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Regulator of calcineurin 1 (Rcan1) controls growth plasticity of adult pancreas

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Abstract

Background & Aims—Growth of exocrine pancreas is regulated by GI hormones, notably cholecystokinin (CCK). CCK-driven pancreatic growth requires calcineurin (CN), which activates NFATs (Nuclear Factor of Activated T-cells), but the genetic underpinnings and feedback mechanisms that regulate this response are not known.

Methods—Pancreatic growth was stimulated by protease inhibitor (PI)-containing chow, which induces secretion of endogenous CCK. Expression profiling of PI-stimulation was performed on Affymetrix 430A chips and CN was inhibited via FK506. Exocrine pancreas-specific overexpression of CN inhibitor Rcan1 (Regulator of Calcineurin 1) was achieved by breeding elastase-Cre(ER) transgenics (TG) with ‘flox-on’ RCAN1 mice.

Results—CN inhibitor FK506 blocked expression of 38 genes, as confirmed by qPCR. The CN-dependent genes were linked to growth-related processes, while their promoters were enriched in NFAT and NFAT/AP1 sites. Multiple NFAT targets, including Rcan1, Rgs2, HB-EGF, Lif and Gem, were validated by ChIP. One of these, a CN feedback inhibitor Rcan1, was induced >50 fold during 1–8h course of pancreatic growth and strongly inhibited (>99%) by FK506. To examine its role in

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pancreatic growth, we overexpressed Rcan1 in an inducible, acinar-specific fashion. Rcan1 overexpression inhibited CN-NFAT signaling, as shown using an NFAT-luciferase reporter and qPCR. Most importantly, the increase in exocrine pancreas size, protein/DNA content and acinar proliferation were all blocked in Rcan1 overexpressing mice.

Conclusions—We profile adaptive pancreatic growth, identify Rcan1 as an important new feedback regulator and firmly establish that CN-NFAT signaling is required for this response.

Keywords

Rcan1; NFAT; calcineurin; pancreas; growth

INTRODUCTION

An adequate supply of pancreatic digestive enzymes is critical for digestion and absorption of food. To maintain its function in response to changes in diet or following injury¹, the normally quiescent exocrine tissue can undergo a rapid expansion. The gastrointestinal hormone cholecystokinin (CCK) is a key regulator of adult pancreatic growth and is necessary and sufficient for this response². Administration of exogenous CCK leads to both increased in pancreatic size *in vivo* and robust proliferation of acinar cells in culture. An alternative model used in this study involves increased endogenous secretion of CCK induced by feeding a chow containing a protease inhibitor (PI)³. CCK signals via its G-protein coupled receptor to activate MAPK, mTOR and calcineurin. How these early signaling events translate into growth, however, is largely unknown.

Calcium/calmodulin-dependent phosphatase calcineurin (CN or PP2B) has been shown to be an important mediator of adaptive responses such as T-cell proliferation, cardiac hypertrophy, osteogenesis and lung maturation⁴. CN activates several substrates, including a family of four Nuclear Factor of Activated T-cells (NFATc1-c4) transcription factors. In a basal state NFATs are heavily phosphorylated and sequestered in the cytoplasm, but upon stimulation rapidly shuttle into the nucleus due to CN-mediated dephosphorylation. The role of CN-NFAT axis in growth has thus far been addressed primarily in the heart. Expression of a constitutively active CN or NFATc4 is sufficient to produce cardiac hypertrophy⁵ and eventually leads to heart failure⁶. Using CN inhibitors CsA and FK506, our laboratory has shown that CN may also be important for CCK-mediated growth of the exocrine pancreas⁷. More recently, we have also shown that CCK activates NFATs both *in vitro* and *in vivo*, in a CN-dependent fashion⁸. Nonetheless, how activation of CN-NFAT leads to growth and the feedback regulation of this adaptive response are still poorly understood.

NFATs are well-characterized transcriptional regulators of inflammation. CN-NFAT pathway is also involved in a range of other responses, but relatively few of these NFAT-regulated genes have been identified. In the initial part of the study we carried out a genome-wide transcriptional profiling of a 2h point of pancreatic growth. We identified and analyzed the transcriptional regulation of 38 genes both increased in response to PI-feeding and blocked by the CN inhibitor FK506. Our efforts then focused on one of the 38 target genes, Regulator of Calcineurin 1 (Rcan1/Dscr1/Mcip1), an endogenous inhibitor of CN activity with several alternative transcripts. In the heart, exon4 transcript is controlled by CN-NFAT whereas exon1 transcript is expressed independent of this pathway⁹. Here, we tested the role of Rcan1 as a feedback inhibitor of the adaptive, and in contrast to muscle largely hyperplastic, growth of exocrine pancreas. We demonstrate a sustained, CN-dependent increase in Rcan1 throughout early course of pancreatic growth. We show that Rcan1 inhibits CCK-stimulated CN-NFAT signaling. Most significantly, we note that with tissue specific, inducible expression of Rcan1, CCK-induced acinar cell proliferation and pancreatic growth are nearly abolished. Our work

shows that Rcan1 is an important new regulator of pancreatic growth and CN-NFAT signaling is required for this response. These results represent the first molecularly-defined feedback mechanism that delimits hormonally-mediated growth of the exocrine pancreas.

EXPERIMENTAL PROCEDURES

Materials

The protease inhibitor (PI) camostat was provided by Ono Pharmaceuticals (Osaka, Japan). TaqMan reverse transcription and Expand™ PCR kits were purchased from Roche (Basel, Switzerland). Antibodies and all other reagents are listed in supplementary materials.

Animals and Treatment

Animal experiments, including PI-feeding and FK506 injections, are outlined in supplementary materials or performed as previously described⁸. To overexpress Rcan1 in pancreatic acini, we bred FLAG-Rcan1 [Rcan1] transgenic mice¹⁰ with Elastase(ER)-Cre [Ela-Cre] mice¹¹, which were generated as previously described. Rosa26-LacZ reporter mice were from Jackson Laboratories. All strains of mice were genotyped by PCR with primers in supplementary Table II (C). β -gal expression was examined as previously described¹².

Cell Culture

NIH 3T3 cells stably transfected with CCK-AR were described previously¹³, HEK-293 and 266-6 cells were obtained from ATCC (Rockville, MD), and all cells cultured as instructed. Pancreatic acini were isolated from 6–8 week old male mice as previously described⁸.

RNA isolation, bioinformatics and microarray analysis

RNA isolation, cDNA synthesis and PCR followed previously described procedures¹⁴. cDNA synthesis, hybridization and fluorescence signal optimization on Affymetrix 430A gene chips was performed at University of Michigan microarray core according to manufacturer's protocols. Detailed methods and computational analysis are outlined in supplementary materials.

Quantitative, real-time RT-PCR and Western blotting

1 μ g of total RNA was reverse transcribed using TaqMAN reagents. Real-time PCR was performed using Bio-Rad I-Cycler IQ, as previously described¹⁴. The primers were designed with Primer3¹⁵ based on gene sequences retrieved from GenBank (supplementary Table IIA). Western blots were performed as previously described⁸.

ChIP and RT-PCR

ChIP in 266-6 cells and isolated pancreatic acini was carried out using ChIP-IT express (Active Motif). The cells were left untreated or stimulated with CCK or A23187 and fixed in either 1% (266-6 cells) or 0.75% formaldehyde (isolated acini) for 10 min at room temperature. Glycine solution was applied, cells were harvested, lysed and sonicated. 25 μ L of each chromatin sample was precleared, blocked with protein G agarose beads and incubated with appropriate antibody. The complex was washed, eluted and cross-links reversed by 4h incubation at 65°C. The samples were then treated with proteinase K for 2h, DNA was purified and used for PCR. The primers used in PCR amplification are in Supplementary Table IIB.

Quantification of Pancreatic Growth

Determination of body weight, protein, DNA concentration and BrdU-based acinar cell proliferation were performed as previously described¹⁶. DAPI-stained nuclei were quantified

using CellProfiler™ software per authors' instructions¹⁷, whereas BrdU-positive cells were counted by hand. The percentage of BrdU labeled cells was a ratio of BrdU-positive/DAPI-stained nuclei, averaged for 3–5 fields per animal.

Luciferase Reporter Assay

NFAT-luciferase adenovirus⁶ was a gift from Dr. JMolkentin (University of Cincinnati). High concentrations ($\geq 10^{11}$ PFU) of NFAT-luciferase, AdRcan1 and control β -gal/EGFP adenovirus were produced as described⁸. For *in vitro* Rcan1 expression, isolated acini were pre-incubated with AdRcan1, then with 5×10^6 PFU of NFAT-luc adenovirus for 1h and finally with CCK for 5½h. For *in vivo* Rcan1 overexpression, isolated acini from TAM-injected Ela-Cre(ER)/Rcan1 or littermate controls were incubated with 5×10^6 PFU of NFAT-luc for 30min and then treated for 5½h. Rcan1-luciferase construct was a gift of Dr. CGlembotski (San Diego State) and transfected into AR42J NIH 3T3 cells with Superfect reagent (Invitrogen). For all luciferase assays, cells were washed with PBS, lysed in reporter lysis buffer (Promega, Madison, WI) and measured in a Berthold luminometer. Each condition was assayed based on >3 separate isolations with 3 replicates per preparation, normalized to protein concentration in the lysate.

Statistics

Comparisons of multiple data sets were analyzed by one-way ANOVA followed by Dunnett's or Bonferroni's post-test carried out on Graphpad Prism and expressed as mean $\pm$ SE. Pair-wise analysis was performed using a two-tailed Student's *t*-test. Large microarray data sets were initially compared by ANOVA with p-values of the groups subject to F-test. False discovery rate and associated q-values were then derived as described¹⁸.

RESULTS

RNA microarray analysis of PI-induced and CN-dependent genes

We performed genome-wide expression profiling of the 2h time point of CCK-driven pancreatic growth, focused on CN-dependent genes in particular. Pancreatic RNA was harvested from mice fasted overnight or fasted and re-fed PI-containing chow for 2h and injected with either 3mg/kg of FK506 or vehicle, then hybridized with Affymetrix 430A genechips (4 mice/group, 16 chips total), as outlined in Figure 1A, top. PI-feeding led to a significant (>3 fold, $q \leq 0.08$) increase in expression of 81 genes and decrease in expression of 2 genes, whereas FK506 significantly (>70%, $q \leq 0.08$) blocked PI-induced expression of 38 genes. The top 12 of the 38 genes, which we termed calcineurin (CN)-dependent, were inhibited >90% by FK506 and are listed in Table I, whereas the remaining 26 and the entire microarray are available in supplementary materials. Underlining the importance of CN for pancreatic growth, CN-dependent genes comprised a third of those induced by PI-feeding (Figure 1A, bottom). A set of six genes induced by PI-feeding and strongly inhibited by FK506 were independently examined by qPCR (Figure 1B). The results closely correlated with changes seen by microarray. As an additional control, we examined CCK-deficient mice. These animals do not exhibit significant pancreatic growth in response to PI and their qPCR expression profile was nearly identical to FK506-treated mice (Figure 1B). Most CN-dependent genes appeared to function in growth, cell-cell communication, transcriptional regulation or negative feedback (Suppl. Table I); statistical analysis of gene ontology (GEO) annotations confirmed these observations (Suppl. Fig. 1). A network association analysis of the 38 CN-dependent and 81 PI-induced genes, showed significant overlap with concepts related to growth, differentiation and stress response (Figure 1C, thick lines). Interestingly, NFAT and AP-1, two transcription factors previously shown to be activated by CCK, were also linked to our two sets of genes.

NFAT regulation of CN-dependent genes

Based above findings, we next assessed the potential role of NFATs as transcriptional regulators of CN-dependent genes in pancreatic growth. We retrieved non-coding regulatory sequences of these 38 genes along with their rat and human orthologs, performed exon-anchored alignments and searched for conserved NFAT binding sites. Any gene with at least one NFAT site conserved across three species was termed as 'NFAT-regulated' and a subset within this group with several closely-spaced sites as 'high-confidence'. Genes which did not contain any sites with 3-way conservation or those that could not be aligned were termed 'unlikely' or 'undetermined', respectively. Independent analysis using two computational tools, MatInspectorTM and rVISTA, produced largely overlapping results (Figure 2A). Subject to the same analysis, nearly all of experimentally established, immune system-related 'gold standard' genes were found to be NFAT-regulated, whereas only a few and no high confidence identifications were found among genes selected at random (Figure 2B). Like many other transcription factors, NFATs are known to control gene expression as parts of larger regulatory 'modules.' Using the frameworker tool in GenomatixTM software, we found several statistically overrepresented modules connected to CN or CN-NFAT signaling (Suppl. Figure 2A). The well-known NFAT/AP1 transcription factor complex was then analyzed analogously to NFATs, identifying several CN-dependent genes that contained this conserved promoter module (Suppl. Figure 2B and C). Computational analysis is a good point of departure, though results may not always reflect the biology. To experimentally test our predictions, we examined several high confidence NFAT-regulated genes with previously known functions relevant to growth via chromatin immunoprecipitation (ChIP) assay. Treatment of the mouse pancreatic 266-6 acinar cells with calcium ionophore A23187 and primary isolated pancreatic acini with CCK both stimulated NFATc1 binding to promoter regions of five CN-dependent genes (Figure 2C and D).

Rcan1 is strongly induced in the course of pancreatic growth

As the gene most sensitive to FK506 and among the top three induced by feeding PI-containing chow (Table I), Rcan1 expression appeared to be tightly linked to pancreatic growth. To examine changes in Rcan1 expression throughout the early time course of pancreatic growth, we obtained RNA and protein from mice either fasted overnight or fasted and refed PI-containing chow for 1–8h. PI-feeding produced a sustained, >50 fold increase in Rcan1 mRNA (Figure 3A), while the expression of Rcan2 and Rcan3, members of the same family independent of CN-NFAT pathway¹⁹, was not altered (Figure 3A). Parallel changes took place for the 26kDa exon4 RCAN1 protein (Figure 3B), whereas levels of the larger exon1 form were not altered (data not shown). CCK also dose-dependently activated Rcan1 promoter-driven luciferase reporter, and this effect was shown to be CN-dependent as it was blocked by FK506 (Figure 3C). As an additional control, we examined the effect of feeding. In fasted mice circulating CCK is typically at 0.5–1 pM, in animals fed standard diet it increases to 3–5 pM, whereas in those fed PI-containing chow to ~15 pM¹⁶. No appreciable increase in Rcan1 expression was noted among feeding controls or CCK-deficient mice (Figure 3D).

Inducible, pancreatic acinar-cell specific Rcan1 overexpression in vivo

The sustained, CCK-dependent increase in expression of Rcan1 prompted us to test if this endogenous regulator of CN can function as a feedback inhibitor of CCK-driven pancreatic growth. To express Rcan1 in an inducible, acinar cell-specific fashion, we crossed mice expressing tamoxifen (TAM)-inducible, elastase promoter-driven Cre recombinase [Ela-Cre (ER)] with 'flox-ON' FLAG-Rcan1 mice. We examined Cre protein using immunohistochemistry and western blots. Cre was expressed in the exocrine pancreas of TAM-injected Ela-Cre(ER) mice, but not in islets or ducts (Suppl Figure 3A) and in the pancreas but not the salivary glands or liver (Suppl Figure 3B). To verify tissue specific activation of Ela-

Cre(ER), we crossed this strain with Rosa26-LacZ reporter mice. Immunohistochemistry for β -gal showed Cre recombinase was activated in >80% of pancreatic acini, but not in islets, blood vessels or ducts (Suppl Figure 3C) of TAM-injected Ela-Cre(ER)/LacZ mice. No β -gal staining was noted in salivary acinar cells of double transgenic or the pancreas and salivary gland of single transgenic controls (data not shown). To assess acinar-specific expression of Rcan1 transgene, we prepared RNA from acini of TAM-injected Ela-Cre(ER)/Rcan1 mice or single transgenics. FLAG-Rcan1 transgene was expressed in acini of double transgenic mice independent of CCK, but not in single transgenic littermates (Suppl Figure 3D). By contrast, acini from uninjected animals showed equal, CCK-inducible expression of endogenous Rcan1 message for all three groups. RCAN1 protein was also strongly expressed in TAM-injected Ela-Cre(ER)/Rcan1 mice (Suppl Figure 3E, lane 2), but not in TAM-injected wild type animals and weakly for vehicle-injected double transgenics; no transgene expression was seen in the liver (Suppl Figure 3E, lanes 4–6). Lastly, to test dose-dependent effects of Rcan1 overexpression, we utilized a second, independently generated line of ‘Flox-ON’ Rcan1 animals¹⁰, called ‘Rcan1(a)’ mice. The double transgenic offspring of the cross between Ela-Cre(ER) and Rcan1(a) mice showed ~8 fold lower transgene expression (data not shown).

Rcan1 is an inhibitor of CCK-driven pancreatic CN-NFAT signaling

Next, we tested if Rcan1 overexpression inhibits CN-NFAT signaling in CCK-mediated pancreatic growth. As a readout of CN-NFAT activation we used a previously characterized NFAT-luciferase (NFAT-luc) reporter⁸. CCK induced a robust, dose-dependent activation of this reporter in primary isolated pancreatic acini incubated with a control β -gal/GFP adenovirus, but not those incubated with Rcan1 virus (Figure 4A). Expression of Rcan1 also blocked the translocation of NFATc1GFP to the nucleus (Suppl Figure 4). Likewise, CCK-induced, dose-dependent response for NFAT-luc reporter in isolated acini of wild type mice was largely abrogated in acini from TAM-injected Ela-Cre(ER)/Rcan1 littermates (Figure 4B). Thus, *in vivo* expression of the Rcan1 led to functional inhibition of CN-NFAT signaling on par with that of adenovirally-mediated overexpression. Acinar-specific overexpression of Rcan1 *in vivo*, however, did not alter CCK-dependent phosphorylation of ribosomal protein S6 and 4E-BP1, two well-known downstream targets of mTOR, a key signal in PI-induced pancreatic growth (results not shown). Interestingly, Rcan1 also inhibited the PI-induced activation of many, but not all of the 38 CN-dependent, FK506 inhibited genes (Figure 4C, compare with Table I and Figure 1B). Upon closer examination, the genes significantly inhibited by Rcan1 in PI-feeding aligned with our computational predictions of NFAT-regulated genes (Figure 2A), implying NFAT-specific skewing of the inhibitory effects of Rcan1.

Rcan1 overexpression blocks pancreatic CCK-driven acinar proliferation

Rcan1 transgene clearly inhibits CN-NFAT signaling hence we asked if acinar cell-specific Rcan1 overexpression was sufficient to inhibit CCK-driven proliferation of pancreatic acinar cells. TAM-injected wild type and Ela-Cre(ER)/Rcan1 mice were fasted and refed with either control or PI-containing chow for 2 days and treated with BrdU. In both Ela-Cre(ER)/Rcan1 and controls fed normal diet, less than 0.5% of cells incorporated BrdU in their nuclei (Figure 5A). In wild-type mice fed a PI-containing diet there was a significant increase in BrdU incorporation and this increase was largely blocked in Ela-Cre(ER)/Rcan1 mice (Figure 6A). In addition, BrdU incorporated into morphologically identifiable, amylase-staining acinar cells (Figure 5B).

Rcan1 overexpression blocks CCK-mediated pancreatic growth

Lastly, we tested if acinar cell-specific overexpression of Rcan1 was also sufficient to inhibit pancreatic growth *in vivo*. We compared TAM-injected Ela-Cre(ER)/Rcan1 and controls fed

either standard or PI-containing chow for up to 10 days. In wild type animals, feeding PI-containing diet led to a large increase in the gross size of the pancreas compared to littermates on a standard chow (Figure 6A). Pancreatic weight relative to BW more than doubled, with an increase that peaked at 7 days (Figure 6B). CCK-driven pancreatic growth induced by PI, however, was completely abolished in *Ela-Cre(ER)/Rcan1* mice throughout the timecourse (Figure 6). A detailed study of 4–6 mice/group at the 7 day point confirmed these findings, with doubling of relative pancreatic weight in TAM-injected wild type and single transgenics fed PI-containing chow, but almost no growth in *Ela-Cre(ER)/Rcan1* littermates (Figure 6C). Interestingly, the *Ela-Cre(ER)/Rcan1(a)* line which expresses lower levels of *Rcan1* showed an intermediate phenotype; increased growth compared to control mice fed standard chow, but a decrease compared to controls fed PI-containing chow (Figure 6C). Pancreatic protein and DNA content measured after 7 days of standard or PI-containing diet showed changes that paralleled growth (Figure 6C and D). These last sets of data point to the hyperplastic nature of pancreatic growth and agrees both with our BrdU incorporation data and previous work⁷.

DISCUSSION

Physiological growth of the pancreas takes place in response to high protein diet, hyperphagia, pregnancy and lactation²⁰. The molecular mechanisms that govern this adaptive response, however, are poorly understood. Here, we examined the expression profile of CCK-mediated pancreatic growth. We focused on CN-NFAT signaling axis, identifying several novel growth-related, NFAT-regulated genes. We also demonstrate that one of these genes, *Rcan1*, functions as a CN/NFAT-dependent feedback inhibitor that controls growth plasticity of adult pancreas.

The results from our expression profile both validate and extend past work. Sets of genes significantly altered in our expression profile significantly overlap with molecular concepts linked to pathways previously shown to be activated CCK, including MAPK-AP1¹⁴, Rho-SRF²¹ and NF- κ B²². Aside from CN-NFAT-*Rcan1*, our work also points to JAK-STAT and its inhibitors *Socs2* and *Socs3* as yet another signal regulating this response (Suppl. Table I and unpublished data). Several of our 38 CN-dependent genes have been tied to regulation of growth, differentiation and metabolism. FGF21 has been shown to be important for hepatic regeneration and β -cell survival²³, HB-EGF in β -cell transdifferentiation and pancreatic cancer²⁴, whereas *Socs3* for proliferation and angiogenesis in the liver²⁵. We posit that these and other membrane-bound paracrine/autocrine mediators alter local microenvironment, drive hyperplasia or differentiation and thereby direct pancreatic growth. Analogous mechanisms have already been shown in the liver following a partial resection²⁶ and the pancreas following pancreatitis²⁷. Broadly, our results argue that CCK-mediated pancreatic growth has much in common with these regenerative responses.

NFATs are best known as transcriptional mediators of inflammation. Their role as regulators of growth or tissue homeostasis in general, however, is not as clear. Our work is one of the most detailed analyses of genome-wide expression that underlies an adaptive response linked to CN-NFAT signals. We predict a sizable new set of NFAT-regulated genes related to pancreatic growth and show that NFATc1 binds to promoters of selected high quality identifications. The time frame of increased NFATc1 binding by ChIP corresponds to CCK-stimulated nuclear shuttling of NFATc1-GFP in acini⁸ and the kinetics of NFAT translocation in other cell types. Lastly, NFATs have intrinsically low affinity for DNA and therefore function within complexes or modules. Known partners of NFATs include GATA-4 in the heart, C/EBP in lung, MEF2 in muscle and AP-1/Foxp3 in immune cells²⁸. Complex-dependent effects dictate timing, affinity and outcome of NFAT binding in a tissue or stimulus-dependent manner. Here, we identify several enriched transcriptional modules among CN-dependent genes, including the well-known synergistic NFAT/AP-1 complex²⁹. As a site of

convergence, transcriptional complexes may explain why inhibition of a single pathway can be sufficient to disrupt an entire physiological response.

Rcan1 expression increased throughout the early course of pancreatic growth and endogenous Rcan1 strongly inhibited CCK-induced CN-NFAT signaling. This led us to formulate the central paradigm of this study: CCK-mediated activation of CN leads to nuclear translocation and activation of NFATs, NFAT-dependent induction of Rcan1 and finally RCAN1-mediated inhibition of CN. The exact means whereby CN-NFAT-RCAN1 axis constrains growth, however, remains unknown. One rationale is that RCAN1 blocks proliferation; an alternative may be that RCAN1 limits angiogenesis. NFATc1 has been shown to regulate vascular endothelial growth factor (VEGF) expression³⁰ and mice that overexpress Rcan1 showed decreased tumor formation due attenuated angiogenesis³¹. Rcan1 is also expressed at a higher level in Down Syndrome (Rcan1 is also known as Down Syndrome Critical Region 1 or DSCR1) and may decrease susceptibility to solid tumors in these individuals. CCK also increased expression and NFATc1 binding to the promoter of Leukemia Inhibitory Protein (Lif). Lif has recently been shown to regulate microvessel density and VEGF expression in retinopathy³². Direct or indirect role of Rcan1 in regulation of angiogenesis is certainly a promising area for future research.

Finally, our work here shows that transgenic overexpression of Rcan1 is sufficient to completely block pancreatic growth. The pre-emptive expression of Rcan1 clamps the CN-NFAT-Rcan1 signal in a permanent “off” position, independent of CCK. This tissue-specific genetic approach circumvents side effects of pharmacological agents like immune suppression, nephro and neurotoxicity, whereas inducible overexpression preempts possible developmental defects or compensatory changes. Rcan1 also inhibited many, but not all of the genes blocked by CN inhibitor FK506. Interestingly, Rcan1 appeared to significantly inhibit only CN-dependent genes which we also computationally predicted to be NFAT-regulated. NFAT-specific skewing of the inhibitory effect of Rcan1 on CN has been suggested elsewhere, but primarily based on biochemical evidence³³.

Recent advances in manipulation of cell fate and plasticity of pancreatic tissue brought renewed promise of treatments for pancreatitis, pancreatic cancer and diabetes. Our work and the expression profiles of pancreatic development³⁴, pancreatitis³⁵ and pancreatic cancer³⁶ are grounds for further integrative research. Relevantly, NFATc1 has been shown to be inappropriately activated in pancreatic cancer³⁷. The role of Rcan1 has yet to be examined, but may well prove to be important based on significant downregulation of its message in at least two microarrays of human pancreatic cancer samples^{38–39}.

In summary, we examined the expression profile of an early point of CCK-mediated pancreatic growth. We computationally and experimentally examined several growth related genes, focusing on endogenous CN regulator Rcan1. Rcan1 showed a strong, sustained expression throughout the growth response and significantly inhibited CCK-simulated CN-NFAT signaling. Most notably, inducible, exocrine-specific expression of Rcan1 *in vivo* completely blocked acinar cell hyperplasia and the accompanying CCK-driven pancreatic growth. Our work identifies an important new feedback mechanism that delimits adaptive pancreatic growth and provides firm evidence that CN-NFAT axis is necessary for this hormonally-mediated, adaptive response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of PI-induced and CN-dependent genes in pancreatic growth (A) Top: experimental design of the microarray experiments. Arrows show biologically meaningful comparisons: genes altered with PI-feeding (red) and inhibited by FK506 (blue). Bottom: PI-feeding significantly (>3fold, $q \leq 0.08$) increased the expression of 81 genes, whereas FK506 significantly (>70%, $q \leq 0.08$) blocked 38 genes. (B) RNA samples were obtained from pancreases of four sets ($n=3-8$ /group) of mice: (1) control mice fasted overnight, (2) mice fasted overnight and refed PI-chow for 2h (3) same as previous, except pretreated twice daily with 3mg/kg of FK506 and (4) CCK-deficient mice fasted overnight and refed PI. Bars represent mean $\pm$ SEM expression by qPCR relative to fasting controls; # $p < 0.05$ and ## $p < 0.01$ versus 2h PI (black bars). (C) Network association of molecular concepts for the 38 CN-dependent and 81 PI-induced genes, represented as two light green nodes. Each node in the network stands for a molecular concept or a set of biologically related genes, color-coded in the legend. The size of the node is proportional to the number of genes and connections signify statistically significant enrichment ($p < 0.005$). Links to concepts related to growth/differentiation are shown in blue.

Figure 2. Delineating NFAT-regulated genes

(A) Proximal promoters (MatInspector™) or regulatory regions (rVISTA) for mouse, rat and human orthologs of 38 CN-dependent genes were obtained and aligned. Of note, MatInspector™ did not automatically retrieve the proximal promoter of the exon4 transcript of RCAN1. With manual retrieval, RCAN1 was a high confidence identification. (B) The promoters of 21 experimentally established, NFAT-regulated genes (gold standard) and a set of 21 random genes analyzed by MatInspector™ were evaluated using the same methods and criteria as (A). (C) Chromatin Immunoprecipitation (ChIP) assay of A23187-stimulated binding of NFATc1 to promoters of five CN-dependent genes computationally predicted to be regulated by NFAT. Studies were carried out in 266-6 cells. Pol II binding was used as a readout of transcriptionally active genes and a primer set for EF1α was used as a control. (D) ChIP assay of CCK-stimulated binding of NFATc1 to the promoters of the same five CN-dependent genes as in (C) carried out in primary isolated mouse acinar cells.

Figure 3. PI-containing chow increases RCAN1 expression

Mice were fasted overnight, refed PI-containing chow for 1–8 hours and sacrificed. (A) mRNA expression of RCAN1, but not RCAN2 or RCAN3, significantly ($**p \leq 0.01$, $n=5-6$) increased with PI-feeding. 18S RNA was used as a control. (B) Exon 4 form of RCAN1 was significantly induced ($*p < 0.05$ or $*p < 0.01$) by PI-feeding with a peak at 2h. Representative western blot for 26kDa form of RCAN1 and cyclophilin A (CycloA) loading control are shown above a bar graph quantifying the results of 3 independent experiments from 5–6 mice per time point. (C) CCK dose-dependently activates Rcan1-luciferase reporter in a CN-dependent fashion in NIH3T3 CCK-AR cells. ($*p \leq 0.05$, $n=5-6$) (D) Representative western blot for the 24kDa RCAN1 form and cyclophilin A (loading control) of pancreases of wild type mice fasted, free fed, fasted overnight and refed either standard or PI-containing diet; CCK-deficient mice were fasted and refed PI-containing diet.

Figure 4. RCAN1 overexpression functionally inhibits CN-NFAT pathway

(A) Dose-response activation of NFAT-luciferase reporter by CCK in isolated acini incubated with either RCAN1 or β -gal/GFP adenovirus ($n=3$, $*p<0.05$ versus time zero; $\#p<0.05$ versus control virus). (B) Dose-response activation of NFAT-luciferase reporter in fresh isolated acini from either TAM-injected Ela-Cre(ER)/Rcan1 or wild type littermates ($n=3$, $*p<0.05$ versus time zero; $\#p<0.05$ versus controls) (C) RNA samples from fasted controls as well as Ela-Cre (ER)/RCAN1 or wild type littermates fasted and fed PI-containing chow for 2h were used for RT-qPCR. All mice were injected with TAM. Shown are the mean $\pm$ SEM induction relative to fasting controls for a set of eight genes ($n=4-7$; $\#p<0.05$ versus 2h PI wild type).

Figure 5. RCAN1 transgene blocks acinar cell proliferation *in vivo*. (A) TAM-injected wild type and Ela-Cre(ER)/RCAN1 mice were fasted and refed standard or PI-containing chow for 2 days, treated with BrdU and sacrificed 2h later. Representative slides of BrdU immunostaining in red, DAPI in blue and overlay in pink (white arrows). The results from experiments on 3–5 mice are quantified as % cells with BrdU staining over total DAPI-staining. ** $p < 0.01$ compared to standard chow; # $p < 0.05$ versus wild type fed PI-containing chow. (B) BrdU (pink nuclei/white arrows) incorporates into morphologically mature, amylase expressing (green) acinar cells.

Figure 6. Acinar-specific overexpression of RCAN1 blocks pancreatic growth

(A) TAM-injected wild type and Ela-Cre(ER)/RCAN1 mice were fed standard or PI-containing chow for 10 days. Representative photographs of the pancreas for age and body weight (BW)-matched mice. Squares are 6×6mm. (B) Relative pancreatic weights of wild type and Ela-Cre(ER)/RCAN1 mice fed a standard or PI-containing chow for 0–10 days. All animals were pre-injected with TAM; n=3–5, **p<0.01 versus day zero; ##p<0.01 vs same day wild type fed PI-containing chow. (C, D, E) Changes in relative pancreatic weight (C), protein (D) and DNA content (E) at 7 days; TAM-injected wild type, Ela-Cre(ER)/RCAN1 and single transgenic littermates (n=4–6) were fed with standard or PI-containing chow (**p<0.01 vs wild type; ##p<0.01 vs. wild type PI-containing chow).

Table I

FK506 inhibits numerous genes induced by PI-feeding

GenBank Accession	Gene Symbol	Product	Functional Annotation	PI Fold induction	% inhibited by FK506
NM_019466.1	Rcan1	regulator of calcineurin 1	calcium signaling, phosphatase regulator, muscle development	30.5	99.5
NM_020013.1	Fgf21	fibroblast growth factor 2	growth factor, extracellular space	33.8	99.2
BI558298	Zfp703	zinc finger protein 703	Unknown, oncogene?	7.97	98.0
NM_032540.1	Kel	Kell blood group	proteolysis	30.2	97.1
NM_007707.1	Socs3	suppressor of cytokine signaling 3	cell growth,, negative regulator of insulin signal	34.5	96.6
NM_009061.1	Rgs2	regulator of G-protein signaling 2	cell cycle, G-protein coupled signaling,	6.34	94.3
NM_009311.1	Tac1	tachykinin 1	inflammatory response, synaptic transmission,, pain	10.3	94.2
NM_007679.1	Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	regulation of transcription	18.9	93.3
NM_013689.1	Tec	cytoplasmic tyrosine kinase	protein phosphorylation, signaling	6.64	92.3
NM_026473.1	Tubb6	Tubulin, beta 6	microtubule-based movement, polymerization	12.7	92.1
U10551.1	Gem	GTP binding protein, expressed in muscle	small G-protein, GTPase signaling	11.2	91.0
NM_008580.1	MAP3K5	mitogen activ. kinase kinase kinase 5	MAPKKK cascade, phosphorylation, apoptosis	15.1	90.6

The top 12 of 38 CN-dependent genes (those inhibited >90% by FK506) identified by genome-wide expression profiling of pancreatic growth, in order of % inhibition by FK506. The entire list is presented in supplementary materials. The columns are GenBank Accession, Mouse Genome Informatics (MGI 3.54) gene symbol, product name, gene ontology (GEO) molecular function annotation, fold induction by protease inhibitor (PI) and % inhibition of that induction by FK506.