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## **TGF- $\beta$ RII knock-down promotes tumor growth and chemoresistance to gemcitabine of pancreatic cancer cells via phosphorylation of STAT3**

Vincent Drubay, Nicolas Skrypek, Lucie Cordiez, Romain Vasseur, Céline Schulz, Nihad Boukrout, Bélinda Duchêne, Lucie Coppin, Isabelle Van Seuningen, Nicolas Jonckheere

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1 **TGF- $\beta$ RII knock-down promotes tumor growth and chemoresistance to gemcitabine of**  
2 **pancreatic cancer cells via phosphorylation of STAT3**

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15

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## Abstract

Pancreatic adenocarcinoma (PDAC) is one of the most deadly cancers in the western countries because of a lack of early diagnostic markers and efficient therapeutics. At the time of diagnosis, more than 80% of patients have metastasis or locally advanced cancer and are therefore not eligible for surgical resection. Pancreatic cancer cell also harbour a high resistance to chemotherapeutic drugs such as gemcitabine that is one of the main palliative treatment for PDAC.

TGF- $\beta$  possesses both tumor-suppressive and oncogenic activities in pancreatic cancer. TGF- $\beta$  signalling pathway plays complex role during carcinogenesis by initially inhibiting epithelial growth and later promoting the progression of advanced tumors and thus emerged as tumor suppressor pathway. TGF- $\beta$  binds to its receptor TGF- $\beta$ RII and activates different pathways: canonical pathway involving the Smad proteins and alternative pathways such as MAPKs. Smad4 is mutated in 50-80% of PDAC. Mutations of TGF- $\beta$ RII also occurs (5-10%). In order to decipher the role of TGF- $\beta$  in carcinogenesis and chemoresistance, we decided to characterize the knocking down of TGF- $\beta$ RII that is the first actor of TGF- $\beta$  signalling. We developed pancreatic cancer cell lines stably invalidated for TGF- $\beta$ RII and studied the impact on biological properties of pancreatic cancer cells both in vitro and in vivo. We show that TGF- $\beta$ RII silencing alters tumor growth and migration as well as resistance to. TGF- $\beta$ RII silencing also leads to S727 STAT3 and S-63 c-Jun phosphorylation, decrease of MRP3 and increase of MRP4 ABC transporter expression and induction of a partial EMT phenotype.

In the future, the better understanding TGF- $\beta$  signaling pathways and underlying cellular mechanisms in chemoresistance to gemcitabine may bring new therapeutic tools to clinicians.

Keywords: TGF- $\beta$ RII receptor, STAT3, metastasis, gemcitabine, ABC transporters

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## Introduction

Pancreatic cancers (PC) are projected to become the second leading cause of cancer-related death by 2030 (Rahib et al., 2014). The survival curve is extremely short (6 months) and the survival rate at 5 years is very low (3%). This dramatic outcome is related to a lack of therapeutic tools and early diagnostic markers which makes pancreatic cancer the most deadly cancer. At the time of diagnosis, more than 80% of PC are already metastatic or locally advanced and only about 10 to 15% of patients are considered eligible for surgical resection (Vincent et al., 2011). Remaining patients that do not benefit of surgery will receive palliative chemotherapy and notably gemcitabine, a fluorinated analog of deoxycytidine that is a major chemotherapeutic drug used in firstline in advanced PC. Unfortunately, PC is characterized by an intrinsic and acquired chemoresistance that lead to relapse and death (Kleeff et al., 2016). Deciphering mechanisms responsible for PC cell resistance to gemcitabine is thus crucial to improve efficacy of the drug and propose more efficient therapies.

TGF- $\beta$  signalling pathway plays a complex role during carcinogenesis. TGF- $\beta$  initially inhibits epithelial growth whereas it appears to promote the progression of advanced tumors and thus emerged as tumor suppressor pathway in pancreatic cancer (Principe et al., 2014). TGF- $\beta$  can act in an autocrine manner or as a paracrine factor secreted by the microenvironment (Derynck et al., 2001). After binding to its receptor TGF- $\beta$ RII, TGF- $\beta$  signals via activation of several pathways. The canonical pathway involves the Smad proteins, but activation of other pathways such as MAPKs, PI3K or small GTPases (Derynck et al., 2001) pathways may also mediate TGF- $\beta$  effects. It is also interesting to note that Smad4/DPC4 (deleted in pancreatic cancer 4) is mutated in 50-80% of PDAC whereas mutations of TGF- $\beta$ RII are less common (5-10%) (Kleeff et al., 2016; TCGA-Network., 2017). We previously showed that TGF- $\beta$  can regulate gene expression via canonical or alternative signalling pathways (Jonckheere et al., 2004).

73 In order to design new therapeutic strategies, it is thus mandatory to better characterize the  
74 signaling pathways and complex gene networks that are altered during carcinogenesis  
75 progression. Therefore, to better understand the role and contribution of TGF- $\beta$ RII in TGF- $\beta$   
76 signalling and biological properties of PC cells in vitro and in vivo, we developed PC cell lines  
77 stably invalidated for TGF- $\beta$ RII. Our results show that TGF- $\beta$ RII silencing alters tumor growth  
78 and migration and increases resistance to gemcitabine in vitro and in vivo. TGF- $\beta$ RII  
79 silencing also leads to STAT3 and c-Jun phosphorylation, alteration of MRP3 and MRP4  
80 ABC transporters expression and induction of a partial EMT phenotype.

81 This work underlies the importance of TGF- $\beta$  signaling pathways and associated cellular  
82 mechanisms as inducers of chemoresistance to gemcitabine and proposes potential new  
83 therapeutic tools to clinicians, surgeons and anatomopathologists for this deadly disease.

84

## 85 **Material and methods**

### 86 **Cell culture**

87 CAPAN-1 and CAPAN-2 PC cell lines were cultured as previously described (Jonckheere et  
88 al., 2004). TGF- $\beta$ RII-knocked down (KD) cells were obtained following stable transfection of  
89 CAPAN-1 and CAPAN-2 cells with four different pGeneClip<sup>TM</sup> puromycin vectors encoding  
90 TGF- $\beta$ RII ShRNA (SA Biosciences<sup>TM</sup>) as previously described (Jonckheere et al., 2012).  
91 The empty vector was used to raise control clones called Non Targeting (NT). Four selected  
92 clones of NT and each TGF- $\beta$ RII-KD cells were pooled in order to avoid clonal variation and  
93 were designated TGF- $\beta$ RIIKD6, TGF- $\beta$ RIIKD7, TGF- $\beta$ RIIKD8 and TGF- $\beta$ RIIKD9. All cells  
94 were maintained in a 37°C incubator with 5% CO<sub>2</sub> and cultured as the parental cells.

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### 96 **qRT-PCR**

97 Total RNA from PC cells was prepared using the NucleoSpin<sup>®</sup> RNA II kit (Macherey Nagel,  
98 Hoerd, Germany). cDNA was prepared as previously described (Van Seuningen et al.,  
99 2000). Semi-quantitative PCR was performed as previously described (Mesquita et al.,  
100 2003). qPCR was performed using SsoFast<sup>TM</sup> Evagreen Supermix kit following the  
101 manufacturer's protocol using the CFX96 real time PCR system (Bio-Rad). Primer  
102 information is given in table 1. Each marker was assayed in triplicate in three independent  
103 experiments. Expression level of genes of interest was normalized to the mRNA level of  
104 GAPDH housekeeping gene.

105

### 106 **Protein extraction and western-blotting**

107 Total cellular extracts were performed as previously described in Van Seuningen et al. (Van  
108 Seuningen et al., 1995) and Jonckheere et al. (Jonckheere et al., 2009). Western-blotting on  
109 nitrocellulose membrane (0.2  $\mu$ m, Whatman) was carried out as previously described  
110 (Piessen et al., 2007). Membranes were incubated with antibodies against STAT3 (79D7,  
111 Cell signalling), phospho S727 STAT3 (9134, signalling), c-Jun (60A8, Cell signalling),  
112 phospho S63 c-Jun (54B3, Cell signalling) and  $\beta$ -actin (AC-15, sigma). Antibodies were

113 diluted in 5% (w/v) non-fat dry milk in Tris-Buffered Saline Tween-20 (TBS-T). Peroxydase-  
114 conjugated secondary antibodies (Sigma-Aldrich) were used and immunoreactive bands  
115 were visualised using the West Pico chemoluminescent substrate (Thermo Scientific, Pierce,  
116 Brebières, France). Chemo-luminescence was visualised using LAS4000 apparatus  
117 (Fujifilm). Density of bands were integrated using Gel analyst software® (Claravision, Paris,  
118 France) and represented as histograms. Three independent experiments were performed.

119

### 120 **Cell proliferation**

121 Cells were seeded at  $1 \times 10^5$  cells per well in 6-well plates. Cells were counted daily using a  
122 Malassez counting chamber using Trypan Blue exclusion dye (Life Technologies) during 96h.  
123 Experiments were performed three times in triplicate.

124

### 125 **Wound healing test**

126 1500 cells were seeded per wells in 96 well plates (Image Lock™ plates, Essen Bioscience)  
127 and cultured until confluence was reached. The wound was realized using IncuCyte wound  
128 maker (Essen BioScience). Cells were washed three times with PBS 1X and complete  
129 medium was added to the cells. Wound widths were analyzed using Incucyte platform (Live-  
130 Cell imaging System, Essen Bioscience) and pictures collected every 2h.

131

### 132 **Cytotoxicity assay**

133 Cells were seeded in growth medium into 96-well plates at a density of  $10^4$  cells per well.  
134 After 24h incubation, the medium was replaced by fresh medium containing gemcitabine at  
135 35 nM and incubated for 72h at 37°C. The viability of cells was determined using the 3-(4,5-  
136 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Sigma-Aldrich) as  
137 previously described (Skrypek et al., 2013). Percentage of viability =  $[(A_{\text{treated}} - A_{\text{blank}})/(A_{\text{neg.}} -$   
138  $A_{\text{blank}})] \times 100$ ; where  $A_{\text{treated}}$  is the average of absorbance in wells containing cells treated with  
139 gemcitabine,  $A_{\text{neg.}}$  is the average of wells containing cells without gemcitabine treatment, and  
140  $A_{\text{blank}}$  is the average of wells containing medium without cells.

141

## 142 **Subcutaneous xenografts**

143 NT or TGF- $\beta$ R2-KD CAPAN-1 ( $10^6$  cells in 100  $\mu$ l Matrigel) and CAPAN-2 ( $2 \times 10^6$ ) cells were  
144 injected subcutaneously (SC) into the flank of seven-week-old male Severe Combined  
145 Immunodeficient (SCID) mice (CB17, Janvier, France). Six mice were used per group. Tumor  
146 size was evaluated weekly by measuring the length (l) and the width (L) and tumor volume  
147 was calculated with the formula ( $l^2 \times L$ ). Once palpable tumors were developed ( $250 \text{ mm}^3$ ),  
148 gemcitabine (15 mg/kg) or PBS (200  $\mu$ l) were injected intra-peritoneously, twice a week. All  
149 procedures were in accordance with the guideline of animal care committee (Comité Ethique  
150 Expérimentation Animale Nord Pas-de-Calais, #122012).

151

## 152 **Immunohistochemistry**

153 Xenografts were fixed in 10% (w/v) buffered formaldehyde, embedded in paraffin, cut at 4  $\mu$ m  
154 thickness and applied on SuperFrost® slides (Menzel-Glaser, Braunschweig, Germany).  
155 Manual IHC was carried out as previously described (van der Sluis et al., 2004). The  
156 antibodies were used as followed: anti-STAT3 (1:200, #483 Santa Cruz), anti-c-Jun (1:200,  
157 60A8 Cell signaling), anti-E-Cadherin (1:200, 3195 Cell signalling) and anti-vimentin (1:200,  
158 sc5741, Santa Cruz). Intensity of staining was graded as weak (1), moderate (2) or strong  
159 (3). The percentage of ductal stained cells was graded as 1 (0–25%), 2 (25–50%), 3 (50–  
160 75%) and 4 (75–100%). Total score was calculated by multiplying the intensity score and  
161 percentage score.

162

## 163 **Expression analysis in CCLE database**

164 TGF- $\beta$ R2, ABCB1/MDR1, ABCC1/2/3/4/5 and ABCG2 z-score expressions were extracted  
165 from databases available at cBioPortal for Cancer Genomics (Cerami et al., 2012; Gao et al.,  
166 2013). The queries were realized in CCLE (44 pancreatic samples, Broad Institute, Novartis  
167 Institutes for Biomedical Research) (Barretina et al., 2012).

168

169 **Statistical analyses**

170 Statistical analyses were performed using the Graphpad Prism 6.0 software (Graphpad  
171 softwares Inc., La Jolla, USA). Differences in data of two samples were analysed by the  
172 student's t test or ANOVA test with selected comparison using tukey post-hoc test and were  
173 considered significant for P-values <0.05 \*, p<0.01 \*\* or p<0.001 \*\*\*.

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## Results

### Generation and characterization of stable TGF- $\beta$ RII-KD cellular clones

Expression of TGF- $\beta$ RI, TGF- $\beta$ RII, TGF- $\beta$ RIII Smad2, Smad3, and Smad7 was confirmed by RT-PCR in CAPAN-1 and CAPAN-2 cells. Wild type Smad4, as it is mutated, is not detected in CAPAN-1 (Jonckheere et al., 2004; Schutte et al., 1996). Altogether this suggests that CAPAN-2 cells harbor a functional TGF- $\beta$  signaling pathway whereas the canonical Smad pathway is not functional in CAPAN-1 cells. Moreover, strong TGF- $\beta$ 1 and mild TGF- $\beta$ 2 mRNA levels were observed in both cell lines suggesting TGF- $\beta$  growth factor autocrine expression (Figure 1A).

We generated CAPAN-1 and CAPAN-2 stable cell lines in which TGF- $\beta$ RII was knocked down (TGF- $\beta$ RII-KD) by a shRNA approach. Four different shRNA sequences were used to establish four different cell lines designated as TGF- $\beta$ RIIKD6, TGF- $\beta$ RIIKD7, TGF- $\beta$ RIIKD8 and TGF- $\beta$ RIIKD9. Using qPCR, we confirmed that TGF- $\beta$ RII mRNA levels are decreased in all CAPAN-1 and CAPAN-2 TGF- $\beta$ RII-KD cells compared to NT control cells ( $p < 0.005$ , \*\*\*) (Figure 1B). We were not able to produce TGF- $\beta$ RIIKD7 cell line in CAPAN-2.

In CAPAN-2 KD cells, the inhibition of TGF- $\beta$ RII expression was correlated with a loss of activity of the Smad binding elements (SBE)-Luc synthetic promoter (Figure 1C). In CAPAN-2 NT cells, TGF- $\beta$  treatment induces a 10-fold increase of SBE-Luc relative activity whereas this effect was lost in TGF- $\beta$ RII-KD cells ( $p < 0.001$ ). As expected, in CAPAN-1 cells mutated for Smad4, we did not observe any activity of SBE-Luc construct with or without TGF- $\beta$  treatment (not shown). Interestingly, TGF- $\beta$ RII knocking down led to decreased TGF- $\beta$ 1 mRNA level in CAPAN-1 TGF- $\beta$ RII-KD cells (44-87% decrease) (Figure 1D) whereas the effect was less pronounced (21-25%) in TGF- $\beta$ RII-KD CAPAN-2 cell lines.

### Involvement of TGF- $\beta$ RII in PC cell biological properties

201 We investigated the effect of TGF- $\beta$ RII silencing on CAPAN-1 and CAPAN-2 proliferation  
202 and migration properties. Cell migration was assessed by wound healing test. In CAPAN-2  
203 NT cells, the wound was entirely closed at 60h. In CAPAN-2 TGF- $\beta$ RII-KD cells, we  
204 observed a strong delay of wound closure that was statistically significant at 16-18h  
205 ( $p < 0.001$ , \*\*\*) (Figure 2A, left panel). Interestingly, we did not observe any statistically  
206 significant difference in wound closure in CAPAN-1 TGF- $\beta$ -RIIKD or NT cells suggesting the  
207 involvement of a functional Smad4 signaling pathway in wound closure (Figure 2A, right  
208 panel). TGF- $\beta$ RII-KD CAPAN-1 or CAPAN-2 cells also showed a trend toward increased  
209 proliferation at 96h compared to the respective NT control cells but that remained not  
210 significant (not shown).

211 In order to determine the role of TGF- $\beta$ RII on pancreatic carcinogenesis in vivo, CAPAN-1/-2  
212 TGF- $\beta$ RII-KD8 and NT SC xenograft studies were carried out. We selected the TGF- $\beta$ RII-  
213 KD8 cell lines for in vivo studies as this cell line harboured the best KD in CAPAN-1 and  
214 CAPAN-2. The results indicate that the tumour volume was significantly higher in  
215 xenografted mice with CAPAN-1 TGF- $\beta$ RII-KD8 compared to CAPAN-1 NT controls. The  
216 relative tumour volume was  $2.26 \pm 0.1 \text{ cm}^3$  when compared to NT control tumour volume  
217 ( $1.66 \pm 0.14 \text{ cm}^3$ ) at day 21. The increase was statistically significant (\*\*,  $p < 0.01$ ). Similar  
218 results were obtained with CAPAN-2 TGF- $\beta$ RII-KD8 xenografts ( $0.423 \pm 0.05$  vs  $0.828 \pm 0.08$   
219  $\text{cm}^3$ ) at day 42 (Figure 2B). Furthermore, we also evaluated the presence of micro-  
220 metastasis in the liver by detecting the presence of human GAPDH in the liver of the mouse  
221 by qPCR (Figure 2C). We detected micro-metastases in 5/7 (71%) CAPAN-2 controls  
222 whereas only 2/10 (20%) of CAPAN-1 TGF- $\beta$ RII-KD8 xenografted mice harboured micro-  
223 metastases. Contingency analysis showed that difference was close to statistical significance  
224 ( $p = 0.058$ ). We did not observe any difference in CAPAN-1 TGF- $\beta$ RII-KD8 (4/6) compared to  
225 CAPAN-1 NT controls (4/6). No human GAPDH mRNA was detected in mice without  
226 xenografts. Our results suggest that TGF- $\beta$ RII signalling is involved in tumor growth and  
227 migration of pancreatic cancer cells both in vitro and in vivo.

228

### 229 **Role of TGF- $\beta$ RII on PC cells sensitivity to gemcitabine**

230 We investigated the effect of TGF- $\beta$ RII silencing on CAPAN-1 and CAPAN-2 cell sensitivity  
231 to gemcitabine. We show that the lack of TGF- $\beta$ RII induces a significant increase of  
232 resistance to gemcitabine treatment in both CAPAN-1 (87-152% increase of survival rate,  
233 Figure 3A) and CAPAN-2 (50-161% increase, Figure 3B) cell lines compared to NT control  
234 cells. All differences were statistically significant. We then carried out SC xenograft of NT or  
235 TGF- $\beta$ RII-KD8 CAPAN-2 cells that were subsequently treated with gemcitabine for 46 days.  
236 Gemcitabine treatment decreased the normalized tumor volume in CAPAN-2 NT xenografts  
237 ( $2.7 \pm 1.02$  vs  $1.4 \pm 0.11$  at D83) compared to initial tumor volume (D36). On the contrary, the  
238 tumor growth was exacerbated in TGF- $\beta$ RII-KD xenografts following gemcitabine treatment  
239 ( $2.9 \pm 1.7$  vs  $4.36 \pm 1$  at D83) (Figure 3C). Altogether, our results suggest that TGF- $\beta$ RII alters  
240 sensitivity of PC cells to gemcitabine both in vitro and in vivo.

241

### 242 **Identification of signalling pathways altered following TGF- $\beta$ RII knocking down**

243 Impact of TGF- $\beta$ RII knocking-down on intracellular signaling was studied using phospho  
244 array that detect relative site-specific phosphorylation of 43 proteins simultaneously (Figure  
245 4). Intensities of each spots for TGF- $\beta$ RII were measured and normalized to the CAPAN-2  
246 NT proteins (Figure 4A). We observed an important increase of phosphorylation of S63 c-Jun  
247 (3.3-fold) and S727STAT3 (1.5-fold) in CAPAN-2 TGF- $\beta$ RII-KD8 compared to NT cells. We  
248 also observed a mild decrease of phosphorylation of Y694 STAT5a (0.6-fold) and  $\beta$ -catenin  
249 (0.5-fold) (Figure 4B). Similar experiments were conducted for CAPAN-1 TGF- $\beta$ RII-KD8 and  
250 NT cells. Only weak variations were observed (30%). By western blotting, we confirmed the  
251 increased of phospho-S727 STAT3 (4.49-fold) (Figure 5A) and phospho-S63 (7-fold)  
252 (supplemental figure 1A) in CAPAN-2 TGF- $\beta$ RII-KD8 cells compared to CAPAN-2 NT cells.

253 Gemcitabine treatment also induced an increase of phospho-S727 STAT3 (1.95-fold) (Figure  
254 5A) and phospho-S63 c-Jun (3.18-fold) (supplemental figure 1A) in NT cells (compared to  
255 untreated cells). This effect was not found in TGF- $\beta$ RII-KD8 cells. We then performed  
256 immunohistochemistry for STAT3 and c-Jun in NT or TGF- $\beta$ RII-KD8 CAPAN-2 SC  
257 xenografts (Figure 5B and supplemental figure 1B). Nuclear and cytoplasmic IHC staining  
258 were scored. We show that STAT3 nuclear H-score in TGF- $\beta$ RII-KD8 tumors was  
259 significantly higher than in NT tumors (\*,  $p=0.0429$ ) (Figure 5C). We also observed that  
260 STAT3 nuclear staining was increased following gemcitabine treatment (\*,  $p=0.0286$ ). A mild  
261 increase of nuclear STAT3 was observed in TGF- $\beta$ RII-KD8 tumors following gemcitabine  
262 treatment but was not statistically significant ( $p=0.33$ ) (Figure 5C). No alteration of c-jun  
263 expression was observed in NT and TGF- $\beta$ RII-KD8 untreated xenograft tumors  
264 (supplemental figure 1B). H score measurement indicates that gemcitabine treatment led to a  
265 significant decrease of cJun staining in TGF- $\beta$ RII-KD8 tumors (Supplemental figure 1C).  
266 Altogether, our results indicate that TGF- $\beta$ RII signalling implicates STAT3 and c-Jun  
267 phosphorylation in pancreatic cancer cells.

268

## 269 **TGF- $\beta$ RII silencing alters the expression of ABC transporters and EMT markers in PC** 270 **cells**

271 To go further and understand which molecular mechanisms could be responsible for the  
272 induced chemoresistance, we investigated the effect of TGF- $\beta$ RII silencing on the expression  
273 of ATP-binding cassette (ABC) transporters that are commonly known to confer resistance to  
274 xenobiotics including chemotherapeutic drugs. Using qPCR, we investigated the expression  
275 of ABCB1/MDR1, ABCC1/MRP1, ABCC2/MRP2, ABCC3/MRP3, ABCC4/MRP4,  
276 ABCC5/MRP5 and ABCG2 in NT and TGF- $\beta$ RII-KD CAPAN-1 and CAPAN-2 cells. MRP1  
277 was not detected. We found that MDR1 (x4.2-fold, \*\*), ABCG2 (x1.9-fold, \*\*\*) and MRP4  
278 (x1.4-fold, \*) mRNA levels were significantly increased in TGF- $\beta$ RII-KD CAPAN-1 cells

279 compared to NT cells (Figure 6A). MRP3 mRNA level was decreased in TGF- $\beta$ RII-KD  
280 CAPAN-1 cells (x0.42-fold, \*\*\*) and CAPAN-2 (x0.65-fold, p=0.13) (Figure 6A). TGF- $\beta$ RII and  
281 ABC transporter expression was analyzed from 44 pancreatic cancer cell lines from CCLE.  
282 We showed that TGF- $\beta$ RII mRNA relative level was correlated with expression of MRP3  
283 (Pearson  $r=0.3856$ ,  $p=0.0097$ ) (Figure 6B) and inversely correlated with MRP4 (Pearson  $r=-$   
284  $0.3691$ ,  $p=0.037$ ) (Figure 6C).

285 Furthermore, TGF- $\beta$  is commonly described as an inducer of epithelial-mesenchymal  
286 transition (EMT) that is associated with chemoresistance (Voulgari & Pintzas, 2009). We  
287 performed vimentin (mesenchymal marker) and E-cadherin (epithelial marker)  
288 immunohistochemical staining on FFPE sections of NT or TGF- $\beta$ RII-KD CAPAN-2 xenografts  
289 treated with gemcitabine to check their status. Surprisingly, we observed a slight increase of  
290 vimentin in TGF- $\beta$ RII-KD CAPAN-2 cells xenografts compared to NT tumors (supplemental  
291 figure 2). Moreover, we found that gemcitabine treatment induced a loss of E-cadherin  
292 staining and a gain of vimentin staining suggesting an EMT phenotype following gemcitabine  
293 treatment.

294 Altogether, these results suggest that TGF- $\beta$ RII silencing alters MRP3 and MRP4 ABC  
295 transporters expression in pancreatic cancer cells and induces a partial EMT phenotype that  
296 could lead to chemoresistance to gemcitabine.

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298

299 **Discussion**

300

301 In the present manuscript, we characterized pancreatic cancer cell lines stably invalidated for  
302 TGF- $\beta$ RII and investigated the consequences on both biological properties and response to  
303 chemotherapy in vitro and in vivo. We show an increase of tumor growth and a reduction of  
304 cell migration. We also show for the first time an increased resistance to gemcitabine that  
305 could be mediated by S727 STAT3 phosphorylation and via deregulation of MRP3 and  
306 MRP4 ABC transporter expression. TGF- $\beta$  signalling pathway has been described as a  
307 double edge sword during carcinogenesis (Akhurst & Derynck, 2001); acting as a tumor  
308 suppressor in the early stages but promoting metastasis in the advanced carcinoma  
309 (Principe et al., 2014). Moses's laboratory generated TGF- $\beta$ RII knock out mice crossed with  
310 Ptf1a-Cre; LSL-KrasG12D and showed that compound mice developed well differentiated  
311 PDAC (Ijichi et al., 2006) mostly highlighting the role as a tumor suppressor. TGF $\beta$ R2  
312 targeting by a monoclonal antibody is also effective at reducing metastasis (Ostapoff et al.,  
313 2014). In our cellular models, we confirmed that TGF- $\beta$ RII inhibition led to an increased  
314 tumor growth in vivo and TGF- $\beta$ RIIKD CAPAN-2 tumors led to less metastasis in the liver.

315 STAT3 targeting has been proposed as a therapeutic target in pancreatic adenocarcinoma  
316 (Sahu et al., 2017). Combined treatments of gemcitabine and a JAK inhibitor (AZD1480) led  
317 to stroma remodeling, increased density of microvessel, enhanced drug delivery and  
318 improved survival of in Ptf1a-Cre; LSL-KrasG12D; TGF- $\beta$ RII<sup>KO</sup> vivo models suggesting an  
319 effect of the treatment via the stroma (Nagathihalli et al., 2015). S727 phosphorylation was  
320 previously studied in prostate carcinogenesis and was shown to promote cell survival and  
321 cell invasion (Qin et al., 2008). In the present work, we also showed that TGF- $\beta$ RII inhibition  
322 led to STAT3 S727 phosphorylation and increased gemcitabine resistance of the tumor cells  
323 suggesting the crucial role of STAT3 in both tumor and stromal cells. STAT3 knockdown was  
324 shown to be associated with increased response to gemcitabine in pancreatic cancer cells

325 (Venkatasubbarao et al., 2013). It is interesting to note that Erlotinib treatment that inhibits  
326 epidermal growth factor receptor (EGFR) tyrosine kinase also inhibited phosphorylation of  
327 STAT3 (Miyabayashi et al., 2013). Among the targeted therapy for PDAC, erlotinib  
328 associated with gemcitabine is the only drug showing statistically significantly improved  
329 survival (Moore et al., 2007).

330 TGF- $\beta$  signaling is mediated through canonical SMAD and non-canonical non-SMAD  
331 pathways (Principe et al., 2014). Accordingly, we only observed increase of c-Jun and  
332 STAT3 phosphorylation in CAPAN-2 TGF- $\beta$ RIIKD cells but not in the CAPAN-1 model that is  
333 SMAD4 mutated. It was previously shown that STAT3-induced senescence requires  
334 functional TGF $\beta$ R signaling and notably a functional SMAD3/SMAD4 pathway. STAT3  
335 promotes SMAD3 nuclear localization (Bryson et al., 2017). We hypothesize that the TGF-  
336  $\beta$ RIIKD-induced gemcitabine resistance, shown in the present manuscript, is mediated by  
337 STAT3 which similarly requires SMAD3/SMAD4 dependent pathway.

338 In Smad4 mutated CAPAN-1 cells we observed an increased expression of MRP4, ABCG2  
339 and MDR1. This increased expression could be responsible for the gemcitabine resistance of  
340 CAPAN-1 TGF- $\beta$ RIIKD cells. The link between ABC transporters and TGF- $\beta$  pathway is  
341 scarcely described. TGF- $\beta$ 1 has been shown to upregulate ABCG2 expression in MiaPACA2  
342 pancreatic cells which is contradictory with our findings (Kali et al., 2017). In breast cancer  
343 cells, silencing of TGF- $\beta$ RII leads to overexpression of multidrug resistance protein ABCG2  
344 and tamoxifen resistance (Busch et al., 2015).

345 TGF- $\beta$  is usually considered as a bona fide inducer of EMT (Wendt et al., 2009). However,  
346 we were surprised to observe that TGF- $\beta$ RII inhibition led to a partial EMT with an increase  
347 of vimentin. STAT3 signaling is linked to cancer cell plasticity and is able to promote EMT  
348 and CSC expansion (Junk et al., 2017). Previous work also showed that IL6, secreted by  
349 pancreatic stellate cells, triggers STAT3 activation in pancreatic cells which subsequently

350 induces EMT via Nrf2 (Wu et al., 2017). Therefore, we hypothesize that the paradoxal EMT  
351 observed in TGF- $\beta$ RII cells is a consequence of the STAT3 phosphorylation on S727.

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474 **Figure legends**

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476 **Figure 1: Establishment of TGF- $\beta$ RII-KD CAPAN-1 and CAPAN-2 cell lines.** (A) Analysis  
477 of mRNA expression of TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ R1, TGF- $\beta$ R2, TGF- $\beta$ R3, Smad2, Smad3,  
478 Smad4, Smad ATP7A, ATP7B and 28S in CAPAN-1, CAPAN-2 cells by RT-PCR. (B)  
479 Analysis of mRNA relative expression of TGF- $\beta$ R2 in NT and TGF- $\beta$ R2-KD CAPAN-1 and  
480 CAPAN-2 cell lines. Expression in NT cells was arbitrarily set to 1. (C) Smad-Binding-  
481 Elements (SBE) relative luciferase activity in untreated and TGF- $\beta$  treated NT and TGF- $\beta$ R2-  
482 KD CAPAN-2 cells. Relative luciferase activity was expressed as a ratio of SBE-Luc  
483 normalized with pGL3 basic activity. (D) Analysis of mRNA relative expression of TGF- $\beta$ 1 in  
484 NT and TGF- $\beta$ R2-KD CAPAN-1 and CAPAN-2 cell lines.

485

486 **Figure 2: TGF- $\beta$ R2 alters tumor growth and migration in pancreatic cancer cells.** (A)  
487 Wound healing closure of NT and TGF- $\beta$ R2-KD CAPAN-1 and CAPAN-2 cell lines using the  
488 IncuCyte™ chamber apparatus. (B) Subcutaneous xenografts of NT/TGF- $\beta$ R2-KD8 CAPAN-  
489 1 and CAPAN-2 cells in scid mice. Tumour growth (mm<sup>3</sup>) was evaluated until sacrifice. \*\*p <  
490 0.01 and \*\*\*p < 0.001 indicate statistical significance of TGF- $\beta$ R2-KD1 compared with the NT  
491 control. Ns: not significant. (C) Evaluation of the presence of micro-metastasis in the liver by  
492 detecting the presence of human GAPDH in the liver of NT and TGF- $\beta$ R2-KD CAPAN-1 and  
493 CAPAN-2 xenografted mice by qPCR.

494

495 **Figure 3: TGF- $\beta$ R2 alters sensitivity to gemcitabine in pancreatic cancer cells in vitro**  
496 **and in vivo.** Survival rates in different TGF- $\beta$ R2-KD CAPAN-1 (A) and CAPAN-2 (B) cell  
497 lines or their NT control cells were measured following treatment with gemcitabine using the  
498 MTT assay. Results are expressed as % of cell survival (/untreated cells). Three independent  
499 experiments were performed. (C) Subcutaneous xenografts of NT and TGF- $\beta$ R2-KD8  
500 CAPAN-2 cells in scid mice. Gemcitabine (15 mg/kg) or PBS (200  $\mu$ l) were injected intra-  
501 peritoneously, twice a week once palpable tumors were developed. Normalized tumor growth

502 is expressed as the ratio of tumor progression relative to tumor volume on the first day of  
503 gemcitabine treatment. Right graph represents tumor growth over time. Left graph represents  
504 final tumor volume at day 83 (normalized as initial tumor volume at D36 equal to 1).

505

506 **Figure 4: Impact of TGF- $\beta$ RII knocking-down on signaling pathways.** (A) Impact of TGF-  
507  $\beta$ RII knocking-down on intracellular signaling was studied using phospho array that detect  
508 relative site-specific phosphorylation of 43 proteins. Boxes highlight spots for S63 c-Jun and  
509 S727 STAT3. (B) Heatmap representing the intensities of each spots (TGF- $\beta$ RII vs NT) that  
510 were measured and normalized to the reference spots for CAPAN-1 and CAPAN-2 cells.

511

512 **Figure 5: TGF- $\beta$ RII knockdown promotes STAT3 phosphorylation and nuclear**  
513 **localisation in CAPAN-2 cells.** (A) STAT3, phospho-S727 STAT3 and  $\beta$ -actin expression  
514 was analysed by western blotting. Bands intensities were quantified by densitometry and  
515 ratios (KD vs NT or treated/untreated) are indicated in the graphs. Expression in NT (for  
516 TGF- $\beta$ RIIKD) or untreated (for gemcitabine/TGF- $\beta$ ) cells was arbitrarily set to 1. (B) IHC  
517 analysis of STAT3 on extracted xenografted NT and TGF- $\beta$ RIIKD tumors. (C) Nuclear and  
518 cytoplasmic IHC staining were scored in NT and TGF- $\beta$ RIIKD xenografted tumors that were  
519 treated with gemcitabine or PBS. \* $p < 0.05$  indicates statistical significance of TGF- $\beta$ RII-KD1  
520 compared with the NT control.

521

522 **Figure 6: TGF- $\beta$ RII silencing alters ABC transporters expression.** (A) mRNA expression  
523 of *TGF- $\beta$ RII*, *c-Jun*, *STAT3*, *MRP1*, *MRP2*, *MRP3*, *MRP4*, *MRP5*, *ABCG2* and *MDR1* was  
524 analyzed in NT and TGF- $\beta$ RII-KD CAPAN-1 and CAPAN-2 cells by qRT-PCR. The  
525 histogram represents the ratio of their expression in TGF- $\beta$ RII-KD compared with NT cells.  
526 Three independent experiments were performed. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$   
527 indicate statistical significance of TGF- $\beta$ RII-KD1 compared with the NT control. TGF- $\beta$ RII,  
528 MRP3 (B) and MRP4 (C) mRNA expression was extracted from pancreatic cell lines from

529 Cancer Cell Line Encyclopedia (CCLE). Statistical analyses of MRP3/TGF $\beta$ RII and  
530 MRP3/TGF- $\beta$ RII correlations were analysed Pearson's correlation coefficient.

531

532 **Table 1 : Primers used for RT-PCR and qPCR experiments**

533

534 **Supplemental material**

535

536 **Supplemental Figure 1: TGF- $\beta$ RII knockdown promotes c-Jun S-63 phosphorylation in**

537 **CAPAN-2 cells.** (A) c-Jun, phospho-S63 c-Jun and  $\beta$ -actin expression was analysed by

538 western blotting. Bands intensities were quantified by densitometry and ratios (KD vs NT or

539 treated/untreated) are indicated in the graphs. Expression in NT (for TGF- $\beta$ RIIKD) or

540 untreated (for gemcitabine/TGF- $\beta$ ) cells was arbitrarily set to 1. (B) IHC analysis of c-Jun on

541 extracted xenografted NT and TGF- $\beta$ RIIKD tumors. (C) IHC staining was scored in NT and

542 TGF- $\beta$ RIIKD xenografted tumors that were treated with gemcitabine or PBS. \* $p < 0.05$  indicate

543 statistical significance of TGF- $\beta$ RII-KD compared with the NT control.

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545 **Supplemental Figure 2: TGF- $\beta$ RII knockdown promotes partial EMT-like phenotype.**

546 IHC analysis of E-cadherin and vimentin on extracted xenografted NT and TGF- $\beta$ RIIKD

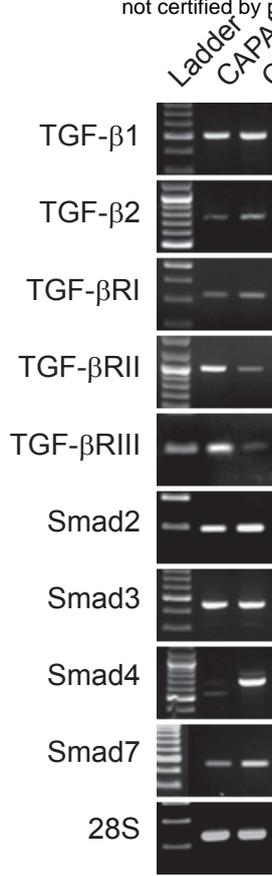
547 tumors. IHC staining was scored in NT and TGF- $\beta$ RIIKD xenografted tumors that were

548 treated with gemcitabine or PBS. \* $p < 0.05$ , \*\* $p < 0.01$  indicate statistical significance of TGF-

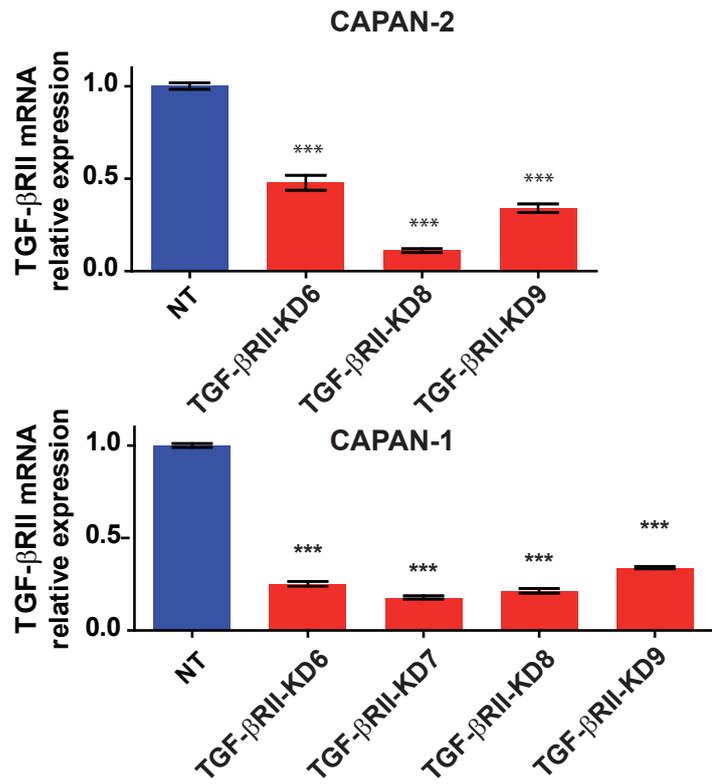
549  $\beta$ RII-KD compared with the NT control.

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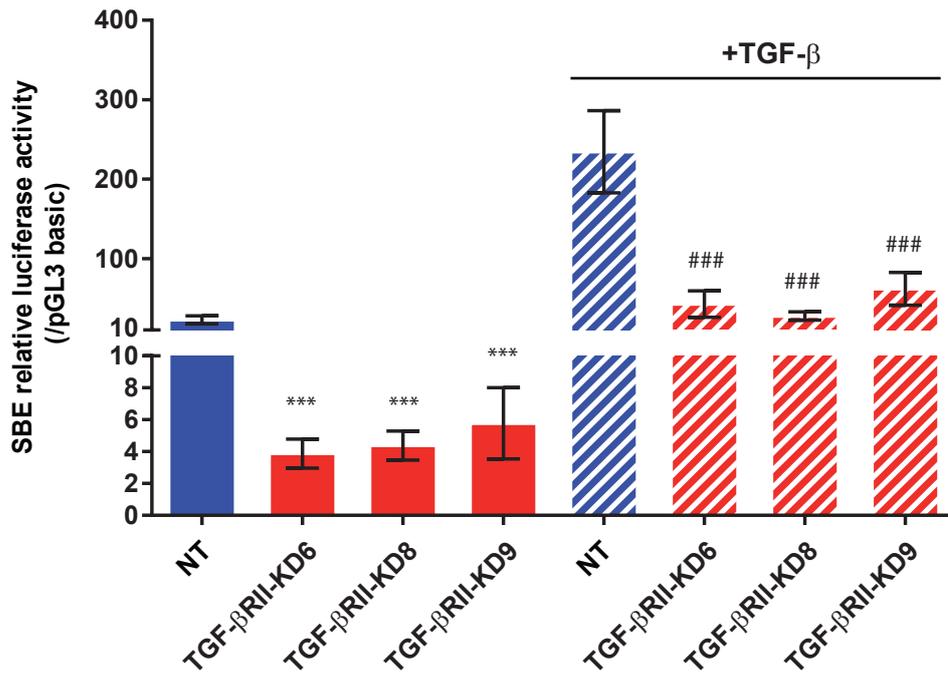
**A**



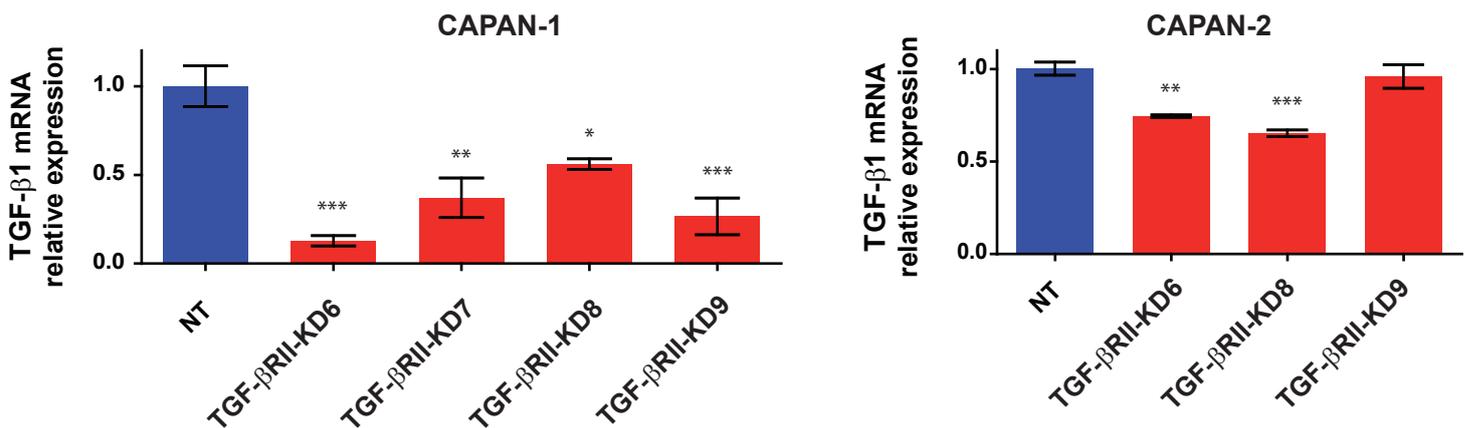
**B**



**C**



**D**



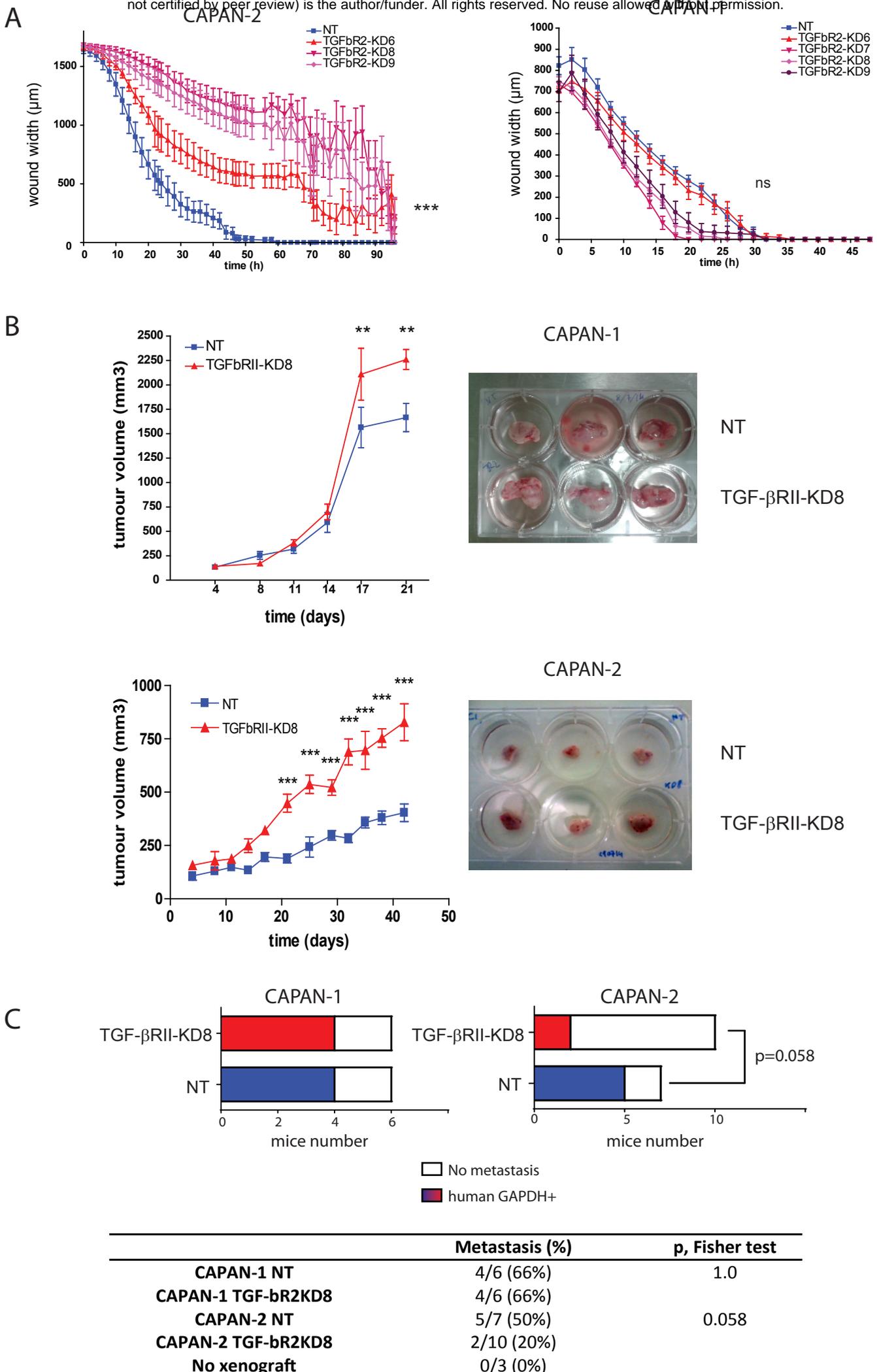
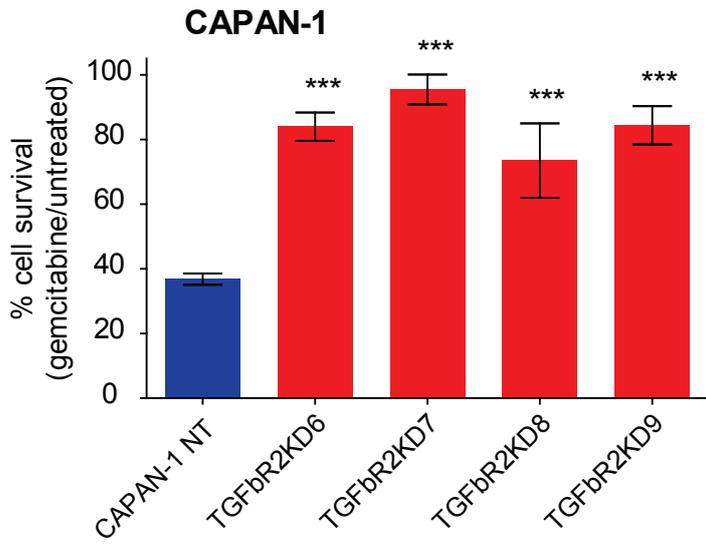
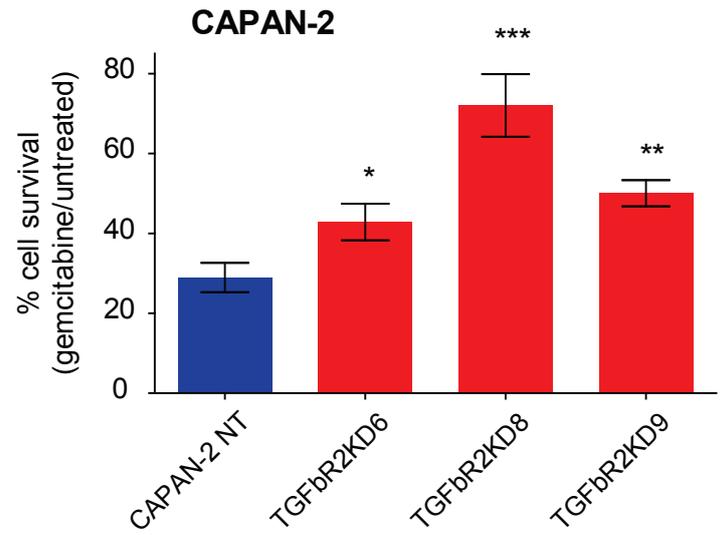


Figure 2

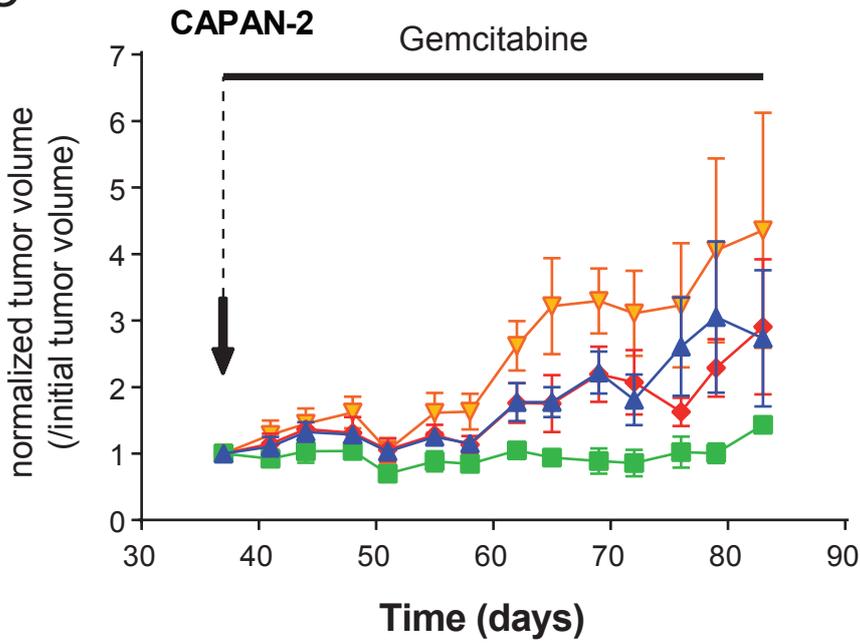
**A**



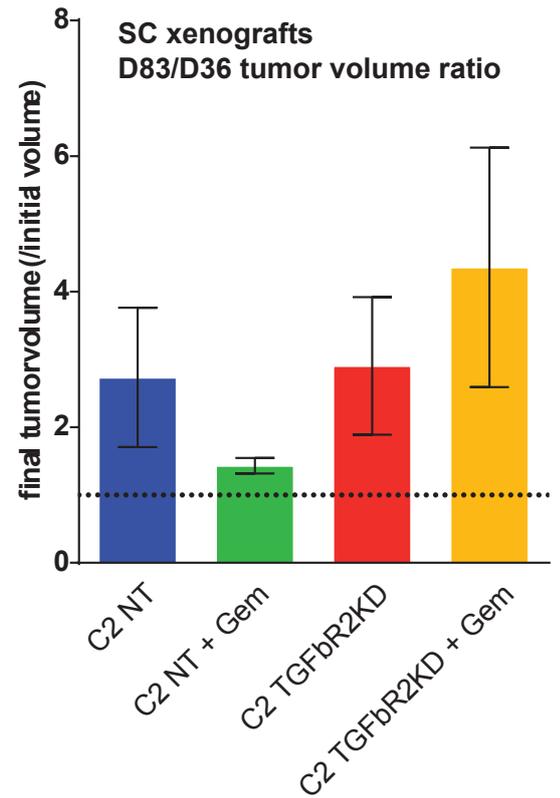
**B**



**C**

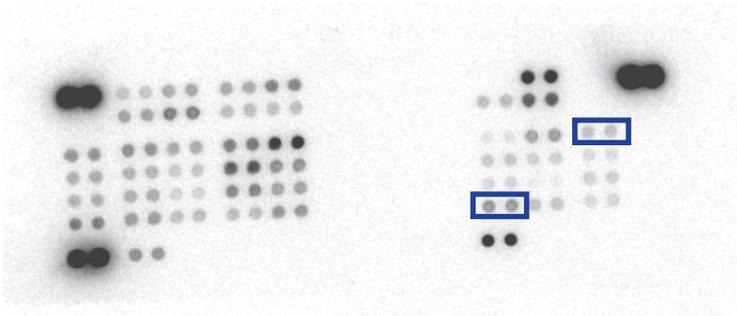


▲ C2 NT                      ◆ C2 TGFbR2KD  
■ C2 NT + Gem              ▼ C2 TGFbR2KD + Gem

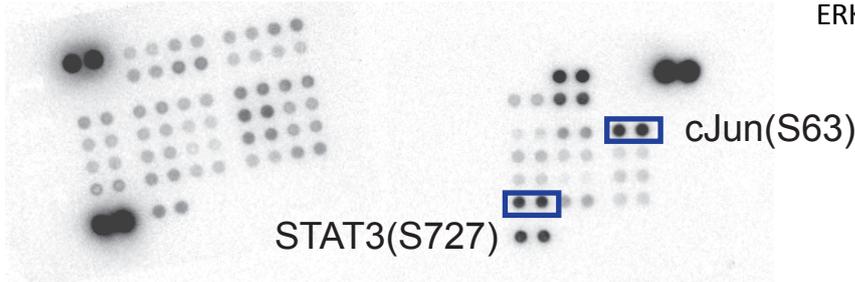


**A**

CAPAN2 NT



CAPAN2 TGF- $\beta$ R2KD



**B**

CAPAN-2 CAPAN-1  
TGF- $\beta$ R2-KD vs NT

Akt 1/2/3 (S473)  
Akt 1/2/3 (T308)  
AMPK alpha1 (T174)  
AMPK alpha2 (T172)  
**beta-Catenin**  
Chk-2 (T68)  
**c-Jun (S63)**  
CREB (S133)  
EGF R (Y1086)  
eNOS (S1177)  
ERK1/2 (T202/Y204, T185/Y187)  
FAK (Y397)  
Fgr (Y412)  
Fyn (Y420)  
GSK-3 alpha/beta (S21/S9)  
Hck (Y411)  
HSP27 (S78/S82)  
HSP60  
JNK 1/2/3 (T183/Y185, T221/Y223)  
Lck (Y394)  
Lyn (Y397)  
MSK1/2 (S376/S360)  
p27 (T198)  
p38 alpha (T180/Y182)  
p53 (S15)  
p53 (S392)  
p53 (S46)  
p70 S6 Kinase (T389)  
p70 S6 Kinase (T421/S424)  
PDGF R beta (Y751)  
PLC gamma-1 (Y783)  
PRAS40 (T246)  
Pyk2 (Y402)  
RSK1/2/3 (S380/S386/S377)  
Src (Y419)  
STAT2 (Y689)  
**STAT3 (S727)**  
STAT3 (Y705)  
**STAT5a (Y694)**  
STAT5a/b (Y694/Y699)  
STAT5b (Y699)  
STAT6 (Y641)  
TOR (S2448)  
WNK-1 (T60)  
Yes (Y426)

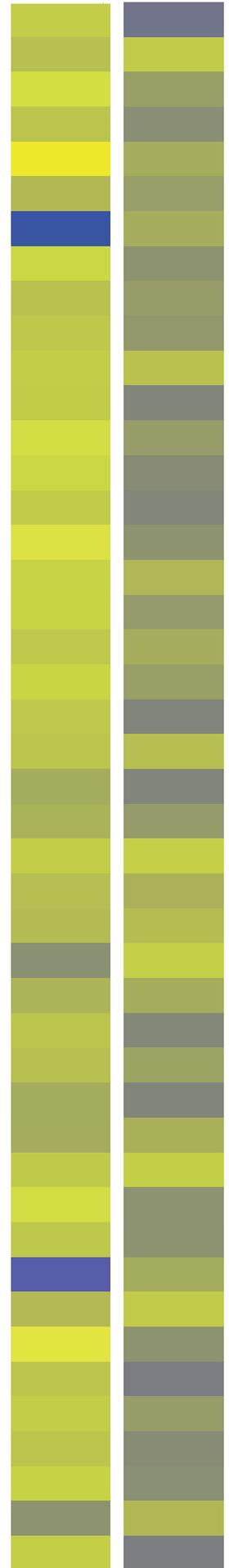


Figure 4

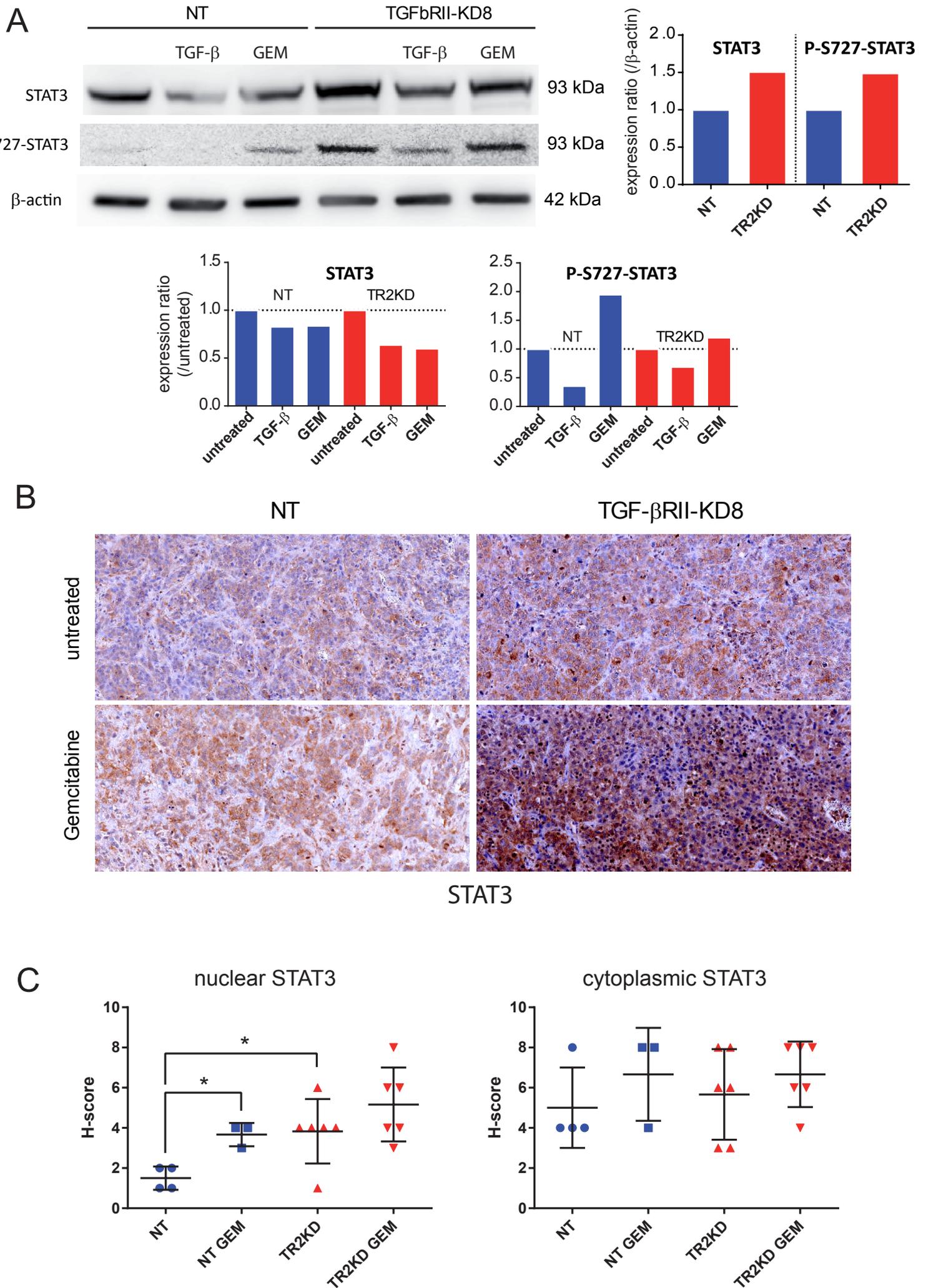
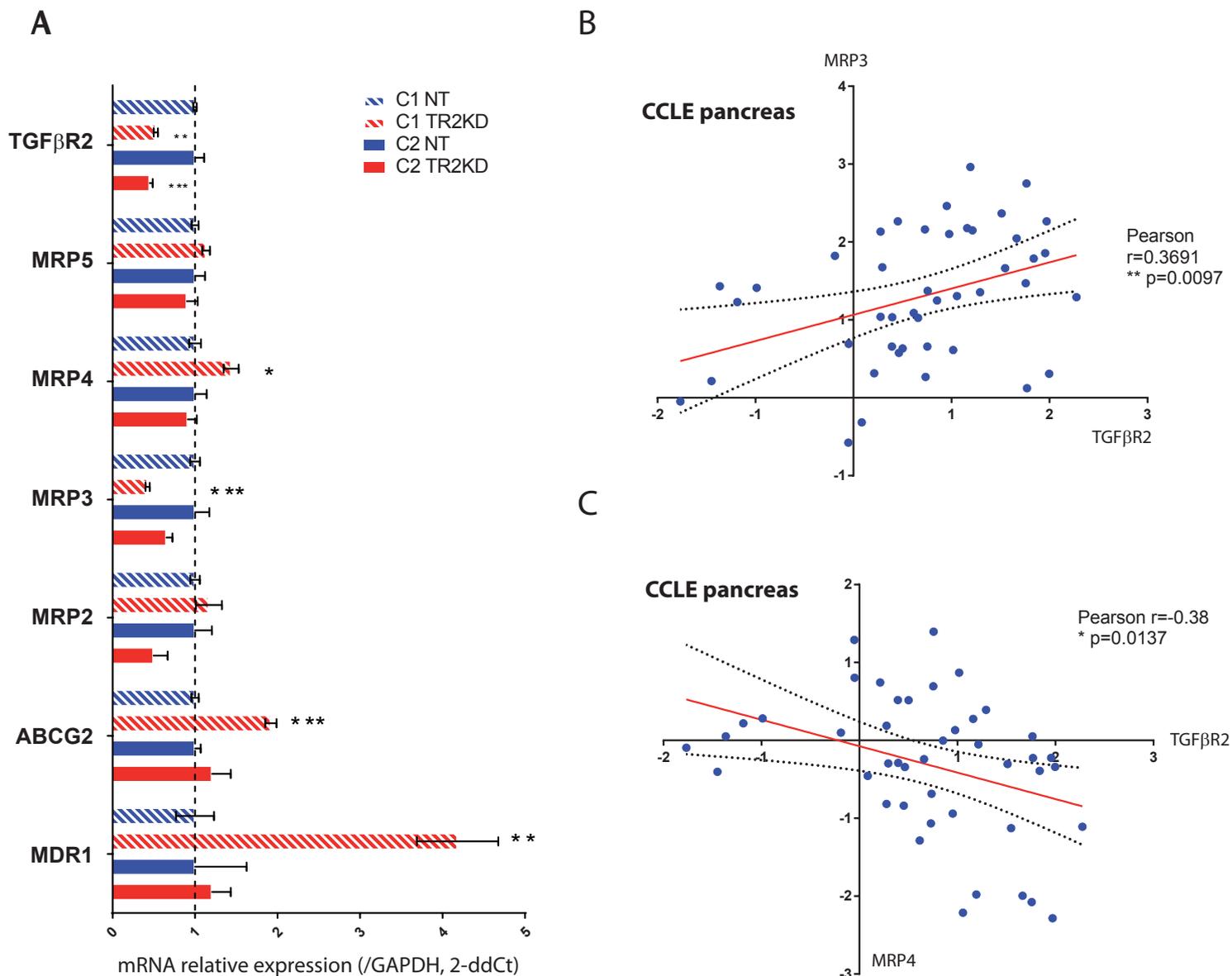


Figure 5

Figure 6



**Table 1 : Primers used for RT-PCR and qPCR experiments**

Gene	Orientation	Sequences of primers (5'-3')	T <sup>o</sup> m (°C)	Expected size
RT-PCR				
<i>28S</i>	Forward Reverse	GCAGGGCGAAGCAGAAGGAAACT TGAGATCGTTTCGGCCCAA	59	231
<i>TGF-β1</i>	Forward Reverse	GAGGTGACCTGGCCACCATTCAT CCAGCCGAGGTCCCTTGCGGA	60	194
<i>TGF-β2</i>	Forward Reverse	GCTTTTCTGATCCTGCATCTG CAATACCTGCAAATCTTGCTTC	56	823
<i>TGF-βRI</i>	Forward Reverse	CTCTCCTTTTTTCTTCAGATCTGC AATCCAACCTCCTTGCCCTT	55	328
<i>TGF-βRII</i>	Forward Reverse	GCCAACAACATCAACCACAACACA TAGTGTTTAGGGAGCCGTCTTCAG	61	1003
<i>TGF-βRIII</i>	Forward Reverse	TGCCTTACTTCTCTTGCCTTAA GCAAAGTGGCATCATATTATT	56	100
<i>Smad2</i>	Forward Reverse	GTCCATCTTGCCATTCACG TGGTGATGGCTTTCTCAAGC	55	192
<i>Smad3</i>	Forward Reverse	GGGCTCCCTCATGTCATCTA GGCTCGCAGTAGGTAAGTGG	60	443
<i>Smad4</i>	Forward Reverse	CTCCTGAGTATTGGTGTTC CTAAAGGTTGTGGGTCTGC	56	796
<i>Smad7</i>	Forward Reverse	GGCTCGCAGTAGGTAAGTGG TTGTTGTCCGAATTGAGCTG	55	448
qPCR				
<i>TGF-β1</i>	Forward Reverse	CACTCTCAAACCTTACGAGACC CGTTGCTAGGGGCGAAGATG	58	131
<i>TGF-βRII</i>	Forward Reverse	AGGAGTATGCCTCTTGGAAGAC AGCCAGTATTGTTTCCCAAC	58	123
<i>Human GADPH</i>	Forward Reverse	CCACATCGCTCAGACACCAT CCAGGCGCCAATACG	58	70
<i>Mouse GADPH</i>	Forward Reverse	AGGTCGGTGTGAACGGATTTG TGTAGACCATGTAGTTGAGGTCA	58	129