Ca2+ Influx Channel Inhibitor SARAF Protects Mice From Acute Pancreatitis

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Abstract

Background & Aims

Pancreatitis is characterized by increased influx of Ca^{2+} into acinar cells, by unknown mechanisms. Inhibitors of Ca^{2+} influx channels could be effective in treating acute pancreatitis, but these have deleterious side effects that can result in death. We investigated the expression patterns and functions of acinar cell Ca^{2+} channels and factors that regulate them during development of acute pancreatitis, along with changes in the channel inactivator store-operated calcium entry–associated regulatory factor (SARAF). We investigated whether SARAF is a target for treatment of acute pancreatitis and its status in human with pancreatitis.

Methods

We generated mice that expressed SARAF tagged with hemagglutinin, using CRISPR/Cas9 gene editing, and isolated acinar cells. We also performed studies with *Saraf*$^{-/-}$ mice, *Saraf* $^{zf/zf}$ mice, mice without disruption of *Saraf* (control mice), and mice that overexpress fluorescently labeled SARAF in acinar cells. We analyzed interactions between stromal interaction molecule 1 (STIM1) and SARAF in HEK cells stimulated with carbachol using fluorescence resonance energy transfer microscopy and immunoprecipitation. Mice were given injections of caerulein or L-arginine to induce pancreatitis. Pancreatic tissues and blood samples were collected and levels of serum amylase, trypsin, tissue damage, inflammatory mediators, and inflammatory cells were measured. We performed quantitative polymerase chain reaction analyses of pancreatic tissues from 6 organ donors without pancreatic disease (controls) and 8 patients with alcohol-associated pancreatitis.

Results

Pancreatic levels of Ca^{2+} influx channels or STIM1 did not differ significantly between acinar cells from mice with vs. without pancreatitis. By contrast, pancreatic levels of *Saraf* messenger RNA

and SARAF protein initially markedly increased but then decreased during cell stimulation or injection of mice with caerulein, resulting in excessive Ca^{2+} influx. STIM1 interacted stably with SARAF following stimulation of HEK or mouse acinar cells with physiologic levels of carbachol, but only transiently following stimulation with pathologic levels of carbachol, leading to excessive Ca^{2+} influx. We observed reduced levels of *SARAF* messenger RNA in pancreatic tissues from patients with pancreatitis, compared with controls. SARAF knockout mice developed more severe pancreatitis than control mice after administration of caerulein or L-arginine, and pancreatic acinar cells from these mice had significant increases in Ca^{2+} influx. Conversely, overexpression of SARAF in acini reduced Ca^{2+} influx, eliminated inflammation, and reduced severity of acute pancreatitis.

Conclusions

In mice with pancreatitis, SARAF initially increases but is then degraded, resulting in excessive, pathological Ca^{2+} influx by acinar cells. SARAF knockout mice develop more severe pancreatitis than control mice, whereas mice that express SARAF from a transgene in acinar cells develop less-severe pancreatitis. SARAF therefore appears to prevent pancreatic damage during development of acute pancreatitis. Strategies to stabilize or restore SARAF to acinar cells might be developed for treatment of pancreatitis.

Key words: Pancreatitis, Ca^{2+} influx channels, STIM1, SARAF, treatment.

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Introduction

Acute and chronic pancreatitis are inflammatory pathologies with no effective treatment[1, 2]. The multiple causes of pancreatitis are alcohol consumption, bile reflex, pancreatic duct obstruction, autoimmunity, genetic mutations and idiopathic causes[3, 4]. Pancreatitis is typified by altered CFTR localization and function in the duct[5-7], increased systemic and pancreatic inflammatory mediators and tissue damage culminating in increased plasma amylase and lipase[1, 2]. These result in injured acinar cells and destruction of the exocrine pancreas. Tissue and cellular analysis in animal models reveal that a major cause of pancreatitis is targeting of secretory granules with their digestive enzymes to the acidic lysosomes, activation of trypsin in acinar cell cytoplasm, and cell damage[8, 9]. A nodal point for all forms of pancreatitis is a prolonged, sustained increase in the concentration of free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$)[10, 11]. Indeed, preventing the Ca^{2+} increase in cellular[12, 13] and animal models[14-16] prevents the cell damage observed in acute pancreatitis.

Sustained increase in $[Ca^{2+}]_i$ requires activation of Ca^{2+} signaling. The physiological Ca^{2+} signal entails receptor-stimulated activation of phospholipase C and generation of IP_3. IP_3 activates the IP_3 receptors (IP_3Rs), primarily at the apical pole of acinar cells to release a small fraction of ER Ca^{2+}. Ca^{2+} release from the ER results in clustering of the ER Ca^{2+} sensor STIM1 at the plasma membrane/endoplasmic reticulum (ER/PM) junctions, where it activates both the Ca^{2+} influx store-operated Orai1 channels[17] and the TRPC channels[18]. The increase in $[Ca^{2+}]_i$ activates the Sarcoplasmic/endoplasmic (SERCA) and plasma membrane (PMCA) Ca^{2+} ATPase pumps, which refill the ER with Ca^{2+} and clear cytoplasmic Ca^{2+}, respectively. This cycle is repeated periodically, resulting in the physiological Ca^{2+} oscillations[19].

To prevent cell toxicity, it is critical to inhibit the Ca^{2+} influx channels, which initiates the phase of Ca^{2+} reduction in each Ca^{2+} spike during Ca^{2+} oscillations. Ca^{2+}-dependent inhibition of Orai1 has been studied extensively[20]. Orai1 activated by STIM1 undergoes two forms of inactivation. Fast Ca^{2+}-dependent inactivation (FCDI) that is completed in about 100 milliseconds, and slow Ca^{2+}-dependent inactivation (SCDI) that reduces channel current by about 80% within 2 minutes of channel opening[20]. Both, FCDI and SCDI are mediated by the Ca^{2+} influx channel regulatory protein SARAF (SOCE-associated regulatory factor)[21-23].

Acute pancreatitis caused by excessive Ca^{2+} influx can be treated by inhibition of Ca^{2+} influx channels or by increasing the activity of the Ca^{2+} influx channels inhibitor SARAF. We have

previously shown that genetic and pharmacological inhibition of TRPC channels prior to induction of acute pancreatitis effectively prevents the disease[15]. However, blocking TRPC channels after induction of acute pancreatitis fails to inhibit the disease. Pharmacological inhibition of Orai1 partially protects against all models of acute pancreatitis tested, but targeted deletion of Orai1 in pancreatic acinar cells results in 70% mortality due to bacteremia and sepsis[13, 16 24]. Pancreatic acinar cells synthesize and secrete significant amounts of antibacterial agents to the gut to control the gut microbiota. Deletion of acinar cell Orai1 inhibits synthesis and secretion of the antibacterial agents to the gut, resulting in lethal dysbiosis[24]. An alternative approach is to target the regulator rather than the channels themselves.

Here, we show that while SARAF interacted with STIM1 to limit Ca^{2+} influx during physiological stimulation, interaction of SARAF with STIM1 was transient during pathological stimulation of acinar cells, resulting in a sustained, toxic Ca^{2+} influx. Pathologic stimulation of acinar cells caused minimal change in the expression of various Ca^{2+} signaling proteins, including STIM1, over a time-course of 6 hrs. By contract, the mRNA and protein level of SARAF increased 20-fold early during pathologic stimulation but then decreased in a time-dependent manner to very low level. Knockdown of SARAF in two mouse models markedly increased Ca^{2+} influx, mitochondrial Ca^{2+} accumulation, inflammation and severity of acute pancreatitis. Conversely, targeted transgenic expression of SARAF in mouse pancreatic acini reduced pathologic Ca^{2+} influx, prevented the inflammation and reduced tissue damage caused by induction of severe acute pancreatitis in two mouse models. These findings suggest that SARAF should be pursued as a potential therapeutic target in acute pancreatitis.

Methods

Generation of SARAF knockout mice: SARAF knockout mice were generated using zinc finger nuclease mediated genome editing (Supplementary Fig. S1A). The zinc finger nuclease construct was custom designed by Sigma (Figs. S1A, lines A and B) and injected into the zygote embryos by the gene targeting core of NIDCR[25]. The embryos were planted in females with FVBNJ and C57bl6 background and the pups were screened for deletions in the genomic DNA. One mouse from each background was selected for breeding. The C57bl6 mice (line B) had deletion of 400 bases in the *SARAF* gene, resulting in a complete knockout. The FVBNJ mice (line A) had a 30 base-pair deletion at the start codon resulting in a truncated SARAF mouse line. The protocol for screening the mice is based on[26] and the primers are listed in supplementary table 1. The mutations and deletions were confirmed by amplifying and sequencing 1000-3000 bases around the targeted sites. Briefly, the PCR product was TA cloned into pCR 2.1 vector from Invitrogen, transformed and 8 colonies were screened for each mouse. The sequences indicated that the mice were heterozygous for the mutations and were crossbred to obtain the knockouts.

Generation of transgenic mice with targeted expression of SARAF-YFP in pancreatic acinar cells: Mice were generated using the rat elastase gene promoter (generously gifted by Dr. David Hui, University of Cincinnati) bases -500/+8[27]. The elastase promoter was

cloned into EcoRI and BamH1 site of the pCMV6-AC-mYFP Tagged Cloning Vector (cat# PS100042) and mouse SARAF cDNA (accession # NM_026432) was inserted between the MluI and NotI sites. An EcoR1 site was engineered after the stop codon of YFP (Fig. S1B). The construct was digested and injected into zygote embryos of FVBNJ strain. Tail genomic DNA from littermates was screened for the construct by PCR and subsequent sequencing by TA cloning of the PCR product. Two positive mice were found, and one was propagated. Specific pancreatic expression was verified by RT-PCR (Fig. S1C). The transgene copy number was calculated using the protocol reported in[28]. Briefly, the concentration of genomic data was determined using Quant-iT dsDNA Assay Kit (Invitrogen, cat. no. Q33130). Taqman probes (# Mm01197901) from Invitrogen was used for qPCR from the genomic DNA and the calculated copy number was 3. The SARAF transgenic mice are named SARAF/TG in the text and the Figures.

Generation of endogenous HA-tagged SARAF mouse: SARAF was endogenously tagged with HA using CRISPR/Cas9. Guide RNA and donor plasmid was designed to target the last exon just before the stop codon (Table S1 and Fig. S1D). The donor plasmid and gRNA were co-injected in the mouse zygotes and implanted. The pups were screened for insertion of the HA tag with the primers in table S1. The PCR product generated 2000 bases upstream and downstream from the target site and TA cloned into pCR2.1 plasmid for sequencing. One of 6 founders one was backcrossed to establish the mouse line. Expression of HA in various mouse tissues is shown in Fig. S1E.

Preparation of Pancreatic acinar cells: Pancreatic acini were prepared from 3- to 6-month-old mice as described previously[24]. Briefly, mice were sacrificed by CO_2 inhalation, and the pancreas was removed and injected with cold solution A containing (mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 pyruvate, 10 HEPES (pH 7.4, 310 mOsm), 0.1% bovine serum albumin, and 0.02% soybean trypsin inhibitor. The tissue was finely minced and digested with collagenase P (0.25 mg/ml) dissolved in solution A. After washing twice, the acini were suspended in solution A without STI and were kept on ice until use.

Measurement of cytoplasmic ($[Ca^{2+}]_i$) and mitochondrial ($[Ca^{2+}]_m$): $[Ca^{2+}]_i$ was measured in acini loaded with Fura2 by incubation with 5 μM Fura2/AM (TEFLabs Inc., Austin, TX) and 0.05% Pluronic F-127 (Invitrogen, Carlsbad, CA) for 30 min at room temperature. Fluorescence was imaged with a TILL system at excitation wavelengths of 340 and 380 nm and emission above 500 nm was collected. $[Ca^{2+}]_m$ was measured by loading the cells with Rhod2 by incubation with 8 μM Rhod2/AM (Life Technologies, Carlsbad, CA) and 0.05% Pluronic F-127 for 15 min at room temperature. The acini were spun down and kept on ice for 30 min. Acini were plated on 0.01% poly-L-lysine coated cover slips and perfused continuously with solution A. Fluorescence was imaged at an excitation wavelength of 587nm with a 60X objective using an Olympus IX81 confocal microscope. Images were digitized at 2-s intervals and analyzed by MetaFlour software.

Measurement of saliva secretion: 4-6-month-old mice were anesthetized by intramuscular injection of ketamine (60 mg/kg) and xylazine (8 mg/kg). Stimulated salivary secretion was initiated by injecting the mice subcutaneously with the indicated mg/kg pilocarpine, and saliva was collected every 5 min for 20-30 min into pre-weighted Eppendorf tubes by capillary drainage. The secreted saliva volume was normalized to body weight and averaged.

Induction of acute pancreatitis: Acute pancreatitis was induced by two separate models, the caerulein and arginine models. Mild acute pancreatitis was induced by 7 hourly intraperitoneal injections of 10 μg/kg caerulein. Severe acute pancreatitis was induced by 8 or by 12 hourly injections of 40 μg/kg caerulein (AnaSpec Inc., Fremont, CA). Mice were euthanized 2 h after the last injection and blood and pancreas were collected. Severe acute pancreatitis was also induced by the arginine model[29] as modified by [30]. Mice were injected intra-peritoneally with 3g/kg of 20%

L Arginine solution every hour for 3 hours. Arginine solution was made in saline and filter sterilized. Control mice were injected with sterile saline (0.85% sodium chloride). The mice were kept on a heating pad in between the injections and for 4 hours after the last injection. The mice were euthanized after 72h. Collected blood serum was used to measure amylase and inflammatory mediators. The pancreatic tissue was collected and immediately embedded in OCT for hematoxylin and eosin(H&E) staining, evaluation of necrosis and immunohistochemistry. Tissue damage was analyzed as detailed before[6].

Measurement of serum amylase and amylase secretion: Blood samples were collected retro-orbitally from the mice and spun down at 1,000g. Amylase activity was measured in the cleared serum. Pancreatic acini were stimulated with 2 or 10 pM CCK-8 or various caerulein concentrations for 30 min at 37°C. The cells were centrifuged for 15 s at 5,000 rpm and the supernatants were collected. The secreted amylase was calculated as the percent of total amylase collected by lysing the cells and granules with 0.1% titox-x100. Amylase activity was measured with a Phadebas kit (Magle Life Sciences, Cambridge, MA) following the manufacturer's instructions and as described before[14].

Measurement of trypsin activity: Trypsin activity was measure in pancreatic extracts prepared by homogenizing the tissue in 1x assay buffer provided in the kit from Abcam (ab102531). The assay was carried out according to the kit protocol. Briefly, 25 µl of supernatant was incubated with the provided trypsin substrate in a 96 well plate and absorbance was measured at 405 nm every 15 min up to 2 hours at 25 ºC. One unit of trypsin activity is defined as amount of trypsin that cleaves the substrate, yielding 1.0 µmol of p-NA per minute at 25°C.

Measurement of necrosis: We used Oil red O stain to indirectly but more quantitively evaluate necrosis, similar to the procedure in[31, 32]. In brief, pancreatic sections were air dried for 30 min and fixed in 10% formalin, washed twice in water and once in 60% isopropanol. Slides were stained by incubation for 10 minutes in Oil red O (Electron Microscopy Sciences, cat #26504-01) and rinsed once in 60% isopropanol. The slides were counterstained with hematoxylin for 5 min and washed in tap water for 10 min and were mounted using pure glycerol (Matsuda et al., 2014). The area stained red was divided by the total area of the section to calculate % damage in each slide. Data was analyzed by MetaMorph software.

Myeloperoxidase (MPO) assay: MPO activity was measured in pancreatic and lung extracts prepared from tissues homogenized in 1x assay buffer provided in the kit from BioAssay Systems, cat# EMPO-100. The samples were spun down and the supernatant was collected. The assay was in a 96 well plate following the manufacturer protocol and used 25 µl of the sample. Fluorescence was measured at excitation of 535 nm and emission of 585 nm at 0 and10 min time points. The activity was calculated as U/L where one unit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1 µmole resorufin per min.

Measurement of inflammatory markers: Serum samples were collected from the WT, $SARAF^{-/-}$ and pancreatic-targeted SARAF transgenic mice before and after inducing pancreatitis. The levels were measured using Luminex assay kit from EMD Millipore for inflammatory mediators (Mcytomag-70K) according to the manufacturer instructions and read on Biorad, Bio-plex 200 system.

Immunofluorescence procedure: Frozen pancreatic tissue sections (8 µm thick) were fixed with cold methanol for 10 min at -20°C. After washing with PBS, the sections were blocked with 20% donkey serum (Jackson ImmunoResearch, West Grove, PA) for 30 min and incubated with primary antibodies: anti-neutrophil (Abcam, Cambridge, MA), anti-CD3 (Abcam, Cambridge, MA), anti-CD68 (Bio-Rad, Hercules, CA), and anti-mB220 (R&D systems, Minneapolis, MN) overnight at 4°C. The sections were incubated with the appropriate fluorescent tagged secondary

antibodies for an hour and were mounted in 40,6-diamidino-2-phenylindole (Electron Microscopy Sciences, Hatfield, PA). The stained tissue sections were imaged with Olympus confocal microscope model IX81 and inflammatory infiltrates were calculated using Volocity software version 6.3.0.

Western blots and Co-IP: Protein expression of SARAF and co-immunoprecipitation of STIM1 and SARAF were determined in pancreatic acini from mice expressing SARAF-HA. For Western blots, after the indicated treatment, the acini were washed twice in PBS and lysed in cold-RIPA buffer (Cell Signaling, Danvers, MA) containing protease inhibitor (Roche, Basel, Switzerland). For co-immunoprecipitation, acini treated as indicated were lysed with ice-cold lysis buffer (10 mM Na_3PO4, 137 mM NaCl, 2.7 mM KCl, 50 mM NaF, 1% Triton X-100, and protease inhibitor cocktail) and incubated on ice for 30 min. After brief sonication, lysates were centrifuged for 20 min at 14,000 rpm and quantified by Bradford protein assay (Bio-Rad, Hercules, CA). Lysates (300 µg protein) were incubated with anti-STIM1 antibodies (BD biosciences, San Jose, CA) overnight at 4°C and then protein G Sepharose beads (GE healthcare, Chicago, IL) were added and incubated for additional 3 h at 4°C. Beads were collected by brief centrifugation, washed three times with lysis buffer and proteins were released with sample buffer for separation by SDS/PAGE gel. Protein expression in mice pancreas was determined in SARAF-HA control mice and mice injected with 40g/kg caerulein twice or 8 times and sacrificed at 90 min and 9 hours, respectively. The pancreata were collected, homogenized and lysed in RIPA buffer and the supernatants were collected for protein analysis. The blots were probed by anti-HA antibodies (cat # 3724S, Cell Signaling, Danvers, MA) and band densities were determined by Photoshop.

FRET measurement: HEK293 cells were plated on glass bottom dishes (MatTek Corporation, Ashland, MA) and transfected with SARAF-YFP (acceptor) and STIM-CFP (donor)[22], using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and incubated for 18 hrs. FRET imaging was carried out using a confocal microscope (FV1000; Olympus) equipped with UplanSApo 60× oil immersion objective (NA 1.35; Olympus) at × 1 zoom. FRET signals were determined on a pixel-by-pixel basis using Fluoview 1000 software and data was analyzed using Microsoft excel.

qPCR: Total mRNA was extracted using TRIZOL according to the manufacturer instructions (Invitrogen). The mRNA (2 µg) was reverse transcribed using cDNA synthesis kit (Life Technologies) with primers for quantitative RT-PCR for SARAF (Mm00509538_m1), STIM1 (Mm01158413_m1), Orai1 (Mm07734349_m1), TRPC3 (Mm00444690_m1), TRPC1 (Mm00441975_m1), TRPC6 (Mm01176083_m1) and GAPDH (Mm99999915_g1) purchased from Applied Biosystems. The fold change in transcript levels was calculated by normalizing the threshold values to GAPDH.

Human pancreas mRNA was extracted from pancreatic tissue collected from volunteers after obtaining written consent at the Dept of Medicine, University of Szeged, Hungary. mRNA from pancreatic tissue of patient with pancreatitis from was either purchased from Origene (CR561065, CR561741, CR561222 and CR560152). Human pancreatic mRNA was also extracted from formalin fixed paraffin embedded samples collected at the Dept of Medicine, University of Szeged as part of pancreatitis analysis in patients. mRNA was extracted using the PureLink™ FFPE RNA Isolation Kit (K156002) and reverse transcribed to cDNA. The primers used for human beta actin (Hs01060665-g1) and SARAF (Hs00903199_m1) were purchased from Life technologies. The fold change in transcript levels was calculated by normalizing the threshold values to beta actin.

Data mining and analysis: The gene expression omnibus (GEO) was searched for human and animal models with pancreatitis and RNA-seq results were found for 3 control and pancreatitis rats and for 3 health human controls and 9 humans with pancreatitis. The GEO was then searched for large datasets (>50 samples) containing data on gene expression in human pancreatic tissue, or in patients with pancreatic disease. Seven datasets were identified:

GSE16515, GSE15471, GSE28735, GSE55643, GSE74629, GSE77858, GSE62452, and GSE91035. Gene expression profiles were imported directly into Partek Genomics Suite, and log2 normalized data were analyzed using 1-way ANOVA with multiple test correction. Expression data for SARAF (TMEM66) were exported for all datasets with significant differential expression (false discovery rate, FDR<0.05) between control samples and disease samples and for rat and human pancreatitis, and graphs were created using Graphpad Prism software.

Statistics: All experiments were repeated at least three times and all data were expressed as mean ± SEM. Statistical significance was determined by means of Student's *t* test or ANOVA as appropriate. p values are listed in the Figures and p values smaller than 0.05 are considered statistically significant.

Results

Ca^{2+} influx proteins and regulators in acute pancreatitis: To search for a potential cause for the pathogenic, uncontrolled Ca^{2+} influx in acute pancreatitis we used *in vitro* and *in vivo* physiological and pathological cell stimulation and determined the change in the level of the key TRPC and Orai1 Ca^{2+} influx channels and their regulators STIM1 and SARAF. Fig. 1A shows FRET measurements between STIM1-CFP and SARAF-YFP in transfected HEK cells stimulated with physiological (0.25 μM) and pathological (100 μM) carbachol. At physiological stimulation, SARAF interaction with STIM1 was maximal after 3 min and remained stable for the duration of cell stimulation. By contrast, at pathological stimulation STIM1-SARAF interaction was maximal at 2 min, did not attend the level measured with physiological stimulation, and then continually declined for the duration of cell stimulation.

To determine if STIM1-SARAF interaction followed the same pattern in native pancreatic acinar cells, we tagged native mouse SARAF with HA using CRISPR/Cas9 gene editing (see methods and Supplementary Figs. S1D and S1E). Freshly isolated acini from these mice were stimulated with 0.1 or 100 μM carbachol for 0.5-10 min and the interaction of SARAF-HA and native STIM1 was measured by co-immunoprecipitation (Co-IP). Example blots and the summary of 4 experiments with cells obtained from 8 mice are shown in Fig. 1B. The pattern observed with the native proteins is the same as with the expressed proteins, indicating that pathological stimulation acutely disrupted the STIM1-SARAF interaction and suggested that this interaction that protects from excessive Ca^{2+} influx is lost early during physiological stimulation.

In further analysis, we probed the mRNA levels of pancreatic acinar Ca^{2+} influx channels[14, 24, 33, 34], *Stim1* and *Saraf* over 6 hrs in response to pathologic stimulation of isolated acini with 100

μM carbachol. The results in Figs. 1C and Fig. S2A show a slight or no increase in *Orai1*, *Trpc1*, *Trpc3* and *Trpc6* mRNA above baseline. *Stim1* mRNA levels increased modestly within 1 hr of stimulation and remained elevated throughout. On the other hand, *Saraf* mRNA markedly increased in the first hour of stimulation but declined after 4 hours and was reduced by about 60% after 6 hours of stimulation. Next, we analyzed the level of SARAF-HA protein in isolated acini and *in vivo*. The sample blots and the averaged experiments with acini in Fig. 1D show that SARAF protein expression increased for the first hour of cell stimulation but was reduced to below the basal level after 2 hours of stimulation and more so after 4 hours of stimulation. Moreover, Fig. 1E shows that as early as 90 min after injecting mice with caerulein to induce acute pancreatitis pancreatic SARAF is nearly completely degraded and remained so after 8 caerulein injection, the time of fully developed severe acute pancreatitis.

Relevance to human: To determine if changes in SARAF expression are relevant to human pancreatitis, first we searched the gene expression omnibus (GEO) for *saraf* mRNA in animal models and *SARAF* mRNA level in human. Fig. S2B shows results found for 3 control rats and 3 rats with alcoholic pancreatitis and Fig. S2C shows the results for 3 healthy volunteers and for 9 patients with various forms of pancreatitis. Although there is a clear trend of reduced SARAF in pancreatitis, false discovery rate (FDR) analysis failed to show statistical significance. Next, we searched for large data sets of more than 50 samples with pancreatic disease and focused on patients with pancreatic ductal adenocarcinoma (PDAC) because of the established connection between PDAC and pancreatitis[35]. The results in Fig. S2D shows significant reduction if SARAF mRNA in multiple data sets of patients with PDAC. Finally, we collected pancreatic mRNA from health volunteers and patients with pancreatitis and analyzed total h*SARAF* mRNA by qPCR. Fig. 1F clearly show reduced h*SARAF* mRNA in pancreatitis. These findings point to the importance of SARAF both in human pancreatic cancer and pancreatitis.

The overall findings in Figs. 1 and S2 with mice and with human indicate that the function of SARAF, a major regulator of Ca^{2+} influx channels, is profoundly compromised during induction of acute pancreatitis. We next used knockout and transgenic mouse models to probe the physiological and pathological consequences of these findings.

Knockout of SARAF in mice increases Ca^{2+} influx and alters Ca^{2+} signaling and cell function: To evaluate the role of SARAF in acute pancreatitis we generated two *saraf* knockout (*Saraf* $^{-/-}$) mouse lines by Zinc Finger Nuclease (*saraf* $^{zf/zf}$ mice, supplementary Fig. S1A, lines A and line B). The effect of *saraf* knockout on Ca^{2+} signaling was tested in *saraf* $^{zf/zf}$ mice with similar

results obtained with lines B (Fig. 2) and A (Fig. S3). Figs. 2A and S3A show that deletion of *saraf* had minimal effect on ER Ca^{2+} content of pancreatic acinar cells, as evidenced by complete Ca^{2+} discharge upon inhibition of SERCA pumps with CPA, although a small but significant increase in ER Ca^{2+} was found in pancreatic acinar cells from *saraf* $^{zf/zf}$ line B (Fig. 2A). Stimulating the acini with 100 μM carbachol to trigger complete Ca^{2+} release showed similar results. No difference in the rate of Ca^{2+} clearance was observed between wild-type and *Saraf* $^{-/-}$ mice. However, receptor and store-operated (SOC) Ca^{2+} influx markedly increased in pancreatic acinar cells from the two *saraf* $^{zf/zf}$ mouse lines (Figs. 2A and S3A).

As a consequence of markedly increased Ca^{2+} influx, the frequency of receptor-evoked Ca^{2+} oscillations was increased in *Saraf* $^{-/-}$ cells (Figs. 2C and S3C). In addition, deletion of *Saraf* increased mitochondrial Ca^{2+} content in resting pancreatic acinar cells (Figs. 2D, S3D and S3E). Mitochondrial Ca^{2+} content ($[Ca^{2+}]_{mito}$) was evaluated by treating acinar cells with the mitochondrial uncoupler tetrachlorosalicylanilide (TCS), which dissipates the mitochondrial membrane potential. Accordingly, after discharge of mitochondrial Ca^{2+} content, receptor stimulation had no further effect on $[Ca^{2+}]_{mito}$. The elevation in basal $[Ca^{2+}]_{mito}$ content resulted in enhanced receptor-evoked $[Ca^{2+}]_{mito}$ release (Figs. 2D and S3E). Receptor stimulation completely discharged $[Ca^{2+}]_{mito}$, as evident from the lack of effect of the TCS when added after carbachol. Finally, Fig. 3E shows that the increased Ca^{2+} influx, $[Ca^{2+}]_{mito}$ and Ca^{2+} oscillations were translated to an enhanced amylase secretion stimulated with physiological agonist concentration. Furthermore, muscarinic stimulation of saliva secretion was higher in live *Saraf* $^{-/-}$ mice (Figs. 2F, S4) upon physiologic (0.05 and 0.25 mg/kg) but not pathogenic (0.5 mg/kg) receptor stimulation.

Deletion of SARAF aggravates acute pancreatitis: The critical and common role of Ca^{2+} influx in multiple forms of acute pancreatitis[1, 10, 11], prompted us to examine the effect of SARAF deletion on acute pancreatitis. We hypothesized that acute pancreatitis would be more severe in *Saraf* $^{-/-}$ mice. As expected, deletion of Saraf did not affect maximally severe acute pancreatitis induced by maximal concentrations of caerulein (not shown). By contrast, Saraf deletion caused higher increase in serum amylase when mild acute pancreatitis was induced using 10 μg/kg caerulein (Fig. 3A). Moreover, Fig. 3B show significantly high basal pancreatic trypsin activity in Saraf $^{-/-}$ mice that did not increase further upon induction of mild acute pancreatitis and was higher than in WT with mild pancreatitis. Evaluation of necrosis by Oil red O staining[31] showed a higher trend in the *Saraf* $^{-/-}$ mice (Fig. 3C). Tissue damage (Fig. 3E) and inflammation (Fig. 3F) were much more severe in in *Saraf* $^{-/-}$ mice. Fig. 3F shows an increase in basal serum IL-1α and IL-10 in the *Saraf* $^{-/-}$ mice. These cytokines and IL-6 increased minimally upon induction of mild acute

pancreatitis in WT mice but were markedly elevated in *Saraf*$^{-/-}$ mice (Fig. 3F). Analysis of inflammatory cells within the pancreas revealed markedly increased infiltration of neutrophils, macrophages, T cells and B cells in the pancreas of *Saraf*$^{-/-}$ mice (Fig. 3G, and example images in Fig. S5). Finally, increased IL6 is associated with increased lung injury[36]. Accordingly, Fig. 3D shows higher lung damage in *Saraf*$^{-/-}$ mice with acute pancreatitis.

Targeted SARAF overexpression in pancreatic acini ameliorates severe acute pancreatitis: The dissociation of STIM1-SARAF interaction and dramatic reduction in SARAF levels in pancreatitis (Fig. 1), raised the question of whether maintaining high SARAF levels could reduce pathological Ca^{2+} influx and ameliorate acute pancreatitis. To examine this question, we developed transgenic mice with targeted overexpression of SARAF in pancreatic acini (refer to as SARAF/TG). Figs. 4A and 4B show that overexpression of SARAF in pancreatic acini had no measurable effect on ER Ca^{2+} content (CPA response) or Ca^{2+} release from the ER (carbachol response), but reduced Ca^{2+} influx by about 50%. The reduced Ca^{2+} influx resulted in decreased Ca^{2+} oscillation frequency (Fig. 4C), but the SARAF/TG acini were able to maintain normal mitochondrial Ca^{2+} content and response to receptor stimulation (Fig. 4D). The reduction in Ca^{2+} influx and Ca^{2+} oscillations by SARAF/TG acini caused reduction in exocytotic amylase release triggered by physiological receptor stimulation (Fig. 4E) and the inhibition of exocytosis by supramaximal receptor stimulation (Fig. 4F) that is associated with pancreatitis.

Fig. 5 compares caerulein-induced severe acute pancreatitis in SARAF/TG mice and wild-type littermates. Fig. 5A shows reduced serum amylase, and Figs. 5B and 5C show reduced pancreatic damage, in the SARAF-TG pancreas compared to wild-type mice. Most likely this was due to a profound reduction in inflammatory mediators in the SARAF/TG mice, which nearly completely suppressed the increase in M-CSF, TNF-α, IL-6 and IL-10 (Fig. 5D). Accordingly, pancreatic overexpression of SARAF reduced pancreatic infiltration of neutrophils, macrophages, T cells and B cells (Fig. 5E and Fig. S6).

To extend h findings in Fig. 5 we tested if the SARAF/TG mice are protected when acute pancreatitis is induced by 12 caerulein injections and in an additional, independent model of acute pancreatitis, the L arginine model[29]. Fig. 6A shows reduced serum amylase and Fig. 6B shows reduced pancreatic trypsin activity in SARAF/TG mice injected with 12 times with caerulein compared with WT mice. The basal and 12 times caerulein-injected MPO level was lower in the SARAF/TG compared with WT mice. Analysis of serum amylase (Fig. 6D) and tissue injury (Fig. 6E) in mice in which acute pancreatitis was induced by L arginine confirmed the protective effect

of SARAF in a second pancreatitis model. In addition, the SARAF/TG mice showed reduced pancreatic necrosis (Fig. 6F) and infiltration of T cells (Fig. 6G) and neutrophils (Fig. 6H) than WT mice when acute pancreatitis was induced by L arginine injections.

Discussion

Pancreatitis is a pathology with significant morbidity and mortality that damages the pancreas and has no effective treatment or cure[3, 4]. Acute pancreatitis involves the release of inflammatory mediators within the pancreas and subsequent acinar cell damage[4]. Chronic pancreatitis is caused by repeated severe or mild acute pancreatitis attacks[1, 3]. Acinar cell damage is a critical driver of acute and chronic pancreatitis. The pancreatic duct is the first line of defense[37, 38], protecting the acini from damaging mediators by secreting HCO_3^--rich fluid[37] . In all forms of pancreatitis, mislocalization and degradation of CFTR[6] breaches the ductal guard, causing retention and activation of digestive enzymes within acinar cells and cell damage[5-7].

Pathologic sustained increase in $[Ca^{2+}]_i$ is a prerequisite for aberrant exocytosis, causing targeting secretory granules to the lysosomes and activation of digestive enzymes within acinar cells in all forms of pancreatitis [1, 10, 11, 15]. Numerous studies have shown that the sustained increase in cytoplasmic Ca^{2+} is due to uncontrolled Ca^{2+} influx. Although the cause of the increased Ca^{2+} influx has never been investigated, inhibition of Ca^{2+} influx channels and loading acinar cells with cytoplasmic Ca^{2+} buffers (EGTA and BAPTA) have been used to demonstrate the role of elevated $[Ca^{2+}]_i$ in cell damage and as potential treatments for pancreatitis[12-16]. However, usage of synthetic Ca^{2+} buffers is not a viable treatment option. Inhibition of Ca^{2+} influx TRPC channels proved ineffective when administered after induction of acute pancreatitis[15]. Inhibition of the main Ca^{2+} influx channel Orai1 is expected to have multiple side effects, since Ca^{2+} influx by Orai1 is essential for numerous cell functions and human mutations in Orai1 results in severe disease[39]. Although short term inhibition of Orai1 reduced the damage observed in acute pancreatitis[16], short term deletion of Orai1 exclusively in pancreatic acini resulted in gut dysbiosis and death[24].

These observations and the discovery of dramatic changes in SARAF in the course of acute pancreatitis led us to reason that partial inhibition of all Ca^{2+} influx channels might be a better approach to control Ca^{2+} influx and pancreatic damage in acute pancreatitis. TRPC[18] and Orai1 channels[39] are activated by STIM1. The SARAF protein interacts with STIM1 to mediate

inactivation of Orai1[21, 23] and likely TRPC channels[40]. A critical finding in Fig. 1 is that SARAF interaction with STIM1 is disrupted by pathological stimulation of acinar cells and that expression levels of SARAF change profoundly during pathological stimulation. Thus, pancreatic pathology is initiated by an early loss of protection by SARAF due to dissociation of SARAF from STIM1 to maintain Orai1 in a fully activate state and is exacerbated in later stages of the disease by nearly complete degradation of SARAF. Protection by SARAF appears to be important in both acute and chronic pancreatitis and is relevant to human, since reduction in SARAF mRNA is observed in human with pancreatitis and with PDAC. This also suggest that SARAF may have a role in pancreatic cancer. Together, these findings indicate that the most dramatic response of acinar cells to a pathological stimulus is an increase in SARAF transcription and translation, likely as a protection against Ca^{2+} toxicity. When this response breaks down pancreatitis develops.

The loss of protection from excessive Ca^{2+} influx due to degradation of SARAF suggests that stabilizing and increasing SARAF expression should be a preferred approach to protecting against acute pancreatitis. This should restore the normal state of Ca^{2+} influx, rather than prominently disrupting channel activity that mediates numerous vital cellular functions. Deletion of SARAF in mice had no noticeable behavioral and physiological phenotype or pancreatic and salivary glands damage, although it enhanced fluid secretion and exocytosis in response to receptor stimulation. This suggests that SARAF functions mainly in the cell-active states. Accordingly, deletion of SARAF exacerbated acute pancreatitis and the inflammation associated with it. Therefore, the fairly mild effect of complete deletion of SARAF (present studies), compared to the severe effects of germline[39] or pancreatic-specific deletion of Orai1[24], suggest that SARAF should be preferred target for treatment of acute pancreatitis. Moreover, stabilization of SARAF restores the basal state rather than removing essential function. The proof-of-concept that increasing SARAF expression could treat acute pancreatitis is evident from the effect of targeted expression of SARAF in the pancreas, which improved pathology in acute pancreatitis while nearly eliminating the inflammation associated with it. Taken together, our findings suggest that induced expression and stabilization of SARAF should be an attractive approach to treating acute pancreatitis.

Figure Legends

Figure 1: STIM1-SARAF interaction and SARAF mRNA and protein in acute pancreatitis

(**A**) FRET was measured between STIM1-CFP and SARAF-YFP expressed in HEK cells together with M3 receptors and stimulated with 0.25 μM (black-physiological) or 100 μM (red-pathological) carbachol. The traces are the average of the indicated number of cells analyzed. (**B**) Pancreatic acini prepared from SARAF-HA expressing mice were stimulated with 0.1 or 100 μM carbachol and used to STIM1-SARAF interaction by Co-IP. Shown are example blots for acini obtained from SARAF-HA mice and from control (CTL) mice and the summary of 4 independent experiments. The (*) in the control lower blot mark a non-specific band (**C**) SARAF (red), STIM1 (blue), Orai1 (green) and TRPC3 (black) mRNA levels normalized to GAPDH mRNA were determined by qPCR in acini stimulated for the indicated times in 5 independent experiments. (**D**) Pancreatic acini prepared from SARAF-HA expressing mice were stimulated with 100 μM carbachol for the indicated times and used to determine changes in SARAF protein. The Figure shows example blots and the numbers in the columns indicate the number of independent experiments. (**E**) Mice expressing SARAF-HA were injected with 40 μg/Kg caerulein 2 and 8 timed to initiate and develop fully blown acute pancreatitis, respectively. Mice were sacrificed 90 min after the second injection or 2 hrs after the 8th injection and the pancreas were rapidly removed to prepare lysates and analyze expression of SARAF. Shown are the blots and averages. (**F**) pancreatic tissue from 6 health and 8 humans with pancreatitis was used to extract RNA and analyze SARAF mRNA. All averages are shown as mean±s.e.m. and in panels (B-F) the p values are listed.

Figure 2: Ca^{2+} signaling and secretion in SARAF$^{-/-}$ mice (SARAF$^{zf/zf}$ mice, line B) pancreatic acini

(**A-B**) Pancreatic acini from wild-type (black traces and columns) and SARAF$^{-/-}$ mice (red traces and columns) loaded with Fura2 and perfused with Ca^{2+}-free solutions containing 0.2 mM EGTA were treated with 25 μM CPA (**A**) or 100 μM carbachol (**B**) and then exposed to solutions containing the indicated Ca^{2+} concentrations to evaluate Ca^{2+} release and Ca^{2+} influx. The traces and columns show the mean±s.e.m of the indicated number of cells from at least 4 independent experiments. (**C**) Fura2-loaded acini from wild-type and SARAF$^{-/-}$ mice were stimulated with 2 pM CCK-8 to evaluate the frequency of Ca^{2+} oscillations. (**D**) Pancreatic acinar cells from wild-type (black traces and columns) and SARAF$^{-/-}$ mice (red traces and columns) loaded with Rhod2 to evaluate mitochondrial Ca^{2+} concentration. The image shows typical Rhod2 loaded acini. The acini were treated first with the uncoupler TCS and then carbachol (upper traces and columns) or with carbachol and then TCS (lower traces and columns). (**E**) Amylase secretion was measured in wild-type and SARAF$^{-/-}$ pancreatic acini stimulated with 10 pM CCK-8 for 30 min. The numbers in columns indicate the number of independent experiments. (**F**) Salivary secretion was measured in 10 wild-type and 10 SARAF$^{-/-}$ mice stimulated with 0.25 mg/kg pilocarpine.

Figure 3: Deletion of SARAF aggravates acute pancreatitis

Mild acute pancreatitis was induced by 7 hourly intraperitoneal injection of 10 µg/Kg of caerulein. The control mice were injected with saline. Severity of pancreatitis was evaluated by analysis of (**A**) serum amylase; (**B**) trypsin activity within the pancreas; (**C**) Necrosis using Oil red O staining; (**D**) MPO activity in lung tissue as a reporter of lung inflammation; (**E**) tissue damage by H&E staining; (**F**) serum inflammatory mediators IL-1α, IL-6 and IL-10 and (**G**) inflammatory cells within the pancreas, Neutrophils, macrophages (CD68), T cells (CD3) and B cells (B220). All incidence reports move severe acute pancreatitis in $SARAF^{-/-}$ mice.

Figure 4: Ca^{2+} signaling and exocytosis in acini with targeted expression of SARAF in pancreatic acinar cells (SARAF/TG mice)

(**A-B**) Pancreatic acini from wild-type (black traces and columns) and SARAF/TG mice (royal blue traces and columns) loaded with Fura2 and perfused with Ca^{2+}-free solutions containing 0.2 mM EGTA were treated with 25 µM CPA (**A**) or 100 µM carbachol (**B**) and then exposed to solutions containing the indicated Ca^{2+} concentrations to evaluate Ca^{2+} release and Ca^{2+} influx. The traces and columns show the mean±s.e.m of the indicated number of cells from at least 4 independent experiments. (**C**) Fura2-loaded acini from wild-type and SARAF/TG mice were stimulated with 2 pM CCK-8 to evaluate the frequency of Ca^{2+} oscillations. (**D**) Pancreatic acini from wild-type (black traces and columns) and SARAF/TG mice (royal blue traces and columns) were loaded with Rhod2 and treated with TCS and then carbachol (upper traces) or with carbachol and then TCS (lower traces). (**E, F**) Amylase secretion was measured in wild-type (black) and SARAF/TG pancreatic acini (royal blue) stimulated with 10 pM CCK-8 (**E**) or the indicated caerulein concentrations (**F**) for 30 min and released amylase was measured as % to total. The numbers in columns indicate the number of independent experiments.

Figure 5: Targeted expression of SARAF in pancreatic acinar cells ameliorates severity of acute pancreatitis in the caerulein model.

Severe acute pancreatitis was induced by 8 hourly intraperitoneal injection of 40 µg/Kg caerulein to WT (gray) or SARAF/TG mice (royal blue). Mice were scarified 2 hrs after the last injection and used to analyze (**A**) serum amylase, (**B-C**) tissue damage (**B,** example images and **C** averages), (**D**) serum inflammatory mediators MC-SF, TNF-α, IL-6 and IL-10 and (**E**) inflammatory cells within the pancreas, Neutrophils, macrophages (CD68), T cells (CD3) and B cells (B220).

Figure 6: Targeted expression of SARAF in pancreatic acinar cells ameliorates severity of acute pancreatitis in caerulein and L arginine models.

(**A-C**) Severe acute pancreatitis was induced by 12 hourly intraperitoneal injection of 40 µg/Kg caerulein to WT (gray) or SARAF/TG mice (royal blue). Mice were scarified 2 hrs after the last injection and used to analyze (**A**) serum amylase, (**B**) trypsin activity within the pancreas and (**C**) MPO within the pancreas as an indication of inflammation. (**D-H**) Severe acute pancreatitis was induced by intraperitoneal injection of L arginine to WT (gray) or SARAF/TG mice (royal blue). Mice were used to analyze (**D**) serum amylase, (**E**) tissue damage, (**F**) necrosis, (**G**) infiltration of T cells and (**H**) infiltration of macrophages.

Supplementary Figure 1: Generation of SARAF knockout and transgenic mice

(**A**) shows the site of the zinc finger-targeted deletion in line A (1-90 of exon 1) and line B (1-392 of exons 1 and 2) and qPCR analysis of SARAF mRNA using the primers in table S1. (**B**) shows the design of the vector used to target expression of SARAF-YFP in pancreatic acini using the elastase promotor and (**C**) is qPCR analysis of SARAF/TG mRNA expression in various epithelial tissues showing exclusive expression in the pancreas. (**D**) Shows the site of insertion of the HA tag in the SARAF gene. (**E**) Western blot analysis of SARAF-HA in various tissues.

Supplementary Figure 2: Effect of pancreatitis on pancreatic expression of mouse TRPC1 and TRPC6, rat and human SARAF and SARAF in PDAC

(**A**) Pancreatic acini were stimulated with 100 µM carbachol for the indicated times and *Trpc1* and *Trpc6* mRNA levels normalized to *Gapdh* mRNA were determined by qPCR in 5 independent experiments, as in Fig. 1C. Note the different mRNA scale in Fig. 1C and this Figure. (**B**) SARAF mRNA in control rats and rats with alcoholic pancreatitis (Data from xxx). (**C**) SARAF mRNA in patients with (red) and without (blue) pancreatitis (Data from xxx). (**D**) SARAF mRNA in patients with (red) and without (blue) PDAC (Results from the four indicated data sets).

Supplementary Figure 3: Ca^{2+} signaling in SARAF$^{-/-}$ mice (line A) pancreatic acini

(**A-B**) Pancreatic acinar cells from WT (black traces) and *Saraf*$^{-/-}$ mice (red traces) loaded with Fura2 and perfused with Ca^{2+}-free solutions containing 0.2 mM EGTA were treated with 25 µM CPA (**A**) or 100 µM carbachol (**B**) and then exposed to solutions containing the indicated Ca^{2+} concentrations. The traces show the mean±s.e.m of the indicated number of cells from at least 4 independent experiments. (**C**) Fura2-loaded acini from WT and *Saraf*$^{-/-}$ mice were stimulated with

2 pM CCK-8 to induce Ca^{2+} oscillations. (**D**) Pancreatic acinar cells from WT (black traces and columns) and *Saraf*$^{-/-}$ mice (red traces and columns) loaded with Rhod2 were treated with TCS and then carbachol (**D**) or with carbachol and then TCS (**E**).

Supplementary Figure 4: Deletion of SARAF increases receptor stimulated salivation

Salivary secretion was measured in 5 WT (black) and 5 *Saraf*$^{-/-}$ *mice* line B by application of 0.05 mg/Kg pilocarpine + 0.02 mg/Kg isoproterenol; of 0.25 mg/Kg pilocarpine + 0.1 mg/Kg isoproterenol; or of 0.5 mg/Kg pilocarpine. Deletion of *Saraf* enhanced salivation at low and intermediate but not at maximal receptor stimulation. The results are mean±s.e.m and p values are given next to the symbols.

Supplementary Figure 5: Example images of inflammatory cells in wild-type and SARAF$^{-/-}$ mice.

The averages from multiple images are shown in Figure 3.

Supplementary Figure 6: Example images of inflammatory cells in wild-type and SARAF/TG mice.

The averages from multiple images are shown in Figure 5.

Supplementary Table 1: Primers used for cloning, genotyping, sequencing of mouse lines.

Transgenic elastase Saraf mice	F :5' GCTGGATGCAACTCAGCTGG 3' R :5' AACTTGTGGCCGTGCACGTCGCCG 3'	1550 bp
Saraf HA tagged mice	F :5' GGGTCTGCCTTGTTGGGTCTC 3' R :5' GCGGTCTGTGCTCAGTCTCAG 3'	
Saraf Ha tagging primers	5'AACATTATGAACAATTTTCATATATTTTGAA AAGCTAATGCAGACTTTGTTTTTCTCATCCTA GGATATGGTGGCACCAGAAGACGGTACCCT TACGACGTTCCAGACTACGCTTAAAATAGGA AATTGAAGGCAAACACTGGATGCAAAGTTTC TGATTTGTCATCACCATCTCTTTAACACCTG GCTAATGGGAATAA3'	530 bp (HA positive) 500 bp (WT)
SARAF sgRNA for HA tagging	5'GAAATTAATACGACTCACTATAGGACGGTA AAATAGGAAATTGAGTTTTAGAGCTAGAAAT AGC 3'	
SARAF zinc finger nuclease mediated knockout on FVBNJ background	F :5' AGATTGGCGCAGGACAAAGT 3' R :5' AGACGGCGCTCGGTG 3'	131 bp (WT) 116 bp (KO)
SARAF zinc finger nuclease mediated knockout on C57bl6 background	F :5' GGCGGGCCCTTCCAGAATTTT 3' R :5' CAGTCGGCCGTGCAGAGCAA 3'	1700 bp (WT) 1344 bp (KO)
Saraf transgenic mice cloning primers	F:5'ACGAATTCTATGAAAAAAAAAGCAATCCT CACTCTTC 3' R:5'ACGGATCCCGAGACCACTGCCCCTTGC CATGAGCGGAA 3'	

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