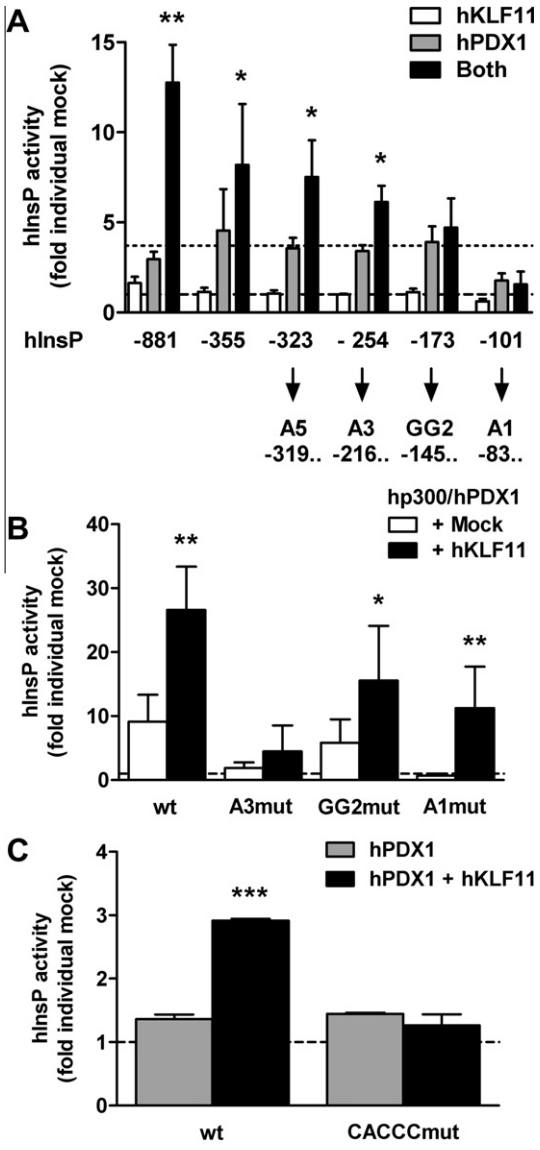

Fig. 3. Effects of hInsP 5'-deletions and mutation of PDX1 binding sites on hPDX1 and hKLF11-induced promoter activity in non-β-cells. **(A)** HEK293 cells were transiently cotransfected with −884 + 54hInsP reporter plasmid or its 5′-deletion constructs, hPDX1 and hKLF11 or pcDNA3.1 empty vector (mock; individual mock level, dashed line). hKLF11 alone was without effect but synergistically enhanced PDX1 transactivation (Both, hPDX1 and hKLF11). hPDX1 alone stimulates hInsP activity in all 5′-deletion fragments tested (dotted line, average induced level of 3.7fold). The very short -101 fragment displayed general reduced activity. hPDX1-induced hInsP activation remained constant but synergistic stimulation by hPDX1 and hKLF11 decreased with shortening of promoter and was abolished at −173 (loss of A3). Means ± SD, $n = 3$; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. individual mock. **(B)** HEK293 cells were transiently cotransfected with wild type (wt) or mutated (mut) −881 + 54hInsP reporter plasmid, hp300, hPDX1 and hKLF11 or pcDNA3.1 (mock). Data were normalised to wt mock without hp300 and hPDX1 (basal mock, dashed line). Compared to individual mock, hKLF11 synergistically enhanced hp300/hPDX1-stimulated wt hInsP activity. The impact of hKLF11 was preserved when A1 or GG2 box were mutated but was completely abolished by mutation of A3. Means ± SD, $n = 5$; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. individual mock. **(C)** HEK293 cells were transiently cotransfected with wild type (wt) or mutated (CACCCmut) −387 + 54hInsP reporter plasmid, hPDX1 and hKLF11 or pcDNA3.1 (mock). Data were normalised to wt basal mock without PDX1 (dashed line). Reduced stimulation by hPDX1 or hPDX1/hKLF11 compared to Fig. 3A is caused by less transfected DNA (0.25 μg instead of 0.5 μg per plasmid). Synergistic stimulation of wt hInsP by hPDX1 and hKLF11 is completely abolished by mutation of CACCC box located at −98 to −88. Means ± SD, $n = 3$; $^{***}p < 0.001$ vs. wt basal mock.

lack hKLF11. Similar to Fig. 1A, overexpression of hKLF11 alone had no impact on promoter activity. Coexpression of hPDX1 alone stimulated hInsP about 3.7fold (dotted line) in all fragments down to −173. Activity of the −101 + 54hInsP fragment was low due to loss of most enhancing elements but was still inducible by hPDX1 to a lesser extent. Coexpression of hKLF11 and hPDX1 together synergistically and significantly stimulated the full length promoter as compared to PDX1 alone. This synergistic stimulation declines continuously with shortening of the promoter, and remains still significant after depletion of A5 (−254 + 54hInsP), but was abolished after removal of A3 (−173 + 54hInsP).

Because this observation suggests a relevant role for the PDX1 binding site A3, we further tested the impact of internally mutated PDX1 binding sites A3, GG2 and A1 within the context of the full length −881 + 54hInsP fragment (Fig. 3B). A5 was not included for absence of effects in 5′-deletion experiments (Fig. 3A). HEK293 cells were transiently cotransfected with wild type (wt) or mutated (mut) −881 + 54hInsP reporter plasmid, hp300, hPDX1 and hKLF11 (0.25 μg each). Mock controls lack hKLF11. Data were normalised to individual basal mock without hp300 and hPDX1 (dashed line). hKLF11-mediated stimulation of hPDX1-induced wild type insulin promoter activity remains preserved after mutation of GG2 or A1 but was completely abolished by mutation of A3.

The CACCC box has been described to be essential for KLF11/p300-mediated stimulation of hInsP activity in INS-1 β-cells (Bonnefond et al., 2011).

We tested the relevance of the CACCC box for hKLF11-mediated stimulation of hInsP activity in HEK293 non-β-cells by site mutation in the context of a −387 + 54hInsP fragment. For comparison, both wt and mutated sequences were cotransfected with hPDX1 alone or in combination with hKLF11 (Fig. 3C). Data were normalised to basal wt mock without hPDX1 (dashed line). Reduced amount of transfected DNA resulted in reduced response compared to Fig. 3A (0.25 μg instead of 0.5 μg/plasmid). However, synergistic stimulation by PDX1 together with KLF11 remains profound in wt hInsP but was completely absent upon CACCC box mutation.

These data demonstrate that synergistic stimulation of hKLF11/hPDX1-mediated hInsP activation in HEK293 non-β-cells requires the CACCC box and the PDX1 binding site A3, while deletion or mutation of the other PDX1 binding sites (A5, GG2 and A1) did not affect the stimulatory impact of KLF11.

4. Discussion

KLF11 has been characterised in different model systems as an activator as well as an inhibitor of the hInsP in β-cells. This indirectly suggests that the specific molecular context may contribute to these variations. Accordingly, the results of the present study demonstrate that hKLF11 inhibits hInsP in β-cells but activates hInsP in HEK293 non-β-cells, which lack the β-cell-specific transcription machinery. hInsP activation in HEK293 non-β-cells requires ectopic expression of the otherwise absent major transcriptional insulin gene activator PDX1. The additionally required transcriptional cofactor p300 is present in HEK293. It has been demonstrated that several KLFs including KLF1 (Zhang and Bieker, 1998; Zhang et al., 2001), KLF2 (SenBanerjee et al., 2004), KLF4 (Feinberg et al., 2005; Geiman et al., 2000) and KLF13 (Song et al., 2003; Song et al., 2002) interact with p300 or its paralogue CBP. Based on these findings, we speculated in our previous report that also KLF11 may interact with p300 in pancreatic β-cells. Meanwhile, zinc finger-binding of KLF11 to p300 has been demonstrated (Fernandez-Zapico et al., 2009). Our present work confirms interactions of hKLF11 with hp300 and further excludes interactions with hPDX1.

p300 levels have been described to be rate limiting for PDX1 function (Stanojevic et al., 2004). Consequently, we observed in HEK293 cells that hPDX1- and hPDX1/hKLF11-induced hInsP activ-

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ition were substantially enhanced if endogenous p300 levels were increased by additional ectopic hp300 expression. Notably, hp300 alone or in combination with hKLF11 did not influence the hInsP in the absence of cotransfected hPDX1. We additionally coexpressed the established adenoviral p300 inhibitor E1A, which binds to p300 and thereby disrupts the formation of the p300:PDX1 transactivation complex (Qiu et al., 2002). Complete suppression of induced hInsP activation by E1A validates that hKLF11-mediated stimulation in HEK293 cells depends on a functional p300:PDX1 transactivation complex.

We further investigated the role of p300 and PDX1 for hKLF11-mediated modulation of hInsP activity in β-cells. In accordance with our previous study (Niu et al., 2007), we found hKLF11-mediated inhibition of hInsP in INS-1E as well as INS-rß-PDX1 and INS-rß-DN-PDX1 β-cells. Interestingly, additional coexpression of hp300 in INS-1E β-cells did not enhance this inhibitory effect but abolished it. Recruitment of KLF11 may inhibit p300 but, upon overproduction, remaining free p300 is still capable to form a functional PDX1 transcription complex. As expected, overexpression of PDX1 did not influence hKLF11-mediated suppression of hInsP since the two molecules did not interact. On the other hand, reduction of functional PDX1 by the inhibitor DN-PDX1 (transctivation domain truncated) substantially reduced hKLF11 function. These data suggest that interactions between KLF11 and p300 suppress PDX1-mediated transactivation of hInsP in β-cells.

The regulatory elements A5, A3, GG2 and A1 are known PDX1 binding sites within the hInsP. Among these sites only the A3 element is required for KLF11 functions in HEK293 non-β-cells since its mutation abolished the observed synergistic stimulatory effect of hPDX1 together with hKLF11. The hInsP also contains two putative binding sites for KLF11; a GC-rich site at −348 to −340 and a CACCC motif at −96 to −88. In previous work, we detected binding of KLF11 to the GC-rich site (Niu et al., 2007) thereby confirming findings of Neve et al. (2005). Though initially expected, 5′-deletion of the GC site did not influence the inhibitory impact of hKLF11 on hInsP activity in β-cells. Conversely, we found a reduced basal hInsP activity and also a reduced inhibitory impact of hKLF11 when the CACCC box was mutated. Our current data reveal that a functional CACCC box is also required for the stimulatory impact of hKLF11 on the hInsP in HEK293 non-β-cells. Since own earlier published gel shift analysis did not detect any binding of KLF11 to the CACCC box, we assume that KLF11 may interact indirectly via cofactors with this site.

Bonnefond et al. (2011) confirmed general loss of hInsP activity after CACCC box mutation, but in contrast demonstrated interactions between KLF11 and this site. They assumed that promoter activity depends on proper binding of KLF11 to the CACCC site while disruption of this binding causes reduced insulin gene expression. In support they demonstrated reduced insulin levels in otherwise healthy, fertile and unobtrusive KLF11−/− mice (Song et al., 2005). However, the global nature of the gene disruption makes it difficult to ascertain whether the reduced insulin gene expression in β-cells is caused by loss of KLF11 binding to the CACCC box or by yet unknown indirect mechanisms. Interestingly, the same study demonstrates decreased KLF11 activation of hInsP in β-cells if the inhibitor DN-p300 is ectopically produced (Bonnefond et al., 2011). This supports the current consensus that KLF11 interacts with p300.

hInsP-related hKLF11 functions require the p300:PDX1 transcriptional complex as well as the PDX1 binding site A3 and also the CACCC box. Functional inactivation of one of these factors is sufficient to abolish the impact of hKLF11. On this account, KLF11 appears to be a modulator of p300:PDX1-mediated hInsP transactivation. In HEK293 non-β-cells, hKLF11 may support the incomplete p300:PDX1 transcription complex in the absence of β-cell-specific cofactors and thereby enhances promoter activity. In β-cells, all specific cofactors are present and interactions between hKLF11 and p300 may impair complex formation and subsequently reduce PDX1-mediated transactivation.

In summary, our data demonstrate that KLF11 can differentially regulate the insulin gene depending on the molecular context. We could consistently uncover that KLF11 regulates hInsP via p300 and PDX1 independent of the molecular environment or type of action (stimulation in non-β-cells vs. inhibition in β-cells). This capability may, at least in part, explain opposite regulation of hInsP found by others in different experimental settings/models. Importantly, KLF11 functions require two regulatory sites within the hInsP; the A3 element and the CACCC box. In conclusion, we postulate that KLF11 and potentially other KLFs with redundant functions have a role in fine-tuning insulin gene expression in certain cellular situations rather than playing a major role as basic transcriptional activators or suppressors of this gene *in vivo*.

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