Upregulation of the ERRγ–VDAC1 axis underlies the molecular pathogenesis of pancreatitis

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Emerging evidence suggest that transcription factors play multiple roles in the development of pancreatitis, a necroinflammatory condition lacking specific therapy. Estrogen-related receptor γ (ERRγ), a pleiotropic transcription factor, has been reported to play a vital role in pancreatic acinar cell (PAC) homeostasis. However, the role of ERRγ in PAC dysfunction remains hitherto unknown. Here, we demonstrated in both mice models and human cohorts that pancreatitis is associated with an increase in ERRγ gene expression via activation of STAT3. Acinar-specific ERRγ haploinsufficiency or pharmacological inhibition of ERRγ significantly impaired the progression of pancreatitis both in vitro and in vivo. Using systematic transcriptomic analysis, we identified that voltage-dependent anion channel 1 (VDAC1) acts as a molecular mediator of ERRγ. Mechanistically, we showed that induction of ERRγ in cultured acinar cells and mouse pancreata enhanced VDAC1 expression by directly binding to specific site of the *Vdac1* gene promoter and resulted in VDAC1 oligomerization. Notably, VDAC1, whose expression and oligomerization were dependent on ERRγ, modulates mitochondrial Ca^{2+} and ROS levels. Inhibition of the ERRγ–VDAC1 axis could alleviate mitochondrial Ca^{2+} accumulation, ROS formation and inhibit progression of pancreatitis. Using two different mouse models of pancreatitis, we showed that pharmacological blockade of ERRγ–VDAC1 pathway has therapeutic benefits in mitigating progression of pancreatitis. Likewise, using PRSS1^{R122H}-Tg mice to mimic human hereditary pancreatitis, we demonstrated that ERRγ inhibitor also alleviated pancreatitis. Our findings highlight the importance of ERRγ in pancreatitis progression and suggests its therapeutic intervention for prevention and treatment of pancreatitis.

nuclear receptor | ERRγ | VDAC1 | mitochondrial Ca^{2+} | pancreatitis

The physiological role of the pancreatic acinar cell (PAC) is to synthesize, transport, store, and secrete digestive enzymes. It relies on normal functions and cross talk among a triad of acinar cell organelles including the endoplasmic reticulum (ER), mitochondria, and the endolysosomal-autophagy system (1). Recent studies have shown that perturbation of the function of these organelles is a critical determinant in pancreatitis and underlie the mechanisms involved in the pathogenesis of acute pancreatitis (AP). AP—owing to premature activation of inactive pancreatic proenzymes inside the PACs and self-digestion of the pancreas—is a necroinflammatory disease associated with high morbidity and mortality lacking specific therapy (1–3). Pancreatitis is a frequent side-effect (in up to 10% of patients) during endoscopic retrograde cholangiopancreatography, a technique used for diagnosing and treating biliary and pancreatic ailments (1). Furthermore, no therapeutic agents are currently in use that can alter the course of the disease.

As demonstrated by studies in genetic and experimental mouse models, the development of AP involves dysregulated autophagy and unresolved inflammation (2–4). Mechanistically, triggers for AP lead to dysfunction and stress of acinar cell organelles, such as the ER, mitochondria (e.g., mitochondrial Ca^{2+} overload and reactive oxygen species (ROS) production), and the endolysosomal–autophagy system (5–8), which in turn precipitates to inappropriate intracellular activation of trypsinogen and inflammatory pathways (9). Sustained mitochondrial Ca^{2+} overload leads to acinar cell damage, death, and pancreatic inflammation, typically associated with AP (10–12). Furthermore, impaired autophagy and ER stress are implicated in mitochondrial dysfunction and accumulation of ROS in the damaged mitochondria (13, 14). Additionally, AP promotes the development of pancreatic ductal adenocarcinoma (PDAC), which remains refractory to current treatment modalities (15–17).

Orphan nuclear receptor ERRγ is a transcription factor that integrates multiple endocrine and metabolic signals (18). Pancreatic β cell-specific ERRγ knockout mice have impaired postnatal β cell maturation (19), whereas our recent results indicate that ERRγ

Significance

Pancreatitis is a necroinflammatory condition lacking specific therapy. Genetic association studies of candidate locus region indicated multiple single-nucleotide variants for Estrogen-related receptor γ (ERRγ) that are associated with chronic pancreatitis. Based on our observation that ERRγ expression is induced in several experimental mouse models as well as in human pancreatitis, we have demonstrated that inhibiting ERRγ activity can be a viable prophylactic as well as therapeutic option to treat pancreatitis. Our findings provide functional support for ERRγ to be a tractable mediator of pancreatitis progression. Therapeutic intervention via ERRγ inhibition showed promising results in alleviating pancreatitis in experimental and human hereditary pancreatitis mimicking models. Therefore, it may be possible to explore ERRγ inhibition as a therapeutic avenue for treating pancreatitis.

deficiency disrupts PAC homeostasis (20). Conversely, aberrant induction of ERRγ contributes to the pathogenesis of many metabolic diseases, as reflected in type two diabetes (21, 22), insulin resistance (23), and alcoholic liver injury (24). The emerging role and the functional plasticity of ERRγ in diverse pathologies in conjunction with the role of ERRγ in the pancreas prompted us to explore its role in AP. We hypothesized that identifying the key drivers of acinar cell dysfunction at the molecular level in models of AP could provide an exploitable target to develop effective strategies for AP diagnosis and therapy.

Here, we report that ERRγ gene expression is up-regulated in mouse models of pancreatitis as well as in patients with acute or chronic pancreatitis. ERRγ overexpression induces VDAC1 gene expression and subsequent oligomerization, which promotes Ca^{2+}-dependent mitochondrial dysfunction, impairment of autophagy, and ER stress. Based on this concept, we further investigated if ERRγ inhibitor can prevent the development of pancreatitis and whether therapeutic administration of this inhibitor can reverse preexisting pancreatitis in caerulein-induced and human hereditary pancreatitis (HP)—mimicking mouse models.

Results

Increased ERRγ Expression Is Associated with Mouse Models of AP. ERRγ-driven transcriptional program has been implicated to directly or indirectly regulate the expression of key metabolic enzymes and major hormones leading to diverse metabolic outcomes (18). Considering the pleiotropic roles of ERRγ in different physiological and pathophysiological condition and to understand the role of ERRγ in acinar cell dysfunction, initially, we tested the hypothesis that aberrant regulation of ERRγ can promote the development of pancreatitis. To that end, we used the widely accepted caerulein hyperstimulation-induced pancreatitis (CER) mouse model (4) as an initial test platform. Initially, we tested the effect of caerulein on ERRγ expression in vivo. We observed a sustained increase in the ERRγ protein level in the pancreas of mice killed at different timepoints following caerulein hyperstimulation up to 24 h (*SI Appendix*, Fig. S1*A*). Similarly, caerulein treatment induced ERRγ protein level in mouse PACs and mouse pancreatic acinar 266-6 cell line (*SI Appendix*, Fig. S1*B*), supporting our in vivo findings in CER model. Based on these observations, we determined the 16-h time point for killing the animal for the CER model for further detailed analysis (*SI Appendix*, Fig. S1*C*). CER was confirmed by aggravated pancreatic histological damage (Fig. 1*A*) and elevated levels of serum amylase (a diagnostic biomarker of AP) and pancreatic trypsin activity (Fig. 1*B*). ERRγ expression was markedly induced at both the mRNA and protein levels (Fig. 1*C*) in this model. Additionally, CER pancreata exhibited robust nuclear accumulation of ERRγ mainly in the acini (Fig. 1*D*).

Next, we examined the effect of bile acid-induced pancreatitis—a well-documented preclinical model of pancreatitis that causes pancreatic oxidative injury, sterile inflammation, and extensive necrosis—on ERRγ expression (*SI Appendix*, Fig. S1*D*). Taurolithocholic acid 3-sulfate (TLCS), which is primarily used to determine bile acid response, is a representative treatment mimicking acute biliary pancreatitis that occurs during ampullary gallstone obstruction (4). Murine model of bile acid-induced pancreatitis was generated by pancreatic ductal infusion of TLCS. Induction of pancreatitis in this TLCS model was confirmed by an aggravation of pancreatic histological damage (*SI Appendix*, Fig. S1*D*) and elevated levels of serum amylase level and pancreatic trypsin activity (Fig. 1*E*). ERRγ expression was found to be markedly induced at both the mRNA and protein levels (Fig. 1*E*) upon TLCS challenge.

Pancreatitis due to alcohol abuse is a painful and potentially fatal condition (25). About one-third of AP cases are alcohol-induced and a majority of pancreatitis patients have a history of chronic alcohol consumption (25). We, therefore, used a clinically relevant mouse model (26) to induce chronic alcohol-induced mild AP (AIP) (*SI Appendix*, Fig. S1*E*). AIP exhibited an increase in serum ALT and serum amylase levels (Fig. 1*F*), which correlated with a robust increase in the ERRγ protein level. Together, these data strongly indicate that ERRγ is aberrantly up-regulated in several cell and mouse models of AP and hence, potentially involved in the pathogenesis of AP.

Next, we sought to determine the upstream signaling pathway that contributes toward ERRγ induction during pancreatitis. Signal transducer and activator of transcription 3 (STAT3) mediates inflammation signaling pathways and is implicated in the pathogenesis of pancreatitis and PDAC (27, 28). Pancreas-specific deletion or inactivation of STAT3 affects the severity of AP in different models, whereas phosphorylation and nuclear translocation of STAT3 links local damage to multi-organ failure. Additionally, STAT3 acts as a transcriptional activator of ERRγ gene expression (22). We observed a robust induction of nuclear (Tyr705), but not mitochondrial (Ser727), phosphorylation of STAT3 in CER pancreata (*SI Appendix*, Fig. S1*F*), which correlates with the induction of ERRγ expression (Fig. 1 *C* and *D*). Chromatin immunoprecipitation (ChIP) assay further confirmed the binding of STAT3 to the STAT3-RE (STAT3 response element) of *Errγ* gene promoter in CER pancreata (*SI Appendix*, Fig. S1*F*). Conversely, knockdown of *Stat3* blocked caerulein-induced *Errγ* mRNA expression and protein level in 266-6 cells (*SI Appendix*, Fig. S1*G*). These findings indicate that STAT3 facilitates ERRγ induction during pancreatitis. Collectively, these findings imply that ERRγ might be a molecular mediator in the pathogenesis of pancreatitis.

ERRγ Haploinsufficiency Protects against the Development of Pancreatitis. As demonstrated by studies in genetic and experimental mouse models, the development of pancreatitis involves dysregulated autophagy and unresolved inflammation (2–4). Furthermore, impaired autophagy and ER stress are implicated in mitochondrial dysfunction and accumulation of ROS in the damaged mitochondria (13). Therefore, to determine whether ERRγ is a molecular regulator of pancreatitis, we measured mitochondrial Ca^{2+} accumulation, ROS level, and lactate dehydrogenase (LDH) release—a measure of necroptosis—upon *Errγ* knockdown (si*Errγ*) in 266-6 cells treated with caerulein. *Errγ* depletion (Fig. 2*A*) reversed acinar cell response to caerulein treatment in vitro, as observed by a significant reduction in mitochondrial Ca^{2+} accumulation, ROS level, and extracellular LDH release (Fig. 2*B*).

Recently, we have demonstrated that under physiological conditions, ERRγ plays a crucial regulatory role to maintain acinar cell homeostasis and identity (20). However, the link between ERRγ and exocrine pancreas disorders, including pancreatitis, remain elusive. Germline deletion of ERRγ results in neonatal lethality, therefore, to specifically investigate the effects of loss of ERRγ during pancreatic disorders, we generated ERRγ conditional knockout mice by crossing *Errγ^f/f* with CAG-CreERT2 transgenic mice (*Errγ^cko*) (*SI Appendix*, Fig. S2*A*). ERRγ deletion was confirmed in mice treated with 75 mg/kg tamoxifen by protein level analysis (*SI Appendix*, Fig. S2*B*). However, to our surprise, we also observed a dramatic decrease in the pancreatic digestive enzyme, amylase (AMY2), protein level. Additionally,
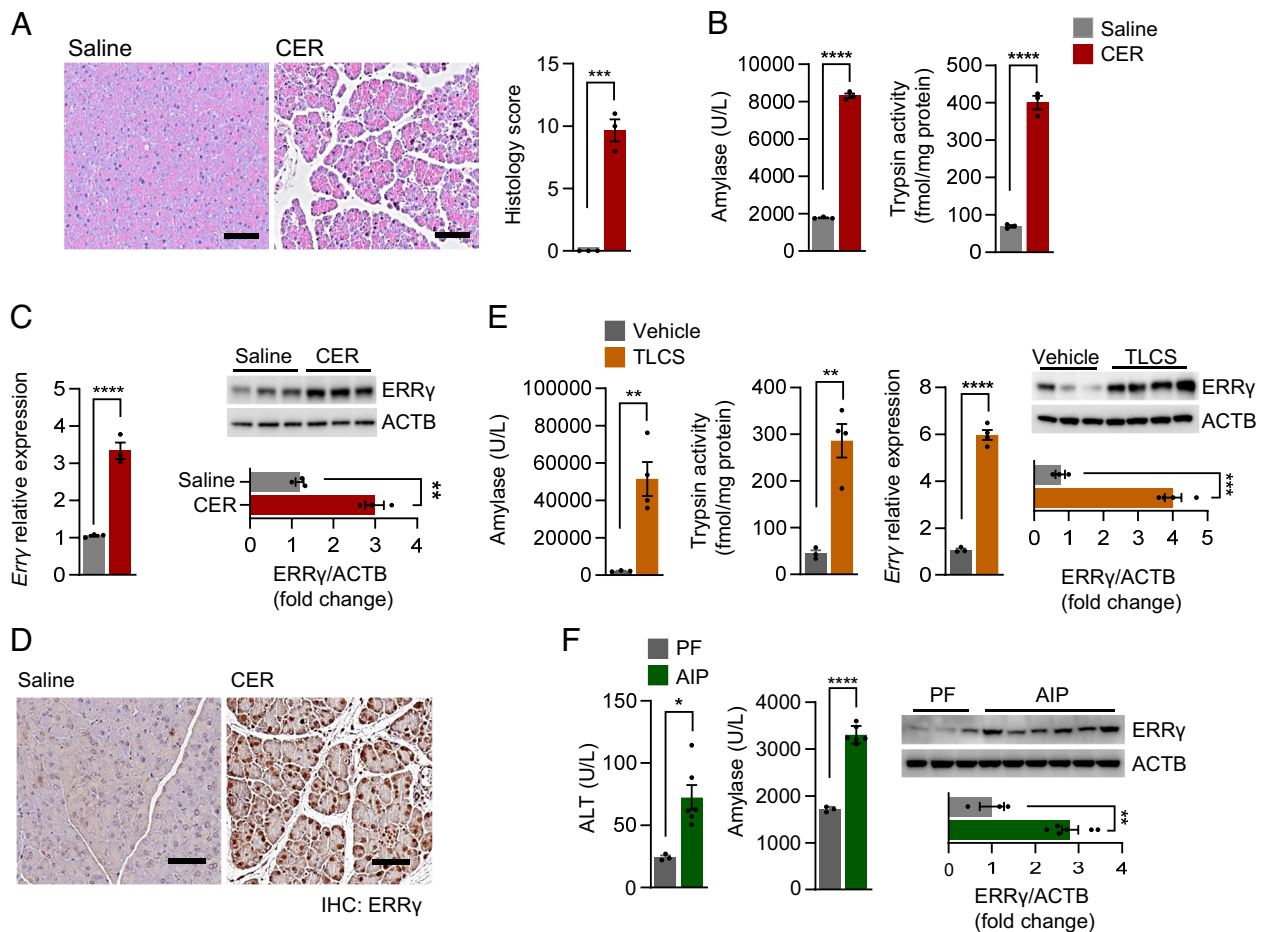
Fig. 1. ERRγ is a molecular regulator of pancreatitis. (*A*) Representative H&E images and histology scoring of the pancreas from saline (Sal) and caerulein hyperstimulation (CER) pancreatitis conditions. Animals were killed 16 h after the first saline or caerulein injection. (*B*) Serum amylase level and intrapancreatic trypsin activity. (*C*) Pancreatic *Errγ* relative mRNA level (*Left*) and immunoblot of ERRγ (*Right*) from mice pancreata. (*D*) Representative IHC staining of ERRγ in pancreas tissues. (n = 3 mice/group; two-sided *t* test). (*E*) Serum amylase level, intrapancreatic trypsin activity, pancreatic *Errγ* relative mRNA level and immunoblot of ERRγ from mice pancreata from vehicle and TLCS pancreatitis conditions. Animals were killed 16 h after the vehicle or TLCS injection. (n = 3 to 4 mice/group; two-sided *t* test). (*F*) Serum ALT, serum amylase level, and immunoblot of ERRγ from mice pancreata from pair-fed (PF) and EtOH-diet fed (AIP) alcohol-induced pancreatitis conditions. Animals were killed 8 h after the acute alcohol binge. (n = 3 to 6 mice/group; two-sided *t* test). Results are representative of those from two independent in vivo experiments. Data represent mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. (Scale bars, 50 μm). (See also *SI Appendix*, Fig. S1).

in accordance with our earlier observations (20), *Errγ^cko* mice exhibited a significant decrease in pancreas weight (*SI Appendix,* Fig. S2C) and severe pancreatic atrophy (*SI Appendix,* Fig. S2D). This was suggestive of a functional plasticity of ERRγ in the pancreas, where either complete absence or aberrant upregulation are detrimental to the organ. Consequently, this pancreatic abnormality in *Errγ^cko* mice precluded us from further use of this mouse line in the context of pancreatitis. To circumvent this issue, we sought to generate a floxed haploinsufficient ERRγ (*Errγ^+/f*) mouse line by crossing *Errγ^f/f* with wild-type (B6) mice (Fig. 2C). PAC-specific ERRγ haploinsufficiency (*Errγ^+/*) was confirmed in mice intraperitoneally injected with adeno-associated virus 8 (AAV8), which contained an enhanced Cre recombinase (iCre) driven by acinar cell-specific elastase1 (Ela1) promoter (Fig. 2C). *Errγ^+/* mice cohort exhibited none of the histologic abnormalities (Fig. 2C) or abrupt loss of pancreas weight (Fig. 2D), which was evident in the *Errγ^cko* mice, further validating our hypothesis that complete absence of ERRγ may have unwanted consequences in the pancreas. As a proof of principle to ensure that the pancreas was specifically targeted and there was no virus spillage into the adjacent tissues, we performed immunoblotting analysis and observed a significant decrease (~50 to 60%) in ERRγ mRNA and protein levels in the whole pancreas but not in the liver

(Fig. 2E). Unlike in *Errγ^cko* mice, AMY protein level remained unchanged in both *Errγ^+/f* and *Errγ^+/* mice. Similarly, CRE expression was detected exclusively in the pancreas, indicating the specificity and efficiency of the procedure. Additionally, analysis of isolated acinar and ductal cells from these mice confirmed that ERRγ knockdown was acini-specific (*SI Appendix*, Fig. S2E).

To determine whether ERRγ haploinsufficiency can prevent the development of pancreatitis, we challenged *Errγ^+/f* and *Errγ^+/* mice with CER. *Errγ^+/* mice reduced all the sequelae of pancreatitis in CER model, which included marked reduction in histological damage (Fig. 2F), serum amylase level, and pancreatic trypsin activity (Fig. 2G). *Errγ^+/* mice exhibited a significant decrease in the PKR-like endoplasmic reticulum kinase (PERK) signaling pathway—a crucial element involved in the integrated stress response system—which was evidenced by decrease in eukaryotic initiation factor 2α subunit (eIF2α) phosphorylation as well as improved autophagic flux as observed from the reduction in sequestosome-1 (SQSTM1/p62) protein level (Fig. 2H). Finally, ERRγ haploinsufficiency also reduced gene expression of inflammation (*Tnfα* and *Il6*), oxidative stress (*Hmox1*) and ER stress (*Chop*) (Fig. 2I) markers as well as the protein level of pancreatic stellate cells' activation marker (αSMA) in the pancreas (*SI Appendix*, Fig. S2F). We conclude that functional plasticity of
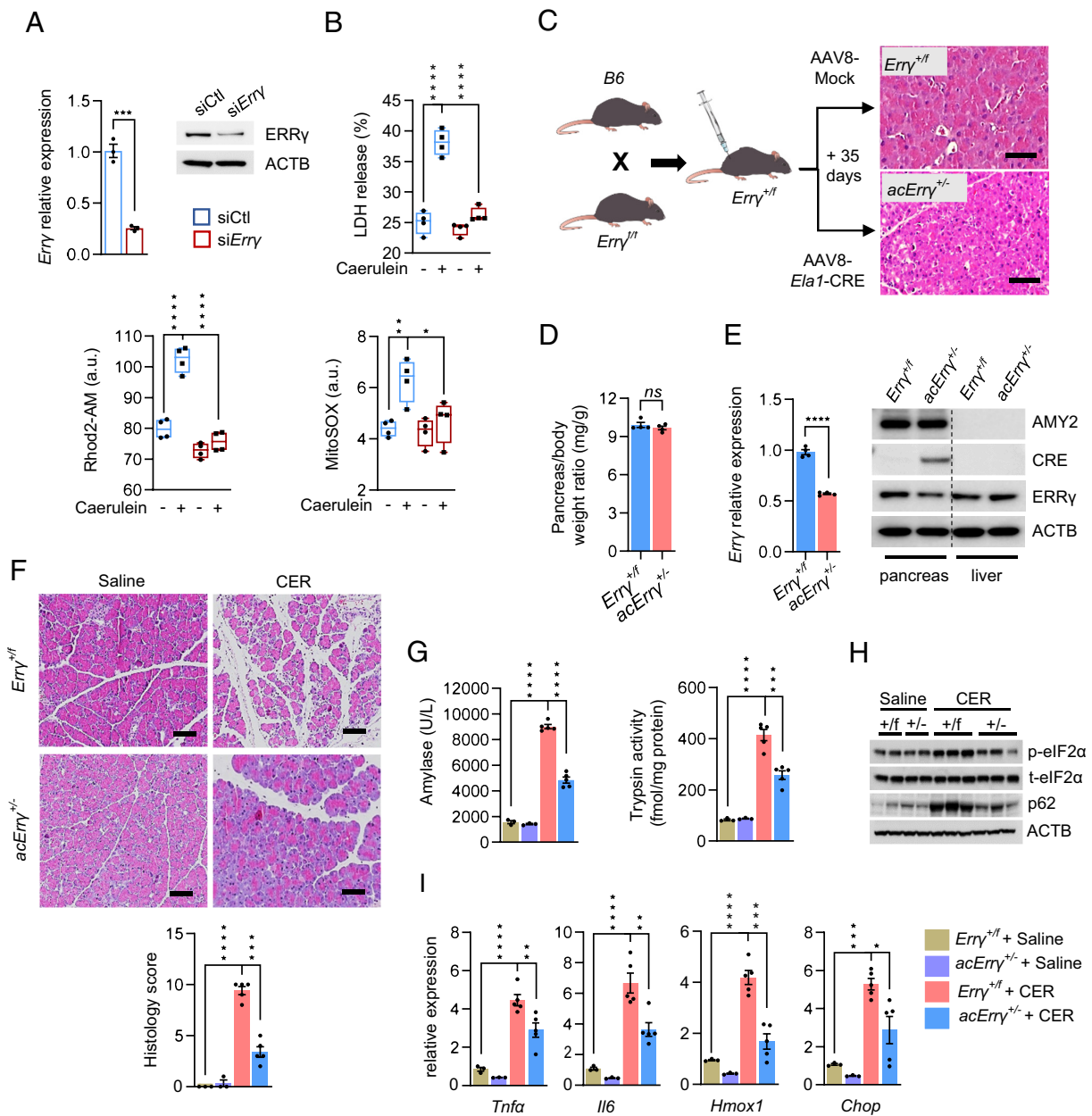
Fig. 2. Acinar cell-specific *Errγ* haploinsufficiency retards the progression of pancreatitis. (*A*) Analysis of ERRγ mRNA expression and protein level in 266-6 cells following knockdown of *Errγ* using targeted siRNA (si*Errγ*) (two-tailed *t* test). (*B*) LDH release, Rhod2-AM fluorescence, and MitoSOX fluorescence in 266-6 cells following treatment with caerulein in the presence or absence of si*Errγ* as indicated (two-way ANOVA analysis). (*C*) The pancreas-specific haplo-insufficient *Errγ* knockout line (ac*Errγ*$^{+/-}$) was induced by crossing C57BL6/J (B6) mice with *Errγ*$^{f/f}$ mice to initially generate *Errγ*$^{+/f}$, followed by intraperitoneal infusion of AAV8-*Ela1*-iCre into *Errγ*$^{+/f}$ mice to specifically, but partially, delete *Errγ* in the whole pancreas. Representative H&E images of the pancreas from *Errγ*$^{+/f}$ and ac*Errγ*$^{+/}$ mice. (*D*) Pancreas weight to body weight ratio of mice in *C*. (*E*) *Errγ* relative mRNA level (*Left*) and immunoblots for AMY2, ERRγ and CRE from the pancreas and liver of mice in *C*. (n = 4 mice/group; two-sided *t* test). (*F*) Representative H&E images and histology scoring of the pancreas from saline (Sal) and caerulein hyperstimulation (CER) pancreatitis conditions in *Errγ*$^{+/f}$ and ac*Errγ*$^{+/}$ mice. Animals were killed 16 h after the first saline or caerulein injection. (*G*) Serum amylase level and intrapancreatic trypsin activity of mice in *F*. (*H*) Immunoblots for ER stress (phospho- and total-eIF2α), and autophagic flux impairment markers (p62) from the pancreas of mice in *F*. (*I*) Pancreatic inflammation (*Tnfα* and *Il6*), oxidative stress (*Hmox1*), and ER stress (*Chop*) markers of mice in *F*. (n = 3 to 5 mice/group; two-way ANOVA analysis). Results in 266-6 cells (*A* and *B*) are representative of those from two to three independent experiments. Results (*C–I*) are representative of those from two independent in vivo experiments. Data represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 n.s. not significant. (Scale bars, 50 μm). (See also *SI Appendix*, Fig. S2).

ERRγ is involved in maintaining PAC homeostasis and a tight regulation of ERRγ level is critical for this balance.

Inhibition of ERRγ Prevents the Development of Pancreatitis. Currently, there are no targeted preventive options for pancreatitis. Therefore, as a proof of principle, we investigated whether prophylactic administration of ERRγ inhibitor (29, 30), DN434 (Fig. 3*A*), could have a favorable impact within the context of CER and TLCS models of pancreatitis. As anticipated, potent

ERRγ inhibition (Fig. 3*B*) by DN434 attenuated the measures of pancreatitis as evidenced by improvement in pancreatic histology (Fig. 3*C*) and reduction in serum amylase and pancreatic trypsin activity (Fig. 3*D*). DN434 treatment also reduced gene expression of inflammation, oxidative stress, and ER stress markers in the pancreas (Fig. 3*E*), as well as reversed autophagic flux impairment and inflammatory cell infiltration, as observed from p62 and F4/80 immunohistochemistry, respectively (Fig. 3*F* and *SI Appendix*, Fig. S3*A*). This protective effect of DN434 led us to
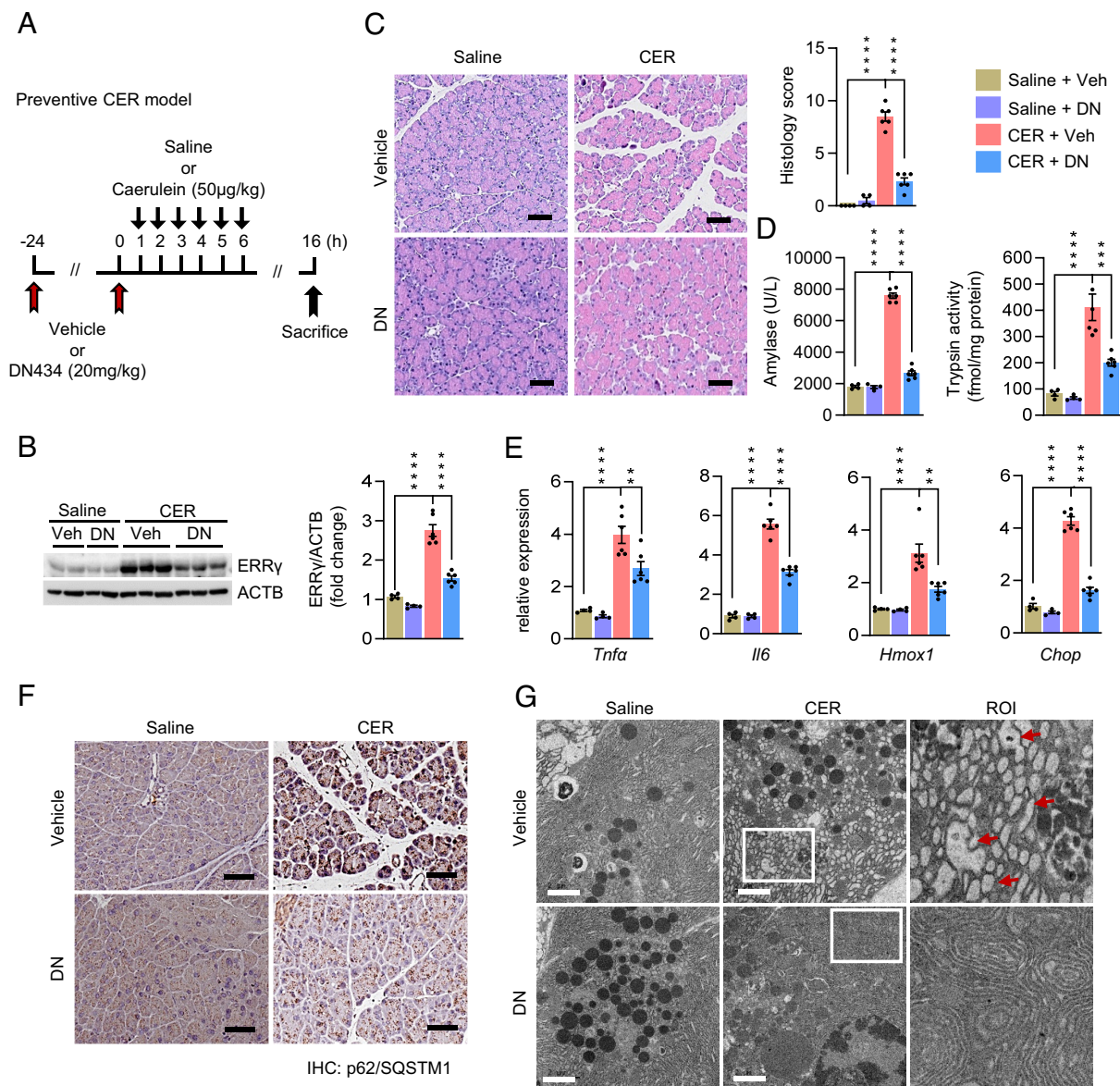
Fig. 3. Pharmacological inhibition of ERRγ prevents the development of pancreatitis. (*A*) Scheme for preventive caerulein hyperstimulation (CER) pancreatitis model in mice. Experimental small molecule inhibitor (DN434; abbreviated as DN; 20 mg/kg) for ERRγ was administered prophylactically, 24 h and 1 h prior to the first caerulein injection. Animals were killed 16 h after the first saline or caerulein injection. (*B*) Immunoblot for ERRγ from the pancreas of mice in *A*. (*C*) Representative H&E images and histology scoring of the pancreas from mice in *A*. (*D*) Serum amylase level and intrapancreatic trypsin activity of mice in *A*. (*E*) Pancreatic inflammation (*Tnfα* and *Il6*), oxidative stress (*Hmox1*), and ER stress (*Chop*) markers of mice in *A*. (*F*) Representative IHC staining of p62/SQSTM1 of the pancreas from mice in *A*. (*G*) Representative EM images (and dotted *Inset*) of the pancreas from mice in *A*. Red arrows: ER. (Scale bar, 2 μm). (n = 4 to 6 mice/group; two-way ANOVA analysis). Results are representative of those from two independent in vivo experiments. Data represent mean ± SEM. **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. ROI; region of interest. (Scale bars, 50 μm). (See also *SI Appendix*, Fig. S3).

hypothesize that ERRγ inhibitor functions through the restoration of acinar cell organelle function. High-resolution transmission electron microscopy (TEM) revealed extensively distended and dilated ER with nearly complete loss of associated ribosomes in CER pancreata, which was reversed upon DN434 treatment (Fig. 3*G*). Similarly, DN434 treatment reversed the effect of caerulein, in vitro, as evidenced by decrease in mitochondrial Ca^{2+} accumulation, ROS level and extracellular LDH release in 266-6 cells (*SI Appendix*, Fig. S3*B*). Consistent with the ameliorative effect on CER model, prophylactic DN434 treatment (*SI Appendix*, Fig. S3*C*) also prevented pancreatic histological damage (*SI Appendix*, Fig. S3*D*), and reduced serum amylase level and pancreatic trypsin activity (*SI Appendix*, Fig. S3*E*) in the TLCS model of pancreatitis. Overall, these findings reinforce the notion that inhibiting ERRγ may be an effective therapeutic route to treat pancreatitis.

VDAC1 Acts as an Effector Gene of ERRγ in Pancreatitis. A frequently encountered problem while working with the isolated PACs in culture is that acinar cells tend to transdifferentiate to ductal-like cells with ductal cell traits if they are kept in culture over a long period of time. To rule out that possibility in the context of our experiments, we initially compared between the freshly isolated cells (day 0) and noninfected cells that were cultured for 4 d for several well-established acinar cell-type and ductal cell-type gene expression markers. Gene expression analysis demonstrated that the acinar cell transcriptome was maintained over time. Overall, these data indicated that till 4 d in culture, acinar cells can maintain their characteristics and do not transdifferentiate into ductal-like cells (*SI Appendix*, Fig. S4*A*). Next, to determine how enhanced ERRγ expression impacts exocrine pancreas function, we performed RNA-seq on isolated PACs overexpressing ERRγ (GSE161757). ERRγ overexpression

allowed us to obtain a nonconfounding picture of ERRγ-driven transcriptional alterations within the acinar cell milieu, while at the same time restricting the plethora of signaling pathways that are activated in pancreatitis in vivo (7, 15). RNA-seq analysis identified 1,083 (713 up-regulated, 370 down-regulated) genes with significant differential expression upon ERRγ overexpression (Fig. 4A). Mitochondrial calcium overload acts as a mediator of

Ca^{2+} toxicity in the acinar cells during pancreatitis (3, 8, 9, 27, 31). Therefore, we initially analyzed the effect of ERRγ overexpression on key genes documented to be involved in mitochondrial Ca^{2+} uptake (Fig. 4B). To further define the pathways underpinning these differences, we performed gene set enrichment analysis. ERRγ overexpression resulted in increased expression of genes involved in mitochondrial membrane protein complex formation,
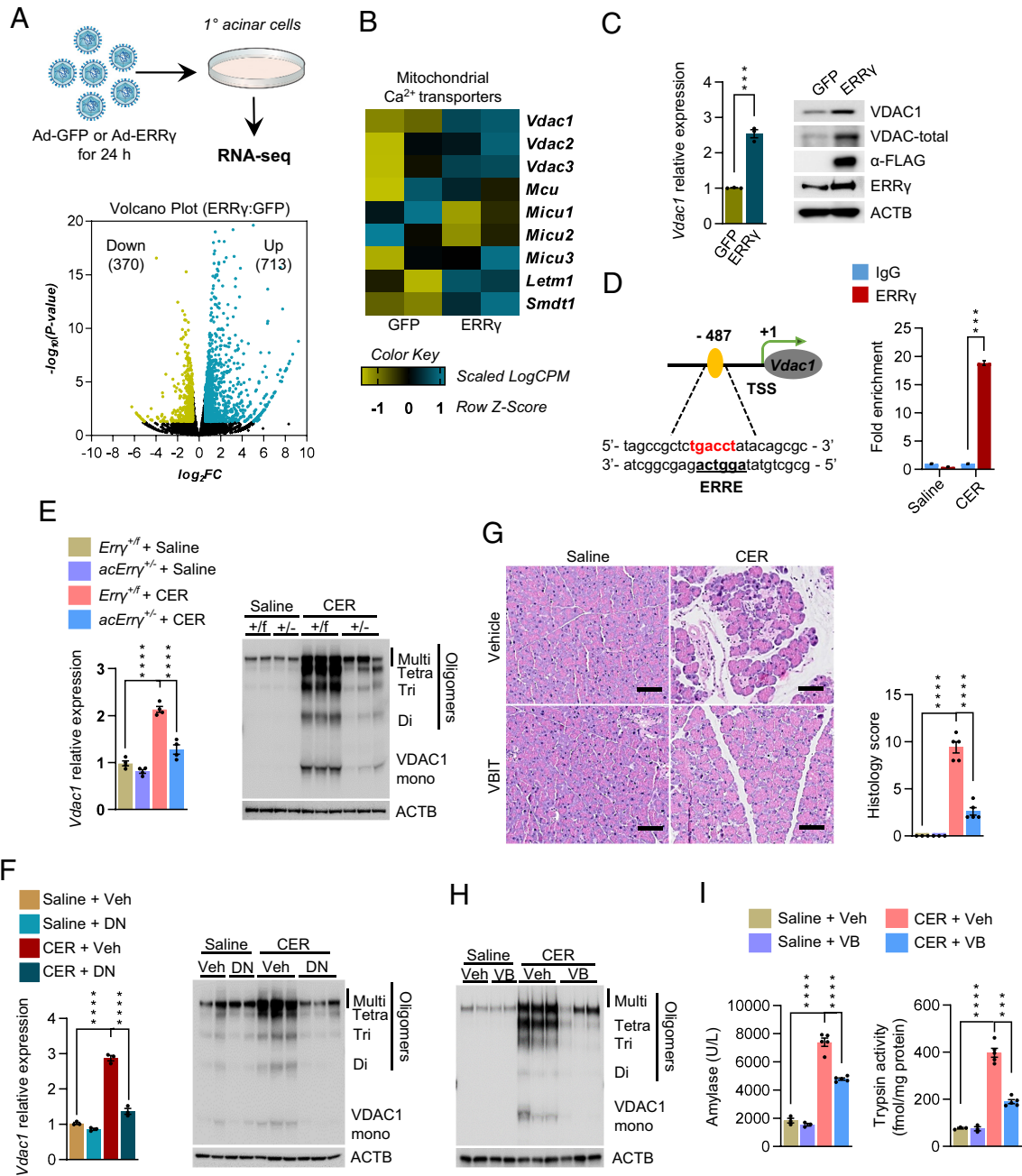

Fig. 4. VDAC1 acts as an effector gene of ERRγ in promoting pancreatitis. (A) Total RNA was isolated from 1° acini cells transduced with adenoviral vectors (Ad) overexpressing GFP or ERRγ for 24 h and analyzed by RNAseq (Top). A volcano plot showing genome-wide changes in mRNA level (Bottom). (B) Heat map depicting the differential expression of genes involved in mitochondrial calcium transport. (C) Analysis of VDAC1 mRNA and protein expression in primary acinar cells following treatment with Ad-ERRγ or Ad-GFP overexpression (10 MOI) for 24 h (two-tailed t test). (D) A putative ERRγ-response element (ERRE) in *Vdac1* gene promoter (Left). In vivo chromatin immunoprecipitation (ChIP)-qPCR analysis of ERRγ binding to *Vdac1* gene promoter of pancreas harvested from saline (Sal) and caerulein hyperstimulation (CER) pancreatitis conditions (n = 3 mice/group; two-tailed t test). (E) Analysis of VDAC1 mRNA and immunoblot of VDAC1 oligomerization from the pancreas of *Errγ*$^{+/f}$ and ac*Errγ*$^{+/}$ mice (n = 3 to 5 mice/group; two-way ANOVA analysis). (F) Analysis of VDAC1 mRNA and immunoblot of VDAC1 oligomerization from the pancreas of preventive caerulein hyperstimulation (CER) pancreatitis model (n = 4 to 6 mice/group; two-way ANOVA analysis). (G) Representative H&E images and histology scoring from the pancreas of mice of preventive caerulein hyperstimulation (CER) pancreatitis model with VDAC1 oligomerization inhibitor, VBIT-12 (20 mg/kg). Animals were killed 16 h after the first saline or caerulein injection. Experimental scheme of this model is described in *SI Appendix*, Fig. S4D. (H) Immunoblot of VDAC1 oligomerization from the pancreas of mice in G. (I) Serum amylase level and intrapancreatic trypsin activity of mice in G. (n = 3 to 5 mice/group; two-way ANOVA analysis). Results are representative of those from two independent experiments. Data represent mean ± SEM. ***$P < 0.001$ and ****$P < 0.0001$. (Scale bars, 50 μm). (See also *SI Appendix*, Fig. S4).

mitochondrial matrix as well as TCA cycle-related genes (*SI Appendix*, Fig. S4*B*).

Induction of *Vdac1* transcript levels was a prominent event in response to ERRγ overexpression in our transcriptomic analysis. VDACs are the most abundant proteins in mitochondrial outer membrane regulating Ca^{2+} influx, metabolism, inflammasome activation (32), and cell death (33, 34). Stimulus-dependent increase in intracellular Ca^{2+} level leads to enhanced *Vdac1* gene expression. The increase in VDAC1 expression shifts the equilibrium to its oligomeric state, allowing cytochrome c release from the mitochondria which results in apoptotic cell death (34). RT-qPCR and immunoblot analyses confirmed the induction of VDAC1 upon ERRγ overexpression at the mRNA and protein level (Fig. 4*C*). Next, we asked if VDAC1 is a direct transcriptional target of ERRγ. A putative ERRE (TGACCT) was found in the *Vdac1* proximal promoter (Fig. 4 *D*, *Left*). ChIP assay confirmed the binding of ERRγ to the ERRE of *Vdac1* in CER pancreata (Fig. 4 *D*, *Right*). We next hypothesized that the increase in VDAC1 transcripts would lead to intracellular Ca^{2+} overload following VDAC1 oligomerization. In the CER model of pancreatitis, *Errγ*$^{+/f}$ mice exhibited strong induction in *Vdac1* mRNA level and increase in VDAC1 oligomerization (Fig. 4*E*). This effect was significantly reversed in *Errγ*$^{+/}$ mice challenged with CER. Expectedly, pharmacological inhibition of ERRγ in the CER model led to similar reduction in *Vdac1* mRNA level and VDAC1 oligomerization (Fig. 4*F*). These findings indicated that ERRγ-dependent VDAC1 expression is required during pancreatitis. Next, we sought to determine whether inhibition of VDAC1 oligomerization by VBIT-12 (35) can protect against caerulein-induced acinar cell damage in vitro and in vivo. While VBIT-12 treatment resulted in reduced mitochondrial Ca^{2+} accumulation, ROS level, and LDH release in 266-6 cells following caerulein challenge (*SI Appendix*, Fig. S4*C*), prophylactic administration of VBIT-12 in the CER model (*SI Appendix*, Fig. S4*D*) reduced pancreatic histological damage (Fig. 4*G*), resulted in a significant reduction of VDAC1 oligomerization (Fig. 4*H*), and lowered serum amylase level and pancreatic trypsin activity (Fig. 4*I*). Interestingly, VDAC1 overexpression counteracted the mitigating effect of *Errγ* knockdown leading to enhanced mitochondrial Ca^{2+} accumulation, ROS level, and LDH release in 266-6 cells upon caerulein challenge (*SI Appendix*, Fig. S4*E*), further confirming the contribution of the ERRγ–VDAC1 axis in pancreatitis progression. Overall, these data support the contention that VDAC1 oligomerization is a key downstream step involved in the ERRγ-mediated pathogenesis of pancreatitis.

Dysregulation of the ERRγ–VDAC1 Axis Is Preserved in Human Pancreatitis. Next, to investigate the potential translational relevance of our findings, we obtained and analyzed pancreata from acute and chronic pancreatitis patients (*SI Appendix*, Table S1). Initial immunofluorescence analysis confirmed that both ERRγ and VDAC1 are expressed in the acinar cells in humans (*SI Appendix*, Fig. S5*A*). Consistent with our in vitro and in vivo data, immunohistochemical analysis indicated that both ERRγ and VDAC1 expression was significantly increased in patients with acute (n = 5) and chronic pancreatitis (n = 5) - compared to normal subjects (n = 5) (Fig. 5*A*). This observation suggests that human pancreatitis correlates with an aberrant increase in ERRγ as well as enhanced VDAC1 expression. These results further suggest the importance of the ERRγ–VDAC1 axis in the pathogenesis of human pancreatitis and provide additional evidence corroborating ERRγ transcriptional regulation in acinar cell function.

ERRγ Inhibition Is an Experimental Therapeutic Route to Treat Pancreatitis. Although, there are no targeted therapeutic interventions for pancreatitis, our current findings indicate

an amenable target with potential therapeutic implications. Accordingly, mice treated with ERRγ inhibitor DN434 (Fig. 5*B*) were refractory to CER-induced histological damage (Fig. 5*C*). Therapeutic administration of DN434 also lowered serum amylase level and pancreatic trypsin activity to near normal (Fig. 5*D*) as well as protein level of pancreatic stellate cells' activation marker (αSMA) in the pancreas (*SI Appendix*, Fig. S5*B*). Recently, we have established a humanized model of HP using PRSS1^{R122H}-Tg mice (36), which offers the opportunity for preclinical evaluation of our drug efficacy. After initiating AP, DN434 was administered twice daily over a 7-d period (Fig. 5*E*). This strategy mimics a clinical therapeutic scenario as the onset of pancreatitis and the start of treatment typically takes a few hours. Compared to vehicle-treated mice, which exhibited histological manifestation of CP along with reduced pancreas weight, DN434 administration abolished the progression of CP (Fig. 5*E*). Taken together, these results indicate that DN434's beneficial therapeutic effect can encompass a variety of patients with pancreatitis.

Overall, our study demonstrates that pancreatic insults, via STAT3 phosphorylation and activation, induce ERRγ gene transcription. ERRγ directly binds to the promoter, and induces, VDAC1 gene transcription and oligomerization. This leads to aberrant increase in mitochondrial Ca^{2+} and ROS levels, ER stress, and impairs autophagic flux. This triad of dysfunctional cellular processes leads to acinar cell damage via intrapancreatic trypsinogen activation, which contributes to the pathogenesis of pancreatitis. Pharmacological inhibition of ERRγ (DN434) blocks the key initiating step involved in the molecular pathogenesis of pancreatitis and protects acinar cells against pancreatic insults (Fig. 5*F*).

Discussion

The mechanism of pancreatitis is complex. Here, we have explored the potential benefits of targeting ERRγ therapeutically to treat pancreatitis. Initially, we demonstrate that ERRγ protein level is robustly induced in human pancreatitis as well as in multiple murine models of pancreatitis induced by different insults, including caerulein, alcohol, and bile acid. Hence, induction of ERRγ is a general feature of pancreatitis. Upon identifying ERRγ as a critical transcription factor that is induced during pancreatitis, we systematically investigated its role in regulating acinar cell homeostasis. Furthermore, using pharmacologic blockade of ERRγ activity by administering an inhibitor, we have highlighted the translational potential of ERRγ inhibitors in the treatment of AP. As such, ERRγ represents an attractive therapeutic target in AP and warrants further comprehensive evaluation for clinical application. These findings underscore how a better understanding of transcriptional regulation can suggest strategies for pancreatitis therapy.

In an apparent contradiction to our earlier observation where we observed that ERRγ transcript level is reduced in two patient cohorts of pancreatitis (20), here we report that ERRγ protein level is significantly induced in a different patient cohort of AP and CP. A plausible explanation for this discrepancy can be drawn from a previous report that demonstrated, using an integrated multilayered omics approach, a remarkable mismatch between the transcript level and protein expression of several genes in response to cellular stress, a phenomenon conserved across different species (37). This can potentially explain the apparent discrepancy between our earlier observation, which was analyzed at the transcript level, and our current study, where we have analyzed ERRγ expression at the protein level. Furthermore, it should be considered that there
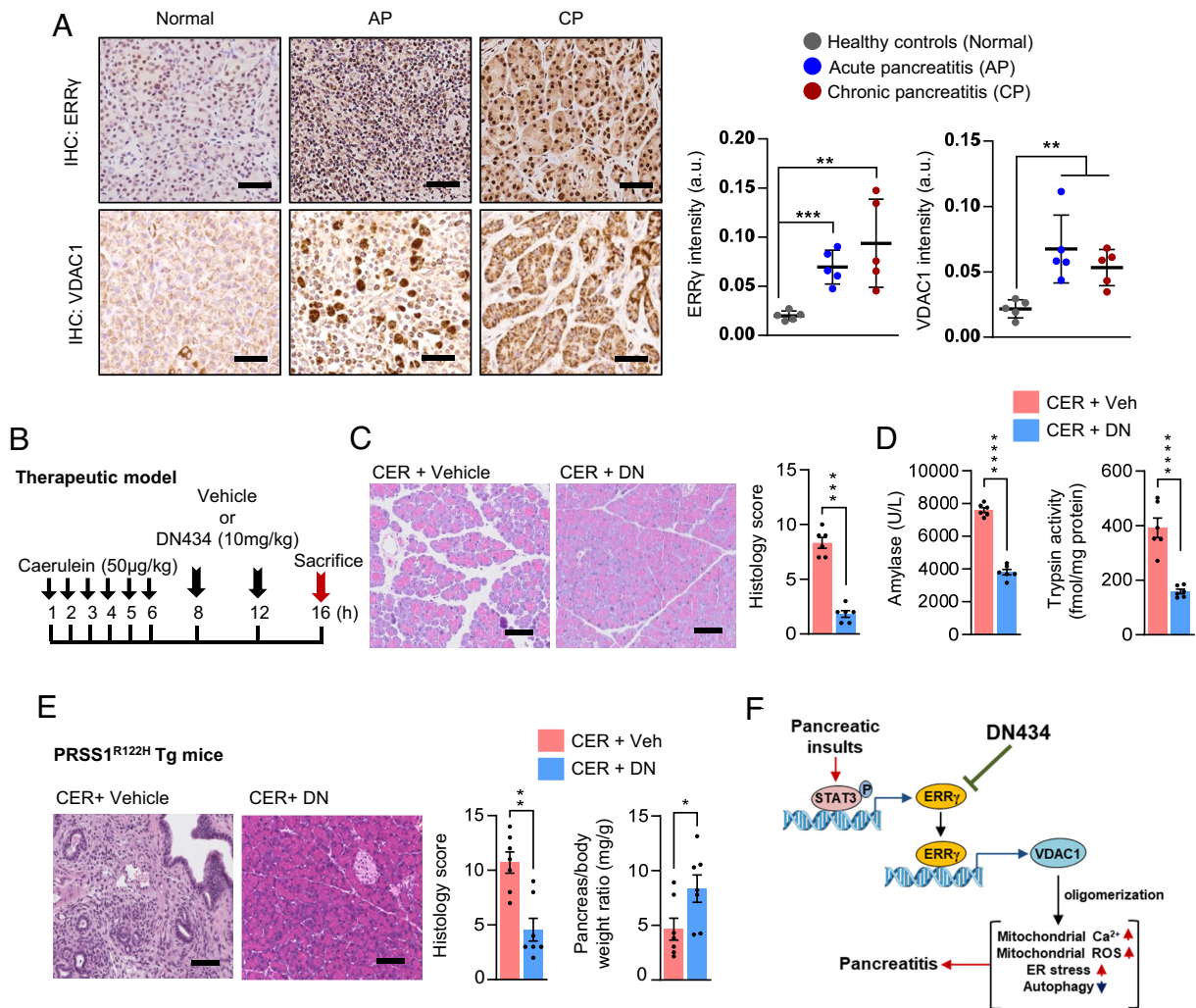
Fig. 5. ERRγ inhibitor is an effective experimental therapeutic in treating pancreatitis. (*A*) Representative IHC staining of ERRγ and VDAC1 in pancreatic tissue from patients and quantification of the samples (n = 5 subjects/group). (*B*) Scheme of pancreatitis induction (CER) and treatment. Pancreatitis was induced by caerulein, and therapeutic drug (DN) was administered twice, 7 and 11 h after the first caerulein injection. (*C*) Representative H&E images and histology scoring of the pancreas from mice in *B*. (*D*) Serum amylase level and intrapancreatic trypsin activity of mice in *B*. (n = 6 mice/group; two-tailed *t*-test) (*E*) Pancreatitis induction (CER) and treatment in PRSS1^R122H mice. Pancreatitis was induced by caerulein, and therapeutic drug (DN) was administered 5 h after the first caerulein injection. Vehicle or DN were given twice daily *(10 mg/kg; b.i.d)* for the next 7 d. Representative H&E images, histology scoring and pancreas weight to body weight ratio of mice after 7 d of treatment. (n = 7 mice/group; two-tailed *t* test). (*F*) Schematic representation of the molecular regulatory role of the ERRγ–VDAC1 axis that contributes toward the pathogenesis of pancreatitis. Results are representative of those from two independent in vivo experiments. Data represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. (Scale bars, 50 μm). (See also *SI Appendix*, Fig. S5 and Table S1).

are practical difficulties in obtaining high-quality pancreatic tissue, therefore, reliable gene expression datasets are extremely limited. In addition, patient characteristics can play a crucial role in the disease pathogenesis and can lead to a significant variation among different cohorts. Considering all these issues, in this study, we have used a dual approach of loss-of-function model as well as pharmacological inhibition model to investigate the mechanistic involvement and functional plasticity of ERRγ in PAC in normal physiology and stress. Earlier, we have analyzed datasets from human genetic studies and evidenced that multiple regulatory single-nucleotide variants within the noncoding region of ERRγ gene was associated with the development of pancreatitis (20). However, genome-wide association studies do not provide a clear functional information underpinning the role of the gene in pathogenesis of the disease. As our initial evidence indicated that ERRγ is required for acinar cell homeostasis under normal conditions, we have specifically investigated the effect of partial loss of ERRγ on acinar cell integrity during pancreatitis.

Here, we have addressed an aspect of disease pathogenesis by elucidating the transcriptional regulatory role of ERRγ in

pancreatitis. To mimic the pathology where different inducers of pancreatitis induce ERRγ gene expression, we utilized an ERRγ overexpression-specific quantitative transcriptomic analysis to identify VDAC1 as a potential transcriptional target of ERRγ. Consistent with previous literature, our data support a common mechanism involving Ca^{2+}-driven organelle dysfunction (impaired autophagy, ER stress, mitochondrial Ca^{2+} accumulation and ROS formation) at the onset of pancreatitis (5–9, 11). Mitochondrial dysfunction is a hallmark in both in vivo and ex vivo experimental and genetic models of pancreatitis (8, 11, 12). In particular, recent studies have been focused on elucidating the mechanisms whereby impaired autophagy and mitochondrial dysfunction in acinar cells cause inflammation in pancreatitis (6, 8, 9). Our functional elucidation further revealed the pathological significance of ERRγ-driven VDAC1 gene transcription and subsequent VDAC1 oligomerization which promotes mitochondrial Ca^{2+} accumulation and ROS formation. This, in conjunction with impaired autophagic flux and ER stress, precipitates toward aberrant trypsinogen activation and acinar cell damage.

A recent study aimed to alter the mitochondrial Ca^{2+} entry pathway via genetic ablation of mitochondrial calcium uniporter (MCU) to reduce the severity of experimental AP (38). This approach failed to reduce the biochemical and histological aberrations associated with local and systemic damage during AP. VDAC1 is the sole mitochondrial calcium channeling protein that is localized in the OMM. Conversely, MCU represents one of the several other calcium channeling proteins residing in the IMM. Thus, based on our current observations, targeting the ERRγ–VDAC1 axis represents a more reasonable approach to alleviate the severity of experimental pancreatitis. However, complete depletion of ERRγ severely compromises pancreatic development, potentially because VDAC1 is crucial not only for mitochondrial metabolism, but also for cell survival (39). Our strategy to control ERRγ transcriptional activity with an inhibitor avoiding developmental effects highlights the importance of fine-tuning ERRγ expression in maintaining exocrine pancreas homeostasis in adulthood. Further evidence of this translational promise comes from the marked correlation of pancreatic ERRγ and VDAC1 protein levels in pancreatic ailments with a clear trend toward higher expression levels from healthy subjects to acute and chronic pancreatitis patients. Finally, to address the therapeutic potential of ERRγ inhibition in pancreatitis, we utilized the humanized mouse model of HP (36), which develops AP and progresses to CP, thereby capturing the whole spectrum of human HP. In this humanized model, in addition to the CER model of pancreatitis, we have demonstrated the efficacy of ERRγ inhibition as a potential therapeutic intervention for pancreatitis.

There are several limitations to this study. ERRγ is expressed in a variety of pancreatic cell-types including acinar cells, ductal cells, and pancreatic β-cells. This raises additional question whether ERRγ is induced in all these cell types as well as in pancreatic stellate cells (PSCs) and immune cells in the context of pancreatitis. Conversely, it is currently unknown whether ERRγ can contribute to PSC or immune cell activation, a key feature observed during sustained and repeated pancreatic injury and inflammation (40). These possibilities warrant in-depth analysis using different cell-type-specific knockout models of ERRγ in the context of aggravated pancreatic pathophysiology, for example, chronic pancreatitis and pancreatic cancer. Regarding mouse models of pancreatitis, taking into consideration that caerulein mimics the sequelae of pancreatitis in humans, it fails to faithfully replicate the exact human pathology. Additionally, although ERRγ mediates the effect of different insults to exacerbate acinar cell dysfunction and ERRγ inhibition mitigates these effects to restore acinar cell homeostasis, it remains to be determined precisely how much does ERRγ contribute to the development of pancreatitis. Due to restricted access to human tissue, most of our study make use of animal models, immortalized PAC line or freshly isolated acinar cells to study AP pathogenesis. These models reproduce the spectrum of human disease severity and help us to understand the cell biology of pancreatitis and the molecular factors involved; they also allow for testing of a potential therapeutic approach. However, proper validation of the correlation between ERRγ expression and human pancreatitis will require extensive analysis of several cohorts of patient samples. Nevertheless, we show that pancreatitis, at the molecular level, can be characterized as an ERRγ-hyperactive state of the exocrine pancreas and that controlling ERRγ transcriptional activity has beneficial effects in various mouse models of pancreatitis. Given that ERRγ is implicated in several metabolic diseases (18) and a druggable target (18, 29, 30), it may be possible to test ERRγ inhibitors in a clinical set-up to treat pancreatitis.

Materials and Methods

Materials and methods are detailed in *SI Appendix*.

Study Approval. All animal experiments were approved by the IACUC at Kyungpook National University School of Medicine, Korea Advanced Institute of Science and Technology, and Mayo Clinic. All mice were housed in a specific pathogen-free facility, mice were maintained under a 12-h light–dark cycle and given free access to food and water, and experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the United States NIH (NIH Publication, 8th Edition, 2011).

Animal Models of Pancreatitis. Eight-to-twelve-week-old male C57BL/6J mice were used for in vivo experiments, unless otherwise mentioned. All animals were provided ad libitum access to food (standard chow diet, Research Diets, New Brunswick, NJ, USA) and water before the study.

In the caerulein hyperstimulation-induced pancreatitis model (CER), pancreatitis was induced by 50 μg/kg caerulein (Sigma-Aldrich, C9026) administered at 6 hourly intraperitoneal injections; control mice received similar injections of saline. For experiments using ERRγ inhibitor (DN200434, DN434), mice were pretreated with DN434 (20 mg/kg, *i.p.*) or vehicle (10% DMSO + 70% of 25% β-cyclodextrin (Sigma-Aldrich, H107) + 20% saline) 24 h prior, followed by a second booster treatment 1 h prior to the first injection of caerulein. Mice were killed 16 h after the first caerulein injection. For experiments using VDAC1 oligomerization inhibitor (VBIT-12; Selleckchem S8936), mice were pretreated with VBIT-12 (20 mg/kg, *p.o.*) or vehicle (10% DMSO + 70% of PEG-400 + 20% Tween-80) 24 h prior, followed a second booster treatment 1 h prior to the first injection of caerulein. Mice were killed 16 h after the first caerulein injection.

Bile acid-induced pancreatitis was induced as described (11) by retrograde injection of the pancreatic duct with 1% TLCS (Sigma Aldrich, T0512) while controls had ductal injection of saline. For experiments using ERRγ inhibitor (DN200434, DN434), mice were pretreated with DN434 (20 mg/kg, *i.p.*) or vehicle (5% DMSO + 95% of 20% PEG400 in saline) 24 h prior, followed a second booster treatment 1 h prior to the TLCS injection. Mice were killed 16 h later.

Alcohol–induced mild model of pancreatitis was induced as described (26) by initially feeding mice with the control Lieber–DeCarli diet ad libitum for 5 d for acclimatization. Thereafter, ethanol (EtOH)-fed group was allowed free access to the ethanol Lieber–DeCarli diet containing 5% (*v/v*) ethanol for 10 d, and control group was pair-fed with the isocaloric control diet. At day 11, ethanol-fed and pair-fed mice were gavaged in the early morning with a single dose of ethanol (5 g/kg body weight) or isocaloric maltose dextrin, respectively, and killed 9 h later.

Experimental Therapeutic Study

For therapeutic study in the CER model, pancreatitis was induced by 50 μg/kg caerulein, administered at 6 hourly intraperitoneal injections. Vehicle or DN434 (10 mg/kg, *i.p.*) were administered twice, 7 h and 11 h after the first caerulein injection. Sixteen hours after the first dose of caerulein, mice were humanely killed, and pancreas was harvested for analysis.

For therapeutic studies in PRSS1^{R122H}-Tg mice, mice were randomly assigned into groups and treated by oral administration with vehicle or DN434 (10 mg/kg, *i.p.*). Drug treatment began 5 h after the first caerulein injection, twice daily over 7 d. Twelve hours after the final dose, mice were humanely killed, and pancreas were harvested for analysis.

RNA-Sequencing (RNAseq) and Bioinformatics Analysis. For each experiment, two biological replicates were used. Samples from different conditions were processed together to prevent batch effects. Quality of total RNA was assessed by the RNA integrity number (RIN) using Agilent Bioanalyzer. All retained RNA samples had a RIN > 8. The total RNA library was subjected to transcriptome sequencing. The sequencing was carried out with Macrogen (www.macrogen.co.kr; Seoul, Korea). Changes in gene expression of the cells transduced with adenovirus constructs overexpressing GFP or ERRγ were compared. Transcripts with fold change of >1.5

($P < 0.05$) were included as differentially expressed genes (DEGs). After identifying the DEGs, gene ontology (GO) analysis was performed using the DAVID bioinformatics program (https://david.ncifcrf.gov) for gene identification and annotation. The annotation results were categorized under biological process, molecular function, and cellular function. To identify the functional groups and molecular pathways associated with the observed DEGs, the RNAseq data were further analyzed using the Kyoto Encyclopedia of Genes and Genomes database (www.genome.jp) and GO Resource. The datasets generated and analyzed during the current study are available in the GEO repository (GSE161757).

Statistics. Data are expressed as mean ± SEM. Unpaired two-tailed Student's t test was used for two-group analyses. A one-way (for one independent variable) or two-way (for two independent variables) ANOVA with Tukey's multiple comparisons test was used for comparison among the different groups on all pairwise combinations (GraphPad Prism 7). A P value of < 0.05 was considered statistically significant.

Data, Materials, and Software Availability. RNAseq datasets have been deposited in Gene Expression Omnibus (GSE161757) (41) and Mendeley Data (https://data.mendeley.com/datasets/d35nnp46v9/1) (42). All other study data are included in the article and/or SI Appendix.

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