NEOPLASIA
www.neoplasia.com

Volume 15 Number 1 January 2013 pp. 11–22 11

WNT5A-NFAT Signaling Mediates Resistance to Apoptosis in Pancreatic Cancer[1,2]

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Abstract

INTRODUCTION: WNT5A belongs to the Wnt family of secreted signaling molecules. Using transcriptional profiling, we previously identified WNT5A as target of the antiapoptotic transcription factor CUX1 and demonstrated high expression levels in pancreatic cancer. However, the impact of WNT5A on drug resistance and the signaling pathways employed by WNT5A remain to be elucidated. *OBJECTIVES:* This project aims to decipher the impact of WNT5A on resistance to apoptosis and the signaling pathways employed by WNT5A in pancreatic cancer. *METHODS:* The impact of WNT5A and its downstream effectors on tumor growth and drug resistance was studied *in vitro* and in xenograft models *in vivo*. Tissue microarrays of pancreatic cancer specimens were employed for immunohistochemical studies. *RESULTS:* Knockdown of WNT5A results in a significant increase in drug-induced apoptosis. In contrast, overexpression of WNT5A or addition of recombinant WNT5A mediates resistance to apoptosis *in vitro*. In our attempt to identify downstream effectors of WNT5A, we identified the transcription factor nuclear factor of activated T cells c2 (NFATc2) as transcriptional target of WNT5A signaling. NFATc2 confers a strong antiapoptotic phenotype mediating at least in part the effects of WNT5A on drug resistance and tumor cell survival. *In vivo*, WNT5A expression leads to resistance to gemcitabine-induced apoptosis in a xenograft model, which is paralleled by upregulation of NFATc2. Both WNT5A and NFATc2 proteins are highly expressed in human pancreatic cancer tissues and their expression levels correlated significantly. *CONCLUSION:* We identified the WNT5A-NFATc2 axis as important mediator of drug resistance in pancreatic cancer.

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Abbreviations: NFAT, nuclear factor of activated T cells; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; MTA, multiple tissue array; siRNA, small interfering RNA; PARP, poly(ADP-ribose) polymerase

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[1]This work was supported by grants from the Deutsche Forschungsgemeinschaft (to P.M. and C.P.), Deutsche Krebshilfe (to P.M.), and Behring-Roentgen Foundation (to P.M.) and by European Commission FP7 grant Health-F2-2010-256974 (EPC-TM-Net to T.M.G. and P.M.). This publication reflects only the authors' views. The European Community is not liable for any use that may be made of the information herein. The authors declare no conflict of interest.

[2]This article refers to supplementary materials, which are designated by Figures W1 to W9 and are available online at www.neoplasia.com.

Received 8 August 2012; Revised 25 November 2012; Accepted 29 November 2012

DOI 10.1593/neo.121312

Introduction

The Wnt family of proteins are secreted cysteine-rich glycoproteins comprising 19 highly conserved members [1] that have been shown to play a pivotal role in embryogenesis, development, and tissue homeostasis. Wnt proteins lack transmembrane domains, are posttranslationally modified by glycosylation [2], and are known to bind to the Frizzled family of transmembrane receptors [3]. Various *Wnt* genes are differentially expressed during embryogenesis and among various tissues, including stem cell compartments of several organs [4].

In addition to their crucial role in embryogenesis and development, several Wnt ligands have been linked to carcinogenesis and tumor progression: High levels of certain Wnt family members such as WNT1, WNT3A, and WNT7A are capable of transforming various cell types into tumor cells. Other Wnt ligands such as WNT4, WNT5A, and WNT6, however, fail to induce cellular transformation [5].

The role of WNT5A in tumorigenesis remains ambiguous. In hematopoietic cells as well as breast and renal cancers, WNT5A has been shown to inhibit tumor cell proliferation [6–8] and was associated with a good prognosis [9]. However, accumulating data in other tumor types indicate that increased WNT5A expression may actually promote cancer progression. For example, WNT5A has been demonstrated as potent enhancer of cell motility and invasiveness in melanomas [10] and is highly expressed in various cancers of the lung, stomach, and prostate [11–13].

The downstream signaling events induced by WNT5A also remain controversial and may be cell type–dependent [14]. Most Wnt members signal through Frizzled receptors and activate the *dishevelled* gene, resulting in the inhibition of glycogen synthase kinase-3 beta (GSK-3β) activity and the subsequent stabilization of its target β-catenin, which results in altered gene transcription. In contrast to this "canonical" pathway, other Wnt members may also signal through "noncanonical" pathways such as the Wnt-calcium pathway involving activation of phospholipase C, protein kinase C (PKC), and calmodulin-dependent protein kinase II (CaMKII) or the planar cell polarity pathway involving cytoskeletal reorganization and activation of Rac/Rho GTPases [14–17].

In many cell types, WNT5A has been described to signal through noncanonical Wnt signaling pathways, although it also has the potential to activate the canonical Wnt signaling pathway depending on the receptor context [14,16,17]. In addition to signaling through the Frizzled receptors, WNT5A may activate receptor tyrosine kinases such as Ror2 and Ryk, which are able to inhibit canonical Wnt signaling [18].

Interestingly, in several cell types such as osteoblasts, mammary epithelial cells, and endothelial cells, a link between WNT5A and the nuclear factor of activated T cells (NFAT) transcription factor family has been proposed [9,19,20]. The NFAT family comprises four members of calcium/calcineurin-regulated proteins particularly recognized for their central roles in gene regulation during T-lymphocyte activation [21]. However, a multitude of studies have demonstrated that NFAT proteins are also expressed in cells outside the immune system, and emerging evidence indicates a key role for two NFAT members, NFATc1 and NFATc2, during carcinogenesis by regulating crucial aspects of neoplastic transformation and tumor progression [21]. Both isoforms are frequently overexpressed in epithelial malignancies, which is associated with a highly malignant phenotype [22].

Recently, we identified WNT5A as an important downstream effector of the homeodomain transcription factor CUX1, also known as CUTL1 [16]. We had shown before that CUX1 mediates tumor invasion and tumor cell survival in solid tumors including pancreatic cancer [23,24]. On the basis of these data, it was the aim of this study to characterize the role of the CUX1 target WNT5A on tumor cell survival and drug resistance and to decipher the signaling events downstream WNT5A.

Materials and Methods

Material and Cell Lines

ImimPC1 cells were kindly provided by F. X. Real (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain) [25]. The PaTu-8988t cell line was received from the German Collection of Cell Lines (DSMZ, Braunschweig, Germany). Both cell lines were derived from human primary ductal adenocarcinomas (PaTu-8988t) or liver metastases of pancreatic ductal adenocarcinomas (ImimPC1) and carry activating K-Ras mutations. Stable PaTu-8988t–NFATc2 cells are a kind gift from V. Ellenrieder (University of Marburg). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Darmstadt, Germany) supplemented with 10% fetal calf serum (Gibco) and 250 μg/ml gentamicin (PAA, Cölbe, Germany). The amphotropic packaging cell line LinX was maintained in Dulbecco's modified Eagle's medium, 10% fetal calf serum, gentamicin, and 100 μg/ml hygromycin B (Roth, Karlsruhe, Germany). All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. Gemcitabine was obtained from Lilly (Bad Homburg, Germany) and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and rWnt5A from R&D Systems (Wiesbaden-Nordenstadt, Germany). Reagents were used at the following working concentrations: TRAIL, 15 ng/ml; gemcitabine, 80 μg/ml; rWNT5A, 500 ng/ml; doxycycline (Sigma-Aldrich, Taufkirchen bei München, Germany), 4 μg/ml; puromycin (Sigma), 1.25 μg/ml; G418 (PAA), 100 μg/ml.

Plasmids and Small Interfering RNA

The open reading frame (ORF) of hWNT5A was amplified by polymerase chain reaction (PCR) from the human cDNA clone MGC:71588 IMAGE:30346200 and cloned into the Tet-repressible expression vector pBig2r [26], which was a kind gift of J. R. Hall, using the *Kpn*I and *Spe*I restriction sites. The pBig2r-WNT5A and empty pBig2r vectors were transfected into PaTu-8988t cells. For generation of stable clones, cells were cultured in the presence of 400 μg/ml hygromycin B. Stable expression of WNT5A was confirmed after incubation with doxycycline (4 μg/ml for 24 hours) through immunoblot analysis. Stable ImimPC1 cells were generated using a retroviral system. For retroviral expression, WNT5A was amplified and cloned into pENTR vector using pENTR/D-TOPO Cloning Kit (Invitrogen, Darmdstadt, Germany) and recombined into a Gateway competent pQCXIP vector (kind gift from T. Stiewe, University of Marburg). The luciferase reporter constructs pNFAT and *cis*NFAT were kindly provided by V. Ellenrieder (University of Marburg). The pGL3-Enhancer as control was purchased from Promega (Mannheim, Germany). Small interfering RNA (siRNA) purchased from Ambion (Darmstadt, Germany) against WNT5A or NFATc2 was transfected at a final concentration of 10 nM using RNAiMAX (Invitrogen) according to the manufacturer's protocol. All results could be verified by two independent silencing sequences.

Retroviral Infection

To produce retroviruses, we transfected LinX packaging cells with 5 μg of retroviral vectors; 48 and 72 hours after transfection, the retrovirus-containing supernatant was harvested, filtered, and supplemented with 8 μg/ml polybrene (Sigma). Target cells were transduced by spin infection at 1500 rpm, 37°C for 1 hour and selected with puromycin and G418.

Luciferase Reporter Assays

ImimPC1 and PaTu-8988t cells seeded in 24-well plates were transfected with luciferase plasmids using Transfast transfection reagent (Promega) according to the manufacturer's instructions and harvested 24 hours after transfection. Luciferase assays were performed using the Luciferase-Reporter Assay System (Promega), as described previously [23].

RNA Isolation, cDNA Synthesis, and Real-Time PCR

RNA isolation and first-strand cDNA synthesis were performed using peqGold RNAPure (Peqlab, Erlangen, Germany) and the Omniscript RT-Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Quantitative real-time PCR analysis was performed using the ABI PRISM 7500 Sequence Detector System and the SYBR Green PCR Master Mix Kit (Applied Biosystems, Darmstadt, Germany). The ribosomal protein RPLPO (NM_001002) was used as internal standard.

Immunoblot Analysis

Cells were lysed in RIPA buffer supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany). Nuclear and cytoplasmic fractions were performed using the ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas, St. Leon-Rot, Germany). Thirty micrograms of lysates was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore, Darmstadt, Germany). Immunoblots were probed with primary antibodies against WNT5A (R&D Systems), NFATc2 (Santa Cruz Biotechnology, Heidelberg, Germany), poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology, Frankfurt am Main, Germany), ORC-2 (Santa Cruz Biotechnology), phospho-β-catenin (Ser-37, Thr-41; Millipore) and β-actin (Sigma). Peroxidase-conjugated secondary antibodies against mouse or rabbit were obtained from Amersham (Freiburg, Germany) and goat from Sigma. Blots were detected by Western Lightning ECL (PerkinElmer, Rodgau, Germany).

Apoptosis Assays

Histone-associated DNA fragments were quantified using Cell Death Detection ELISA^{PLUS} (Roche) according to the manufacturer's instructions. Caspase-3 and -7 activity was measured using Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions. The luminescence is proportional to the amount of caspase activity. PARP cleavage was assessed with a specific antibody detecting cleaved PARP (Cell Signaling).

Fluorescence-activated Cell Sorting (FACS)

FACS analysis was used to determine DNA fragmentation of propidium iodide–stained nuclei as described before [24,27]. In brief, cells were stained with propidium iodide after RNase treatment and analyzed in a FACScan flow cytometer (BD Biosciences, Heidelberg, Germany). Cell cycle was analyzed using the FlowJo software (Tree Star, Ashland, OR). Apoptosis assays by annexin V staining were performed according to the manufacturer's instructions (BD Biosciences).

Immunohistochemistry

For expression analysis of WNT5A and NFATc1, a multiple tissue array (MTA) that was generated in the Department of Pathology, Technical University of Dresden according to the guidelines of the local ethics committee was used. Immunohistochemical analysis was performed as previously described [24]. In short, paraffin sections were stained after antigen retrieval (microwave in antigen unmasking solution; Vector Laboratories, Burlingame, CA) with rabbit polyclonal anti-WNT5A (1:100; Abcam, Cambridge, United Kingdom) or NFATc2 (1:200; Abcam). Antibody binding was visualized using a biotinylated secondary antibody, avidin-conjugated peroxidase (ABC method; Vector Laboratories), and 3,3′-diaminobenzidine tetrachloride as a substrate and hematoxylin as counterstain. The MTAs were analyzed semiquantitatively as – (negative), + (25–50% positive tumor cells), ++ (50–75% positive tumor cells), +++ (>75% positive tumor cells).

Animals and Xenografts

Athymic female nude mice were purchased from Charles River (Sulzfeld, Germany). Mice were housed in a climate-controlled SPF facility. All animal experiments were approved by the local government authorities and were performed according to the guidelines of the animal welfare committee.

Nude mice were injected subcutaneously with 1×10^6 ImimPC1 cells dispersed in 0.1 ml of normal saline at each flank. Six mice per group were injected with cells stably transfected with either WNT5A or empty vector. When tumors reached a volume of approximately 100 mm^3, mice were randomized into two groups and injected twice a week with gemcitabine [25 ng/g body weight intraperitoneally (i.p.) dissolved in phosphate-buffered saline (PBS)] or PBS only as control. Tumor growth was measured using a caliper twice weekly for a total of 20 days.

Statistical Analysis

Statistical analysis of tumor growth was performed with GraphPad Prism software using two-way analysis of variance. All numerical data are reported as the means ± SD. Correlation of WNT5A and NFAT expression on the tissue microarrays was calculated with IBM SPSS19 (IBM, Ehningen, Germany) using Spearman-Rho analysis.

Results

Knockdown of WNT5A Enhances Drug- and TRAIL-Induced Apoptosis in Pancreatic Cancer Cell Lines

We first evaluated the effect of WNT5A on basal as well as drug- and TRAIL-induced apoptosis in pancreatic cancer cell lines. As apoptosis-inducing drug, we used the nucleoside analog gemcitabine, which is commonly applied in pancreatic cancer patients. Knockdown of WNT5A by specific siRNA significantly induced basal as well as gemcitabine-induced apoptosis rates in PaTu-8988t cells, as determined by assaying different hallmarks of apoptosis including histone-associated DNA fragmentation using a specific ELISA (Figure 1*A*) and effector caspase-3/7 activity using a specific assay that measures cleavage of a luminogenic caspase-3/7 substrate (Figure 1*B*), cleavage of PARP (Figure 1*C*), and annexin V staining (Figure 1*D*). The effects of WNT5A knockdown on apoptosis could be corroborated in other pancreatic cancer cell lines such as ImimPC1 cells, as determined by PARP cleavage (Figure W1*A*). In addition, all assays could be confirmed by the use of two independent WNT5A-specific siRNA oligonucleotides, which both led to a marked knockdown of WNT5A protein expression and had been validated previously (Figure W1, *B* and *C*) [16].

Chemotherapeutic drugs characteristically trigger apoptosis through induction of DNA damage and activation of the intrinsic apoptosis pathway. By comparison, TRAIL is known to induce the death receptor pathway of apoptosis through binding to its cognate surface

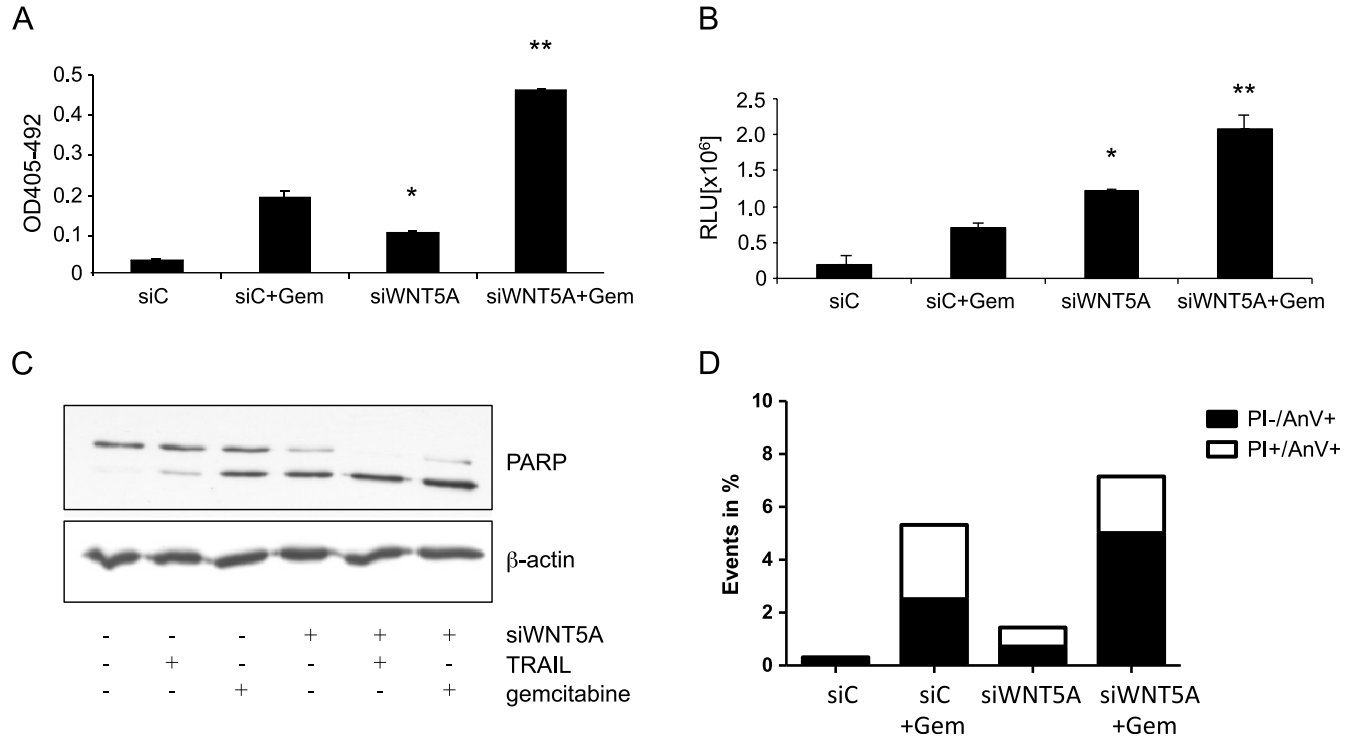

Figure 1. Knockdown of WNT5A induces apoptosis. (A) PaTu-8988t cells were transiently transfected with WNT5A siRNA (siWNT5A) or control siRNA (siC), and apoptosis was induced with gemcitabine for 24 hours. Apoptosis was determined by quantification of histone-bound DNA fragmentation using a specific ELISA. (B) Apoptosis after knockdown of WNT5A by siRNA and apoptosis induced by gemcitabine, as assessed by a caspase-3/7 activation assay. Percentage of apoptotic cells is shown as mean of three independent experiments ± SD. *$P < .05$ as compared with control cells; **$P < .05$ as compared with drug-treated control cells. (C) PARP cleavage after WNT5A knockdown by siRNA and gemcitabine (48 hours)– or TRAIL (12 hours)–induced apoptosis was assayed by a PARP-specific antibody detecting the cleaved PARP (lower band). To control for equal loading, the blots were reprobed with β-actin antibody. (D) Annexin V FACS analysis ± WNT5A siRNA (siWNT5A) or control siRNA (siC) ± gemcitabine (48 hours). Apoptosis was determined by Annexin V (AnV) and propidium iodide (PI) staining and subsequent FACS analysis. Percentage of early apoptotic cells (PI−/AnV+) and late apoptotic/necrotic cells (PI+/AnV+) is shown as percentage of events. Data are representative for three independent experiments.

receptors (extrinsic pathway). Similar to gemcitabine-induced apoptosis, knockdown of WNT5A sensitized to apoptosis induced by TRAIL, as determined by FACS sub-G1 analysis (Figure W2, *A* and *B*), PARP cleavage (Figures 1*C* and W1*A*), and caspase-3/7 activity assays (Figure W3) in PaTu-8988t and ImimPC1 cells. These results indicate that down-regulation of WNT5A by siRNA effectively enhances the sensitivity of pancreatic cancer cell lines to apoptosis induced by different stimuli that trigger apoptosis through extrinsic or intrinsic pathways.

Expression of WNT5A Protects from Drug-Induced Apoptosis

To exclude the possibility that only knockdown of endogenous WNT5A affects apoptosis, we examined whether overexpression of WNT5A protects from drug-induced apoptosis. For that purpose, we generated PaTu-8988t cells stably expressing WNT5A in a Tet-repressible manner: In these cells, addition of doxycycline decreased stably overexpressed WNT5A to normal levels (Figure W4*A*). Using this repressible cell system, we could show that overexpressed WNT5A significantly decreased apoptosis induced by incubation with gemcitabine for 24 or 48 hours (Figure 2*A*). Similar to these findings, addition of exogenous recombinant WNT5A led also to a significant protection from gemcitabine-induced apoptosis (Figure 2*B*) that could be corroborated in both PaTu-8988t and ImimPC1 by PARP cleavage assays (Figure W4, *B* and *C*). These data indicate that WNT5A is able to rescue from drug-induced apoptosis. A similar trend was observed in TRAIL-induced apoptosis, which, however, did not reach statistical significance (Figure W4*D*).

WNT5A Rescues from Drug-Induced Apoptosis In Vivo

To corroborate our *in vitro* findings implicating WNT5A as regulator of resistance to apoptosis *in vivo*, we used xenograft experiments in nude mice. Because ImimPC1 cells are known to form subcutaneous xenograft tumors, we generated ImimPC1 cell lines stably overexpressing WNT5A or empty control vectors and injected them into nude mice. After tumors reached a volume of approximately 100 mm^3, i.p. treatment with gemcitabine or PBS was started. WNT5A overexpression significantly abolished the growth inhibitory effect of gemcitabine treatment compared to control xenografts (Figure 2*C*). In contrast, in the absence of gemcitabine, tumor growth was not significantly accelerated by WNT5A (Figure W4*E*). In addition, the impact of WNT5A on gemcitabine-induced growth inhibition was paralleled by a strong up-regulation of antiapoptotic proteins such as BCL2, supporting the effect of WNT5A on resistance to drug-induced apoptosis (Figure 2*D*).

WNT5A Regulates NFATc2 mRNA

WNT5A acts as secreted ligand in autocrine or paracrine manner. The intracellular signaling events following WNT5A stimulation have been controversially discussed and appear to be cell type–dependent.

Therefore, we aimed to elucidate the intracellular signaling pathways activated by WNT5A in pancreatic cancer. Previous reports suggested an interaction between WNT5A and NFAT signaling in several cell types such as osteoblasts, mammary epithelial cells, and endothelial cells [9,19,20]. Moreover, we could previously demonstrate high expression levels of NFATc2 in pancreatic cancer tissues [28]. In our search for downstream effectors of WNT5A in pancreatic cancer, we therefore investigated a possible link between WNT5A and NFATc2. After knockdown of WNT5A by siRNA in PaTu-8988t cells, we indeed observed a significant decrease of NFATc2 mRNA, as measured by quantitative real-time PCR (Figure 3*A*). These results could be confirmed on promoter level by luciferase assays: Knockdown of WNT5A leads to a marked decrease in NFATc2 promoter activity (Figure 3*B*). In contrast, incubation with recombinant WNT5A increased NFATc2 mRNA peaking after a 4-hour incubation (Figure 3*C*). Likewise, addition of WNT5A increased NFATc2 promoter activity in PaTu-8988t cells (Figure 3*D*). These results could also be confirmed in ImimPC1 cells (Figure W5).

WNT5A Regulates NFATc2 on Protein Level

To verify the regulation of NFATc2 by WNT5A on protein level, we suppressed WNT5A by siRNA and analyzed NFATc2 protein expression by Western blot. WNT5A knockdown led to a marked reduction of NFATc2 protein both in PaTu-8988t cells (Figure 4*A*) and ImimPC1 cells (Figure W6*A*). Because NFATc2 is known to shuttle from the cytoplasm to the nucleus following dephosphorylation by calcineurin, we generated nuclear extracts after knockdown of WNT5A and tested for potential effects of WNT5A on NFATc2 localization.

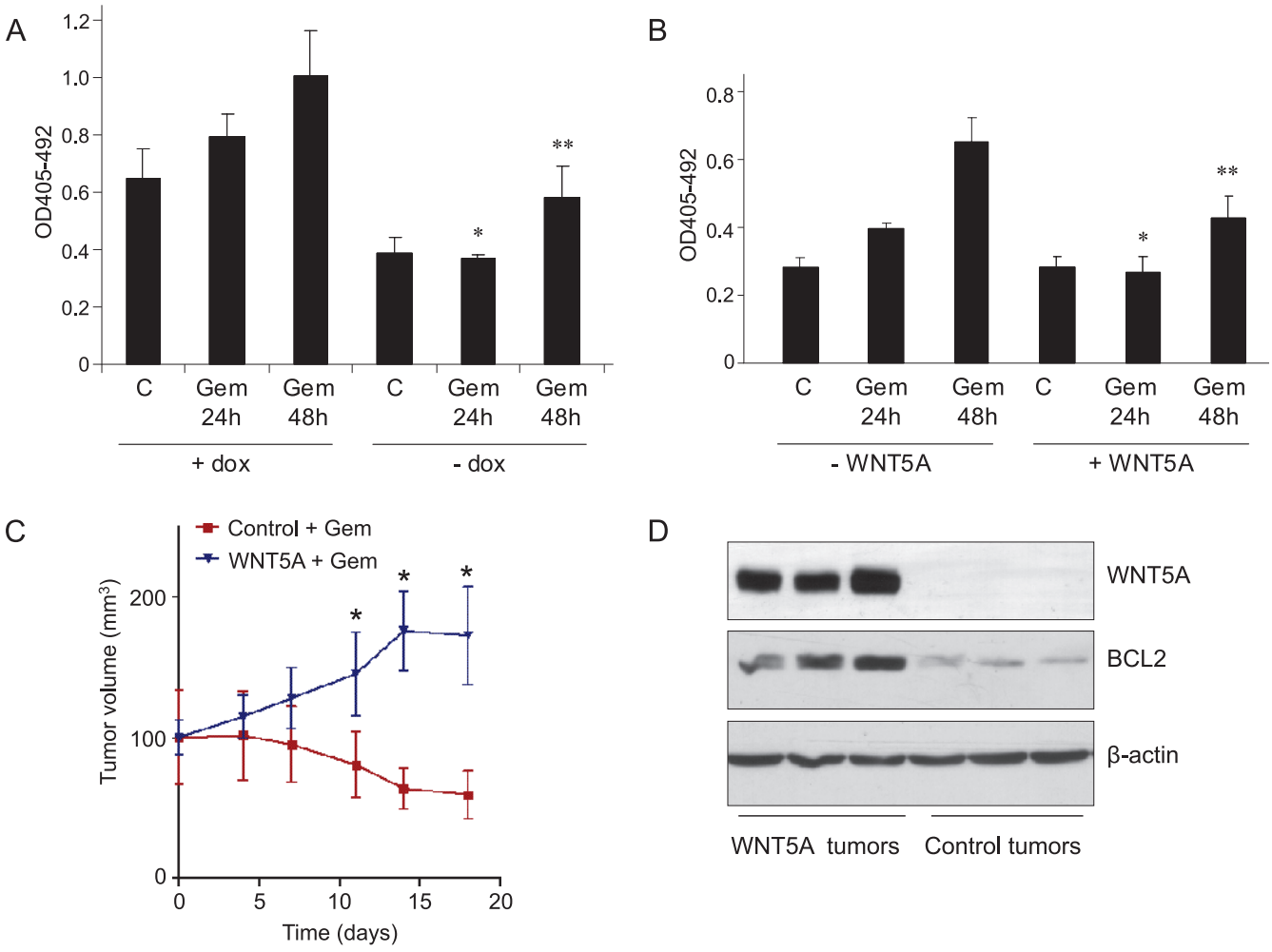

Figure 2. WNT5A rescues from drug-induced apoptosis. (A) PaTu-8988t cells with Tet-repressible WNT5A expression were incubated in the presence (low WNT5A) or absence (high WNT5A) of doxycycline (dox) and exposed to gemcitabine for 24 or 48 hours. Apoptosis was determined by quantification of histone-bound DNA fragmentation using a specific ELISA. *$P < .05$ as compared with +dox cells treated with gemcitabine for 24 hours; **$P < .05$ as compared with +dox cells treated with gemcitabine for 48 hours. (B) PaTu-8988t cells were incubated with recombinant WNT5A and exposed to gemcitabine for 24 or 48 hours. Apoptosis was determined by quantification of histone-bound DNA fragmentation using a specific ELISA. *$P < .05$ as compared with control cells treated with gemcitabine for 24 hours; **$P < .05$ as compared with control cells treated with gemcitabine for 48 hours. Data are representative for three independent experiments. (C) Tumor growth of subcutaneous ImimPC1 cells stably overexpressing WNT5A or empty control vectors ($n = 6$ each). After the tumors had reached a volume of approximately 100 mm^3, treatment with gemcitabine i.p. or PBS i.p. was started and tumor volume was determined for 20 days. *$P < .05$ as compared with control xenografts. (D) Expression of WNT5A and BCL2 in protein lysates of gemcitabine-treated WNT5A-overexpressing or mock-transfected ImimPC1 xenografts, for which material for protein lysates was available.

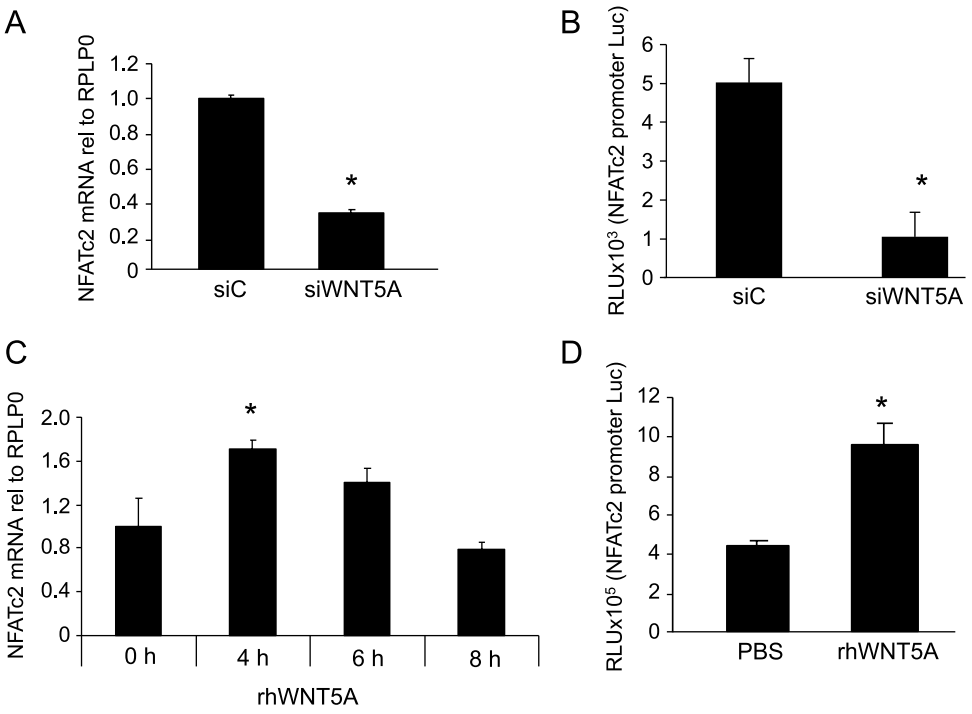

Figure 3. WNT5A induces NFATc2 mRNA. (A) PaTu-8988t cells were transiently transfected with WNT5A siRNA (siWNT5A) or control siRNA (siC). NFATc2 mRNA was determined by quantitative real-time PCR normalized to RPLPO mRNA. (B) PaTu-8988t cells ± WNT5A siRNA or control siRNA were cotransfected with a luciferase plasmid containing an NFATc2 promoter fragment. Promoter activation was measured by luciferase assay. (C) NFATc2 mRNA was determined by quantitative real-time PCR after incubation with rhWNT5A for the indicated time points. (D) NFATc2 promoter activity was determined by luciferase assay after transfection with the NFATc2 promoter luciferase plasmid and incubation with rhWNTA for 24 hours. *$P < .05$ as compared with untreated control cells. Data are representative for three independent experiments.

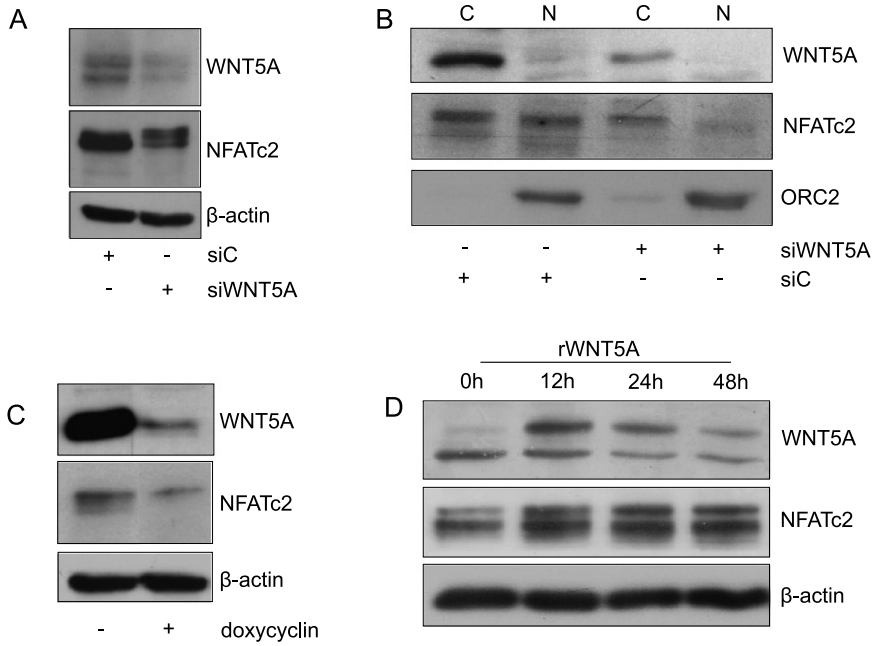

Figure 4. WNT5A induces NFATc2 protein. (A) PaTu-8988t cells were transfected with WNT5A siRNA (siWNT5A) or control siRNA (siC), and NFATc2 protein was determined by Western blot. (B) NFATc2 protein was measured in nuclear and cytoplasmic preparations from PaTu-8988t cells ± WNT5A siRNA or control siRNA. ORC2 was used as a loading control in the nuclear fractions. (C) PaTu-8988t cells with Tet-repressible WNT5A expression were incubated ± doxycycline for 24 hours. NFATc2 protein levels were detected by Western blot. (D) rhWNT5A was added to PaTu-8988t cells for various time points and NFATc2 protein levels were analyzed by Western blot.

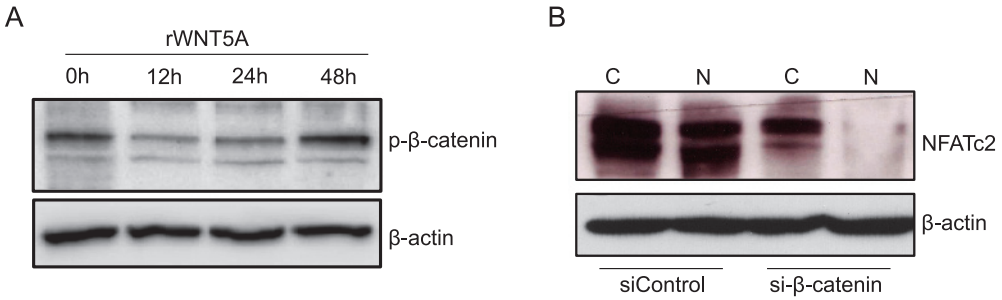

Figure 5. WNT5A regulates NFATc2 through β-catenin–dependent signaling pathways. (A) PaTu-8988t cells were incubated with rhWNT5A for the indicated time points, and transcriptionally inactive β-catenin (phosphorylated Ser-37, Thr-41) was determined using a phospho-specific antibody. (B) PaTu-8988t cells were transiently transfected with siRNA against β-catenin (si-β-catenin) or nonsilencing control (siControl), and NFATc2 protein levels were detected by Western blot after separation of nuclear (N) or cytoplasmic (C) proteins.

At baseline, NFATc2 protein levels were detected in the nucleus and cytoplasm, both of which were markedly reduced after WNT5A knockdown. Therefore, we could rule out a relevant WNT5A-dependent nuclear-cytoplasmic shuttling (Figure 4*B*).

To confirm that NFATc2 protein is upregulated after WNT5A overexpression, we used PaTu-8988t cells stably expressing Tet-repressible WNT5A. Elevated WNT5A levels in the absence of doxycycline clearly correlated with increased NFATc2 protein levels (Figure 4*C*). Likewise, stable overexpression of WNT5A in ImimPC1 cells resulted in increased NFATc2 levels (Figure W6*B*). Moreover, exogenously added recombinant WNT5A led to an increase in NFATc2 protein within 12 hours in both PaTu-8988t cells (Figure 4*D*) and ImimPC1 cells (Figure W6*C*).

In analogy to these *in vitro* findings, expression levels of the WNT5A target NFATc2 in the WNT5A overexpressing xenograft tumors described above were markedly increased (Figure W6*C*), confirming that NFATc2 is upregulated by WNT5A *in vivo*.

WNT5A Regulates NFATc2 through β-Catenin–Dependent Mechanisms

Previously, we showed that WNT5A signaling leading to enhanced migration and invasion in pancreatic cancer cell lines involves β-catenin–dependent canonical signaling mechanisms [16]. Furthermore, we would not find evidence of noncanonical, calcium-dependent mechanisms or alterations in the cell polarity pathways in pancreatic cancer cells, as suggested before by reports in other cell systems [16]. On the

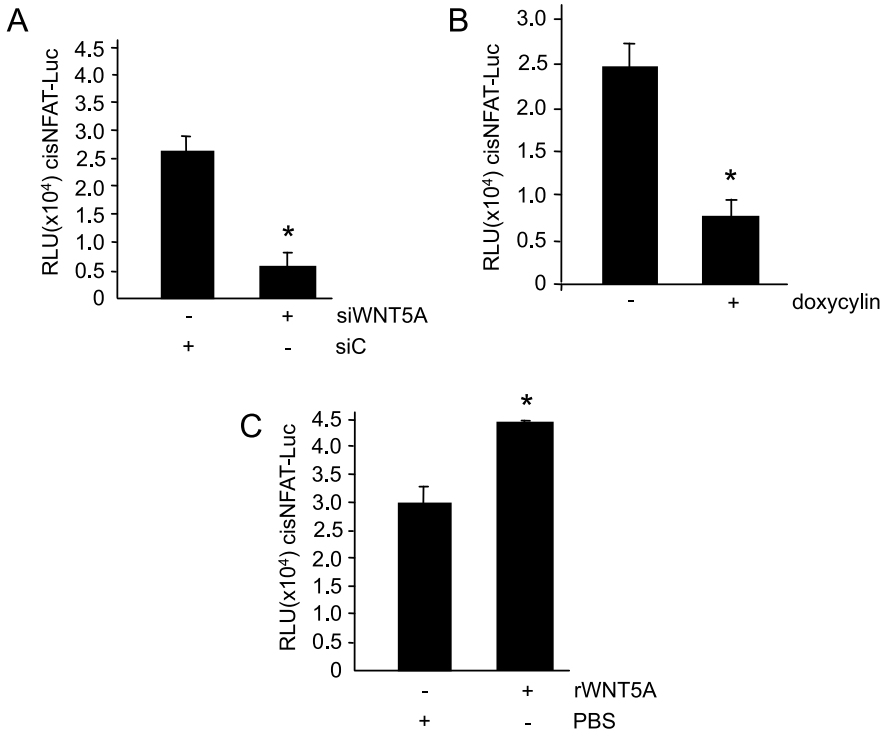

Figure 6. WNT5A induces transcriptional activation of NFATc2. (A) PaTu-8988t cells were transiently transfected with WNT5A siRNA (siWNT5A) or control siRNA (siC) and cotransfected with a *cis*NFAT luciferase plasmid (*cis*NFAT-Luc) containing an NFAT DNA binding motif. NFAT transcriptional activation was measured by luciferase assay. (B) PaTu-8988t cells with Tet-repressible WNT5A expression were transfected with *cis*NFAT-Luc ± doxycycline for 24 hours. (C) rhWNT5A was added to PaTu-8988t cells after transfection with *cis*NFAT-Luc. *$P < .05$ as compared with untreated control cells. Data are representative for three independent experiments.

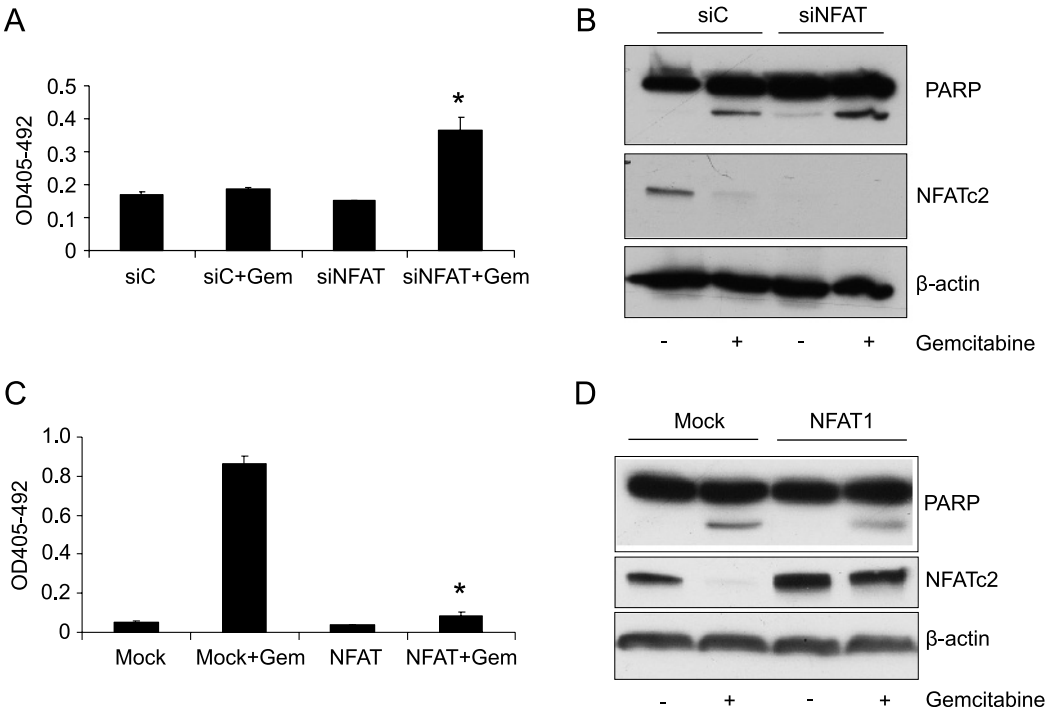

Figure 7. (A, B) NFATc2 knockdown stimulates drug-induced apoptosis. PaTu-8988t cells were transiently transfected with NFATc2 siRNA (siNFAT) or control siRNA (siC), and apoptosis was induced with gemcitabine for 48 hours. Apoptosis was determined by quantification of histone-bound DNA fragmentation by specific ELISA (A) and PARP cleavage (B). (C, D) NFATc2 mediates resistance to drug-induced apoptosis. PaTu-8988t cells stably overexpressing NFATc2 and mock-transfected control cells were incubated with gemcitabine for 48 hours, and apoptosis was assessed by ELISA (C) and PARP cleavage (D). Data are representative of three independent experiments and presented as means ± SD. *$P < .05$ as compared with control cells + gemcitabine.

basis of these data, we aimed to investigate whether the regulation of NFATc2 by WNT5A involves β-catenin–dependent signaling. Both pancreatic cancer cell lines, PaTu-8988t and ImimPC1, expressed receptors for canonical Wnt signaling such as LRP5 and various Frizzled receptors but did not or only weakly express the noncanonical receptors Ror2 or Ryk (Figure W7). Recombinant WNT5A led to a marked decrease in phosphorylated β-catenin (Figure 5*A*), which indicates its increased activity and nuclear translocation, as shown previously [16]. In addition, WNT5A enhanced β-catenin–dependent canonical transcriptional activity, as assayed by measuring TOP/FOPFlash luciferase signals (Figure W8*A*). Furthermore, knockdown of β-catenin by siRNA led to a marked decrease in both nuclear and cytoplasmic NFATc2 protein levels, indicating a β-catenin–dependent regulation of NFATc2 (Figure 5*B*).

WNT5A Regulates NFATc2 Transcriptional Activity

After having demonstrated that WNT5A induces NFATc2 on promoter, mRNA, and protein levels, we aimed to confirm that

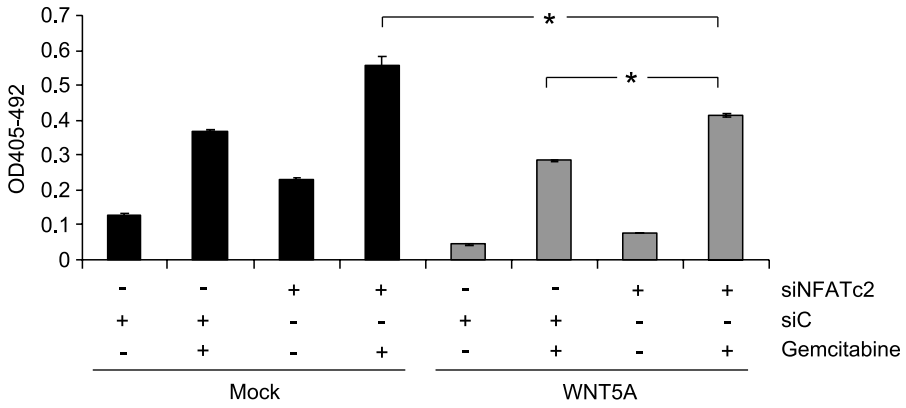

Figure 8. NFATc2 knockdown decreases WNT5A-mediated protection from apoptosis. ImimPC1 cells stably overexpressing WNT5A or empty control vector were transiently transfected with NFATc2 siRNA (siNFAT) or control siRNA (siC), and apoptosis was induced by gemcitabine. Apoptosis was determined by quantification of histone-bound DNA fragmentation by ELISA. *$P < .05$ as compared with control cells, as indicated. Data are representative for three independent experiments.

WNT5A stimulation also translates into increased NFAT-dependent transcriptional activity. For that purpose, we used a luciferase-coupled NFAT-responsive promoter fragment, *cis*NFAT-Luc. After WNT5A knockdown, NFAT transcriptional activity was significantly reduced in both PaTu-8988t (Figure 6*A*) and ImimPC1 cells (Figure W8*B*). Conversely, WNT5A overexpression (Figure 6*B*) and incubation with recombinant WNT5A led to an increase in NFAT transcriptional activity (Figures 6*C* and W8*C*).

NFATc2 Rescues from Drug-Induced Apoptosis

On the basis of these data, we were interested whether NFATc2 is able to mediate the antiapoptotic effects of WNT5A. For that purpose, we examined drug-induced apoptosis after knockdown and overexpression of NFATc2.

In analogy to the effects described for WNT5A, knockdown of NFATc2 resulted in a significant increase of gemcitabine-induced apoptosis, as assessed by histone-associated DNA fragmentation (Figure 7*A*). This was paralleled by a significant increase in PARP cleavage after NFATc2 knockdown (Figure 7*B*). Similar results were observed in ImimPC1 cells (Figure W9*A*) and with two independent NFATc2 siRNA silencing sequences (Figure W9*B*). In analogy to the previous data with WNT5A, stable overexpression of NFATc2 in PaTu-8988t cells led to a significant resistance to apoptosis induced by gemcitabine, as measured by DNA fragmentation (Figure 7*C*) and PARP cleavage (Figure 7*D*).

Interestingly, addition of gemcitabine led to a marked decrease in endogenous NFATc2 protein levels in PaTu-8988t cells (Figure 7, *B* and *D*) but not in ImimPC1 cells (Figure W9*C*). The molecular mechanism underlying this phenomenon remains to be elucidated. In analogy to the findings in gemcitabine-induced apoptosis, NFATc2 also reduced TRAIL-induced apoptosis (Figure W9, *C* and *D*).

To assess the importance of NFATc2 as downstream mediator of WNT5A-induced antiapoptotic effects, we used ImimPC1 cells stably overexpressing WNT5A and transiently reduced NFATc2 levels by siRNA (Figure 8). As expected, WNT5A reduced gemcitabine-induced apoptosis. Knockdown of NFATc2 significantly impaired but did not completely abolish the protective effect of WNT5A (Figure 8). These data indicate that NFATc2 mediates a significant proportion of the antiapoptotic actions of WNT5A in pancreatic cancer cells. However, additional effector pathways downstream WNT5A might be involved as well.

WNT5A and NFATc2 Are Coexpressed in Pancreatic Cancer Tissues

To assess the protein expression of both WNT5A and NFATc2 in pancreatic cancer tissues, we used MTAs containing 143 different pancreatic tissues. Immunohistochemical analysis was performed for both WNT5A and NFATc2. Expression levels of both proteins were evaluated independently from each other in a semiquantitative manner. Both WNT5A and NFATc2 were strongly expressed in

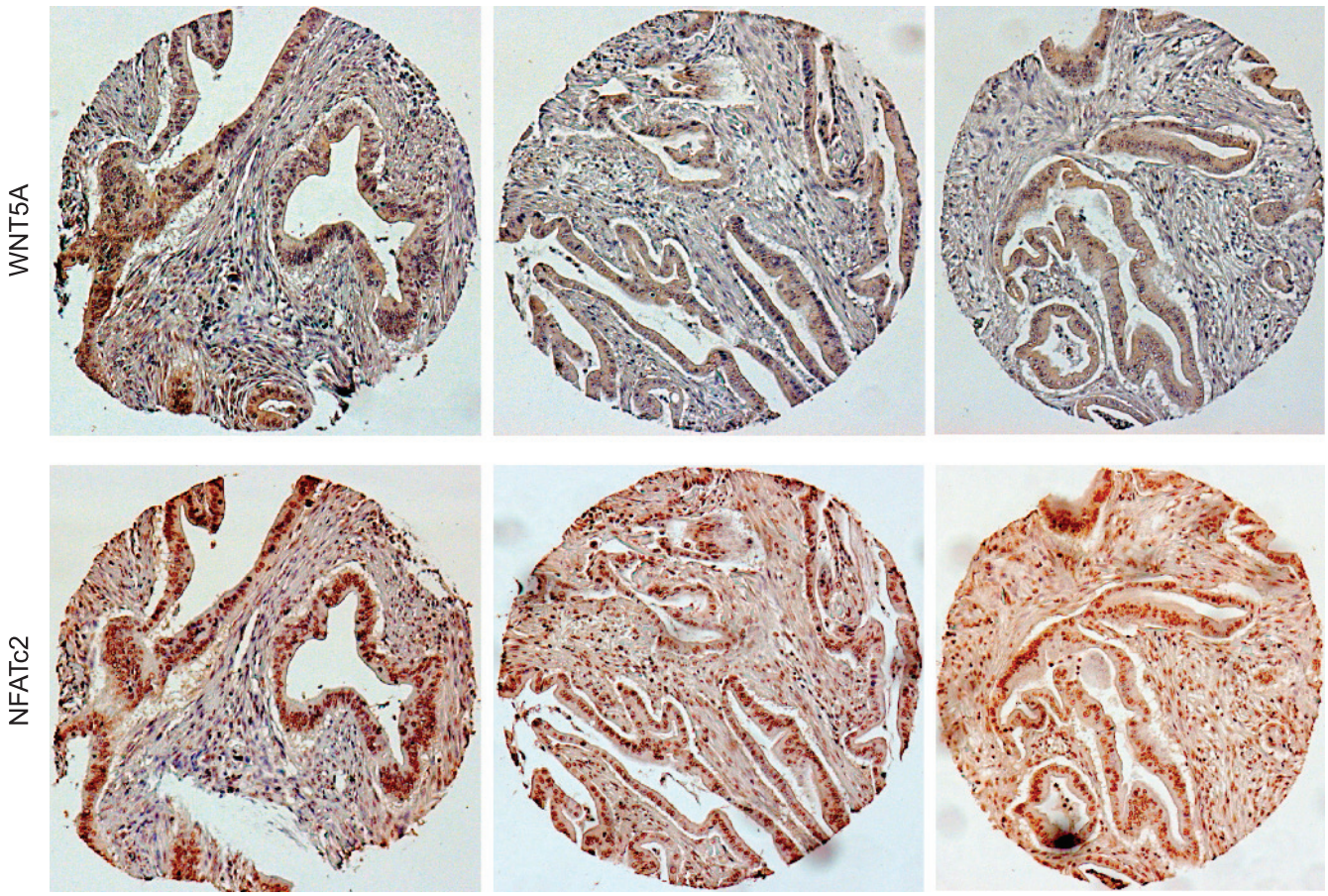

Figure 9. Representative immunohistochemical expression of WNT5A (upper row) and NFATc2 (bottom row) in two adjacent sections each of three pancreatic cancer tissues demonstrating a significant correlation between WNT5A and NFATc2 expression.

43% and 39% of the tissues, respectively. Interestingly, WNT5A and NFATc2 expression correlated significantly with each other ($P < .01$; Figure 9).

Discussion

In this study, we could demonstrate that WNT5A plays an important role in protecting pancreatic cancer cells from apoptosis induced by TRAIL and the nucleoside analog gemcitabine both *in vitro* and *in vivo*. The pro-survival effect of WNT5A was associated with transcriptional up-regulation of NFATc2 that also conferred a strong antiapoptotic phenotype in pancreatic cancer cells.

In the literature, the role of WNT5A in tumors has been a matter of controversy and the data on its effect on tumor cell survival and metastasis remain conflicting.

On the one hand, WNT5A has been shown to act as tumor suppressor by antagonizing the transforming capacities of other Wnts [29]. Furthermore, WNT5A inhibits proliferation of leukemic B cells, and WNT5A hemizygous mice have been reported to develop myeloid leukemias and B cell lymphomas [8]. In humans, epigenetic silencing of WNT5A confers a poor prognosis in patients with acute myeloid leukemia [30]. In line with these findings in hematological malignancies, WNT5A has been shown to inhibit proliferation and invasiveness of colorectal and thyroid cancer cells [31,32], supporting the notion that WNT5A might act as a tumor suppressor.

In other cancer entities, however, increased WNT5A expression is associated with tumor progression and worsened prognosis: In melanoma, WNT5A expression was an independent risk factor for reduced survival [33]. Likewise, WNT5A was correlated with aggressiveness of gastric [34] and ovarian cancers [35].

These conflicting data suggest that the effect of WNT5A on cancer progression is cell type–dependent. To date, the molecular basis underlying these cell type–specific functions of WNT5A remains elusive.

Previously, we identified WNT5A as transcriptional target of the homeodomain transcription factor CUX1 and described WNT5A as important mediator of CUX1-induced tumor cell motility and invasion [16,23]. The effect of WNT5A on tumor cell motility and metastasis was confirmed by other reports in various tumor types such as melanoma [10] and gastric cancer cells [34].

In analogy to our recent findings implicating CUX1 as major regulator of drug resistance *in vitro* and *in vivo* [24], we now present evidence implicating WNT5A as important effector pathway within the CUX1-dependent transcriptional network mediating pancreatic cancer cell survival. This is in line with several reports indicating a possible role of WNT5A in regulating cell survival and resistance to apoptosis: Vuga et al. recently showed that WNT5A increased resistance to H_2O_2-induced apoptosis in isolated fibroblasts from patients with fibrotic interstitial lung disease [36]. Furthermore, WNT5A inhibited serum starvation-induced apoptosis in HEK293 cells [37] and in osteoblasts [38]. These data, however, are contrasted by other reports showing opposing results: Liang et al. proposed that loss of WNT5A inhibits apoptosis of $CD4^+CD8^+$ thymocytes, whereas exogenous WNT5A increases apoptosis of fetal thymocytes in culture [39].

The diversity of WNT5A effects observed in different cell systems suggests that different receptor equipment and/or the recruitment of different intracellular cascades might modulate WNT5A action in a given cell type. Traditionally, WNT5A has been proposed to signal through β-catenin–independent, noncanonical cascades including the calcium-dependent or planar cell polarity pathway [3]. Interestingly, we previously failed to detect alterations in calcium-dependent signaling such as CaMKII or PKC activation, nor did we observe planar cell polarity–related signals such as c-Jun N-terminal kinase (JNK) activation following WNT5A stimulation in pancreatic cancer cells [16]. However, we found an increase in canonical signaling including GSK-3β phosphorylation and β-catenin translocation after WNT5A activation [16]. These findings are in line with other reports that WNT5A might indeed be able to stimulate β-catenin transcriptional activation, depending on a distinct receptor context that requires the presence of the Wnt receptors Frizzled-4 and low-density lipoprotein receptor-related protein 5 (LRP-5) and the absence of the recently identified Wnt receptor ROR2 [3,17].

The activation levels of β-catenin–dependent transcription following WNT5A stimulation in pancreatic cancer cells is generally moderate, though significant [16]. Therefore, we aimed to search for additional downstream effectors that might be able to mediate the profound action of WNT5A on tumor cell survival and resistance to drug-induced apoptosis in pancreatic cancer. One of the potential candidates in this context is the transcriptional regulator NFAT. The NFAT family of transcription factors comprises a group of four proteins that play a critical role in the function of many vertebrate tissues [21]. Besides their well-documented role in T cell activation, NFAT proteins are being increasingly recognized for their implications in tumorigenesis, in particular NFATc1 and NFATc2 [40]. Both NFATc1 and NFATc2 have been described to be highly expressed in pancreatic cancer [28]. NFATc1 has been shown to activate several drivers of tumorigenesis including c-myc and cyclin D1 in pancreatic cancer [31]. NFATc2 has also been associated with tumor progression, e.g., by promoting breast cancer invasion [41]. However, its role in cancer is still controversial and growth inhibitory and proapoptotic actions have also been reported [42].

Several reports in the literature have suggested a link between WNT5A and NFAT signaling: In *Xenopus*, a regulation of NFAT by noncanonical Wnt signaling has been described [43]. In mammalian cells including endothelial cells [20], osteoblasts [20], and chondrocytes [44], WNT5A has also been associated with increased transcriptional activation of NFAT. In Bcr-Abl–positive leukemias, Gregory et al. recently identified NFAT as downstream target of WNT5A in an RNA interference–based synthetic lethal screen [45]. To our knowledge, our report describing NFAT as downstream target of WNT5A is the first evidence of this link in solid tumors.

Likewise, the role of NFAT signaling in apoptosis has been discussed controversially and appears also to be strictly dependent on the cell type and on the NFAT family member: In T cells, NFATc2 induces apoptosis by activation of CD95L transcription [46]. Likewise, Alvarez et al. reported that tumor necrosis factor–α causes cell death in neuronal cells through activation of NFAT signaling [47]. In contrast, NFATc4 was also shown to protect from apoptosis in cortical neurons [48]. In renal tubular cells, NFATc4 was induced by carboplatin leading to increased apoptosis, which is assumed to mediate carboplatin-induced renal toxicity [49]. In fibroblasts, NFATc2 induced apoptosis, whereas NFATc1 induced transformation [42]. NFATc1 was also shown to protect chronic myeloid leukemia (CML) cells from imatinib-induced cell death [45]. To our knowledge, this is the first report describing an antiapoptotic effect of NFATc2 in tumors.

Interestingly, some of the reports describing NFAT activation following WNT5A stimulation in other cell types showed a calcium dependency of NFAT activation, which is in line with the known calcium-dependent NFAT activation pathway through the phosphatase calcineurin. As described above, we previously had failed to detect differences in CaMKII or PKC activation following WNT5A. We

now performed additional experiments to examine involvement of calcium-dependent signaling events in pancreatic cancer cells downstream WNT5A but again were unable to detect changes in intracellular calcium concentrations as determined by FURA-2 and G5A-aequorin measurements or activation of calcineurin activity assays following WNT5A stimulation, despite of the substantial activation of NFATc2 signaling (Griesmann et al., unpublished). Instead, we observed that NFATc2 is significantly induced in pancreatic cancer through β-catenin–dependent mechanisms leading to transcriptional up-regulation involving the NFATc2 promoter. Interestingly, luciferase assays after cotransfection of lymphoid enhancer-binding factor-1 (LEF1) expression plasmids did not reveal a significant regulation of the wild-type NFATc2 promoter fragment by LEF1 (Griesmann et al., unpublished). Therefore, we cannot rule out that other members or isoforms of the TCF/LEF transcriptional complex are involved in the β-catenin–dependent regulation of the NFATc2 promoter.

In summary, our data indicate that the secreted Wnt ligand WNT5A represents an important survival factor downstream of the transcription factor CUX1. In turn, WNT5A induces a transcriptional signaling cascade depending on NFATc2 and thereby mediates resistance to apoptosis in pancreatic cancer. Further studies are needed to decipher the apparent controversial effects of WNT5A signaling in the different tumor entities. It remains to be investigated whether the transcriptional up-regulation of NFAT, which mediates at least in part the antiapoptotic phenotype induced by WNT5A, can be also confirmed in the tumor entities responding differently to WNT5A. Given the high expression levels of WNT5A and NFATc2 in pancreatic cancer tissues that we have described previously [16,28], both appear to be promising candidates for therapeutic targeting to overcome the highly drug-resistant phenotype of pancreatic cancer.

Acknowledgments

We are grateful to Eva Bug and Eleni Aprin for excellent technical assistance.

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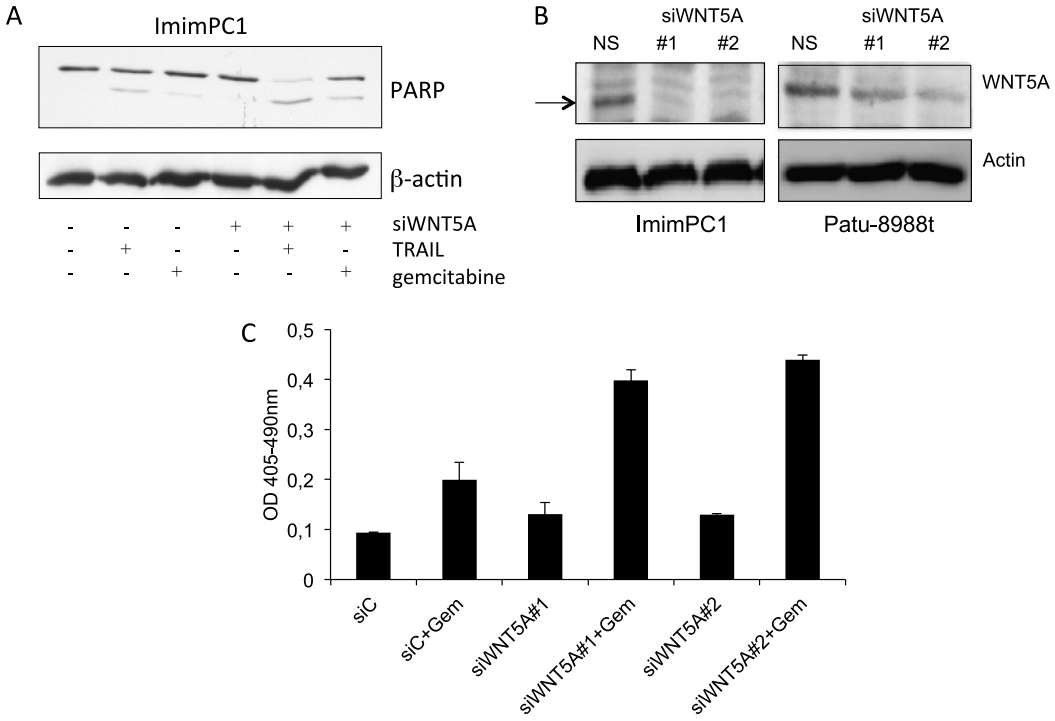

Figure W1. (A) Knockdown of WNT5A induces apoptosis in ImimPC1 cells, as determined by PARP cleavage. ImimPC1 cells were transiently transfected with WNT5A siRNA (siWNT5A) or control siRNA (siC), and apoptosis was induced with TRAIL for 12 hours or gemcitabine for 48 hours. PARP cleavage was determined by Western blot using a specific antibody detecting uncleaved (upper band) and cleaved (lower band) PARPs. To control for equal loading, the blots were reprobed with β-actin antibody. (B) Western blot demonstrating knockdown of WNT5A protein by two different independent silencing sequences (#1 and #2) in ImimPC1 and PaTu-8988t cells. (C) Quantification of basal and gemcitabine-induced apoptosis after knockdown of WNT5A using two independent WNT5A siRNA sequences (siWNT5A#1 and siWNT5A#2) in ImimPC1 cells. Representative quantification of histone-bound DNA fragmentation using a specific ELISA.

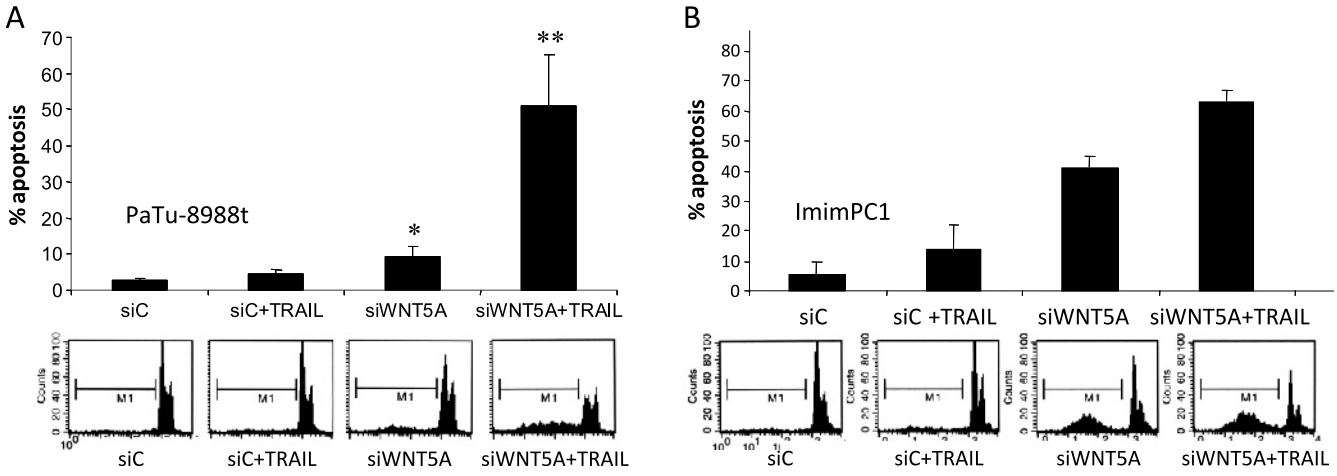

Figure W2. Knockdown of WNT5A induces apoptosis, as determined by sub-G1 analysis. (A) PaTu-8988t and (B) ImimPC1 cells were transiently transfected with WNT5A siRNA (siWNT5A) or control siRNA (siC), and apoptosis was induced with TRAIL for 12 hours. Apoptosis was determined by FACS sub-G1 analysis. Percentage of apoptotic cells is shown as mean of three independent experiments ± SD. *$P < .05$ as compared with control cells; **$P < .05$ as compared with drug-treated control cells.

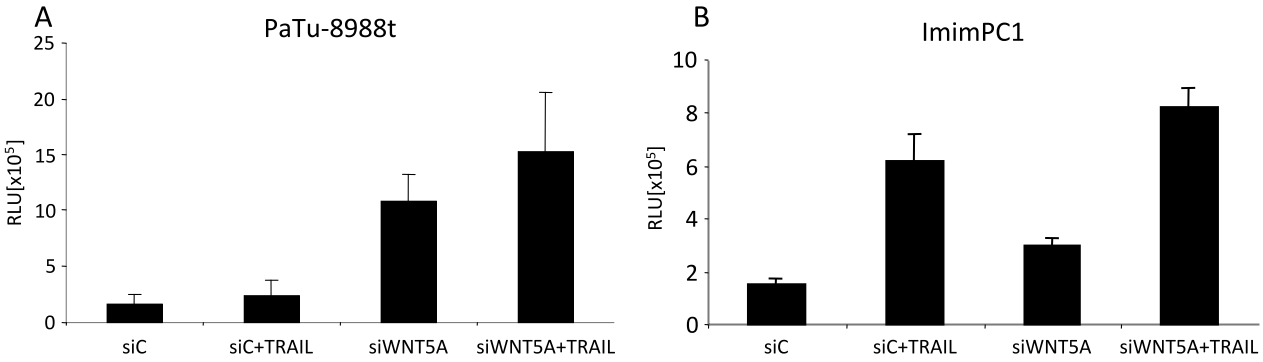

Figure W3. Knockdown of WNT5A induces apoptosis, as determined by measuring caspase-3/7 activity. (A) PaTu-8988t and (B) ImimPC1 cells were transiently transfected with WNT5A siRNA (siWNT5A) or control siRNA (siC), and apoptosis was induced with TRAIL for 12 hours. Effector caspase-3/7 activity was measured using a specific assay that determines cleavage of a luminogenic caspase-3/7 substrate. Data are representative for three independent experiments.

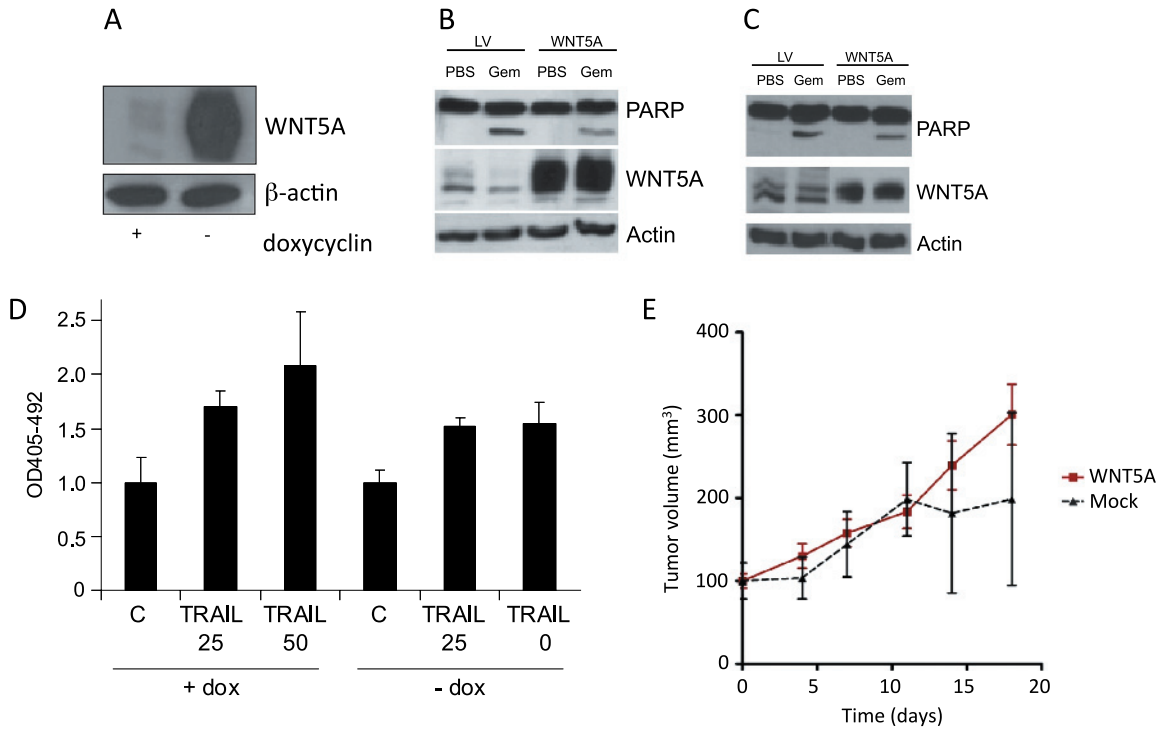

Figure W4. (A) PaTu-8988t cells with Tet-repressible WNT5A expression were incubated in the presence (+) or absence (−) of doxycycline for 24 hours. WNT5A levels were detected by Western blot; β-actin was used as loading control. (B, C) PaTu-8988t cells (B) and ImimPC1 cells (C) were incubated with rhWNT5A and/or gemcitabine, and apoptosis was measured by PARP cleavage. (D) PaTu-8988t cells with Tet-repressible WNT5A expression were incubated in the presence (low WNT5A) or absence (high WNT5A) of doxycycline and exposed to TRAIL for 12 hours. Apoptosis was determined by quantification of histone-bound DNA fragmentation using a specific ELISA. (E) Tumor growth of subcutaneous ImimPC1 cells stably overexpressing WNT5A or empty control vectors ($n = 6$ each). Tumor volume was determined for 20 days after the tumors had reached a volume of approximately 100 mm^3.

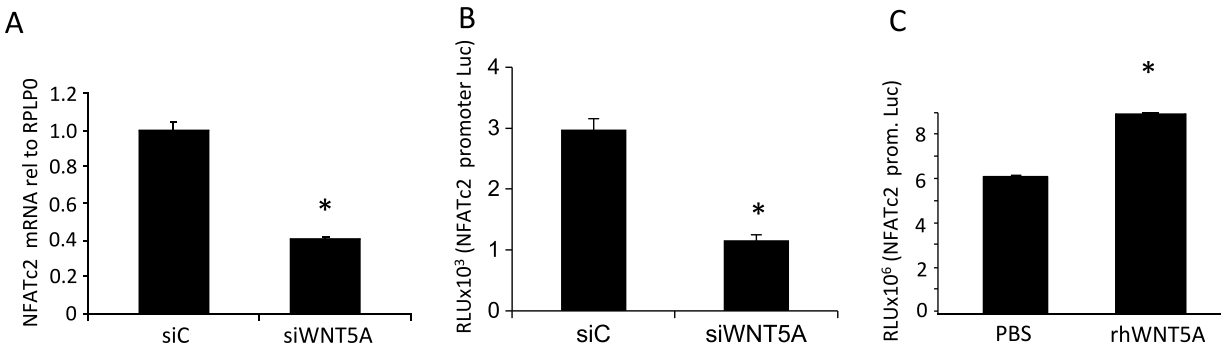

Figure W5. WNT5A induces NFATc2 mRNA in ImimPC1 cells. (A) ImimPC1 cells were transiently transfected with WNT5A siRNA (siWNT5A) or control siRNA (siC). NFATc2 mRNA was determined by quantitative real-time PCR and normalized to RPLPO mRNA. (B) ImimPC1 cells ± WNT5A siRNA (siWNT5A) or control siRNA (siC) were cotransfected with a luciferase plasmid containing an NFATc2 promoter fragment. Promoter activation was measured by luciferase assay. (C) NFATc2 promoter activity was determined after incubation with rhWNTA for 24 hours. *$P < .05$ as compared with untreated control cells.

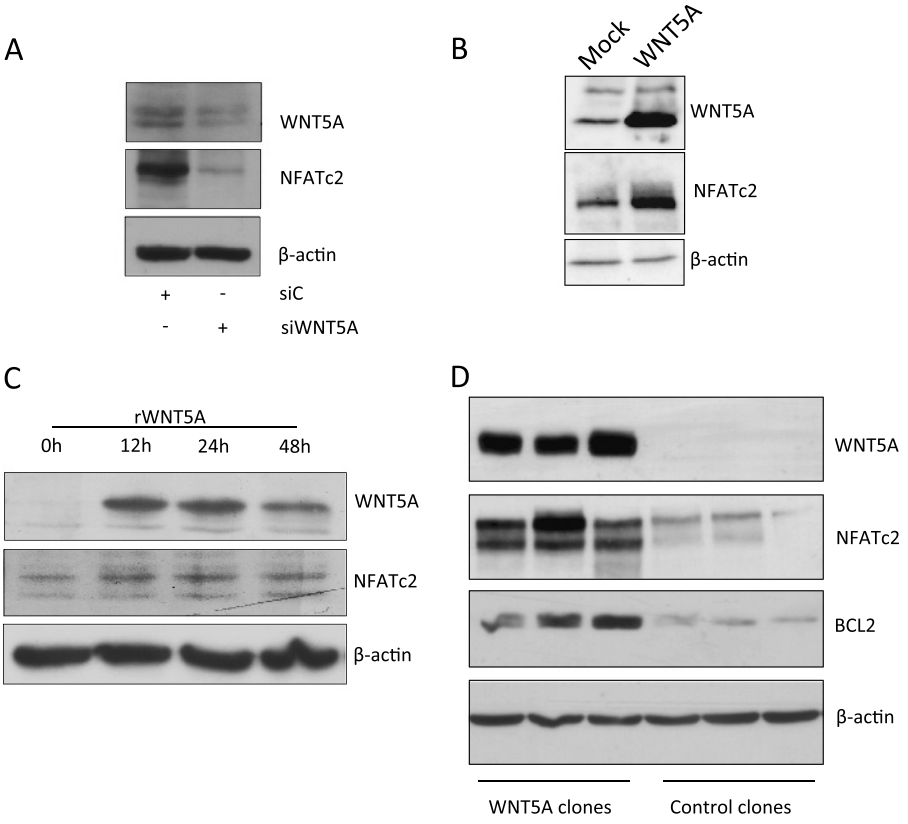

Figure W6. WNT5A induces NFATc2 protein in ImimPC1 cells. (A) ImimPC1 cells were transiently transfected with WNT5A siRNA (siWNT5A) or control siRNA (siC). NFATc2 protein was determined by Western blot; β-actin was used as loading control. (B) ImimPC1 cells were stably transfected with WNT5A or empty vector (mock), and NFATc2 protein levels were determined by Western blot; β-actin was used as loading control. (C) Recombinant human WNT5A was added to ImimPC1 cells for various time points, and NFATc2 protein levels were analyzed by Western blot. (D) WNT5A overexpression in ImimPC1 xenografts is associated with enhanced NFATc2 expression, as determined by Western blot in the gemcitabine-treated xenograft tissues for which sufficient material for protein lysates was available.

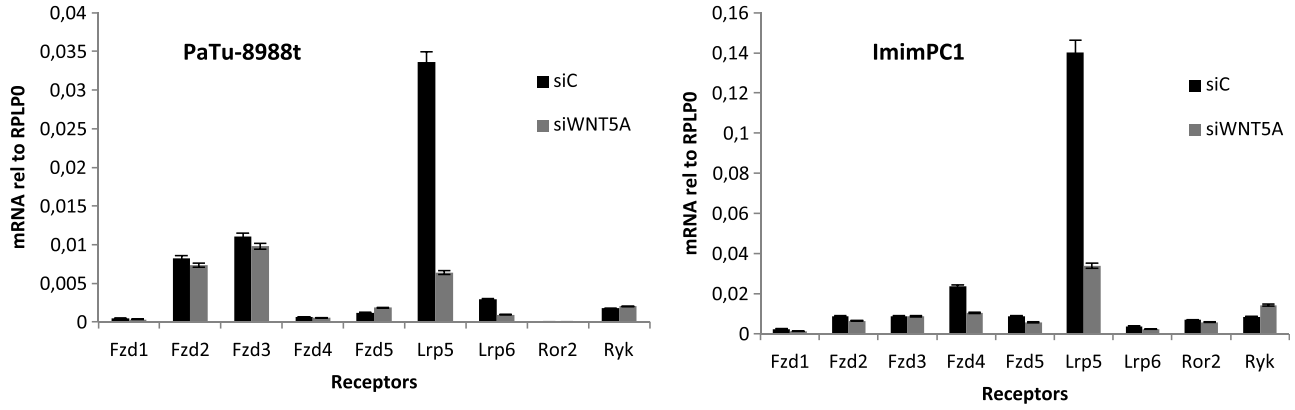

Figure W7. mRNA expression levels of different putative WNT5A receptors in PaTu-8988t and ImimPC1 cells, as determined by quantitative real-time PCR after transient transfection with siRNA against WNT5A (siWNT5A) or nonsilencing control (siC). Fzd, Frizzled receptors; Lrp, low-density lipoprotein receptor–related protein.

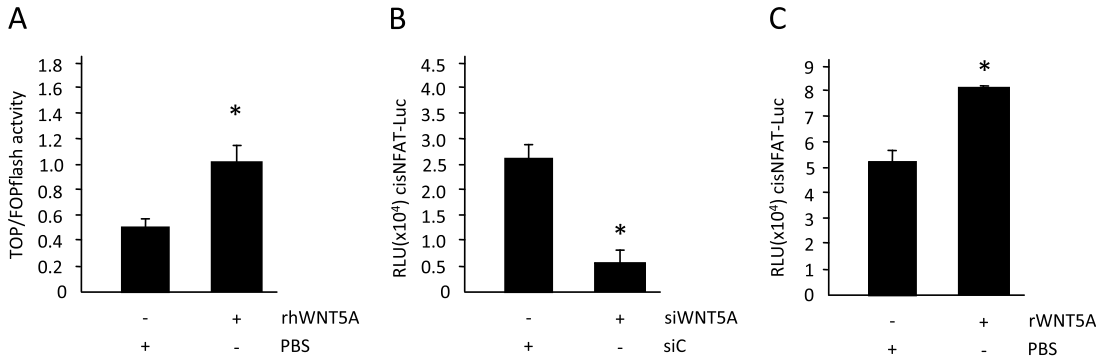

Figure W8. WNT5A involves canonical signaling and induces transcriptional activation of NFATc2 in ImimPC1 cells. (A) TOP/FOPflash indicating canonical Wnt signaling activity ± recombinant human WNT5A (rhWNT5A). (B) ImimPC1 cells were transiently transfected with WNT5A siRNA (siWNT5A) or control siRNA (siC) and cotransfected with a *cis*NFAT luciferase plasmid (cisNFATLuc) containing an NFAT DNA binding motif. NFAT transcriptional activation was measured by luciferase assay. (C) rhWNT5A was added to ImimPC1 cells after transfection with *cis*NFAT-Luc. NFAT transcriptional activation was measured by luciferase assay. **P* < .05 as compared with untreated control cells.

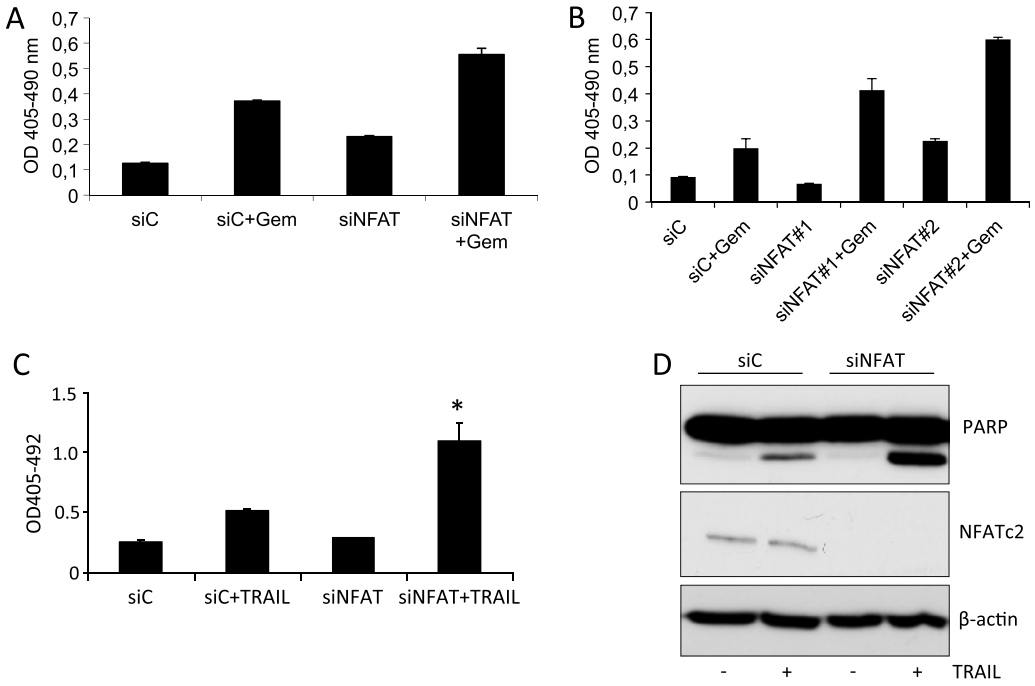

Figure W9. (A) NFATc2 knockdown stimulates gemcitabine-induced apoptosis in ImimPC1 cells, as determined by quantification of histone-bound DNA fragmentation by specific ELISA. (B) NFATc2 knockdown induced by two independent silencing sequences (siNFAT#1 and siNFAT#2) stimulates gemcitabine-induced apoptosis in PaTu-8988t cells, as determined by quantification of histone-bound DNA fragmentation by specific ELISA. (C, D) NFATc2 knockdown stimulates TRAIL-induced apoptosis. ImimPC1 cells were transiently transfected with NFATc2 siRNA (siNFAT) or control siRNA (siC), and apoptosis was induced with TRAIL for 12 hours. Apoptosis was determined by quantification of histone-bound DNA fragmentation by specific ELISA (C) and PARP cleavage (D). To control for equal loading, we reprobed the blots with β-actin antibody.