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Extracellular acidification stimulates GPR68 mediated IL-8 production in human pancreatic β cells

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Acute or chronic metabolic complications such as diabetic ketoacidosis are often associated with extracellular acidification and pancreatic β -cell dysfunction. However, the mechanisms by which human β -cells sense and respond to acidic pH remain elusive. In this study, using the recently developed human β -cell line EndoC- β H2, we demonstrate that β -cells respond to extracellular acidification through GPR68, which is the predominant proton sensing receptor of human β -cells. Using gain- and loss-of-function studies, we provide evidence that the β -cell enriched transcription factor RFX6 is a major regulator of GPR68. Further, we show that acidic pH stimulates the production and secretion of the chemokine IL-8 by β -cells through NF- κ B activation. Blocking of GPR68 or NF- κ B activity severely attenuated acidification induced IL-8 production. Thus, we provide mechanistic insights into GPR68 mediated β -cell response to acidic microenvironment, which could be a new target to protect β -cell against acidosis induced inflammation.

In biological systems, cells actively partake in maintaining homeostasis of their environmental milieu within a precise range of physiological parameters. Cellular systems also foster the unique ability to respond and adapt to physiological stress, preserving survival and function. Signal transduction across cell membrane, through surface receptors is fundamental to detect and respond to changes in the local milieu¹. Protons (H^+) represent an important component of the extracellular milieu². The extracellular fluids and blood pH are tightly regulated and maintained judiciously at ~ 7.4 but under many patho-physiological circumstances such as inflammation, ischemia and tumor formation, acidosis occurs in the localized microenvironment³.

Cells sense extracellular protons concentration by a number of mechanisms^{4,5}. Ion channels such as transient receptor potential V1 and acid-sensing ion channels (ASICs) represent one sensing mechanism. Such channels are predominantly expressed on sensory neurons and act as proton sensors for pain and nociception signals^{6,7}. A sub-family of G protein-coupled receptors (GPCR) represents a second type of proton sensing mechanism. This includes four members: GPR4, GPR68 (or Ovarian cancer G protein-coupled receptor 1, OGR1), GPR65 (or T-cell death-associated gene 8, TDAG8) and GPR132 (or G2A). These receptors sense moderate extracellular pH within a narrow range (pH 6.0 to 7.6) and signal via a variety of intracellular pathways. For example, GPR68 is coupled to the $G_{q/11}$ -phospholipase-C/ Ca^{2+} pathway, whereas GPR4 and GPR65 are coupled to the G_s -adenyl-cyclase/cAMP pathway^{8,9}.

Insulin-producing pancreatic β -cells are highly differentiated cells that play a critical role in maintaining glucose homeostasis. They are factories dedicated to produce and secrete insulin in a tightly regulated fashion¹⁰. β -cells sense a myriad of circulating factors such as glucose, neurotransmitters and hormones that regulate their function under physiological conditions¹¹. They are also sensitive to inflammatory cytokines that are implicated in their destruction in type 1 diabetes (T1D)^{12,13}. A recurring complication of T1D is diabetic ketoacidosis (DKA) resulting in ketonemia and metabolic acidosis¹⁴ with extracellular acidification of the pancreatic microenvironment^{15,16}. However, the mechanism by which human β -cells sense proton concentration and transmit their signal remains largely unknown. It is likely that moderate acidosis in the pancreatic microenvironment is

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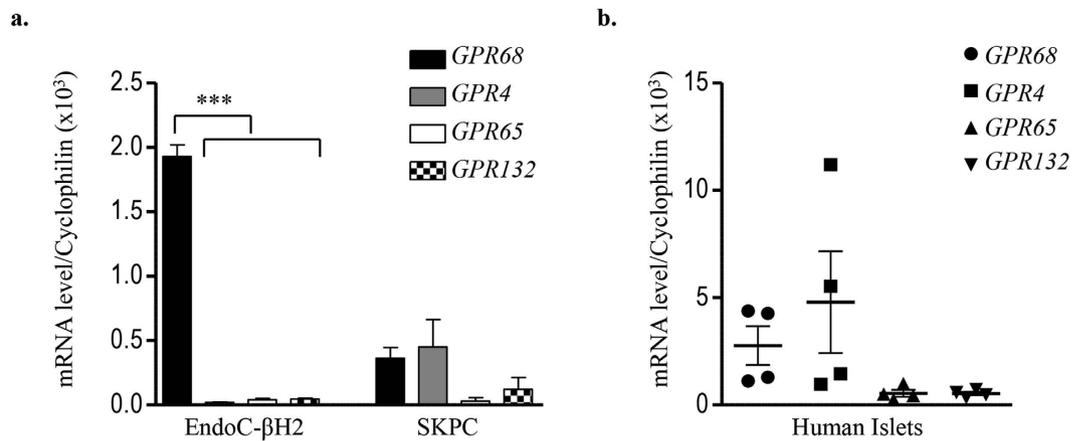


Figure 1. Expression of proton sensing GPCRs in EndoC-βH2, SKPC and human islets. Transcript levels of proton sensing GPCRs (*GPR68*, *GPR4*, *GPR65* and *GPR132*) determined by RT-qPCR in (a) EndoC-βH2 cells compared with ductal carcinoma SKPC cell-line; (b) human islet preparation. Data represented as mean values ± SEM of at least 3 independent experiments. *** $p < 0.001$; (one-way ANOVA, followed by a Tukey's multiple comparisons post-test).

primarily sensed through the proton sensing GPCR because i) ASICs ion channels are not reported to be present in islets,^{17,18} ii) TRPV1 channels, even though reported to be expressed in some β cell-lines, sense acidic pH (pH 4–5)^{17,19–21}. Information is limited on the expression and function of proton sensing GPCRs in pancreatic β-cells. Impaired glucose-stimulated insulin secretion has been described in *GPR68* knockout mice, however the role of proton sensing GPCRs in human β-cells remains to be explored²².

Here, we provide evidence that *GPR68* is the predominant proton sensing receptor expressed by human β-cells. Its expression is tightly regulated by *RFX6*, a β-cell enriched transcription factor²³. We also show using the human β cell line EndoC-βH2²⁴ that extracellular acidification activates *GPR68*, inducing the production and secretion of the chemokine IL-8 through NF-κB activation. In conclusion, proton sensing via *GPR68* is a novel mechanism for the induction of inflammatory response in human pancreatic β-cell.

Results

The proton-sensing receptor *GPR68*, a target of *RFX6*, is expressed in EndoC-βH2 cells and human islets. Our previously published transcriptomic analyses (GEO No: GSE48101) indicated that EndoC-βH2 cells express mRNA coding for the proton-sensing receptor *GPR68*²⁴. We validated these data by Real-Time-quantitative PCR (RT-qPCR) that indicated that *GPR68* mRNA expression was enriched in EndoC-βH2 cells compared to the duct cell line SKPC (Fig. 1a). Transient transfection of EGFP tagged human *GPR68* construct in EndoC-βH2 cells showed its predominant localization on the plasma membrane (Supplementary Fig. 1). *GPR68* was almost the sole proton sensing GPCR expressed in EndoC-βH2 cells, the other ones (*GPR4*, *GPR65*, *GPR132*) being expressed at nearly undetectable levels (Fig. 1a). Similar data were obtained using human islet preparations that expressed *GPR68*, but not *GPR65* and *GPR132* (Fig. 1b). Of note, *GPR4* was detected in human islets and not in EndoC-βH2 cells (Fig. 1a), which could be due to its expression by non β-cells present in human islet preparations like endothelial cells^{25,26}.

RFX6 is a key transcription factor highly expressed in β-cells and required for their function. Our previous transcriptomic analyses indicated that siRNA-mediated *RFX6* knock-down decreased *GPR68* expression in EndoC-βH2 cells [FC, -3.85 ; $p = 7.78 \cdot 10^{-5}$] (GEO No: GSE59049) without effecting the expression of other proton sensing receptors²³. Further validation by RT-qPCR showed that decreased expression level of *RFX6* mRNA ($63.92 \pm 10.5\%$) was consistently accompanied by decrease in the level of *GPR68* mRNA ($59.73 \pm 15\%$) in EndoC-βH2 cells (Fig. 2a). Similar results were obtained in human islets where decreased *RFX6* expression ($79.34 \pm 13\%$) resulted in a $42.15 \pm 10\%$ decrease of *GPR68* transcripts (Fig. 2b). Additionally, overexpression of wt*RFX6* but not p.V506G mutant *RFX6*²³, increased the expression of *GPR68* transcripts (Fig. 2c). *GPR68* expression was also enhanced following transfection of EndoC-βH2 cells with a trans-activation domain VP16 conjugated *RFX6* (Fig. 2d), while transfection of a trans-repression domain conjugated KRAB-*RFX6* in EndoC-βH2 cells resulted in a decreased expression of *GPR68* (Fig. 2e). *GPR68* is thus the major proton sensor expressed in β-cells, its expression being tightly controlled by *RFX6*.

GPR68 is involved in proton-induced inositol phosphate (IP) production in Human β-cells.

GPR68 is a proton-sensing $G_{q/11}$ coupled receptor that stimulates IP formation to elicit pH dependent responses⁸. To examine if acidification of the extracellular medium activates $G_{q/11}$ pathway in EndoC-βH2 cells, we incubated cells at either physiological pH 7.4 or acidic pH 6.4. Buffered pH media did not alter cell morphology, viability (Supplementary Fig. 2a–c) or insulin secretion in EndoC-βH2 cells (Supplementary Fig. 3). Acidic pH 6.4 induced a significant increase in IP formation (Fig. 3a). This effect was blocked by YM-254890, a selective $G_{q/11}$ inhibitor²⁷, demonstrating the selective role of $G_{q/11}$ pathway in pH dependent responses in EndoC-βH2 (Fig. 3a). On the other hand, proton did not modulate cAMP production measured at pH 7.4 or 6.4 (Fig. 3b),

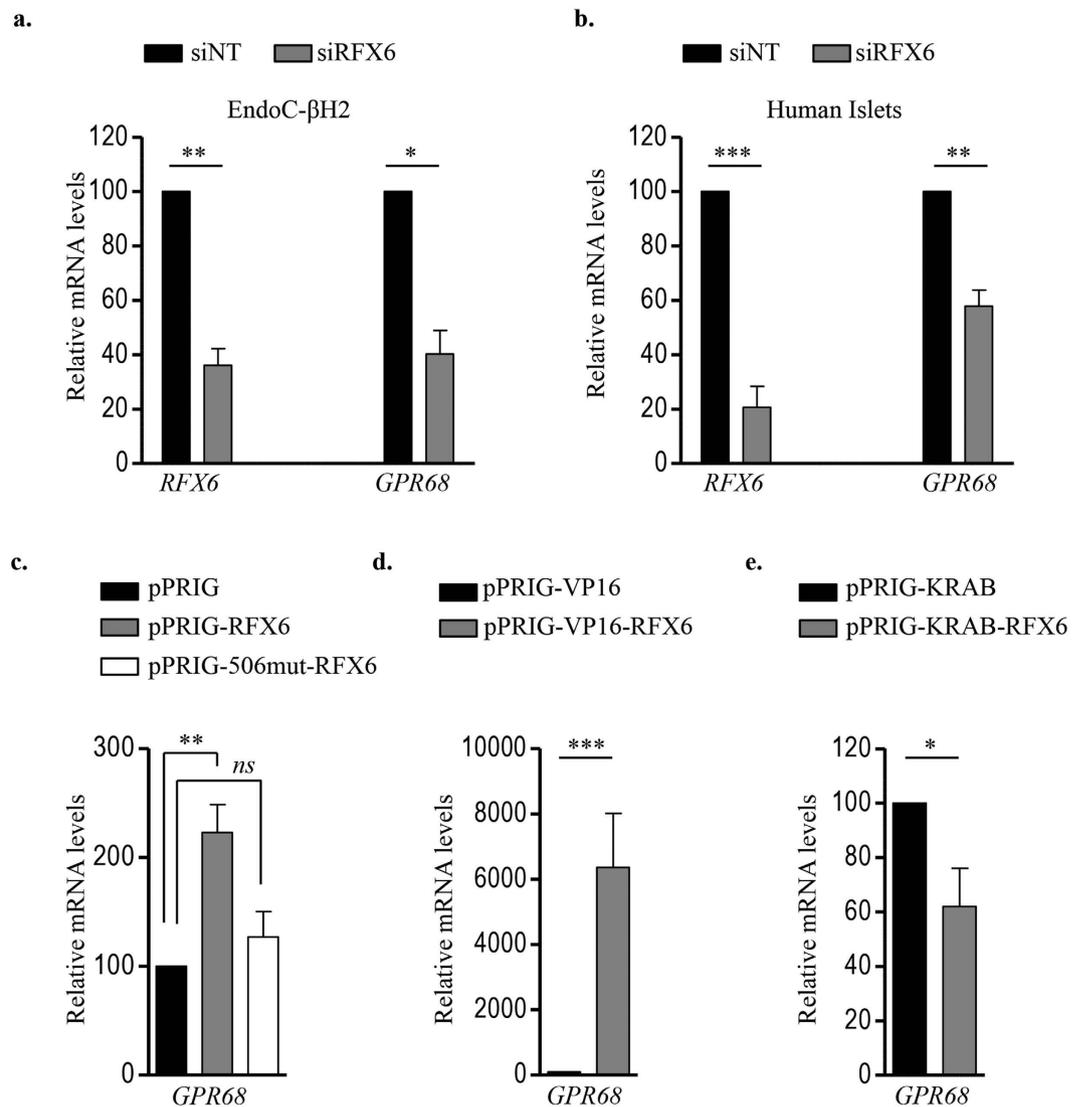


Figure 2. RFX6 regulates GPR68 expression in human β -cells. EndoC- β H2 cells (a) and adult human islets (b) were transfected with control non-target siRNA (siNT) or siRNA targeting *RFX6* (siRFX6). *GPR68* expression was analyzed 72 h post transfection by RT-qPCR. Data are expressed as percentage of siNT transfected cells. (c–e) wtRFX6 and Mut506RFX6 (one-way ANOVA) (c), transactivation domain VP16-conjugated RFX6 (d) and transcriptional repression domain KRAB-conjugated RFX6 (e) were expressed in EndoC- β H2 cells using bicistronic constructs with IRES-EGFP. GFP⁺ cells were FACS isolated 48 h post-transfection and *GPR68* expression was analyzed by RT-qPCR. Data are mean \pm SEM of 3–5 experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and ns, not significant.

further indicating that G_s-coupled receptors such as GPR4 and GPR132 are not involved in proton sensing in EndoC- β H2 cells. We next show that acidic pH-stimulated IP production was GPR68-dependent. Indeed control (siNT) treated β -cells sensed normally the extracellular acidic pH (6.4) and responded by increasing IP formation. In contrast siRNA-mediated GPR68 depletion significantly decreased this induction (Fig. 3c). Accordingly, siRFX6 treatment lead to decreased *GPR68* expression also inhibited proton induced IP formation (Fig. 3c). Thus, GPR68 is involved in proton-induced IP production in EndoC- β H2 cells.

EndoC- β H2 cells express and secrete the pro-inflammatory cytokine IL-8 upon exposure to acidic pH.

Extracellular acidic microenvironment has been reported to induce the expression of pro- and anti-inflammatory cytokines in a variety of cell types^{3,28}. We examined the expression of selected cyto/chemokines by EndoC- β H2 cells exposed to acidic pH. RT-qPCR analysis of cells incubated at pH 7.4 or pH 6.4 for 24 h showed the induction of *IL-8* transcripts in cells exposed to acidic pH (Fig. 4a). At all intermediate pH tested between 7.4 and 6.4, *IL-8* mRNA expression increased while pH decreased (Fig. 4b). Low pH-induced *IL-8* mRNA expression was detected as early as 8 h following low pH exposition and increased at later time points (24 and 48 h) (Fig. 4c). Following incubation at pH 6.4, *IL-8* protein was detected in the conditioned medium of EndoC- β H2 cells. PMA, a strong inducer of *IL-8* in human EndoC- β H2 cell model, was used as positive inducer

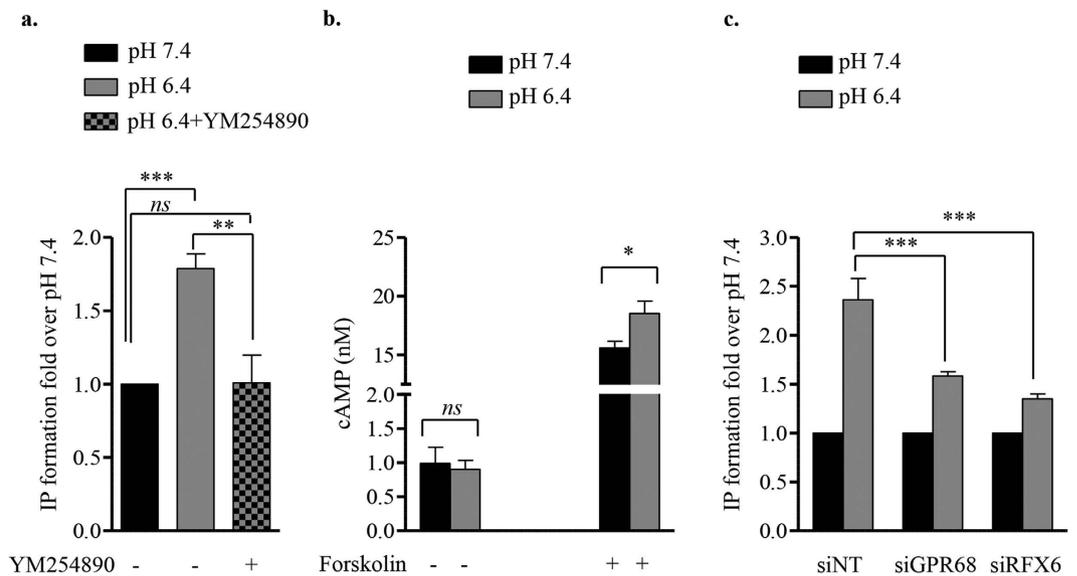


Figure 3. Extracellular pH modulates IP production but not cAMP accumulation in EndoC-βH2 cells. (a) IP formation was determined in EndoC-βH2 cells incubated at pH 7.4 and 6.4. The $G_{q/11}$ -selective inhibitor compound YM-254890 (100 nM) was also tested. Results are expressed as fold change over the IP values at pH 7.4. (b) For cAMP formation assay, EndoC-βH2 cells were incubated for 30 min with and without Forskolin (25 μM) at pH 7.4 or 6.4. Results are expressed as cAMP accumulation (nM) per 5,000 cells. (c) EndoC-βH2 cells were transfected with control siRNA (siNT), siRNA targeting either *GPR68* (siGPR68) or *RFX6* (siRFX6). 72 h post transfection, cells were incubated at pH 7.4 or 6.4 for 30 min and analyzed for the IP formation. Results are expressed as fold change over the IP values at pH 7.4. Data are mean ± SEM of 3–5 experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and *ns*, not significant (one-way ANOVA, followed by a Tukey's multiple comparisons post-test).

(Fig. 4d). pH dependent accumulation of IL-8 in EndoC-βH2 cells was further confirmed by immunostaining (Fig. 4e).

To test whether acidification induced *IL-8* mRNA expression is mediated through GPR68, EndoC-βH2 cells were first incubated at acidic pH in the presence of the selective $G_{q/11}$ inhibitor YM-254890. Under these conditions, the induction of *IL-8* mRNA upon acidic pH is dramatically dampened (Fig. 5a). Moreover both GPR68 siRNA and RFX6 siRNA also decreased acidic pH-induced *IL-8* mRNA induction (Fig. 5b).

IL-8 induction in β-cells by extracellular acidification is NF-κB dependent. NF-κB is a central mediator of inflammatory response²⁹ and RELA, a major subunit of NF-κB complex, is a mediator of *IL-8* transcription³⁰. As EndoC-βH2 cells secrete inflammatory cytokine *IL-8* in response to acidification of their medium, we investigated whether *IL-8* production requires NF-κB activation. We showed nuclear translocation of RELA, a subunit of NF-κB complex upon acidic pH treatment in EndoC-βH2 cells (Fig. 6a). EMSA performed using cellular extracts from EndoC-βH2 showed that DNA-binding activity of NF-κB increased in a time-dependent manner when cells were exposed to acidic pH 6.4 (Fig. 6b). Consistent with these results, cell treatment with JSH-23, a potent NF-κB activation inhibitor II, significantly decreased acidic pH induced *IL-8* mRNA expression (Fig. 6c). Moreover, a siRNA that efficiently targeted *RELA* (Fig. 6d, left), decreased the acidic pH-mediated *IL-8* mRNA induction (Fig. 6d, right). Thus, in EndoC-βH2 cells low pH induces the up-regulation of *IL-8* mRNA through the activation of NF-κB complex.

IL-8 secreted by human β-cells in acidic conditions attracts neutrophils. *IL-8* is a chemotactic pro-inflammatory cytokine that mediates the recruitment and activation of neutrophils during inflammation³¹. We examined if *IL-8* secreted by EndoC-βH2 cells exposed to acidic pH induces neutrophil chemotaxis. We performed *in-vitro* migration assay using CD16-positive human blood neutrophils and EndoC-βH2 cell conditioned media as chemo-attractant (Fig. 7a). When compared to pH7.4 condition medium, acidic conditioned medium significantly attracted blood neutrophils. This chemotactic migration was abrogated by anti-*IL-8* antibody indicating that neutrophil migration to acidic conditioned medium is dependent on the presence of *IL-8* (Fig. 7b).

Discussion

We and others recently showed that in adult human and mouse pancreatic β-cells, the transcription factor RFX6 controls insulin secretion by modulating calcium homeostasis^{23,32}. In the present study, we demonstrate that RFX6 plays a pivotal role in extracellular proton sensing by regulating the expression of the G-protein coupled receptor GPR68 in human β-cells. We next demonstrate that extracellular signal acidification activates GPR68 which induces the production of inflammatory chemokine *IL-8* through activation of the NF-κB complex.

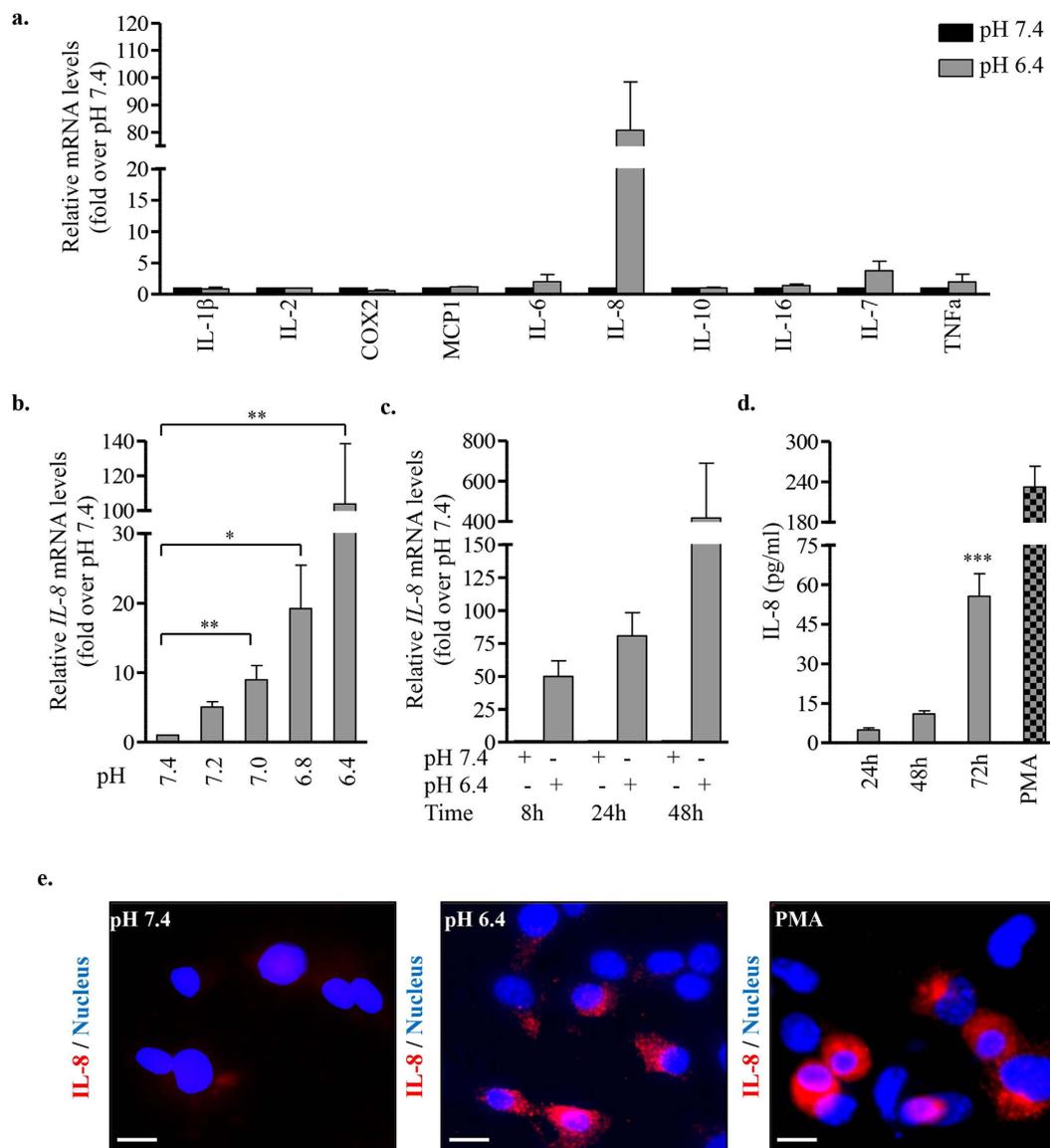


Figure 4. Acidic extracellular pH induces the expression and secretion of IL-8 by EndoC- β H2 cells.

(a) EndoC- β H2 cells were cultured at pH 7.4 or 6.4 for 24 h and screened by RT-qPCR for the expression of selected pro/anti-inflammatory cytokines. (b) IL-8 expression was determined by RT-qPCR in EndoC- β H2 cells cultured at different pH for 24 h (one-way ANOVA, post test for linear trend ($p = 0.0008$)). (c) IL-8 expression was determined by RT-qPCR in EndoC- β H2 cells cultured at pH 7.4 or 6.4 for the indicated time points (one-way ANOVA, post test for linear trend ($p = 0.067$)). (d) EndoC- β H2 cells were cultured at pH 7.4 or 6.4 for 24 h/48 h/72 h or with PMA (100 ng/ml for 12 h) (one-way ANOVA). Secreted IL-8 was quantified by ELISA. (e) Immunostaining for IL-8 in EndoC- β H2 cells cultured at pH 7.4 or 6.4 for 72 h or with PMA (100 ng/ml) for 12 h. Scale bar 10 μ m. Data are mean \pm SEM of 3–5 experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

GPR68 is one of the 4 known proton sensing GPCRs together with GPR4, 65 and 132^{5,8}. Our results indicate that GPR68 is the predominant GPCR of this family in the human β cell-line EndoC- β H2 cells. This appears to be also the case in primary human β -cells. Indeed, human islet preparations that contain β -cells, but also some endocrine and non endocrine pancreatic cells, express GPR68 but no GPR65 and GPR132 (our present data and ref. 25). GPR4 expression is also detected in human islet preparations. However, while GPR68 remains expressed in islet β -cell enriched fractions, GPR4 expression levels collapse in such fraction²⁵. Such data suggest that GPR68 is expressed in primary human β -cells, while GPR4 is expressed in non β -cells in islet preparations.

Signaling via GPCR plays major role in response to neurotransmitters, hormones and environmental stimulants^{33,34}. This is also the case in pancreatic β -cells, where signaling mediated by a number of GPCRs regulate β -cell expansion and function^{35–37}. However, limited information is available on the regulation of GPCR expression^{38,39}. Here, using gain- and loss-of-function approaches, we provide strong evidence that RFX6 is a major positive regulator of GPR68 expression. RFX6 knockdown down-regulates GPR68 in EndoC- β H2 cells and in human islets. Likewise, overexpression of wtRFX6 enhances GPR68 expression. Finally, converting RFX6 into a

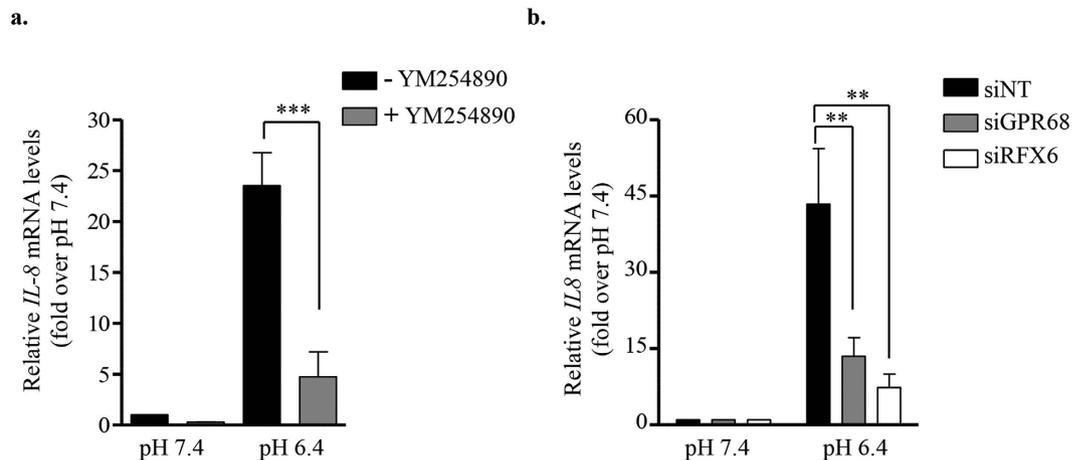


Figure 5. IL-8 induction upon extracellular acidification is RFX6- and GPR68-dependent. (a) EndoC- β H2 cells were cultured for 24 h at pH 7.4 or 6.4 with or without YM-254890 (100 nM), a $G_{q/11}$ -selective inhibitor compound. *IL-8* expression was analyzed by RT-qPCR. (b) EndoC- β H2 cells were transfected with siRNA (siNT), siGPR68 or siRFX6. After 48 h, cells were cultured for an additional 24 h period at pH 7.4 or 6.4 and next analyzed for the expression of *IL-8* by RT-qPCR. Data are mean \pm SEM of 3–5 experiments. ** $p < 0.01$; *** $p < 0.001$ (one-way ANOVA, followed by a Tukey's multiple comparisons post-test).

constitutive transcriptional activator (VP16-RFX6) or repressor (KRAB-RFX6) leads to chimeric proteins able to increase or decrease, respectively, *GPR68* expression. This regulation could be mediated by the direct binding of RFX6 to the X-box motifs on -1 K proximal promoter of *GPR68* as revealed by promoter analysis using MatInspector (data not shown).

Previous data indicated that glucose-stimulated insulin secretion is attenuated in *GPR68*-deficient mice, suggesting that rodent β -cells sense protons via *GPR68*²². Here, we show that in human β -cells, *GPR68* is expressed and is functional. Specifically, in human β -cells, extracellular acidification, through proton-sensing *GPR68*- $G_{q/11}$ receptor stimulates IP production and activates NF- κ B, giving rise to the production and secretion of the inflammatory chemokine *IL-8*. This last point couldn't have been observed in rodent models, as *IL-8* is not expressed by rodent cells and considered as a dead gene in murine genome⁴⁰. Of note, the expression of *CXCL1*, the functional homologs of *IL-8* in rodents⁴¹, is not induced at low pH in the rodent β -cell line MIN6 (our unpublished data). This highlights the need to use human β -cell models such as EndoC- β H2 cells, limits the use of murine models and further confirms the major differences between rodent and human β -cells^{42,43}.

In physiological conditions, interstitial pancreatic pH is maintained at ~ 7.4 while it drops to 7.0 or even below in a number of patho-physiological conditions. This is the case in chronic pancreatitis⁴⁴, or in pancreatic adenocarcinoma^{45–48}. Extracellular pancreatic acidification is also observed in T1D patients with ketoacidosis^{15,16}. Whether and how low extracellular pH acts on human β -cells remains poorly studied. Here, we demonstrate that in human beta cells, *GPR68* senses protons and signals by activating NF- κ B complexes, resulting in *IL-8* production and secretion. Interestingly, accumulating evidence indicate elevated levels of *IL-8* in pancreas-related pathologies such as chronic pancreatitis⁴⁹, pancreatic adenocarcinoma samples^{50,51} but also T1D patients with severe ketoacidosis episodes⁵². In this last case, *IL-8*, by acting as a chemo-attractant, would recruit neutrophils for further inflammatory responses and human β -cell destruction. In T1D, β -cells are destroyed by an auto-immune reaction and it had been thought that no β -cells can resist this aggression. However, recent data indicate that in subgroups of T1D patients, insulin-secreting β -cells that escaped destruction, remain present years after diagnostic^{53,54}. A hypothesis would be that such patients had not developed ketoacidosis-induced *IL-8* production.

In conclusion, we have identified RFX6 as an important transcriptional regulator of the proton-sensing receptor *GRP68* in adult human β -cells. We also demonstrated that through *GPR68*/ $G_{q/11}$ and NF- κ B activation, extracellular acidification induces inflammatory chemokine *IL-8* production. Earlier reports demonstrated the effectiveness of *GPR4* antagonist to block inflammatory response to acidosis in endothelial cells^{55,56}. Moreover very recent reports have characterized specific modulators of *GPR68*⁵⁷. In this context, identifying *GPR68* antagonists could be novel therapeutic targets for the improvement of inflammatory conditions associated with pancreatic acidosis.

Methods

Culture of human cell lines and islets. EndoC- β H2 cells²⁴ were cultured in low-glucose (1g/L) Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing L-glutamine and sodium pyruvate, supplemented with 2% BSA fraction V (Roche Diagnostics), 50 μ M 2-mercaptoethanol, 10 mM nicotinamide (Calbiochem), 5.5 μ g/ml transferrin (Sigma-Aldrich), 6.7 ng/ml selenite (Sigma-Aldrich), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were seeded on Matrigel (1%) / fibronectin (2 μ g/ml) (Sigma-Aldrich) coated plates and cultured at 37 $^{\circ}$ C and 5% CO_2 . The human duct cell line SKPC⁵⁸ was cultured in high glucose DMEM (4.5 g/L) supplemented with 10% fetal calf serum (Biowest), 100 U/ml penicillin and 100 μ g/ml streptomycin. Human islets were isolated and maintained as described⁵⁹.

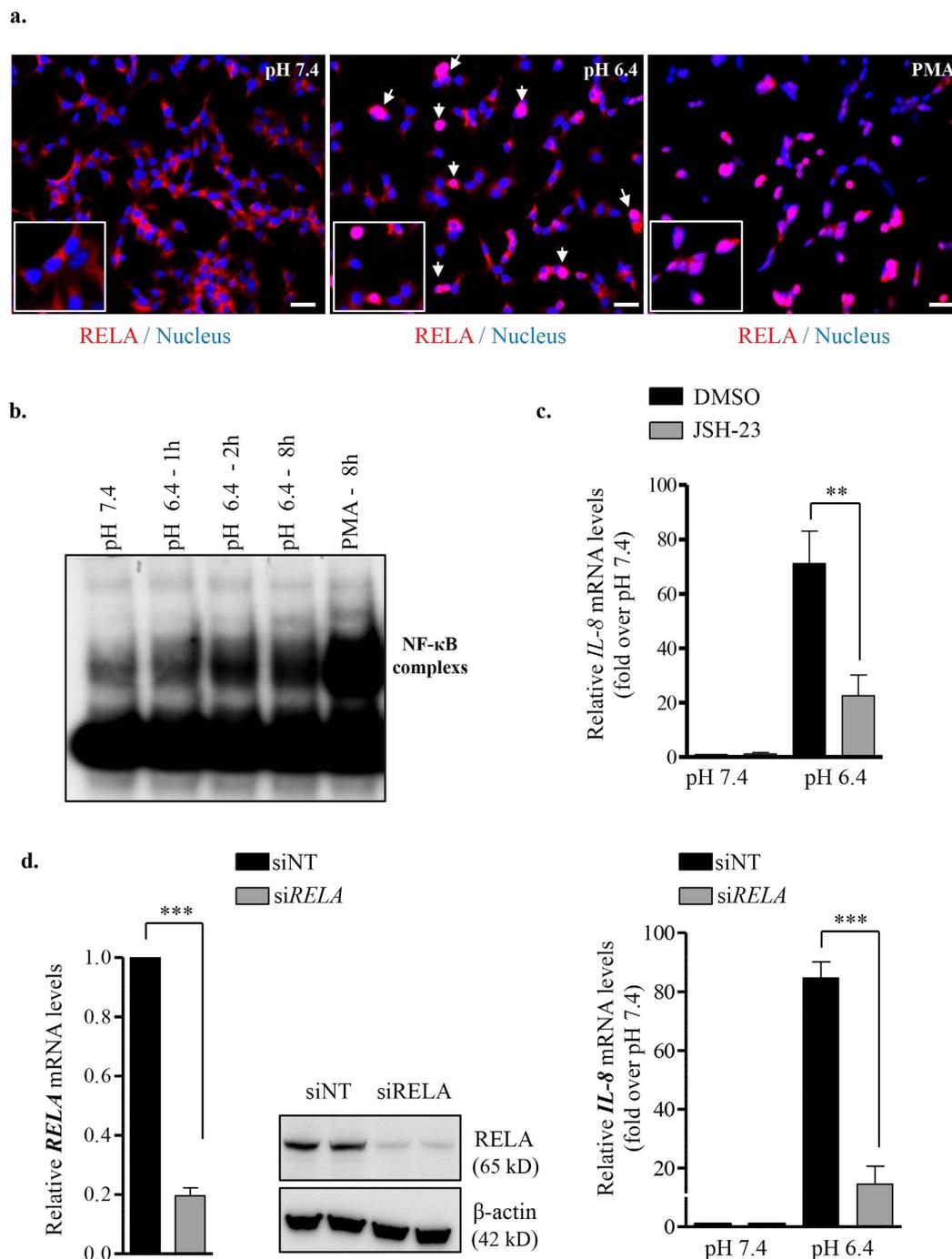


Figure 6. IL-8 induction in EndoC- β H2 cells by extracellular acidification is NF- κ B dependent.

(a) EndoC- β H2 cells were cultured at pH 7.4 or 6.4 for 12 h or with PMA (100 ng/ml for 8 h) and analyzed by immunofluorescence for the nuclear accumulation of RELA (p65) component of NF- κ B complex. Scale bar 10 μ m. (b) EndoC- β H2 cells were cultured at pH 7.4 for 8 h, at pH 6.4 for 1, 2 and 8 h or with PMA (100 ng/ml for 8 h). Proteins were extracted and used for Electrophoretic mobility shift assay (EMSA) analysis using a [32 P] radiolabeled NF- κ B probe. (c) EndoC- β H2 cells were treated with 1 μ M NF- κ B Activation inhibitor-II, JSH-23 for 24 h and analyzed for *IL-8* expression by RT-qPCR (one-way ANOVA). (d) EndoC- β H2 cells were transfected with either control siRNA (siNT) or siRNA targeted *RELA* (siRELA). After 48 h, siNT or siRELA transfected cells were cultured for 24 h at pH 7.4 or 6.4. Efficient *RELA* knock-down was verified by RT-qPCR and immunoblot analysis (left panel). The effect of pH on *IL-8* expression following *RELA* was quantified by RT-qPCR (right panel) (one-way ANOVA). Data are mean \pm SEM of 3–5 experiments. ** $p < 0.01$ and *** $p < 0.001$.

Preparation of buffered culture media. Culture media at different pH were prepared as described^{8,60}. Briefly, low glucose DMEM without sodium bicarbonate (Sigma, D2902) was buffered with HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid; Sigma, H0887), EPPS

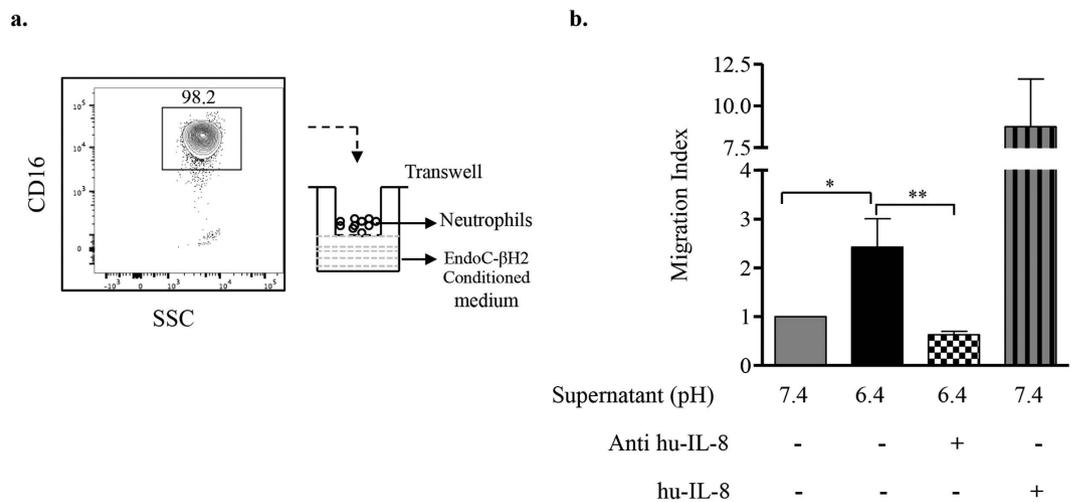


Figure 7. IL-8 secreted by EndoC-βH2 cells in acidic conditions attracts neutrophils. (a) Neutrophils were isolated from whole blood using MACSxpress kit, analyzed for the expression of CD16 and used for *in-vitro* transwell migration assay. (b) Neutrophil chemotaxis was tested using conditioned media from EndoC-βH2 cells cultured at pH 7.4 or pH 6.4 for 72 h. Acidic pH 6.4 conditioned medium pre-treated for 10 min with Anti-human IL-8 (1 μg/ml) was also used as well as pH 7.4 conditioned medium supplemented with recombinant human-IL-8 (50 ng/ml). Data are represented as migration index, calculated by assigning a value of 1 to the number of migrating neutrophils towards pH 7.4-conditioned medium. Data are mean ± SEM of 4 blood donors. * $p < 0.05$; ** $p < 0.01$ (one-way ANOVA, followed by a Tukey's multiple comparisons post-test).

(4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid; Sigma, E9502) and MES (2-(N-Morpholino) ethanesulfonic acid; Sigma, M3671) (8mM each) and pH was adjusted with HCl/NaOH. They were kept iso-osmotic by adding NaCl and NaHCO₃. The pH stability was monitored at the initiation and completion of the experiments.

RNA isolation, reverse transcription and RT-qPCR. Total RNA was extracted from EndoC-βH2 cells using RNeasy Plus Micro kit (Qiagen). First strand cDNA was prepared using Maxima First Strand cDNA synthesis kit (ThermoFisher). RT-qPCR was performed using Power SYBR Green mix (Applied Biosystems) with ABI Prism 7300 sequence detector (Applied Biosystems). *Cyclophilin A* transcript levels were used for normalization of each target gene. The custom primers were designed with IDT Primer-Quest online software and the amplification efficiency for each primer was determined with serial dilution of total cDNA from EndoC-βH2/human islets cDNA. Primer sequences are listed in Supplementary Table.

siRNA Transfection. EndoC-βH2 cells were transfected using Lipofectamin RNAiMAX (life technologies) and ON-TARGET^{plus} siRNA SMARTpool for human *RFX6/GPR68/RELA* gene (40 nM) or ON-TARGET^{plus} Non-targeting pool (siNT) (Dharmacon, Thermo Scientific) as described²³. Human islet samples were partially dissociated with Accutase (PAA Laboratories) and siRNA transfections were performed as described²³.

DNA Transfection. Human RFX6 constructs (pRIG-RFX6, pRIG-Mut506RFX6, pRIG-KRAB-RFX6 and pRIG-VP16-RFX6)²³ were used in this study. The MGC Human GPR68 cDNAclone (Clone ID: 6971805) was purchased (Open Biosystems; Thermo Scientific) and sub-cloned into pEGFP-N1 (Clontech). EndoC-βH2 cells were transiently transfected with DNA using Lipofectamin2000 (Invitrogen) following manufacturer's instructions in Opti-MEM. GFP-positive cells were FACS sorted 24–48 h post transfections and RNA expression was analyzed by RT-qPCR.

Electrophoretic mobility shift assay for NF-κB. EndoC-βH2 cells were cultured at pH 7.4 (8 h) or 6.4 (1, 2 and 8 h) or with PMA (100 ng/ml at pH 7.4 for 8 h). Cellular extracts were prepared and NF-κB activation was analyzed by electrophoretic mobility shift assay (EMSA) using the human immunodeficiency virus long terminal repeat tandem κB oligonucleotide as κB probe⁶¹.

IP and cAMP formation assay. IP formation was quantified with HTRF (Homogeneous Time-Resolved Fluorescence) based "Cisbio IP-One Tb" (Cisbio, Bagnols-sur-Cèze, France) assay kit, following manufacturer's instructions. EndoC-βH2 cell suspensions (5×10^4 cells) were treated in 384-well plate (16 μl volume) with modified stimulation buffer (10 mM Hepes, 10 mM MES, 1 mM CaCl₂, 0.5 mM MgCl₂, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl) at pH 7.4 or 6.4 for 60 min at 37 °C. Where indicated, cells were pretreated with YM-254890, a selective G_{αq/11} inhibitor²⁷ for 30 min prior to incubation with the IP stimulation buffer and maintained throughout the IP determination. IP measurements were performed in triplicates and experiments were repeated at least three times. Samples were read on a TECAN Infinite F500 (Tecan Group, Ltd., Männedorf, Switzerland) with excitation at 320 nm and emission at both 620 nm and 665 nm.

cAMP activity was measured using a cAMP-HTRF assay kit (Cisbio) following manufacturer's instructions. EndoC- β H2 cells (5×10^3) were treated in 384-well plate (12 μ l final volume) with stimulation buffer (PBS containing 10 mM of each HEPES, MES, and 0.5 mM IBMX, at pH 7.4 or 6.4) for 30 min at room temperature. Cells were lysed using kit lysis buffer and cAMP was then measured in 384 well plates (HTRF) with *TECAN* Infinite F500.

Immunocytochemistry and immunoblotting. EndoC- β H2 cells were cultured on Matrigel/fibronectin coated 4-well chambers slide (Nunc Lab-Tek) and processed for IL-8 and RELA immunostaining as described⁶² using anti-IL-8 (1:1,000; BD554717; BD Biosciences) or anti-RELA (1:200; sc8008; Santa Cruz Biotechnology) antibodies. Images were acquired with a Leica Leitz fluorescent microscope equipped with cooled 3-chip charge coupled device camera (Hamamatsu C5810; Hamamatsu) and processed using ImageJ software.

For immunoblot assays, total cellular proteins were prepared as described²³. Proteins (25 μ g) were resolved by SDS PAGE, immunoblotted with antibodies against RELA (sc8008, 1/250 dilution) and Actin (1/1000, Sigma-Aldrich). Membranes were incubated with species-specific HRP-linked secondary antibodies (1:5000) and visualization was performed following ECL exposure.

IL-8 Elisa. Secreted IL-8 protein levels were determined using commercially available Human IL-8 ELISA MAX Deluxe kit (BioLegend #431504) as per manufacturer's instructions. EndoC- β H2 cells were treated with pH 7.4 or 6.4 or with 100 ng/ml PMA (at pH7.4) and culture supernatants were collected and stored for ELISA.

Neutrophil migration assay. Blood samples were obtained from the pediatric endocrinology and diabetes center at Necker Enfants-Malades hospital, Paris, France in accordance with the approved guidelines. All the experimental protocols were approved by the local ethic committee (CPP - Paris Ile de France, France). Informed consent was obtained from all subjects. Neutrophils were isolated using a MACSxpress human neutrophil isolation kit (Miltenyi Biotec). Red Blood Cell lysis buffer was used to remove residual erythrocytes. The purity of isolated neutrophils was consistently between 98–99% based on CD16 staining. Chemotaxis assay was performed in 24-well micro chemotaxis chamber using 6.5 mm Transwell with 3 μ m PVP-free polycarbonate filter membrane (Costar). Neutrophils (2×10^5 cells in 200 μ l PBS) in upper chamber were allowed to migrate towards 500 μ l of conditioned medium produced during 72 h by EndoC- β H2 cultured at pH 7.4 or 6.4. In some experiments, conditioned medium was supplemented with Anti-human IL-8 (1 μ g/ml for 10 min; BD554717; BD Bioscience) or with recombinant human-IL-8 (50 ng/ml; BioLegend). After 2 h at 37 °C, migrating cells were recovered with Accutase (Sigma) in the lower chamber and numbered by flow cytometry. Results are expressed as migration index: number of migrating neutrophils in a defined condition divided by number of migrating neutrophils towards pH 7.4 conditioned medium.

Statistics. Graphs were constructed by using PRISM software (version 5.02 GraphPad). Quantitative data are presented as the mean \pm SEM from at least three independent experiments, unless indicated. For comparison between two mean values, statistical significances were estimated using two-tailed Student's *t*-test. For comparison between three or more values, one-way ANOVA was used with Tukey's multiple comparisons post-test. Statistical significance was set at $p < 0.05$.

References

- Griffith, L. G. & Swartz, M. a. Capturing complex 3 D tissue physiology *in vitro*. *Nat. Rev. Mol. Cell Biol.* **7**, 211–24 (2006).
- DeCoursey, T. E. Voltage-gated proton channels: molecular biology, physiology, and pathophysiology of the H(V) family. *Physiol. Rev.* **93**, 599–652 (2013).
- Lardner, A. The effects of extracellular pH on immune function. *J. Leukoc. Biol.* **69**, 522–530 (2001).
- Wemmie, J. a., Price, M. P. & Welsh, M. J. Acid-sensing ion channels: advances, questions and therapeutic opportunities. *Trends Neurosci.* **29**, 578–586 (2006).
- Okajima, F. Regulation of inflammation by extracellular acidification and proton-sensing [GPCRs.]. *Cell. Signal.* **25**, 2263–2271 (2013).
- Morales-Lázaro, S. L., Simon, S. A. & Rosenbaum, T. The role of endogenous molecules in modulating pain through transient receptor potential vanilloid 1 (TRPV1). *J. Physiol.* **591**, 3109–21 (2013).
- Wemmie, J. a., Taugher, R. J. & Kreple, C. J. Acid-sensing ion channels in pain and disease. *Nat. Rev. Neurosci.* **14**, 461–471 (2013).
- Ludwig, M.-G. *et al.* Proton-sensing G-protein-coupled receptors. *Nature* **425**, 93–8 (2003).
- Wang, J.-Q. *et al.* TDAG8 is a proton-sensing and psychosine-sensitive G-protein-coupled receptor. *J. Biol. Chem.* **279**, 45626–33 (2004).
- Ferrannini, E. The stunned beta cell: a brief history. *Cell Metab.* **11**, 349–52 (2010).
- Rorsman, P. & Braun, M. Regulation of insulin secretion in human pancreatic islets. *Annu. Rev. Physiol.* **75**, 155–79 (2013).
- Atkinson, M. A., Eisenbarth, G. S. & Michels, A. W. Type 1 diabetes. *Lancet* **383**, 69–82 (2014).
- Cnop, M. *et al.* Mechanisms of Pancreatic -Cell Death in Type 1 and Type 2 Diabetes: Many Differences, Few Similarities. *Diabetes* **54**, S97–S107 (2005).
- Chiasson, J. L. *et al.* Diagnosis and treatment of diabetic ketoacidosis and the hyperglycemic hyperosmolar state. *Cmaj* **168**, 859–866 (2003).
- Efstathiou, S. P. *et al.* A mortality prediction model in diabetic ketoacidosis. *Clin. Endocrinol. (Oxf)*. **57**, 595–601 (2002).
- English, P. & Williams, G. Hyperglycaemic crises and lactic acidosis in diabetes mellitus. *Postgrad. Med. J.* **80**, 253–61 (2004).
- Mogi, C., Nakakura, T. & Okajima, F. Role of extracellular proton-sensing OGR1 in regulation of insulin secretion and pancreatic β -cell functions. *Endocr. J.* **61**, 101–10 (2014).
- Huang, S.-J., Yang, W.-S., Lin, Y.-W., Wang, H.-C. & Chen, C.-C. Increase of insulin sensitivity and reversal of age-dependent glucose intolerance with inhibition of ASIC3. *Biochem. Biophys. Res. Commun.* **371**, 729–34 (2008).
- Tominaga, M. & Tominaga, T. Structure and function of TRPV1. *Pflugers Arch. Eur. J. Physiol.* **451**, 143–150 (2005).
- Akiba, Y. *et al.* Transient receptor potential vanilloid subfamily 1 expressed in pancreatic islet beta cells modulates insulin secretion in rats. *Biochem. Biophys. Res. Commun.* **321**, 219–25 (2004).
- Razavi, R. *et al.* TRPV1+ Sensory Neurons Control β Cell Stress and Islet Inflammation in Autoimmune Diabetes. *Cell* **127**, 1123–1135 (2006).

22. Nakakura, T. *et al.* Deficiency of proton-sensing ovarian cancer g protein-coupled receptor 1 attenuates glucose-stimulated insulin secretion. *Endocrinology* **153**, 4171–80 (2012).
23. Chandra, V. *et al.* RFX6 regulates insulin secretion by modulating Ca²⁺ homeostasis in human β cells. *Cell Rep.* **9**, 2206–18 (2014).
24. Scharfmann, R. *et al.* Development of a conditionally immortalized human pancreatic β cell line. *J. Clin. Invest.* **124**, 2087–98 (2014).
25. Nica, A. C. *et al.* Cell-type, allelic, and genetic signatures in the human pancreatic beta cell transcriptome. *Genome Res.* **23**, 1554–1562 (2013).
26. Kim, K.-S. *et al.* GPR4 plays a critical role in endothelial cell function and mediates the effects of sphingosylphosphorylcholine. *FASEB J.* **19**, 819–21 (2005).
27. Takasaki, J. *et al.* A novel Galphaq/11-selective inhibitor. *J. Biol. Chem.* **279**, 47438–45 (2004).
28. Tomura, H. *et al.* Prostaglandin I(2) production and cAMP accumulation in response to acidic extracellular pH through OGR1 in human aortic smooth muscle cells. *J. Biol. Chem.* **280**, 34458–64 (2005).
29. Lawrence, T. The Nuclear Factor NF- κ B Pathway in Inflammation. *About Cold Spring Harb. Perspect. Biol.* **1**, 1–10 (2009).
30. Kunsch, C. & Rosen, C. a. NF-kappa B subunit-specific regulation of the interleukin-8 promoter. *Mol. Cell. Biol.* **13**, 6137–6146 (1993).
31. Baggiolini, M. & Clark-Lewis, I. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett.* **307**, 97–101 (1992).
32. Piccand, J. *et al.* Rfx6 Maintains the Functional Identity of Adult Pancreatic β Cells. *Cell Rep.* **9**, 2219–32 (2014).
33. Rosenbaum, D. M., Rasmussen, S. G. F. & Kobilka, B. K. The structure and function of G-protein-coupled receptors. *Nature* **459**, 356–363 (2009).
34. Katritch, V., Cherezov, V. & Stevens, R. C. Structure-function of the G protein-coupled receptor superfamily. *Annu. Rev. Pharmacol. Toxicol.* **53**, 531–56 (2013).
35. Drucker, D. J. Incretin action in the pancreas: Potential promise, possible perils, and pathological pitfalls. *Diabetes* **62**, 3316–3323 (2013).
36. Zhao, Z. *et al.* Repurposing cAMP-Modulating Medications to Promote β -Cell Replication. *Mol. Endocrinol.* **me20141120** (2014). doi: 10.1210/me.2014-1120
37. Berger, M. *et al.* G α i/o-coupled receptor signaling restricts pancreatic β -cell expansion. *Proc. Natl. Acad. Sci. USA* **112**, 2888–93 (2015).
38. Wang, H., Brun, T., Kataoka, K., Sharma, A. J. & Wollheim, C. B. MAFA controls genes implicated in insulin biosynthesis and secretion. *Diabetologia* **50**, 348–358 (2007).
39. Gupta, D., Peshavaria, M., Monga, N., Jetton, T. L. & Leahy, J. L. Physiologic and Pharmacologic Modulation of Glucose-Dependent Insulinotropic Polypeptide (GIP) Receptor Expression in β -Cells by Peroxisome Proliferator-Activated Receptor (PPAR)-g Signaling. *Diabetes* **59**, 1445–1450 (2010).
40. Guénet, J. L. The mouse genome. *Genome Res.* **15**, 1729–1740 (2005).
41. Hol, J., Wilhelmssen, L. & Haraldsen, G. The murine IL-8 homologues KC, MIP-2, and LIX are found in endothelial cytoplasmic granules but not in Weibel-Palade bodies. *J. Leukoc. Biol.* **87**, 501–508 (2010).
42. Scharfmann, R., Rachdi, L. & Ravassard, P. Concise review: in search of unlimited sources of functional human pancreatic beta cells. *Stem Cells Transl. Med.* **2**, 61–7 (2013).
43. Caicedo, A. Paracrine and autocrine interactions in the human islet: More than meets the eye. *Seminars in Cell and Developmental Biology* **24**, 11–21 (2013).
44. Patel, A. G. *et al.* Pancreatic interstitial pH in human and feline chronic pancreatitis. *Gastroenterology* **109**, 1639–1645 (1995).
45. Gatenby, R. a & Gillies, R. J. Why do cancers have high aerobic glycolysis? *Nat. Rev. Cancer* **4**, 891–899 (2004).
46. Cruz-Monserrate, Z. *et al.* Targeting pancreatic ductal adenocarcinoma acidic microenvironment. *Sci. Rep.* **4**, 4410 (2014).
47. Son, J. *et al.* Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* **496**, 101–5 (2013).
48. Ying, H. *et al.* Oncogenic kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell* **149**, 656–670 (2012).
49. Di Sebastiano, P. *et al.* Expression of interleukin 8 (IL-8) and substance P in human chronic pancreatitis. *Gut* **47**, 423–428 (2000).
50. Hussain, F. *et al.* The expression of IL-8 and IL-8 receptors in pancreatic adenocarcinomas and pancreatic neuroendocrine tumours. *Cytokine* **49**, 134–40 (2010).
51. Chen, Y. *et al.* Interleukin-8, a promising predictor for prognosis of pancreatic cancer. *World J. Gastroenterol.* **18**, 1123–9 (2012).
52. Omatsu, T. *et al.* CXCL1/CXCL8 (GRO α /IL-8) in human diabetic ketoacidosis plasma facilitates leukocyte recruitment to cerebrovascular endothelium *in vitro*. *Am. J. Physiol. Endocrinol. Metab.* **306**, E1077–84 (2014).
53. Keenan, H. A. *et al.* Residual insulin production and pancreatic β -cell turnover after 50 years of diabetes: Joslin Medalist Study. *Diabetes* **59**, 2846–53 (2010).
54. Davis, A. K. *et al.* Prevalence of detectable C-Peptide according to age at diagnosis and duration of type 1 diabetes. *Diabetes Care* **38**, 476–81 (2015).
55. Dong, L. *et al.* Acidosis activation of the proton-sensing GPR4 receptor stimulates vascular endothelial cell inflammatory responses revealed by transcriptome analysis. *PLoS One* **8**, e61991 (2013).
56. Justus, C. R., Dong, L. & Yang, L. V. Acidic tumor microenvironment and pH-sensing G protein-coupled receptors. *Front. Physiol.* **4** DEC, 1–9 (2013).
57. Huang, X.-P. *et al.* Allosteric ligands for the pharmacologically dark receptors GPR68 and GPR65. *Nature* **527**, 477–483 (2015).
58. Vila, M. R., Lloreta, J. & Real, F. X. Normal human pancreas cultures display functional ductal characteristics. *Lab. Invest.* **71**, 423–431 (1994).
59. Ravassard, P. *et al.* Technical advance A genetically engineered human pancreatic β cell line exhibiting glucose-inducible insulin secretion. **121**, 3589–3597 (2011).
60. Simpson, N. E., Bennett, L. K., Papas, K. K., Sambanis, A. & Constantinidis, I. Effects of pH on murine insulinoma betaTC3 cells. *Biochem. Biophys. Res. Commun.* **273**, 937–941 (2000).
61. Cormier, F. *et al.* Frequent engagement of RelB activation is critical for cell survival in multiple myeloma. *PLoS One* **8**, e59127 (2013).
62. Kasper, C. A. *et al.* Cell-cell propagation of NF- κ B transcription factor and MAP kinase activation amplifies innate immunity against bacterial infection. *Immunity* **33**, 804–16 (2010).

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Author Contributions

V.C. designed research, performed experiments, analyzed data and wrote the manuscript. A.K. performed IP and cAMP assays. P.R. performed experiments and participates in manuscript preparation. F.C. performed EMSA experiments and contributed to the manuscript writing. C.R. participates in FACS based experiments. R.J. and M.A. analyzed data and participates in manuscript writing. O.A. designed experiments, analyzed data and participates in manuscript writing. R.S. designed research, analyzed data and wrote the manuscript.

Additional Information

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