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Restricted Heterochromatin Formation Links NFATc2 Repressor Activity With Growth Promotion in Pancreatic Cancer

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Abstract

BACKGROUND & AIMS—Transcriptional silencing of the p15^{INK4b} tumor suppressor pathway overcomes cellular protection against unrestrained proliferation in cancer. Here we show a novel pathway involving the oncogenic transcription factor nuclear factor of activated T cells (NFAT) c2 targeting a p15^{INK4b}-mediated failsafe mechanism to promote pancreatic cancer tumor growth.

METHODS—Immunohistochemistry, real-time polymerase chain reaction, immunoblotting, and immunofluorescence microscopy were used for expression studies. Cancer growth was assessed in vitro by [³H]thymidine incorporation, colony formation assays, and in vivo using xenograft tumor models. Protein-protein interactions, promoter regulation, and local histone modifications were analyzed by immunoprecipitation, DNA pull-down, reporter, and chromatin immunoprecipitation assays.

RESULTS—Our study uncovered induction of NFATc2 in late-stage pancreatic intraepithelial neoplasia lesions with increased expression in tumor cell nuclei of advanced cancers. In the nucleus, NFATc2 targets the p15^{INK4b} promoter for inducible heterochromatin formation and silencing. NFATc2 binding to its cognate promoter site induces stepwise recruitment of the histone methyltransferase Suv39H1, causes local H3K9 trimethylation, and allows docking of heterochromatin protein HP1 γ to the repressor complex. Conversely, inactivation of NFATc2 disrupts this repressor complex assembly and local heterochromatin formation, resulting in restoration of p15^{INK4b} expression and inhibition of pancreatic cancer growth in vitro and in vivo.

CONCLUSIONS—Here we describe a novel mechanism for NFATc2-mediated gene regulation and identify a functional link among its repressor activity, the silencing of the suppressor pathway p15^{INK4b}, and its pancreatic cancer growth regulatory functions. Thus, we provide evidence that inactivation of oncogenic NFATc2 might be an attractive strategy in treatment of pancreatic cancer.

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Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.11.001.

Conflicts of interest

The authors disclose no conflicts.

Keywords

Pancreatic Cancer; NFAT; p15^{INK4b}; Gene Silencing; Heterochromatin

Pancreatic ductal adenocarcinoma is among the most devastating human malignancies.¹ It is commonly diagnosed at advanced, already metastatic, and hence incurable stages. The poor prognosis is largely attributable to its aggressive biology and the propensity of tumor cells to rapidly grow and disseminate into distant organs. The current model of pancreatic carcinogenesis suggests that invasive ductal adenocarcinomas arise from histologically well-defined noninvasive lesions, called pancreatic intraepithelial neoplasias (PanINs), through a series of genetic, epigenetic, and signaling events that eventually overcome cell autonomous tumor suppressor mechanisms to promote carcinogenesis.^{2,3}

One of the most important and potent antitumor mechanisms in mammalian cells is mediated by the INK4/ARF family of p15^{INK4b}, p16^{INK4a}, and p19^{ARF} (mouse homologue to human p14^{ARF}) tumor suppressor genes.⁴ Whereas p19^{ARF} primarily works through inhibition of MDM2-mediated p53 destabilization, p15^{INK4b} and p16^{INK4a} proteins guard against unrestrained proliferation through inhibition of cyclin-dependent kinases 4 and 6, thus maintaining retinoblastoma family proteins in a hypophosphorylated, growth suppressive state.⁵ The induction of INK4 proteins therefore allows normal cells to rapidly respond to cellular stress signals, DNA damage, and oncogenic signaling activation with induction of a G₁ growth arrest.⁴ Depending on the mode, magnitude, and duration of a cell signal, p15^{INK4b} and p16^{INK4a} expression can cause a transient G₁ cell cycle arrest or a permanent state of senescence. However, during development and progression of pancreatic cancer, INK4 proteins are frequently silenced by genetic or epigenetic events.⁶ Clearly, genetic deletion of p16^{INK4a} or its silencing by CpG island hypermethylation are early events in pancreatic carcinogenesis already found with high frequency at stages of premalignant PanIN lesions.^{6,7} In contrast, the p15^{INK4b} tumor suppressor gene is rarely depleted by genetic inactivation or hypermethylation of its gene locus.⁸ Rather, p15^{INK4b} expression and function are tightly controlled by mitogenic and antiproliferative stimuli; hence, silencing of its expression during cancer progression might originate from signaling-induced promoter repression.

Here we report a novel mechanism regulating the silencing of p15^{INK4b} in pancreatic cancer cells involving the oncogenic transcription factor nuclear factor of activated T cells (NFATc2). We show ectopic expression of this calcium/calcineurin responsive transcription factor in early precursor lesions and with increased frequency and intensity in tumor cells of advanced stages that is accompanied by silencing of the p15^{INK4b} tumor suppressor pathway.⁹ On activation, nuclear NFATc2 targets the histone methyltransferase Suv39H1 to the p15^{INK4b} promoter for local histone H3K9 trimethylation. This creates a platform for heterochromatin protein (HP) 1 γ binding and leads to promoter-restricted transition of accessible (open) euchromatin to compacted (closed) and transcriptionally silenced heterochromatin. Conversely, inactivation of NFATc2 prevents p15^{INK4b} promoter silencing and instead restores expression of this master tumor suppressor in cancer cells in vitro and in vivo. Together, this report describes a novel mechanism of NFATc2-mediated gene regulation and identifies a functional link between its previously unappreciated transcriptional repressor activity and silencing of the p15^{INK4b} tumor suppressor pathway in pancreatic cancer. In addition, we provide evidence that inactivation of NFATc2 might be an attractive strategy to reactivate antitumor defense mechanisms in pancreatic cancer.

Materials and Methods

Cell Lines, Plasmids, Reverse-Transcription Polymerase Chain Reaction Analysis, and Small Interfering RNA Oligonucleotides

Detailed descriptions on cell lines, plasmid generation, small interfering RNA (siRNA) oligonucleotides, and transfection protocols used in this study are available in Supplementary Materials and Methods.

Generation of Stable Cell Lines, Microscopic Analysis, Protein Analysis, Promoter Studies, Growth Assays, and Xenograft Models

Supplementary Materials and Methods contains details of antibodies as well as protocols of standard techniques, including generation of viral vectors and stable cell lines, fluorescence microscopy, immunohistochemistry, subcellular fractionation, isolation of proteins from tumor tissues, coimmunoprecipitation, immunoblotting, chromatin immunoprecipitation (ChIP) assays, DNA pull-down assays, proliferation assays, reporter gene assays, soft agar assays, xenograft experiments, and statistical analysis, performed as described previously.¹⁰⁻¹²

Results

Ectopic Expression of the Calcium/Calcineurin Responsive NFATc2 Transcription Factor During Pancreatic Carcinogenesis

Immunohistochemical analysis revealed that NFATc2 was detectable neither in acinar or ductal cells of healthy pancreas nor in early PanIN-1 lesions (Figure 1A). However, we found positive NFATc2 staining in advanced PanIN-2 and PanIN-3 precursor lesions and in the majority of invasively growing cancers (19/23; Figure 1A and B). Depending on its phosphorylation level, NFATc2 can shuttle between the cytosol and the nuclear fraction.¹³ When dephosphorylated by the phosphatase calcineurin, cytoplasmic NFATc2 becomes active and translocates into the nucleus to modulate gene expression. We found NFATc2 widely distributed throughout ductal cells in preinvasive PanIN-2 and PanIN-3 lesions. More important, however, NFATc2 was exclusively or predominantly expressed in the nuclear compartment of invasively growing tumor cells in the majority of positive tumor samples (14/19), thus indicating that NFATc2 is present in an active state in advanced human exocrine pancreatic cancer (Figure 1A and B). We were not able to find a significant correlation between nuclear NFATc2 localization and clinical parameters such as tumor stages and gradings (TNM) (data not shown). Consistent with increased expression of NFATc2 in cancer cells, we found high levels of both the messenger RNA and protein in a significant portion of established human pancreatic cancer cells, as evidenced by Western blot analysis and real-time polymerase chain reaction (PCR) (Figure 1C and D). Together, these results indicate that NFATc2 expression is induced during pancreatic carcinogenesis and reaches maximum expression levels in the nucleus of invasive cancer cells, pointing to a high activity of this transcription factor state in cancer.

NFATc2 Promotes Cancer Growth and p15^{INK4b} Silencing In Vitro and In Vivo

Administration of the calcium ionophore ionomycin caused increased [³H]thymidine incorporation in pancreatic cancer cells (PaTu-8988t and IMIM-PC1 cells), and this effect was abolished following genetic depletion of NFATc2 (Figure 2A and B). Similarly, stable retroviral expression of NFATc2 in Suit007, Suit028, and PaTu-8988t cells increased cell proliferation and colony formation (Figure 2C and D and Supplementary Figure 1A and B) and promoted tumor growth in xenograft tumors (Figure 3A). Interestingly, NFATc2-mediated growth promotion was accompanied by silencing of the p15^{INK4b} tumor suppressor pathway both in vivo and in vitro (Figure 3B and C) and resulted in increased

expression of D-type cyclins and their partner kinases cyclin-dependent kinases 4 and 6 (Supplementary Figure 1C and D). Conversely, genetic depletion of this transcription factor caused release of p15^{INK4b} expression both on messenger RNA and protein levels (Figure 3D and Supplementary Figure 2A), reduced expression levels of D-type cyclins, and prevented cells from growth promotion by ionomycin (Figure 2A and B and Supplementary Figures 1E, 1F, and 2A). Immunohistochemical analyses of serial human pancreatic cancer sections for NFATc2 and p15^{INK4b} expression finally showed an inverse correlation between NFATc2 and p15^{INK4b} expression (Figure 3E). Taken together, these results provided first evidence for NFATc2 repressor activity in cancer cells and suggested a role in silencing of the p15^{INK4b} tumor suppressor pathway in pancreatic cancer.

NFATc2 Targets the p15^{INK4b} Promoter for Silencing

We next questioned whether in growing pancreatic cancer cells the mechanism underlying NFATc2-mediated repression of p15^{INK4b} is mediated through the silencing of its promoter. ChIP assays showed NFATc2 promoter occupancy under serum-free conditions, and this binding was further enhanced on mitogenic stimulation with 10% fetal calf serum (Figure 4A). Consistent with p15^{INK4b} promoter regulation by NFATc2, reporter gene assays displayed dose-dependent repression of the full-length p15^{INK4b} promoter (Figure 4B). Further analysis showed that deletion of the p15^{INK4b} promoter to nucleotide -166 rendered the promoter insensitive to NFATc2-mediated silencing, indicative of the existence of an NFATc2-responsive repression element located in the distal promoter region beyond base pair -165 (Figure 4C and D). Computer-based sequence analysis of the -751/-165 promoter region confirmed this hypothesis and revealed the presence of a single NFAT (GGAAA) recognition site within the distal p15^{INK4b} promoter. This site, located at -593/-589 base pairs, was disrupted in the context of the full-length promoter through site-directed mutagenesis (p15^{INK4b} NFAT-mut) and used for further promoter analysis. The functional relevance of the -593/-589 NFAT binding element was confirmed by DNA pull-down experiments and additional reporter gene assays, showing that mutational disruption of this site (p15^{INK4b} NFAT-mut) rendered the p15^{INK4b} promoter resistant to inducible NFATc2 binding and repression (Figure 4E and F and Supplementary Figure 2B and C). Together, these experiments defined a novel repressor function for NFATc2 and identified the p15^{INK4b} promoter as an NFATc2 target in pancreatic cancer cells.

Heterochromatin Formation Underlies NFATc2-Mediated Silencing of p15^{INK4b} Pancreatic Cancer Cells

Regulation of gene transcription requires posttranslational modifications of histones, which, in concert with chromatin remodeling factors, shape the structure of chromatin.¹⁴ Euchromatin is characterized by weak DNA staining and corresponds to less compacted chromatin, whereas heterochromatin is compacted and stains intensively with DNA dyes. Consistent with previous observations, immunofluorescence microscopy indicated a cytoplasmic localization of NFATc2 in unstimulated cancer cells (Supplementary Figure 2D). Activation of NFATc2, however, caused nuclear translocation of the factor with a predominant punctual localization pattern in euchromatic regions (Supplementary Figure 2D). We then questioned whether euchromatic NFATc2 recruits heterochromatin forming complexes to the p15^{INK4b} promoter for silencing of the tumor suppressor. The following observations indicated that this is indeed the case. First, activation of NFATc2 binding to the p15^{INK4b} promoter, as shown in Figure 4A, was associated with deacetylation of histone 3 (Supplementary Figure 2E) and recruitment of the Suv39H1 histone methyltransferase and a strong induction of histone 3 lysine 9 trimethylation (H3K9-me), a mark associated with gene silencing and heterochromatin formation (Figure 5A). Second and even more important, coimmunoprecipitation experiments and DNA pull-down (Figure 5B) revealed that NFATc2 forms a complex with Suv39H1 and recruits it to the p15^{INK4b} promoter via

the -593/-589 binding site. Additionally, disruption of the NFATc2 promoter-binding site or siRNA depletion of NFATc2 abolished Suv39H1 recruitment to the p15^{INK4b} promoter and subsequent trimethylation of H3K9 without affecting Suv39H1 and H3K9-me protein expression levels (Figure 5B [right panel], Figure 5C, and Supplementary Figure 3A). Most importantly, NFATc2-dependent regulation of H3K9-me could be observed neither on transcriptional start site/exon I and intron within the *INK4b* locus nor on the p14^{ARF} promoter, clearly high-lighting that the heterochromatin formation is restricted to the p15^{INK4b} regulatory sequence (Supplementary Figure 3B–D and data not shown). In contrast, depletion of Suv39H1 did not affect NFATc2 recruitment to the p15^{INK4b} promoter (Supplementary Figure 3E), suggesting that NFATc2 recruits Suv39H1 for subsequent gene repression. Consistent with these findings, pharmacologic inhibition of Suv39H1 using chaetocin or siRNA-mediated depletion abrogated H3K9 trimethylation and subsequent NFATc2-mediated repression of the p15^{INK4b} promoter (Figure 5D–F and Supplementary Figure 3F). Together, these data identified an NFATc2-Suv39H1-dependent transcriptional repression mechanism at the p15^{INK4b} promoter.

HP1 γ Is Required for Sustained Silencing of the p15^{INK4b} Promoter

HP1 family members are critical mediators of repressor complex formation and maintenance, thus greatly contributing to gene silencing.¹⁵ Growing evidence indicates that the three known HP1 isoforms may not fulfill identical functions and instead show differences in their nuclear distribution pattern and nuclear activities. In contrast to HP1 α and HP1 β , which are almost exclusively found in the heterochromatin area, HP1 γ is localized in both heterochromatic and euchromatic regions, where it contributes to transcription complex stabilization.^{16–18} We found HP1 γ widely distributed throughout the nuclei of pancreatic cancer cells before and after growth stimulation (Figure 6A). However, after serum stimulation, HP1 γ partially colocalizes with NFATc2 in euchromatic foci and interacts with the repressor in an Suv39H1-dependent manner (Figure 6A and B). In fact, depletion of Suv39H1 prevented HP1 γ from both interaction with NFATc2 and docking to the p15^{INK4b} promoter (Figure 6B and C). Similarly, genetic knockdown of NFATc2 not only disabled Suv39H1 recruitment and local H3K9 trimethylation, as shown in Figure 5C, but prevented HP1 γ promoter interaction (Supplementary Figure 4A). Functionally, HP1 γ binding to the repressor complex is essential for heterochromatin stabilization and sufficient promoter silencing by NFATc2. Accordingly, DNA pull-down assays showed inducible NFATc2-Suv39H1 complex formation at the p15^{INK4b} promoter, and this complex remained stable for at least 3 hours when performed in the presence of HP1 γ (Figure 6D). However, depletion of HP1 γ destabilized repressor complex formation at the p15^{INK4b} promoter in vitro (Figure 6D) and in vivo (Figure 6E); hence, sufficient binding of neither NFATc2 or Suv39H1 nor local H3K9 trimethylation was observed in ChIP assays performed 3 hours after stimulation (Figure 6E). Consistent with destabilization of the repressor complex, depletion of HP1 γ caused a loss of NFATc2-mediated p15^{INK4b} promoter silencing (Figure 6F and Supplementary Figure 4B). Together, these results defined a novel NFATc2 silencing mechanism in which the binding of this transcription factor to the p15^{INK4b} promoter induces local heterochromatin formation involving the stepwise recruitment of Suv39H1, trimethylation of H3K9, and docking of HP1 γ .

Inactivation of NFATc2 Restores Expression of p15^{INK4b} and Inhibits Growth of Pancreatic Cancer

Finally, to define the biological relevance of our mechanistic findings, we analyzed the effect of NFATc2 inactivation on p15^{INK4b} expression and pancreatic tumor growth in vivo. For this purpose, we subcutaneously injected IMIM-PC1 pancreatic cancer cells with high NFATc2 expression levels into the flanks of nude mice. After 2 weeks, mice were treated intraperitoneally with a widely used specific inhibitor of calcineurin/NFAT signaling,

cyclosporin A (CsA), or dimethyl sulfoxide 5 times weekly. Xenograft tumor growth was significantly delayed on administration of CsA, which was associated with increased p15^{INK4b} messenger RNA levels (Figure 7A and B). We complemented this experiment using a second NFAT inhibitor and treated IMIM-PC1 tumor cell-bearing nude mice with the GSK-3 β inhibitor AR-A014418 (3 times per week). The GSK-3 β serine kinase stabilizes NFATc2 in cancer cells and protects the transcription factor from rapid ubiquitination and proteasomal degradation. Inhibition of GSK-3 β , in turn, promotes NFATc2 inhibition and degradation in cancer cells.¹² Consistent with the CsA results, treatment of tumor-bearing mice with AR-A014418 caused loss of NFATc2 expression and a significant retardation of tumor growth over time, reaching statistical significance after 2 weeks (Supplementary Figure 4C). Moreover, loss of NFATc2 expression abrogated p15^{INK4b} silencing and restored expression of this tumor suppressor gene and cancer defense mechanism in all animals, as shown by the decrease in tumor growth (Supplementary Figure 4D and E).

Taken together, in congruency with our biochemical and molecular analysis, these results showed that promotion of pancreatic cancer growth by NFATc2 involves silencing of the p15^{INK4b} tumor suppressor pathway. In addition, our xenograft tumor models revealed that inactivation of NFATc2 rescues p15^{INK4b} and blocks tumor growth in vivo. Thus, our results indicate that specific targeting of oncogenic NFATc2 signaling and transcription can restore the function of a critical cellular defense mechanism against mitogenic stimuli and therefore NFAT inactivation might be an attractive approach in the treatment of pancreatic cancer.

Discussion

Here we report a novel mechanism of p15^{INK4b} tumor suppressor pathway disruption, which is operative in pancreatic cancer and links genetic activation of the oncogenic NFATc2 transcription factor with growth promotion. NFATc2 was originally identified as a master regulator of gene expression during T-lymphocyte activation.¹⁹ It determines cell fate decisions and adaptation in numerous cell systems inside and outside the immune system and controls growth and survival in different cancer types.²⁰ Ectopic expression and activation of NFATc2 are frequently found in human malignancies and in particular in those with an aggressive and fast-growing phenotype.²⁰ Genetic alterations of the NFATc2 gene have been reported in several cancers of epithelial and nonepithelial origins. For instance, chromosomal translocation of NFATc2 and in-frame fusion with the EWSR1 oncogene have been found in some forms of Ewing sarcomas, a highly aggressive bone malignancy affecting children.²¹ In addition, the NFATc2 gene resides in the critical minimal region of genome amplification in breast and colon cancer and is highly amplified in a significant portion of pancreatic cancers, as revealed by genomic DNA-chip hybridization experiments.²² Consistent with chromosomal amplification of the factor in pancreatic carcinogenesis, we found induction of NFATc2 in premalignant PanIN precursor lesions and with increased frequency in the nuclei of invasively growing tumor cells.

Activation of NFATc2 in pancreatic cancer cells follows mitogenic stimulation and a rise in intracellular calcium concentrations.^{11,23} We have previously shown that NFATc2 targets the c-Myc promoter for a local histone acetylation relay switch involving p300/CBP activation.¹¹ We now extend our analysis on NFATc2-regulated transcription in pancreatic cancer and show that in addition to its well-known activating functions, NFATc2 can work as a transcriptional repressor protein to promote cancer cell proliferation. Notably, we provide evidence that NFATc2 and p15^{INK4b} are inversely expressed in human pancreatic cancer and additionally show that active NFATc2 localizes to euchromatic foci and targets the p15^{INK4b} tumor suppressor gene for inducible and reversible facultative heterochromatin formation and silencing. In detail, NFATc2 binding to its DNA recognition sequence

induces local deacetylation of histone 3, allows binding of the methyltransferase Suv39H1 for H3K9 trimethylation, and finally causes stabilization of the repressor complex through recruitment of HP1 γ . Sequential ChIP experiments revealed that NFATc2-DNA binding is the first critical step in inducible p15^{INK4b} promoter repression; therefore, depletion of the transcription factor or mutational inactivation of the NFAT promoter binding element disrupted recruitment of Suv39H1 and consequently prevented H3K9 trimethylation and local chromatin condensation of the p15^{INK4b} promoter. Another essential step in p15^{INK4b} silencing is the docking of HP1 γ that interacts with and stabilizes local repressor complex formation on the promoter.

HP1 is a class of 3 multifunctional chromatin-associated adapter proteins (HP1 α , HP1 β , and HP1 γ) that prevent initiation of gene transcription by establishing a compact heterochromatin structure and/or recruiting DNA methyltransferases. In contrast to HP1 α and HP1 β , which are primarily present in constitutive heterochromatin to maintain long-term gene silencing, HP1 γ is found in heterochromatin and euchromatin, where it can contribute to both transcriptional activation and repression, depending on the nuclear context and the chromatin complexes with which it interacts.²⁴ In pancreatic cancer cells, HP1 γ is widely distributed throughout the nucleus but colocalizes with NFATc2 in euchromatic foci on growth stimulation. Its interaction with NFATc2 repressor complexes is dependent on the presence and activation of Suv39H1, requires prior H3K9 trimethylation, and is a key event for sufficient silencing of p15^{INK4b} through its ability to stabilize facultative heterochromatin structures. Facultative heterochromatin, defined by euchromatic regions that undergo transformation into compact heterochromatic-like structures, can cover an entire chromosome, span large genomic distances, or as shown here be restricted to defined promoter regions.²⁵ Increasing evidence indicates that local formation of heterochromatin structures at selected promoter sites is an important mechanism in inducible and reversible gene silencing under both physiologic conditions and in pathologic disorders.^{26,27} For instance, hypophosphorylated retinoblastoma protein represses transcription of E2F regulated S-phase genes (eg, cyclin E) through reversible transition of open euchromatin structure to closed heterochromatin foci.²⁸ Similar to NFATc2-driven silencing of the p15^{INK4b} promoter, retinoblastoma protein blocks transcription of E2F target genes through H3K9 trimethylation and subsequent recruitment of heterochromatin stabilizing adaptor proteins.²⁹ However, our findings show for the first time that promoter-restricted heterochromatin formation can also mediate inducible silencing of key tumor suppressor pathways in pancreatic cancer and thus can be used by oncogenic signaling and transcription factors to force cancer growth and progression. We also show that the oncogenic transcription factor NFATc2 takes advantage of this mechanism and stimulates growth of pancreatic cancer through inducible silencing of the p15^{INK4b} tumor suppressor and cell cycle inhibitor. Inactivation of NFATc2, on the other hand, prevents repressor complex binding and chromatin condensation at the p15^{INK4b} promoter, restores expression and function of this bona fide tumor suppressor, and finally causes tumor growth suppression in vivo.

Posttranslational modifications of transcription factors such as phosphorylation, ubiquitylation, or sumoylation commonly determine their functions. Nayak et al showed a sumoylation-mediated repressor function of NFATc1³⁰; however, we failed to confirm a requirement of NFATc2 sumoylation for p15^{INK4b} silencing (data not shown). It remains elusive whether other modifications modulate NFATc2 transcriptional activity, because our own observations indicate that phosphorylation at particular serine residues, for instance, enhances transcriptional activity (Baumgart et al, May 2011, unpublished data). The action of transcription factors is moreover controlled by partner protein recruitment. NFATc2 interacts with p300 histone methyl transferase and Elk-1 to activate c-Myc¹¹ while it recruits Suv39H1 and HP1 γ to silence p15^{INK4b}. In T cells, recruitment of different partners leads to

the induction of a distinct cytokine pattern that in turn determines the mode of immune response.³¹ Therefore, we suggest that interaction with different transcriptional partners can convert NFAT proteins from activators to site-specific repressors, which would provide an important mechanism to control and fine-tune cancer growth.

In conclusion, this study identifies NFATc2 as a transcriptional repressor protein that promotes growth of pancreatic cancer through silencing of the p15^{INK4b} master tumor suppressor pathway. We were also able to unravel the underlying mechanism of silencing and show that NFATc2 directly targets the p15^{INK4b} promoter for stepwise histone modification and local heterochromatin complex formation. Finally, we provide evidence that targeting of the calcineurin-NFATc2 pathway causes reactivation of the p15^{INK4} tumor suppressor pathway and thus might be an attractive novel strategy in pancreatic cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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S.B. and E.G. equally contributed to the manuscript and should be considered first authors.

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Abbreviations used in this paper

ChIP	chromatin immunoprecipitation
CsA	cyclosporin A
H3K9-me	histone 3 lysine 9 trimethylation
HP	heterochromatin protein
NFAT	nuclear factor of activated T cells
PanIN	pancreatic intraepithelial neoplasia
PCR	polymerase chain reaction
siRNA	small interfering RNA

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Figure 1.

NFATc2 is widely expressed in human pancreatic cancer. (A) Immunohistochemical stainings of NFATc2 in sections of human pancreatic tissues to show that overexpression of NFATc2 is induced in PanIN-2 lesions and is highest in invasively growing cancers. (B) Classification of pancreatic cancers according to NFATc2 expression and localization. (C) Western blot analysis illustrates NFATc2 expression levels in different epithelial pancreatic cancer cell lines. (D) Quantitative real-time PCR to analyze the NFATc2 messenger RNA expression levels in different pancreatic cancer cells. The human cyclophilin gene was used as housekeeping gene control. Highest expression levels of NFATc2 were detected in PaTu8988t and IMIM-PC1 cells.

Figure 2.

NFATc2 promotes growth of pancreatic cancer. (*A* and *B*) Proliferation of (*A*) PaTu8988t and (*B*) IMIM-PC1 cells after genetic depletion of NFATc2 in the presence or absence of ionomycin ($0.5 \mu\text{mol/L}$) was assessed by [^3H]thymidine incorporation. Successful knockdown was shown by immunoblotting. (*C* and *D*) Suit007 pancreatic cancer cells stably expressing wild-type NFATc2 were subjected to (*C*) [^3H]thymidine incorporation assay or (*D*) seeded in soft agar. After 10 days, colonies were enumerated. Data are representative of triplicate experiments and displayed $\pm\text{SD}$.

Figure 3.

NFATc2 blunts p15^{INK4b} expression in vitro and in vivo. (A) Female athymic nude mice were subcutaneously injected with Suit028 cells stably expressing NFATc2 (n = 6) or mock control cells (n = 6). Tumor sizes were measured weekly, and tumor volumes were determined after 5 weeks. Bars represent mean tumor volumes $\pm$ SEM. **P* = .04. Proteins from xenograft tissues were stained for HA-NFATc2 to prove successful transduction as well as for NFATc2 to confirm overexpression of NFATc2 in Suit028 cells with endogenous low levels of NFATc2. (B) Immunohistochemical analysis to show that NFATc2 and p15^{INK4b} are inversely expressed in Suit028 tumors. (C) Western blot analysis and quantitative real-time PCR to show the reduced p15^{INK4b} expression in NFATc2-

overexpressing Suit007 cells. (D) PaTu8988t cells were transfected with NFATc2 siRNA or control siRNA and subjected to Western blot analysis and quantitative real-time PCR to confirm the induction of p15^{INK4b} expression after NFATc2 knockdown. (E) Immunohistochemical analysis of serial sections of human pancreatic tissues to show the inverse expression of NFATc2 and p15^{INK4b} in normal pancreatic tissue, precursor PanIN lesions, and pancreatic cancer.

Figure 4.

NFATc2 binds to the p15^{INK4b} promoter to mediate its silencing. (A) ChIP analysis using a specific NFATc2 antibody was performed in PaTu8988t cells after 1 hour of treatment with 10% fetal calf serum (FCS). In vivo binding of NFATc2 to the proximal p15^{INK4b} promoter was determined by quantitative real-time PCR using primers specific for the wild-type (wt) p15^{INK4b} promoter region harboring the NFATc2 binding site. (B) PaTu8988t cells were transfected with the wt p15^{INK4b} promoter construct along with increasing amounts of wt NFATc2. Firefly luciferase activities were normalized to Renilla luciferase activity and expressed as fold induction relative to the activity of untreated p15^{INK4b} promoter. (C) Schematic representation of the -751/+75 human full-length p15^{INK4b} promoter construct (wt p15^{INK4b}) and the indicated deletion constructs (p15^{INK4b} del I-III). The GGAAA NFAT consensus sequence is depicted in the wt p15^{INK4b} promoter construct and also displays the NFAT binding site mutation shown as p15^{INK4b} NFAT-mut. (D) PaTu8988t cells were transfected with the wt p15^{INK4b} or the indicated deletion constructs (p15^{INK4b} del I-III), and responsiveness was measured in the presence or absence of NFATc2. Reporter

gene activities are expressed as fold induction related to the control transfected p15^{INK4b} promoter constructs. (E) DNA pull-down experiment to characterize the binding of NFATc2 on the proximal p15^{INK4b} promoter using nuclear extracts from PaTu8988t cells along with either wt p15^{INK4b} or p15^{INK4b} NFAT-mut oligonucleotide sequences. NFATc2 binding was analyzed by immunoblotting. (F) The luciferase activities of the wt p15^{INK4b} and p15^{INK4b} NFAT-mut promoter constructs in PaTu8988t cells were measured in the presence or absence of NFATc2. Reporter gene activities are expressed as fold activation of the p15^{INK4b} promoter constructs relative to the induction without NFATc2.

Figure 5.

NFATc2 is required for Suv39H1-mediated H3K9 trimethylation and subsequent silencing of the p15^{INK4b} promoter. (A) ChIP analysis was performed in PaTu8988t cells following 1 hour of treatment with 10% fetal calf serum (FCS) using specific NFATc2, H3K9-me, and Suv39H1 antibodies. (B) (Left panel) Coimmunoprecipitation to show the physical interaction between endogenous Suv39H1 and exogenous HA-NFATc2 proteins. (Right panel) DNA pull-down experiment was performed to confirm the requirement of the NFAT binding site for interaction and binding of NFATc2 and Suv39H1 on the p15^{INK4b} promoter. PaTu8988t nuclear lysates were incubated with either wild-type (wt) p15^{INK4b} or p15^{INK4b} NFAT-mut oligonucleotide sequences and subsequently subjected to immunoblotting. (C)

Reduced NFATc2, Suv39H1 (*left panel*), and H3K9-me (*right panel*) binding to the wt p15^{INK4b} promoter region following transient silencing of NFATc2 in the absence of serum was shown by ChIP assay. Successful knockdown of NFATc2 was confirmed by immunoblotting. H3K9-me values were normalized to native H3. (*D* and *E*) Suv39H1-dependent trimethylation of the wt p15^{INK4b} promoter on H3K9 was assessed by ChIP analysis on treatment of PaTu8988t cells with 400 nmol chaetocin to block methyltransferase activity of Suv39H1 (*D*) or transfection of Suv39H1 siRNA under serum-free conditions (*E*). Successful treatment and knockdown were evidenced by immunoblotting. (*F*) IMIM-PC1 cells were transfected with wt p15^{INK4b} promoter along with or without NFATc2 and subjected to luciferase reporter assay following treatment with chaetocin in the absence of serum to show that the NFATc2-mediated silencing of the p15^{INK4b} promoter is dependent on the methyltransferase activity of Suv39H1.

Figure 6.

HP1 γ stabilizes the repression complex to silence p15^{INK4b} expression. (A) Immunofluorescence staining was performed on transfection of PaTu8988t cells with HA-NFATc2 and GFP-HP1 γ constructs and 1 hour of stimulation with 10% fetal calf serum (FCS). Arrows indicate the colocalization of HA-NFATc2 (red signal) and GFP-HP1 γ (green signal) in euchromatic foci (weak 4,6-diamidino-2-phenylindole [DAPI] staining within the nucleus) of the nucleus. Nuclei were visualized by DAPI (blue signals) staining. (B) Coimmunoprecipitation to show the Suv39H1 dependency for NFATc2 and HP1 γ physical interaction in PaTu8988t cells. (C) PaTu8988t cells were subjected to ChIP analysis after knockdown of Suv39H1 to confirm the requirement of Suv39H1 for HP1 γ binding to

the wild-type (wt) p15^{INK4b} promoter. (D) DNA pull-down experiment showing the destabilization of NFATc2 and Suv39H1 binding to the wt p15^{INK4b} promoter after knockdown of HP1 γ . Successful knockdown is shown by Western blot analysis (*lower panel*). (E) ChIP analysis was performed to determine HP1 γ (*left panel*) or NFATc2, Suv39H1, and H3K9-me (*right panel*) binding to the wt p15^{INK4b} promoter following silencing of HP1 γ . Successful knockdown was confirmed by Western blotting. (F) Reporter gene assay to prove that the abolishment of HP1 γ expression reverses p15^{INK4b} silencing by NFATc2. Reporter gene activities were expressed as fold luciferase activity related to Renilla luciferase activity $\pm$ SD.

Figure 7.

Pharmacologic targeting of NFATc2 signaling reduces growth of pancreatic cancer and allows re-expression of p15^{INK4b} in vivo. (*A* and *B*) IMIM-PC1 cells bearing nude mice were treated intraperitoneally with either 5 mg/kg of the calcineurin inhibitor CsA (n = 5) or 75% dimethyl sulfoxide (DMSO) as a vector control (n = 7). Mice were killed after a treatment period of 2 weeks and tumors were extracted. (*A*) The mean tumor volume of DMSO- and CsA-treated mice normalized to vector control $\pm$ SEM. **P* = .01. (*B*) Messenger RNA was isolated from IMIM-PC1 tumor tissues and quantitative real-time polymerase chain reaction was performed to show the induction of p15^{INK4b} messenger RNA expression following pharmacologic inhibition of NFATc2 shuttling. Mouse 1 was arbitrarily compared with mouse 3 and mouse 2 with mouse 4, respectively.