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NFATc1 Links EGFR Signaling to Induction of Sox9 Transcription and Acinar–Ductal Transdifferentiation in the Pancreas

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Abstract

Background & Aims—Oncogenic Kras mutation is a defining genetic alteration in pancreatic ductal adenocarcinoma (PDAC), but is not sufficient to promote cancer formation on its own.

Secondary events, such as inflammation-induced signaling via the epidermal growth factor receptor (EGFR) and expression of the *SOX9* gene, are required for tumor formation. In this study

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Conflict of interest

The authors declare that they do not have any conflict of interest.

Author Contributions

N.-M. C., G.S., A.K., G.-Y.L., P.S., J.-S.Z., L.R., S.N., D.D.B., B.K., S.J., V.E. and E.H. designed and performed experiments and analyzed results. A.K., V.E. and E.H. assembled results and wrote the paper. J.S. and M.H. provided tools and edited the manuscript.

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we sought to identify the underlying mechanisms which link EGFR signaling to *Sox9* gene induction during acinar–ductal metaplasia (ADM), a transdifferentiation process that precedes pancreatic carcinogenesis.

Methods—We analyzed pancreatic tissues from *Kras*^{G12D};*pdx1-Cre* and *Kras*^{G12D};*NFATc1*^{-/-};*pdx1-Cre* mice after intraperitoneal administration of caerulein or dimethyl sulfoxide (controls). Pharmacological inhibition of NFATc1 activation was achieved by application of cyclosporin A. Induction of EGFR signaling and its effects on expression of NFATc1 or SOX9 were investigated by quantitative reverse transcription PCR, immunoblot, and immunohistochemical analyses of mouse and human tissues and acinar cell explants. Interactions between NFATc1 and partner proteins and effects on DNA binding or chromatin modifications were studied using co-immunoprecipitation and chromatin immunoprecipitation assays in acinar cell explants and mouse tissue.

Results—EGFR activation induced NFATc1 expression in metaplastic tissues from patients with chronic pancreatitis and in pancreatic tissues from *Kras*^{G12D} mice and promoted complex-formation with c-Jun in dedifferentiating acinar cells, thereby stimulating the transcription of ductal gene signatures to provoke ADM. This process involved NFATc1:c-Jun-mediated activation of *Sox9* transcription in converting acinar cells. Pharmacological inhibition of NFATc1 or disruption of the *Nfatc1* gene inhibited EGFR-mediated induction of *Sox9* transcription and blocked acinar–ductal transdifferentiation and pancreatic cancer initiation.

Conclusion—Our findings identify an EGFR-NFATc1-*Sox9* signaling cascade as a critical mediator of inflammation-induced PDAC initiation and suggest that disruption of this pathway may offer a novel chemopreventive target for high-risk pancreatitis patients.

Keywords

signal transduction; mouse model; transcriptional regulation; ChIP; NFATc1; EGFR

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies with a five-year survival rate below 5%¹. PDAC is believed to arise from three different precursor lesions, with pancreatic intraepithelial neoplasia (PanIN) being the best described. Activating mutations in the *Kras* oncogene are detected in early PanIN lesions and in more than 90% of advanced human PDAC, thus leading to the current paradigm that this genetic alteration is crucial for PDAC initiation². Lineage-tracing studies in genetically engineered mice (GEM) have shown that acinar cells expressing mutant *Kras*^{G12D} lose their differentiation status and acquire a duct-like phenotype^{3–7}. This process, termed acinar-to-ductal metaplasia (ADM), is now appreciated as an initial step in pancreatic carcinogenesis. ADM can evolve into PanIN lesions and eventually progress to metastatic PDAC^{3,5,7,8}. However, ADM formation and progression occurs with a low penetrance and a long latency, unless *Kras*^{G12D} mice are exposed to chemically induced pancreatitis^{8,9}. Thus, *Kras*^{G12D} expression alone is not sufficient for pancreatic cancer initiation in GEM, but rather requires poorly defined secondary events such as inflammatory signals to drive carcinogenesis^{8–10}.

In line with this, epidemiological studies revealed chronic pancreatitis as a major risk factor for PDAC development in humans^{10–12}.

Metaplastic lesions often express high levels of tyrosine kinase receptors such as EGFR and its natural ligands EGF and TGF α in patients with chronic pancreatitis or early stage tumors^{11–14}. While transgenic expression of EGFR ligands provoked ADM formation in animal models^{13–16}, EGFR inactivation using either genetic or pharmacological approaches preserved acinar cells in a well-differentiated state and suppressed metaplastic transformation by Kras^{G12D} and inflammation^{15,16}. While accumulating evidence from mouse and human studies supports an essential role of EGFR signaling activation in inflammation-driven metaplasia and cancer initiation, the molecular mechanisms linking EGFR signaling with acinar cell dedifferentiation and ADM formation remain elusive.

Nuclear Factor of Activated T cells c1 (NFATc1) belongs to a family of Ca²⁺/calcineurin-responsive transcription factors primarily recognized for their central roles in T cell activation¹⁷. In recent years it became clear that NFATc1 expression and function is not restricted to the immune system and rather plays important roles in gene regulation during transformation and cancer progression^{18–20}. We observed ectopic expression and nuclear accumulation of NFATc1 in more than 60% of human pancreatic cancers and particularly in tumors with peritumoral inflammation²¹. In GEM models, transgenic expression of nuclear NFATc1 cooperates with Kras^{G12D} in pancreatic carcinogenesis, resulting in rapid formation of precursor lesions and progression toward invasive PDACs²².

Here we sought to determine whether nuclear NFATc1 activation bridges among EGFR signaling, ADM and pancreatic cancer initiation. By taking advantage of an established experimental pancreatitis model in GEM, our studies identified a previously unknown EGFR-NFATc1 signaling and transcription pathway with essential functions in inflammation-driven carcinogenesis. EGFR signaling causes a robust induction of NFATc1 expression. In cooperation with partner proteins, NFATc1 controls a signaling network, which is required for promotion of acinar-to-ductal conversion both in animals and acinar cell explants. Finally, genetic or pharmacological inactivation of NFATc1 prevents inflammation/EGFR-mediated ADM in the pancreas.

Materials and Methods

Animals

Generation and characterization of *pdx1-Cre*, *LSL-Kras^{G12D}*, and NFATc1^{fl/fl} mice have been described before^{23–26}. Mouse strains were interbred to obtain: *Kras^{G12D};pdx1-Cre* and *Kras^{G12D};NFATc1^{+/+};pdx1-Cre* mice. All strains were of C57Bl/6 background. Sex was irrelevant for ADM development. Caerulein (50 μ g/kg, 3 times per week, AnaSpec), Cyclosporin A (5mg/kg, 5 $\times$ /week) or DMSO were administered by intraperitoneal injection at the age of 8 weeks for a duration of 4 weeks. All animal experiments were carried out using protocols approved by the Institutional Animal Care and Use Committee at the Philipps-University of Marburg.

Acinar cell isolation

Acinar cell isolation was performed as described previously²⁷. Briefly, the whole pancreas was dissected and incubated at 37°C in a collagenase VIII-containing solution. The minced pancreas was passed through a 100µm nylon filter. After repetitive washing acini were exposed to culture solution containing 30% FCS. Acinar cells were cultured in collagen supplemented with 0.1% FCS and, unless otherwise indicated, treated with DMSO, CsA (0.5µM), TGFα (50ng/ml), FK506 (10nM) or EGF (20ng/ml). Ducts were counted after the indicated time points using brightfield microscopy and Hoechst33324 staining to show cell viability.

Chromatin immunoprecipitation (ChIP assay)

ChIP in 266-6 acinar cells and in pancreatic tissue was performed as described previously²⁸. For detailed information please refer to Supplementary Materials and Methods.

Results

NFATc1 is induced in EGFR-mediated acinar-to-ductal metaplasia

Transdifferentiation of acinar cells into the cells with duct-like structures is induced by oncogenic *Kras* mutation and activation of the EGFR signaling pathway^{15,29}. Consistently, we found increased phosphorylation levels of EGFR and its downstream ERK/MAP kinase in metaplastic areas of human pancreatitis (Figure 1A, Supplementary Figure 1A). Importantly, immunohistochemical analysis also displayed a robust increase in NFATc1 expression in the areas of ductal metaplasia (Figure 1A, Supplementary Figure 1A). To examine whether NFATc1 plays a role in pancreatitis-induced ADM, we treated *Kras*^{G12D} mice with caerulein, a well-established inducer of inflammatory pancreatic damage^{29,30}. A 4-week treatment resulted in severe exocrine pancreatic injury with disordered acinar structure and induction of ADM (Figure 1B). As predicted, progressive ADM formation was associated with an activation of the EGFR signaling pathway. Immunohistochemistry and immunoblot analysis confirmed phosphorylation of both EGFR and ERK/MAP kinase (Figure 1B and C). Caerulein treatment also induced high levels of EGF (Figure 1C), an EGFR ligand frequently overexpressed in human pancreatitis.

To ascertain the *in vivo* functions of NFATc1, we explored changes in its expression levels and pattern in the pancreata of *Kras*^{G12D} mice. NFATc1 expression in *Kras*^{G12D} control animals was predominantly limited to the cytoplasm of exocrine cells, indicating NFATc1 transcriptional inactivity in acinar cells (Figure 1B, Supplementary Figure 1B). However, as reported previously³¹, and consistent with findings in human pancreatitis, NFATc1 was highly induced in the exocrine pancreas in response to caerulein administration (Figure 1B and C). Of note, NFATc1 expression was specifically detected in the nuclei of ADM lesions (Supplementary Figure 1B). Together, these experiments provided evidence for a link between EGFR signaling and NFATc1 activation during early stages of cancer initiation.

NFATc1 is required for EGFR-signaling induced ADM

Cellular adaptation in response to external stimuli has previously been attributed to NFAT signaling and transcription in various tissues^{22,30}. To investigate whether NFATc1 is directly involved in EGFR signaling-induced acinar-to-ductal transdifferentiation, we analyzed NFATc1 expression and function in acinar cells undergoing ductal conversion in response to EGFR stimulation. As predicted from previous studies, activation of EGFR signaling via stimulation with either EGF or TGF α strongly promoted conversion of acinar cells into duct-like cells (Figure 2A and B, Supplementary Figure 2A and B). Importantly, acinar-to-ductal transdifferentiation was accompanied by a strong induction of NFATc1 mRNA expression and increased protein levels in acinar cells (Figure 2C and Supplementary Figure 2C). Strikingly, either depletion of NFATc1 or administration of the clinically utilized calcineurin inhibitors cyclosporine A (CsA) or FK506, which prevent NFAT activation, blocked acinar-to-ductal conversion even upon activation of EGFR signaling (Figure 2A-B, Supplementary Figure 2A,B,D). As a consequence, duct formation was inhibited and expression of ductal differentiation genes, such as cytokeratins 7 and 19 and E-cadherin (Figure 2D and E, Supplementary Figures 2E and 3) were decreased following NFATc1 depletion, while amylase expression was maintained in response to NFATc1 inhibition (Figure 2F). Together, these studies highlight the important role of NFATc1 during EGFR signaling-induced acinar cell reprogramming, and suggest that NFATc1 activation is required for the acquisition of a premalignant duct-like phenotype.

EGFR signaling mediated activation of NFATc1 involves complex formation with c-Jun

In order to regulate changes in gene expression, NFAT proteins interact with other DNA binding partners frequently occupying adjacent sequences on common target gene promoters. We have previously examined genome-wide NFATc1 occupancy in pancreatic cancer cells using ChIP-seq^{22,32} and have consistently observed composite binding sites between NFAT and AP1, a heterodimeric transcription factor consisting of members of c-Jun and c-Fos family on numerous target genes. In fact, 61.8% of the 6273 identified NFATc1 binding peaks were enriched for consensus AP1 binding sites (NFATc1:AP1) (Figure 3A). Notably, GREAT analyses revealed an enrichment of EGF- or related pathways for genes containing NFAT:AP1 overlapping sites (Supplementary Table 1 and 2).

Based on these data, we examined whether combinatorial NFATc1:AP1-dependent transcription links EGFR signaling activation with gene regulation during acinar-to-ductal conversion. Consistently, EGF treatment caused a time-dependent activation of the AP1 factors c-Fos and c-Jun, as evidenced by a progressive increase in MAP kinase-dependent phosphorylation in an acinar cell line and in acinar cell explants (Figure 3B,C and Supplementary Figure 4A–C and 5A–D). We then tested whether EGFR signaling activation stimulates transcription from NFATc1:AP1 responsive promoters. To do so, we employed primary pancreatic cancer cells from *Kras*^{G12D};*p53*^{wt};*Egfr*^{-/-} mice lacking EGFR expression. Consistent with its dependence on the EGFR-MAPK pathway, introduction of c-Jun displayed only marginal effects on NFATc1 mediated promoter transactivation (Figure 3C). However, NFATc1:c-Jun transcriptional activity was strongly enhanced upon restoration of the EGFR signaling pathway following transfection of cells with a constitutively active mutant receptor (Figure 3C). In contrast, NFATc1 activity remained

unaffected by c-Fos expression (Figure 3C), even upon EGFR signaling activation thereby confirming the essential function of c-Jun in NFATc1 mediated promoter transactivation. Consistent with a role for c-Jun in acinar-to-ductal transdifferentiation, c-Jun induction and phosphorylation were detected in response to caerulein treatment (Figure 3D and Supplementary Figure 5E) and immunohistochemical analyses demonstrated high levels of phosphorylated c-Jun in ductal-like lesions of *Kras*^{G12D} mice (Figure 3E). Finally, co-immunoprecipitation studies confirmed inducible NFATc1:c-Jun interactions both in cultured acinar cell explants undergoing EGFR-induced ductal conversion (Supplementary Figure 5F) and in transforming *Kras*^{G12D} mice pancreata (Figure 3F). In summary these findings support a role for EGFR signaling in inducing NFATc1 transcriptional activity in transdifferentiating acinar cells by promoting NFATc1:c-Jun complex formation.

EGFR signaling induces NFATc1-dependent Sox9 expression in pancreatic carcinogenesis

The establishment and maintenance of pancreatic metaplastic lesions depend on the expression and activation of transcription factor machinery that drives acinar transdifferentiation toward a ductal phenotype. Recently, Sox9 has been implicated in ADM formation and pancreatic cancer initiation^{32,33}. In line with these observations, acinar cell explants revealed an induction of Sox9 expression in response to EGF treatment (Figure 4A–C). A luciferase reporter assay in primary pancreatic cancer cells from mice with combined constitutive activation of *Kras* and mutation of the tumor suppressor p53 (*Kras*^{G12D};*Trp53*^{R172H}) displayed transactivation of the *Sox9* promoter upon EGF treatment (Figure 4D), confirming *Sox9* as a downstream target of EGFR signaling. Interestingly, depletion of NFATc1 rescued EGFR-dependent induction of *Sox9* transactivation and expression (Figure 4). Thus, these data identify *Sox9*, a known mediator of pancreatic cancer initiation and progression, as an important NFATc1 target gene downstream of EGFR signaling.

NFATc1:c-Jun complex formation is required for transcriptional activation of Sox9

Detailed sequence analyses revealed the presence of three NFATc1 consensus sequences adjacent to AP1 binding sites within the proximal promoter region of the *Sox9* gene (Figure 5A). We examined basal and inducible NFATc1 and c-Jun promoter recruitment in EGF-treated *Kras*^{G12D} acinar cells by performing ChIP analyses following activation of EGFR signaling. Interestingly, EGFR signaling induced NFATc1 occupancy at the –825 bp and +370 bp sites (Figure 5A). Importantly, inactivation of NFATc1 with CsA resulted in decreased c-Jun recruitment and reduced occupancy of the active histone mark H3K4me3 on the *Sox9* promoter suggesting that NFATc1 plays an essential role in EGFR-driven *Sox9* gene expression (Figure 5B). Similarly, depletion of c-Jun significantly decreased NFATc1 recruitment to the *Sox9* promoter and reduced EGF-induced *Sox9* promoter activation as displayed by decreased RNA Polymerase II recruitment (Figure 5C and Supplementary Figure 5G). Interestingly, in line with the repressive effect of c-Fos on NFATc1 mediated promoter transactivation (Figure 3C), overexpression of c-Fos diminished NFATc1 occupancy and activity of the *Sox9* promoter in *Kras*^{G12D};*p53*^{wt};*Egfr*^{+/–} cells in response to EGFR signaling activation (Figure 5D), indicating that c-Fos impairs binding of the NFATc1:c-Jun complex to target gene promoters. Taken together, these findings suggest that EGFR signaling activates a hierarchical transcriptional cascade in which NFATc1:c-Jun

complexes specifically drive *Sox9* gene expression during the process of acinar-to-ductal conversion.

NFATc1 deletion prevents inflammation-induced ADM *in vivo*

To further examine the importance of NFATc1 for *Sox9* induction in the context of ADM, we crossed *Kras*^{G12D} mice with *NFATc1*^{fl/fl}; *pdx1-Cre* animals to obtain mice with pancreas-specific deletion of *NFATc1*. *Kras*^{G12D}; *NFATc1*^{-/-} mice were born at the expected Mendelian frequency without any gross alterations in pancreatic development or acinar cell specification, rendering it a suitable GEM model to investigate NFATc1-dependent transcription and function in the context of EGFR signaling (Supplementary Figure 6A, B). In agreement with our pharmacological and siRNA-mediated approaches, acinar cell explants from *Kras*^{G12D}; *NFATc1*^{-/-} mice expressed low levels of the ductal markers cytokeratin 7 and 19 upon EGF treatment (Supplementary Figure 6C–E), indicating impaired ADM formation in the absence of NFATc1. Most importantly, loss of NFATc1 rendered the *Sox9* gene insensitive to caerulein-induced transcriptional activation (Figure 4A, C).

To verify the biological relevance of this novel pathway in acinar cell conversion and cancer initiation, we employed the various GEM models and monitored caerulein-induced acinar-to-ductal transdifferentiation following genetic or pharmacological inactivation of NFATc1. NFATc1 staining confirmed absence of the transcription factor in NFATc1-deficient mice (Figure 6A and Supplementary Figure 7). Strikingly, while caerulein caused a massive acceleration of ductal-like lesions in 3-month old *Kras*^{G12D} mice, loss of NFATc1 rendered acinar cells insensitive to inflammation-induced acinar-to-ductal transdifferentiation (Figure 6A, B and Supplementary Figure 7). Immunohistochemistry and immunoblot analyses confirmed stable expression levels of acinar cell differentiation markers (e.g. amylase) in NFATc1-deficient mice, while ductal differentiation markers were not induced (Figure 6A,D and Supplementary Figure 7).

Consistent with a critical role of *Sox9* in NFATc1-mediated acinar-to-ductal conversion, we confirmed a strong induction of *Sox9* expression upon transdifferentiation both at the RNA and protein levels (Figure 6A and D–E). Furthermore and consistent with a cooperative role of NFATc1 and c-Jun in the induction of *Sox9* gene expression in acinar cells, we observed inducible NFATc1 and c-Jun binding to and increased activation of the *Sox9* promoter in *Kras*^{G12D} pancreata upon caerulein treatment (Figure 6F). Thus, disrupted NFATc1:c-Jun complex formation as a consequence of genetic inactivation of *NFATc1* disables *Sox9* transcriptional induction and subsequent ductal transdifferentiation in acinar cells despite the presence of oncogenic *Kras* mutations and activation of EGFR signaling. In summary, our studies identify NFATc1/c-Jun as a critical transcription factor complex essential for the integration of EGFR and inflammatory signals that promote acinar-to-ductal transdifferentiation in the pancreas. Significantly, NFATc1 is induced by cell autonomous EGFR signaling and this pathway is operative both in human and GEM models of carcinogenesis, specifically when exposed to an inflammatory environment.

Discussion

Acinar transdifferentiation toward a duct-like phenotype represents a defining response of acinar cells to stress signals, including inflammation³⁴ and is considered to be the initial step in pancreatic carcinogenesis³⁵. In the absence of oncogenic signaling, ADM represents a regenerative program that allows restoration of pancreatic tissue integrity³⁶. However, in the presence of oncogenic *Kras* mutation, the acinar cell lacks the regenerative potential following tissue damage and undergoes a transition from ductal-like lesions to PanINs, thus promoting pancreatic carcinogenesis³⁷. Over the last few years, multiple studies have shown that singular oncogenic activation of *Kras* is not sufficient to induce PDAC initiation^{4,38}. *Kras* activation rather requires complementary signals such as inflammation to fully induce pancreatic carcinogenesis^{8,29}. Previous work revealed mechanistic details of the inflammatory signaling program as an inducer of cellular plasticity in the pancreas. Upregulation of epidermal growth factor receptors in the acinar cells has long been observed following chronic inflammation and in PDAC^{11,12}. A central role of EGFR signaling during ADM induction and progression to neoplastic precursor lesions in response to inflammatory signals has been demonstrated recently^{15,16,27}. However the selection of EGFR-dependent targets in the context of acinar-to-ductal transdifferentiation remains poorly understood.

The first evidence connecting EGFR-signaling to NFAT transcription factors in carcinogenesis came from expression analysis showing that both proteins are highly induced in pancreatic cancer samples³⁹. Furthermore, recent work showed EGFR-dependent and NFAT-mediated *Cox2* expression in colorectal tumorigenesis demonstrating a functional relevance for this hypothesized link⁴⁰. The identification of *Cox2* as an EGFR/NFAT target already points toward critical functions of the EGFR/NFAT cascade for the integration of inflammatory signals during carcinogenesis. In particular, during pancreatic cancer formation NFAT proteins have shown to play a central role in the integration of environmental signals into oncogenic transcriptional processes that mediate proliferation, cellular differentiation and adaptation to inflammation^{21,22,28,30}. In this study, we show that sustained pancreatic inflammation induced a strong up-regulation of NFATc1 in metaplastic areas of both murine and human chronic pancreatitis models. Detailed *in vitro* studies employing acinar cell extracts confirmed increased NFATc1 expression as a result of EGFR activation. Notably, NFATc1 depletion by pharmacological or genetic approaches resulted in reduced EGFR-mediated expression of ductal gene expression signatures and impaired acinar cell transdifferentiation *in vitro* and *in vivo*. Most importantly, pancreatic cancer initiation in response to inflammation could be significantly blocked in *Kras*^{G12D};NFATc1^{-/-} mice, highlighting the biological significance of the previously unrecognized EGFR-NFATc1 axis in pancreatic cancer initiation.

Characteristically, sufficient DNA binding on target gene promoters and enhancers by NFAT transcription factors requires interaction with partner proteins^{21,28,41}. In the case of the NFAT proteins, the selection of cooperating transcription factors defines NFATc1 target gene specificity and the resulting cellular functions⁴². For instance, interaction with GATA factors is mandatory for NFAT-induced cardiac cell differentiation^{43,44}, while T-cell activation and proliferation requires cooperation with members of the AP1 (c-Jun/c-Fos) family of bZIP proteins⁴³. Notably, recent studies further implicated NFATc1:AP1

cooperativity in adaptive gene regulation and confirmed a role for this interaction in various aspects of carcinogenesis⁴⁵. ChIP-sequencing analysis revealed that more than half of the NFATc1-bound genetic regions possess composite DNA elements that are putatively bound by AP1 proteins²². Consensus NFAT:AP1 binding elements were preferentially found in genes implicated in cytokine/growth factor signaling regulation, growth promotion, and tumor-matrix interactions²², suggesting that a primary function of NFAT:AP1 in complexes in cancer may be to integrate environmental signals with tumor-promoting cell-specific gene responses. Here, we show that inflammation-induced pancreatic cancer initiation involves NFATc1:c-Jun complex formation in de-differentiated pancreatic lesions. Importantly, physical NFATc1:c-Jun association was only visible in the presence of EGF or caerulein, stressing, that upstream signals highly influence NFATc1 partner protein selection and the mode of transcriptional activity on target genes.

Inflammation-induced EGFR-activation has previously been linked to elevation of Sox9 expression levels. EGFR activation in response to epithelial injury induces Sox9 expression during the development of urothelial cancer⁴⁶, thereby describing a functional link between EGFR signaling and Sox9 expression in carcinogenesis. Most importantly, in a recent report Sox9 has been identified as a critical transcription factor in acinar cell dedifferentiation and ductal conversion³². While loss of Sox9 during acinar cell transdifferentiation resulted in reversed ductal gene expression signatures and blocked ADM formation and progression into preneoplastic lesions, ectopic induction of Sox9 in acinar cells in *Kras*^{G12D};*Sox9* mice accelerated *Kras*^{G12D}-driven carcinogenesis and gave rise to a pancreatic phenotype that resembles morphological alterations of *Kras*^{G12D} mice with conditional activation of NFATc1 (*Kras*^{G12D};*NFATc1*), including severe acinar cell destruction and progressive formation of ductal-like lesions^{22,32}. Although the relevance of the EGFR-Sox9 axis has been demonstrated in multiple cellular contexts, the underlying mechanism linking inflammation-induced EGFR activation and Sox9 induction in pancreatic cancer initiation remained elusive. Herein we show that the EGFR-mediated induction of NFATc1 expression during this process provides a link between EGFR activation and Sox9-controlled effects during acinar cell transdifferentiation. This is evidenced by increased *Sox9* promoter activation and elevated Sox9 expression levels upon EGFR-induced NFATc1 activation. Consequently, high Sox9 expression levels were likewise found in primary *Kras*^{G12D};*NFATc1* tumor cells, whereas pharmacological inhibition or genetic depletion of NFATc1 caused a loss of *Sox9* expression even upon EGFR activation. Promoter and ChIP analyses confirmed NFATc1 recruitment to specific *Sox9* promoter regions in response to EGFR activation. Significantly, *Sox9* promoter activation was dependent on cooperative NFATc1:c-Jun binding, as sequential depletion of the transcription factors rendered the *Sox9* promoter insensitive to EGFR activation, resulting in decreased *Sox9* expression and impairment of acinar-to-ductal transdifferentiation. The identification of *Sox9* as a direct target gene of NFATc1 in pancreatic carcinogenesis is also supported by results from a recently published genome-wide ChIP-Seq analysis showing a recruitment of NFATc1 to the *Sox9* promoter in primary pancreatic tumor cells derived from *Kras*^{G12D};*NFATc1* mice²². The activation of the NFATc1-Sox9 axis is not restricted to epithelial regeneration or cancer initiation since recent work identified NFATc1 recruitment to the *Sox9* promoter during tracheal cartilage development⁴⁷.

In summary, our work reveals a previously uncharacterized EGFR-NFATc1 signaling and transcription pathway that bridges EGFR signaling with Sox9 expression during ADM and pancreatic cancer initiation. Utilizing a well-established chronic pancreatitis model in GEM as well as experimental approaches in acinar cell explants, we found that EGFR-signaling induces strong NFATc1 expression in metaplastic pancreatic cells. EGFR-mediated activation of NFATc1 involves complex formation with c-Jun on the *Sox9* promoter, rendering it transcriptionally active. Given the abundance of experimental and clinical evidence linking EGFR-NFATc1 activation with chronic pancreatitis and cancer initiation, we propose that disruption of the pathway is translationally relevant, and identifies new targets and preventive strategies in patients at high risk for developing PDAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. EGFR signaling induces NFATc1 expression

(A–B) Analysis of H&E and immunohistochemical (IHC) staining in (A) human chronic pancreatitis samples and (B) 12 weeks old *Kras^{G12D}* mice after administration of caerulein (scale bars: 100 μ m).

(C) Western Blot analysis of indicated proteins in pancreatic tissue of *Kras^{G12D}* mice.

Figure 2. EGFR signaling mediated acinar-to-ductal transdifferentiation requires activation of NFATc1

(A) TGFα-treated acinar cell explants from *Kras*^{wt} mice in the presence or absence of CsA (0.5 μM) or FK506 (10 nM) were analyzed for duct formation 72 hours after cell isolation (scale bar: 100 μM).

(B) Duct formation was determined following shRNA-mediated depletion of NFATc1 in wild-type mice following TGFα treatment for 5 days (n=3, mean ± SD, scale bars: 100 μm). * indicates p<0.05 as compared to shcontrol alone, ** indicates p<0.05 as compared to shcontrol + TGFα.

(C) Western Blot analysis shows NFATc1 expression in acinar explants after the indicated treatments.

(D–F) QRT-PCR reveals CK-19 expression in response to EGF treatment following siRNA-mediated depletion of NFATc1 in acinar cells from *Kras*^{G12D} mice (D) and mRNA expression of the indicated markers following pharmacological inhibition with FK506 (10 nM) (E) or CsA (0.5 μM) (F) in acinar cell explants from *Kras*^{wt} mice (n=3, mean ± SD).

Figure 3. EGFR signaling induces NFATc1:c-Jun complex formation in acinar-ductal transdifferentiation

(A) Pie chart displays portion of AP1 binding sites within NFATc1 peaks identified by ChIP-Seq. Motif analyses on NFATc1 binding using RSAT peak-motifs shows AP1 binding motif. Statistical significance is denoted with $-\log_{10}$ p-value.

(B) EGF-treated acinar 266-6 cells and acinar cell explants from *Kras^{wt}* mice were analyzed by immunoblotting.

(C) Luciferase reporter assay shows activity of an NFAT-responsive promoter construct in *Kras*^{G12D}; *p53*^{wt/}; *Egfr*^{-/-} cells after expression of indicated constructs (n=3 mean $\pm$ SD).

(D) Protein expression of c-Jun and p-c-Jun in pancreatic lysates of caerulein-challenged *Kras^{G12D}* mice.

(E) Immunohistochemistry displays expression and phosphorylation of c-Jun in caerulein treated 3 months old *Kras*^{G12D} mice (Scale bars: 100 μ m).

(F) Co-immunoprecipitation demonstrates NFATc1/c-Jun complex formation in tissue from caerulein-treated 3 months old *Kras*^{G12D} mice.

Figure 4. EGFR signaling activity involves NFATc1-dependent induction of Sox9

(A) Western Blot analysis showing NFATc1 and Sox9 expression in EGF-treated acinar cell explants in *Kras^{G12D}* or *Kras^{G12D};NFATc1^{Δ/Δ}* mice.

(B) QRT-PCR shows Sox9 mRNA expression in acinar cell explants of *Kras^{G12D}* mice following the designated treatments (n=3, mean values $\pm$ SD).

(C) QRT-PCR displays Sox9 expression in *Kras^{G12D}* versus *Kras^{G12D};NFATc1^{Δ/Δ}* mice in response to EGF treatment.

(D) Luciferase reporter assay in primary pancreatic tumor cells from *Kras^{G12D};Trp53^{R172H}* mice to show Sox9 promoter activity in response to EGF treatment in the presence and absence of NFATc1 (n=3 mean $\pm$ SD).

Figure 5. Transcriptional activation of Sox9 requires NFATc1:c-Jun complex formation on the Sox9 promoter

(A) Cartoon displays putative NFAT and predicted AP1 binding sites in the mouse Sox9 promoter. Promoter mapping reveals NFATc1 binding to the Sox9 promoter.

(B) ChIP assays to determine occupancy of NFATc1, c-Jun and H3K4me3 on the Sox9 promoter in 266-6 cells in response to EGF and/or CsA (3h). NFATc1 and c-Jun: +370 bp; H3K4me3: -825 bp (mean $\pm$ SD).

(C) ChIP assays to demonstrate NFATc1 and RNA Pol II occupancy on the Sox9 promoter (+370 bp) in c-Jun depleted 266-6 cells after treatment with EGF for 3h.

(D) ChIP analyses in primary pancreatic *Kras*^{G12D}; *p53*^{wt/wt}; *Egfr*^{-/-} cells demonstrates NFATc1 binding and H3K4me3 as well as RNA Pol II occupancy on the Sox9 promoter (-825 bp) following transfection of EGFR +/- c-Fos.

Figure 6. Genetic ablation of NFATc1 prevents inflammation-induced acinar-to-ductal transdifferentiation *in vivo*

(A–E) Mice with indicated genotypes were challenged with caerulein and CsA as indicated. Expression analyses and quantification of ductal-like lesions were performed after 4 weeks of treatment.

(A) H&E staining and immunohistochemistry (scale bars: 100 μ m).

(B) Quantification of ductal like lesions (n=5, mean $\pm$ SD).

(C) E-cadherin mRNA expression in pancreatic tissue.

(D–E) Western Blot analysis and Sox9 mRNA (n=4, mean $\pm$ SD) expression in pancreatic tissues.

(F) ChIP analysis in caerulein-treated *Kras*^{G12D} mice shows NFATc1 and c-Jun occupancy (+370 bp) and H3K4me3 (–825 bp) binding to Sox9 promoter.