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Gemcitabine-induced TIMP1 attenuates therapy response and promotes tumor growth and liver metastasis in pancreatic cancer

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Running Title: TIMP1 promotes pancreatic cancer progression

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ABSTRACT

Gemcitabine constitutes one of the backbones for chemotherapy treatment in pancreatic ductal adenocarcinoma (PDAC), but patients often respond poorly to this agent. Molecular markers downstream of gemcitabine treatment in pre-clinical models may provide an insight into resistance mechanisms. Using cytokine arrays, we identified potential secretory biomarkers of gemcitabine resistance (response) in the transgenic KRasG12D; Trp53R172H; Pdx-1 Cre (KPC) mouse model of PDAC. We verified the oncogenic role of the cytokine tissue inhibitor of matrix metalloproteinases 1 (TIMP1) in primary pancreatic tumors and metastases using both in vitro techniques and animal models. We identified potential pathways affected downstream of TIMP1 using the Illumina Human H12 array. Our findings were validated in both primary and metastatic models of pancreatic cancer. Gemcitabine increased inflammatory cytokines including TIMP1 in the KPC mouse model. TIMP1 was upregulated in patients with pancreatic intraepithelial neoplasias grade 3 and PDAC lesions relative to matched normal pancreatic tissue. Additionally, TIMP1 played a role in tumor clonogenic survival and vascular density, while TIMP1 inhibition resensitized tumors to gemcitabine and radiotherapy. We observed a linear relationship between TIMP-1 expression, liver metastatic burden, and infiltration by CD11b+Gr1+ myeloid cells and CD4+CD25+FOXP3+ Tregs, whereas the presence of tumor cells was required for immune cell infiltration. Overall, our results identify TIMP1 upregulation as a resistance mechanism to gemcitabine and provide a rationale for combining chemo/radiotherapy with TIMP1 inhibitors in PDAC.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive cancers with dismal prognosis for overall survival, due to late presentation and limited responses to therapy (1,2). About 80% of cases are inoperable at diagnosis leaving chemotherapy and/or chemoradiotherapy as the main treatment options. Gemcitabine constitutes one of the chemotherapy backbones but patients commonly show either poor or even complete lack of response to this agent (1). Gemcitabine is a DNA nucleoside analog that irreversibly inhibits ribonucleotide reductase (RNR) activity, preventing the synthesis of dioxyribonucleotides required for DNA replication and repair (3). Treatment induces DNA damage and apoptosis of growing cells, thus is frequently employed in combinatorial chemotherapy regimens to treat ovarian, breast and bladder cancers in addition to pancreatic cancer. While patients have been shown to benefit from gemcitabine, poor responses in pancreas imply that intrinsic resistance and poor patient response to this chemotherapeutic limits efficacy (4). In studies addressing gemcitabine sensitivity, it was recently shown that acute cytokine release following DNA damage may contribute to resistance acquired during the course of treatment as well as to treatment failure (5).

A number of cytokines have been associated with PDAC and have been investigated for potential as early diagnostic or predictive biomarkers, and are beginning to demonstrate some power in diagnosis (1,4). TIMP1 was originally highlighted as having potential to distinguish patients with PDAC from controls, however, subsequent evidence demonstrated that although patients display elevated levels, TIMP1 does not identify early disease (6).

Tissue inhibitor of matrix metalloproteases (TIMP1) is known to inhibit metalloproteases (MMPs) disintegrin and metalloproteinase domain-containing protein 10 and 17 (ADAM-10 and ADAM-17) (7). TIMP1 has also been shown to activate pro-survival signalling, independent of its MMP inhibitory activity, through binding with its receptor CD63 and subsequent engagement of integrin ß1 mediated activation of PI3K signalling (8-11).

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TIMP1 can influence a number of tumorigenic biological processes, such as proliferation, apoptosis and metastasis (7,12,13). Although TIMP1 expression is suggested as a prognostic marker in several malignancies (14-16), and its expression is associated with hyperproliferation of K-Ras (G12D) mutated pancreatic cells (17), the functional role of TIMP1 within the PDAC microenvironment remains largely unexplored. Intriguingly, tumor derived TIMP1 has been proposed to support neutrophil infiltration to the normal liver and the creation of a premetastatic niche to which circulating tumor cells adhere and seed metastasis in colorectal cancer (13). Here, we found that TIMP1 is directly elevated in response to gemcitabine treatment that was predominantly associated with a pro-tumorigenic phenotype.

MATERIALS AND METHODS

Cell lines

KPC cells derived from pancreatic tumors of $KRas^{G12D}$; $Trp53^{R172H}$; Pdx-1 Cre (KPC) mice and cultured in Dulbecco's Mimimum Essential medium (DMEM) supplemented with 10% FCS and authenticated as previously reported (18,19). The human PDAC cell line PANC1 was purchased from European Collection of Authenticated Cell Cultures (ECACC) and cultured in DMEM supplemented with 10% FCS. PANC1 cells were authenticated by STR profiling. To generate stable knockdowns (KD) of mouse TIMP1 and human TIMP1, KPC and PANC1 cells were transduced with lentivirus-mediated shRNA or the control vector (MISSION shRNA, Sigma). To generate stable overexpression, PANC1 cells were transduced with retroviral particles. The p6610 MSCV-IP N-HAonly TIMP1 (retroviral plasmid) was purchased from Addgene (catalogue number 35009) and used to generate live retroviral particles in the packaging cell line HEK 293T. The retroviral particles were then used to transduce PANC1 wild-type (WT) and knockdown (KD) cells, alongside an empty retroviral control vector. Immortalized Pancreatic CAF-Stellate Cells were obtained from Neuromics, and cultured in VitroPlus III, low serum, complete (Neuromics). Pancreatic CAFS were phenotypically tested

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by Vitro Biopharma for a range of markers including CD105, CD90, CD44, CD326, CD133, FAP, GFAP, FSP1, α-SMA and Vimentin. KPC and PANC1 cells were selected with 5 µg/ml and 2.5 µg/ml of puromycin, respectively. All cell lines were obtained in 2015, and fresh aliquots were thawed and used up for a limited amount of time to the 6^{th} passage, except gemcitabine-resistant cells that were maintained for two months in cell culture. All cell lines in our lab were tested for Mycoplasma every 4 weeks using the MycoAlert kit (Lonza).

Animal models

All animal experiments were carried out according to the UK Animal (Scientific Procedures) act of 1986. SCID and C57BL/6 mice were purchased from Charles River Laboratories (Kent, UK). KPC transgenic mice (20) were bred at the premises of the University of Oxford. Genotyping was carried out at Transnetyx Inc. (Tennessee, USA). When KPC mice reached 12 weeks of age, palpation, sonography and/or magnetic resonance imaging (MRI) was used to identify and measure the size of intraabdominal pancreatic tumors in KPC mice.

For the subcutaneous models, human PANC1 and murine KPC cells (1 X 10^6 cells in 100µl of phosphate buffered saline (PBS) were injected subcutaneously into SCID (immunosuppressive) and C57BL/6 (immunocompetent) mice, respectively. Mice were randomised when tumors reached 100mm^3. Where specified, mice were treated with Gemcitabine (100mg/kg i.p.) or 8Gy. Tumor volume was measured by callipers as described (21). Non-tumor bearing C57BL/6 mice were also treated with Gemcitabine.

For the liver metastasis model, SCID or C57BL/6 mice were injected intrasplenically with 5 x 10^5 PANC1 or KPC cells, respectively, in PBS as previously described [26]. C57BL/6 mice were sacrificed at day 14 after inoculation, whereas SCID mice were imaged by MRI and sacrificed when liver metastasis was observed 21-26 days after inoculation. For all animal experiments n=5-7mice were randomised in each experimental group.

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Magnetic resonance imaging

The magnetic resonance imaging method (22), including its technical characteristics, is described in detail in Supplementary methods .

Cytokine arrays, phosphokinase and ELISA

Proteome profiler antibody array and phosphokinase array (R&D Systems) were performed according to manufacturer's protocol.

Human and mouse TIMP1 Quantikine and DuoSet ELISA kits (R&D Systems) were used according to manufacturer's protocol.

Immunohistochemistry/Immunofluorescence, Western blot and Flow Cytometry

Tissue sections were stained for haematoxylin/eosin and CD31 staining, and scored as described (21,23). Immunofluorescence, Western blot and flow cytometry are described in Supplementary Methods.

Clonogenic survival, migration and invasion assays

Clonogenic survival was carried out as reported (24). Wound scratches across a monolayer of KPC and PANC1 cells were conducted out using a sterile 200 µl pipette tip. Bright field images were taken at 16 and 22 hours until wound closure. Boyden invasion chamber inserts (Costar, 12µm pore size) were coated with 100µg/cm^2 of Matrigel. Cells were seeded onto the chamber insert and placed into a 24-well plate containing a cell line monolayer acting as an attractant in its respective media, and invasion was measured after 16 hours.

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Microarray and Oncomine analysis, RT-qPCR and invasion towards CAFS

These assays are described in Supplementary Methods. In the microarray experiment, standard MIAME guidelines were followed. The full microarray data (GSE94891) are available on GEO.

Statistical analysis

GraphPad Prism 5 (San Diego, USA) was used for all statistical analyses besides Kaplan-Meier survival curves for which and IBM SPSS software Version 21 (SPSS Inc., Chicago, IL, USA) was used. By default, values were expressed as mean ± standard deviation and statistical significance was measured using 1-way ANOVA with Bonferroni post-test, or using unpaired t-test. The log-rank test was used to compare Kaplan-Meier curves, while unpaired non-parametric comparisons were made using the Mann-Whitney test where appropriate. In all tests, p-values <0.05 were considered significant.

RESULTS

Identification of TIMP1 as a Gemcitabine response gene

To gain a better understanding of cytokine release following DNA damage we treated transgenic KPC mice bearing tumor volumes between 70 and 120mm^3 with saline or Gemcitabine (100mg/kg, i.p.) at day 1, 4 and 8. To identify both early and sustained response genes following Gemcitabine treatment, mice were divided into three groups; control mice were treated with saline and culled at their terminal end point. Gemcitabine 'short-term' mice were sacrificed at day 9 from treatment initiation i.e. 1 day upon completion of Gemcitabine, and Gemcitabine 'long-term' mice were allowed to progress and were sacrificed at their terminal end point **(Figure 1a).** There was no clear separation, in the mice treated with Gemcitabine relative to control treated mice, suggesting no survival advantage with gemcitabine **(Figure 1b, Supplementary Figure 1a)**. We did not cull any mice at day 9 in the

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saline group but believe this is irrelevant to the present work as some of the transgenic KPC mice became terminally ill at this time point. However, the mean tumor volume was only marginally decreased in the Gemcitabine short term group and remained the same at the terminal endpoint compared to controls **(Supplementary Figures 1b and 1c respectively)**. To identify potential secreted biomarkers that could reveal signalling mechanisms downstream of Gemcitabine treatment, cytokine profiling was carried out on the serum from the three groups **(Figures 1c)**. Several cytokines including TIMP1, interleukin 6 (IL-6), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF) and; chemokine (C-X-C motif) ligand 13 (CXCL13)), were upregulated early upon treatment with Gemcitabine. The levels of TIMP1 were particularly high and its increased levels were sustained until mice reached their terminal end point. TIMP1 levels in serum of KPC mice after Gemcitabine were confirmed by ELISA in n=6 mice **(Figure 1d).** Immunohistochemical staining for TIMP1 also confirmed elevated expression of TIMP1 in PDAC tumors following Gemcitabine treatment, irrespective of stromal content of the tumors **(Figure 1e),** as quantified in **(Supplementary Figure 1d)**. In addition, TIMP1 mRNA levels were found to be higher in tumors following Gemcitabine treatment **(Supplementary Figure 1e).** Alternatively, there was no increase in TIMP1 levels in the serum of non-tumor bearing mice following Gemcitabine treatment **(Supplementary Figure 1f)** indicating the tumor to be the source of TIMP1**.** Furthermore, to determine whether TIMP1 played a role in Gemcitabine resistance, Gemcitabine-resistant KPC cells were generated by culturing cells in progressively increasing concentrations of the drug over a period of 2 months, confirmed by clonogenic survival assay **(Supplementary Figure 1g).** Compared to the parental sensitive cell line (KPC^S), Gemcitabine-resistant cells (KPC^R) showed higher expression of TIMP1 in both conditioned media **(Figure 1f)** and lysates **(Figure 1g).** Moreover, TIMP1 levels further increased in conditioned media **(Figure 1f)** following treatment with Gemcitabine in both the sensitive and the resistant cell lines. In addition, online datamining confirmed the increased expression of TIMP1 in Gemcitabine-resistant lung cancer

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cells, relative to Gemcitabine-sensitive lung cancer cell lines **(Figure 1h)** (25). These data indicate that TIMP1 is induced by Gemcitabine and that TIMP1 levels are intrinsically higher in Gemcitabine-resistant cells compared to its sensitive counterparts, and may play a role in tumor cell resistance to Gemcitabine.

TIMP1 is a potential marker of PDAC

Interestingly, analysis of microarray data from 4 different PDAC cohorts revealed a significant upregulation of TIMP1 in human PDAC relative to normal matched pancreatic tissue. In the Iacobuzio-Donahue dataset (26) comprising 12 resected infiltrating PDACs compared to 5 samples of normal pancreas, TIMP1 is among the top 6% of genes upregulated in PDAC versus normal pancreas (p=0.003) **(Figure 2a).** In the Segara dataset comprising multiple samples of pancreatic tissue from 11 surgical resections and six matched normal appearing pancreas tissue (27),TIMP1 is in the top 5% of genes upregulated in PDAC relative to normal tissue (p=4.95E-4) **(Figure 2b).** In the Badea dataset (28) comparing samples of whole PDAC tissue and matched samples of normal pancreas (n=39), TIMP1 was overexpressed in the top 1% of upregulated genes (p=5.03E-15) **(Figure 2c).** Buchholz et al (29) microdissected surgically resected tissue to remove normal tissue effects and in this dataset we observe TIMP1 is upregulated 3-fold and belongs to the top 2% of upregulated genes in both PanIN-3 lesions and PDAC compared to normal duct and acinar cells, PanIN-1b and PanIN-2 lesions **(Figure 2d)**. This indicates that TIMP1 is upregulated at the later stages of PDAC progression, in agreement with recent reports recent that have clearly dismissed the TIMP1 as a marker of early disease (6).

TIMP1 knockdown decreases clonogenic survival and enhances therapy response

To elucidate the role of TIMP1, we stably knocked down TIMP1 expression using five different lentiviral shRNA constructs each in the human PDAC cell line PANC1 and the

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murine PDAC cell line KPC **(Supplementary Figure 2a and 2b)**; The cell lines with the highest knockdowns were confirmed by qPCR **(Supplementary Figure 2c and 2d)** as well as by western blotting **(Supplementary Figure 2e)** besides ELISA **(Figures 3a and b)**. TIMP1 knockdown resulted in a decrease in colony formation in both cell lines **(Figures 3c and 3d)**; quantified in **(Supplementary Figures 2f and 2g)**. In addition, the surviving fraction in TIMP1 knockdown sensitized both cell lines to Gemcitabine and radiation compared to untreated/DMSO controls, (**Figures 3e-h)**. We overexpressed TIMP1 retrovirally in our WT and KD cell line alongside the appropriate empty retroviral vector **(Figure 3i)**. Overexpression of TIMP1 resulted in a rescue of cell growth and desensitised cells to radiation and Gemcitabine **(Figure 3j-l)**. Representative images are shown in **(Supplementary Figure 2h-j).** We injected PANC1 and KPC TIMP1-WT and KD subcutaneously into SCID and C57bl/6 mice respectively and noted that the time it took for the tumors to reach a volume of 300 mm^3 was significantly longer in the TIMP1 KD group compared to TIMP1 WT cells **(Figure 3m)**. We found no differences in the amount of necrosis or stroma between the two groups in PANC1 and KPC tumors **(Supplementary Figure 2k-n).** With the KPC cells the time taken for tumors to reach maximum volume ($700mm^3$) was significantly longer in the TIMP1 KD group **(Figures 3n)**. To confirm whether our *in vitro* chemo/radiosensitisation was replicated *in vivo*, we treated mice with 8Gy or 100mg/kg Gemcitabine. In keeping with our in vitro data, tumor growth was significantly delayed **(Figure 3o and 3p).** Taken together, these results show that TIMP1 facilitates primary tumor growth, associated with lower response to Gemcitabine and radiation both *in vitro* and in our *in vivo* subcutaneous models.

Cell adhesion, chemotaxis and inflammatory pathways are altered by TIMP1 expression.

To identify potential tumorigenic pathways affected by TIMP1 signalling, we carried out a microarray and analysed gene expression in TIMP1 WT versus KD PANC1 cells. In total, 136 differentially regulated genes were identified **(Figure 4).** Gene annotation studies indicated

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enriched expression of inflammatory transcripts, cell adhesion transcripts and chemotaxis in TIMP1 KD cells **(Table 1)**. Representative genes were validated by real-time qPCR **(Supplementary Figures 3a-d).**

TIMP1 knockdown results in decreased migration and liver metastasis

To verify whether the role of TIMP1 in chemotaxis translated to an increased migratory capacity we compared TIMP1 WT and KD cells in a wound scratch assay. TIMP1 expression resulted in a stronger migratory phenotype in both PANC1 **(Figure 5a)** and KPC cells **(Figure 5b)** in the scratch wound assay quantified in **(Supplementary Figure 4a)**. Also, both PANC1 and KPC TIMP1-KD cells showed decreased invasive capacity towards CAFs compared to their WT counterparts **(Supplementary Figure 4b).** TIMP1 did not affect the expression of phosphokinases in PANC1 cells in-vitro **(Supplementary Figure 4c).** TIMP1 has been previously reported to enhance angiogenesis (30,31). In our primary subcutaneous animal models, tumors derived from both PANC1 and KPC TIMP1 KD cells showed decreased blood vessel density staining compared to WT groups **(Figure 5c and d).** As our gene enrichment analysis identified transcripts involved in cell adhesion and chemotaxis, we examined whether TIMP1 expression in PDAC cells could affect their metastatic potential *in vivo*. As the liver is the most common site of metastatic spread in PDAC, we injected PANC1 TIMP1 WT and KD cells intrasplenically into SCID mice as reported (32). MRI imaging was used to monitor liver metastasis (**Supplementary Figure 4d and 4e).** TIMP1 KD resulted in significantly reduced hepatic metastatic burden **(Figure 5e-f)** with lower vascular density within the metastatic regions **(Figure 5g).** To verify our results in a different mouse model, we injected KPC TIMP1 WT and KD cells intrasplenically into C57Bl/6 mice. The hepatic metastatic burden was significantly lower following TIMP1 suppression **(Figure 5h).** H&E staining confirmed the presence of metastasis in the liver **(Figure 5i)** and blood vessel density was also significantly reduced **(Figure 5j)**. To address pathways that may be involved in the metastastic process we

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performed a protein array chip, but found that pathway activation was indistinguishable from controls **(Supplementary Figure 4c)**

TIMP1 level in blood has been considered as a prognostic factor in patients with liver metastasis (33). We harvested blood from C57Bl/6 mice at days 4, 7 and 14 post-intrasplenic injection of KPC cells and found significantly increased circulating TIMP1 with increased metastatic burden **(Figure 6a).** We conclude that TIMP1 expression correlates with enhanced vascular density and is essential for tumor cell migration and metastatic growth.

TIMP1 promotes myeloid cell recruitment and Tregs in liver metastasis

TIMP1 can promote immunosuppression (13). Our microarray data identified inflammatory pathways altered by TIMP1. Hence, we queried whether there were any differences in immunosuppressive myeloid populations in the tumor microenvironment that are known to promote malignant growth. While we failed to identify significant changes in primary subcutaneous PANC1 and KPC mouse models following TIMP1 suppression **(Supplementary Figure 5a-b),** we noted distinct changes in the immune profile of metastatic livers. When we examined the immune profile in livers harbouring metastases at day 4, 7 and 14 following intrasplenic injection of KPC cells in C57Bl/6 mice, no macroscopic metastasis was observed at day 4 or day 7 but metastases were evident by day 14 and CD45+CXCR2+ neutrophils increased with progressive metastatic development **(Figure 6b)**. Taken together, these results suggest that increasing TIMP1 levels during metastasis formation are associated with increased liver infiltration by myeloid cells.

When we investigated the immune profile in liver metastases following intrasplenic injection of TIMP1 WT and KD PANC1 cells in SCID mice **(Figure 5e)** we noted a significant decrease in CD11b+Gr1+ myeloid derived suppressor cells (MDSCs) and neutrophil infiltration following TIMP1 suppression **(Figure 6c).** Similarly, MDSCs and CD4+CD25+FoxP3+ regulatory T cells were decreased in liver metastases of C57Bl/6 mice intrasplenically injected with TIMP1

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KD KPC cells compared to WT controls **(Figure 6d)**. Taken together, our results suggest that the presence of TIMP1 expressing cells is associated with hepatic recruitment of immunosuppressive populations. To query whether the differences in immune cell infiltration was due to differences in metastatic burden, we compared immune cell infiltration in metastasis of equal size and found most of the MDSCs to indeed localise to the tumor and to be of equal percentage. This is indicative of a linear relationship between TIMP1 expression, liver metastatic burden and immune cell infiltration **(Supplementary Figure 5c and 5d).** This would imply that the presence of tumor cells themselves may be required for immune cell infiltration.

DISCUSSION

Gemcitabine constitutes one of the backbones in the treatment of advanced PDAC but patients often respond poorly to this agent. We identified TIMP1 as a Gemcitabine response gene in serum taken from transgenic KPC mice. Immunohistochemical staining revealed increased TIMP1 protein expression independently of the stromal content in tumor after gemcitabine treatment. This was also confirmed by transcriptional analysis showing an increase in TIMP1 mRNA levels in tumors from gemcitabine-treated mice. Although no direct evidence in PDAC, previous analysis of 264 breast cancer patients demonstrated longer overall survival after Gemcitabine-incorporating chemotherapy in patients with TIMP1-negative tumors compared to TIMP1-positive cases (33). Additional studies have correlated high TIMP1 expression with rapid tumor progression after treatment with chemotherapy agents such as cyclophosphamide methotrexate, 5-fluorouracil, epirubicin and doxorubicin in breast and colon cancers (34,35). Furthermore, we found TIMP1 expression to be among the top 1% of upregulated genes in human PDAC cohorts and it was significantly upregulated in human PDAC compared to normal pancreatic tissue. Of note, treatment of non-tumor bearing mice with gemcitabine did

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not alter TIMP1 levels, suggesting that the presence of tumor cells is necessary for TIMP1 upregulation upon chemotherapy with gemcitabine. These data indicate that TIMP1 expression is correlated with tumor progression and decreased response to Gemcitabine in PDAC.

We found that TIMP1 expression is important for tumor cell survival as evidenced from decreased colony formation following stable knockdown of TIMP1 in both human and murine PDAC cell lines. Also, overexpression of TIMP1 enhanced clonogenic survival in untreated cells, and also after gemcitabine and radiotherapy that further supports its pro-tumorigenic role. TIMP1 can activate pro-survival and invasive signalling through engaging CD63, activating PI3K/Akt signalling (8-11). Besides this, TIMP1 can regulate cell proliferation by translocating to the nucleus and interacting with the zinc finger protein PLZF (36). Of note, TIMP1 expression occurs downstream of the K-Ras signalling in the transgenic KPC model (17). Inhibition of TIMP1 resulted in improved response of PDAC cells to ionizing radiation and Gemcitabine. Accordingly, Oncomine database analysis revealed significant TIMP1 upregulation in gemcitabine-resistant lung cancer cell lines compared to gemcitabine-sensitive counterparts. This is in line with our findings and others demonstrating a correlation between TIMP1 and decreased sensitivity of cell lines to chemotherapy (37).

Previous studies have reported a link between TIMP1 and angiogenesis. TIMP1-overexpressing tumors displayed increased VEGF-A expression (38), augmented growth rates with increased vessel density (39), and metastatic colonisation in murine models of lung cancer (40). Inhibition of secreted TIMP1 decreased myofibroblast-induced angiogenesis (41). Also, TIMP1 positive cells have been observed adjacent to CD34+ blood vessels in colorectal liver metastasis (42). In agreement, we now show that TIMP1 suppression results in decreased vessel density, both in human and murine PDAC xenograft and liver metastasis models. Furthermore, we identified alterations in chemotaxis pathways upon knockdown of TIMP1, thus supporting a protumorigenic and prometastatic role for TIMP1 via alteration of chemotaxis and the possible promotion of angiogenesis.

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We also found that expression of genes playing a role in inflammatory pathways appeared most dependent on TIMP1 levels. Inflammation is a critical component of tumor progression (43). The protumorigenic role of TIMP1 has been previously attributed to its cytokine-like functions that form an inflammatory milieu (44). In addition to TIMP1 upregulation, we noted an increase in the inflammatory cytokine IL-6 expression following Gemcitabine treatment in KPC mice. Of note, IL-6 promotes progression of PanINs into PDAC (45,46), whereas TIMP1 expression is downstream of IL-6 in rat hepatocytes(45). Also, the TIMP1 promoter is a direct target of the inflammatory mediator NF-kB (45). Thus, administration of Gemcitabine appears to induce an inflammatory response, including TIMP1 upregulation in PDAC.

TIMP1 can promote an immunosuppressive environment via SDF-1-dependent recruitment of neutrophils to the liver and creating a premetastatic niche (13). Suppression of TIMP1 reduced PDAC cell migration, and also invasion of PDAC cells towards CAFs (immortalised PSCs), indicating a pro-metastatic role for TIMP1. Furthermore, by using an intrasplenic injection model, we observe that circulating PDAC tumor cells require TIMP1 expression to support hepatic colonization associated with recruitment of CXCR2-positive neutrophils, CD11b+Gr1+ MDSCs and CD4+CD25+FOXP3+ regulatory T cells to the liver. Inhibition of CXCR2 abrogates metastatic seeding (19), whereas Tregs are often encountered in fibrotic tissue and can promote liver metastases (47,48). Notably, examination of small liver metastases with comparable size between TIMP1 WT and KD groups failed to reveal a significant difference of MDSCs or Tregs between the two groups, indicative of a linear relationship between TIMP1 expression, liver metastatic burden and immune cell infiltration. This is suggestive that the presence of tumor cells is essential for immune cell infiltration. (1,49,50).

To summarise, treatment of PDAC with Gemcitabine led to upregulation of the cytokine TIMP1. TIMP1 expression correlates with tumor progression in a number of patient cohorts analysed. Suppression of TIMP1 enhanced tumor response to Gemcitabine and radiotherapy, and significantly decreased clonogenic survival, migration and invasion both *in vitro* and *in*

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vivo. Importantly, TIMP1 suppression was associated with decreased vascular density in the tumor xenografts and liver metastasis, and reduced liver infiltration by immunosuppressive cell populations resulting in an overall reduction in liver metastasis formation. Our study therefore provides a rationale for the design and addition of TIMP1 specific inhibitors to chemo/radiotherapy.

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Table 1. Gene Ontology enrichment analysis (biological processes)

Inflammatory response (p= 0.00011)	
BMP2	bone morphogenetic protein 2
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1
S100A8	S100 calcium binding protein A8
S100A9	S100 calcium binding protein A9
AOX1	aldehyde oxidase 1
AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1)
CCR4	chemokine (C-C motif) receptor 4
TPST1	tyrosylprotein sulfotransferase 1
RIPK2	receptor-interacting serine-threonine kinase 2
Cell Adhesion (p= 0.03002)	
FEZ1	fasciculation and elongation protein zeta 1 (zygin I)
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1
SRPX	sushi-repeat containing protein, X-linked
COL8A1	collagen, type VIII, alpha 1
AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1)
ITGA6	integrin, alpha 6
ITGA1	integrin, alpha 1
PTPRU	protein tyrosine phosphatase, receptor type, U
Chemotaxis (p= 0.01593)	
CCR4	C-C motif chemokine receptor 4
DEFB1	defensin beta 1
CXCL16	C-X-C motif chemokine ligand 16
S100A8	S100 calcium binding protein A8
S100A9	S100 calcium binding protein A9

* Top three biological processes (inflammatory response, cell adhesion and chemotaxis) identified to be enriched by the gene ontology enrichment analysis and their associated differentially expressed genes (HGNC symbol and description). The enrichment p-value is corrected for multiple testing using false discovery rate (FDR).

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FIGURE LEGENDS

Figure 1. Identification of TIMP1 as a Gemcitabine response gene ***(A)*** Schematic representation of Gemcitabine treatment given to transgenic KPC mice when tumors reached 70-120mm^3. Mice were sacrificed either short-term (24h) or long term (3-7 weeks; when tumors reached 500mm^3 or due to sickness) upon completion of Gemcitabine treatment. ***(B)*** Kaplan-Meier curve showing probability of survival following Gemcitabine treatment versus the untreated control group. ***(C)*** Cytokine arrays and quantification showing protein expression short-term and long-term upon completion of Gemcitabine treatment in serum from representative KPC mice from A. ***(D)*** ELISA showing TIMP1 levels in serum from all KPC mice (n=6) shown in (A). ***(E)*** *H&E* and TIMP1expression in control and Gemcitabine-treated tumors, (whole face section and selected regions). ELISA showing TIMP1 levels in ***(F)*** conditioned media and ***(G)*** lysates obtained from the parental KPC cell line (KPC^S) and the Gemcitabine-resistant KPC cell line (KPC^R). (H) Oncomine data showing significantly higher TIMP1 expression in gemcitabine-resistant compared to gemcitabine-sensitive lung cancer cells. Log rank test used for Figure 1b, unpaired two-tailed T-test in Figures 1d, e and f, 1-tailed Mann-Whitney used in Figure 1h.*P<0.05; **P<0.01; ***P<0.001.

Figure 2. TIMP1 is a potential marker of PDAC. Log2 median centred ratio of TIMP1 mRNA expression in publicly-available datasets using Oncomine in ***(A)***n=12 human PDAC tissues and 5 samples from the normal pancreas;***(B)*** n=11 surgical resections of human PDAC and six matched normal pancreatic tissue; ***(C)***n=39 samples of human PDAC and matched normal pancreatic tissue, and ***(D)*** surgically resected tissue, microdissected to separate out six normal pancreatic duct specimens, six PanIN-1b lesions, eight PanIN-2 lesions, ten PanIN-3 lesions and eight human PDAC specimens.**P<0.01; ***P<0.001.

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Figure 3. TIMP1 knockdown results in decreased clonogenic survival and sensitization of cancer cells to chemotherapy and radiotherapy. ELISA showing TIMP1 expression in ***(A)*** PANC1 cells and ***(B)*** KPC cells having a stable knockdown of TIMP1 and cells transfected with a control empty vector (EV); ***(C)*** and ***(D)*** Clonogenic surviving fractions inPANC1 and KPC cells described in A and B, respectively relative to untreated/DMSO controls respectively;***(E-H)*** Clonogenic surviving fractions in PANC1 and KPC cells after radiation and Gemcitabine, relative to untreated/DMSO controls respectively; ***(I)*** Western blot showing stable overexpression of TIMP1 in WT and KD cells. ***(J-L)*** Clonogenic survival assay in PANC1 cells described in I. ***(M)*** Time to maximum tumor volume and to 300mm^3 in PANC1 TIMP1 WT and KD cells injected subcutaneously into SCID mice; ***(N-P)*** Time taken to 300mm^3 in KPC TIMP1 WT and KD cells injected into C57Bl/6 mice in untreated, irradiated 8Gy and Gemcitabine-treated (100mg/kg, i.p.) mice. Treatment was initiated when tumors reached 100mm^3. Unpaired two-tailed T-test were performed for Figures 3a, b, e, f, g, h, j, k, and l. Log rank and Mann-Whitney (two-tailed) non-parametric tests used for Figures 3m-p. *P<0.05; **P<0.01; ***P<0.001.

Figure 4. Differential expression analysis reveals that expression of 136 genes is significantly altered in TIMP1 KD. Gene ontology enrichment analysis using GeneCodis identified enrichment of the biological processes cell adhesion, chemotaxis and inflammatory response. ***(A)*** Heatmap of the top50 differently expressed genes in PANC1 TIMP1 WT vs TIMP1 KD cells on the Illumina Human H12 array.

Figure 5. TIMP1 knockdown results in decreased migration and metastasis Migration wound scratch assays in ***(A)*** PANC1 TIMP1 WT and KD cells, and ***(B)*** KPC TIMP1WT and KD cells. CD31 immunofluorescence images taken at 10xmagnification showing blood vessel density, and quantification in***(C)*** PANC1 TIMP1 WT and KD xenografts in SCID mice, and

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(D) KPC TIMP1 WT and KD allografts in C57Bl/6 mice; ***(E)*** Representative whole liver images of SCID mice resected 14 days after intrasplenic injection of PANC1 TIMP1 WT and KD cells, together with the corresponding liver weights as a percentage of total body weight;***(F)*** Representative H&E staining at 10x and 20x magnification and **(G)** the corresponding CD31 images at10x and 20x magnification of the matched areas for CD31;***(H)*** Representative whole liver images of C57Bl/6 mice resected 14 days after intrasplenic injection of KPCTIMP1WT and KD cells with the corresponding liver weights as a percentage of total body weight;***(I)*** Representative H&E staining at 10x magnification and ***(J)*** the corresponding CD31 images at 10x and 20x magnification of the matched areas for CD31. Unpaired two tailed T-test were performed for Figures 5c and d. Mann-Whitney (two-tailed) non-parametric tests used for Figures 3e and h. *P<0.05; **P<0.01; ***P<0.001.

Figure 6. TIMP1enhances the recruitment of MDSCs and Tregs in liver metastases *(A)* Time-course of TIMP1 levels in serum of KPC parental cells injected intrasplenically into C57Bl/6 ***(B)*** FACS analysis quantifications of immune marker populations in livers of mice described in A; FACS analysis quantifications of immune myeloid cell populations in livers of ***(C)*** SCID mice injected intrasplenically with PANC1 TIMP1-WT and KD cells and ***(D)*** C57Bl/6 mice injected with KPC TIMP1WT or KD cells. Y-axis illustrates the % of gated cells as indicated. Unpaired two-tailed T-test were performed for Figure 6. *P<0.05; **P<0.01; ***P<0.001.

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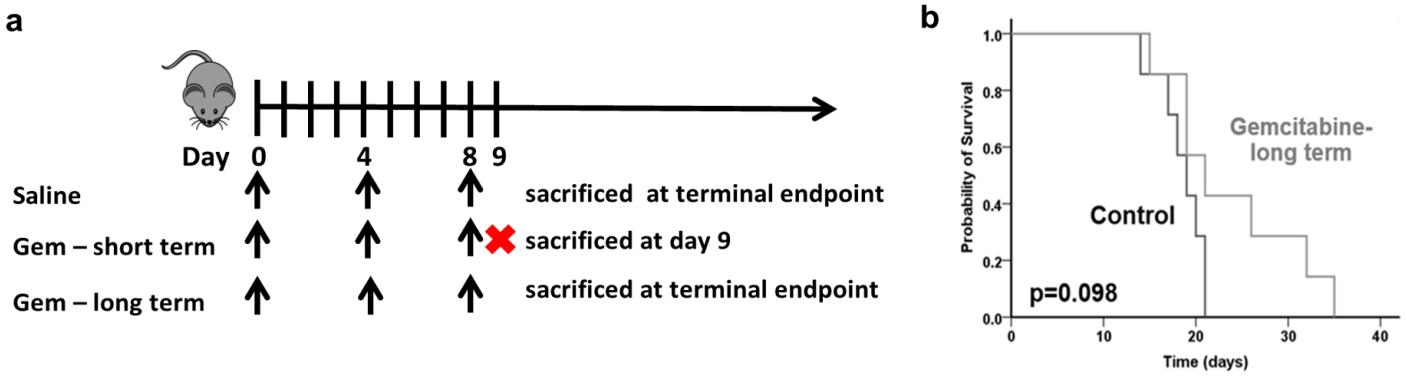
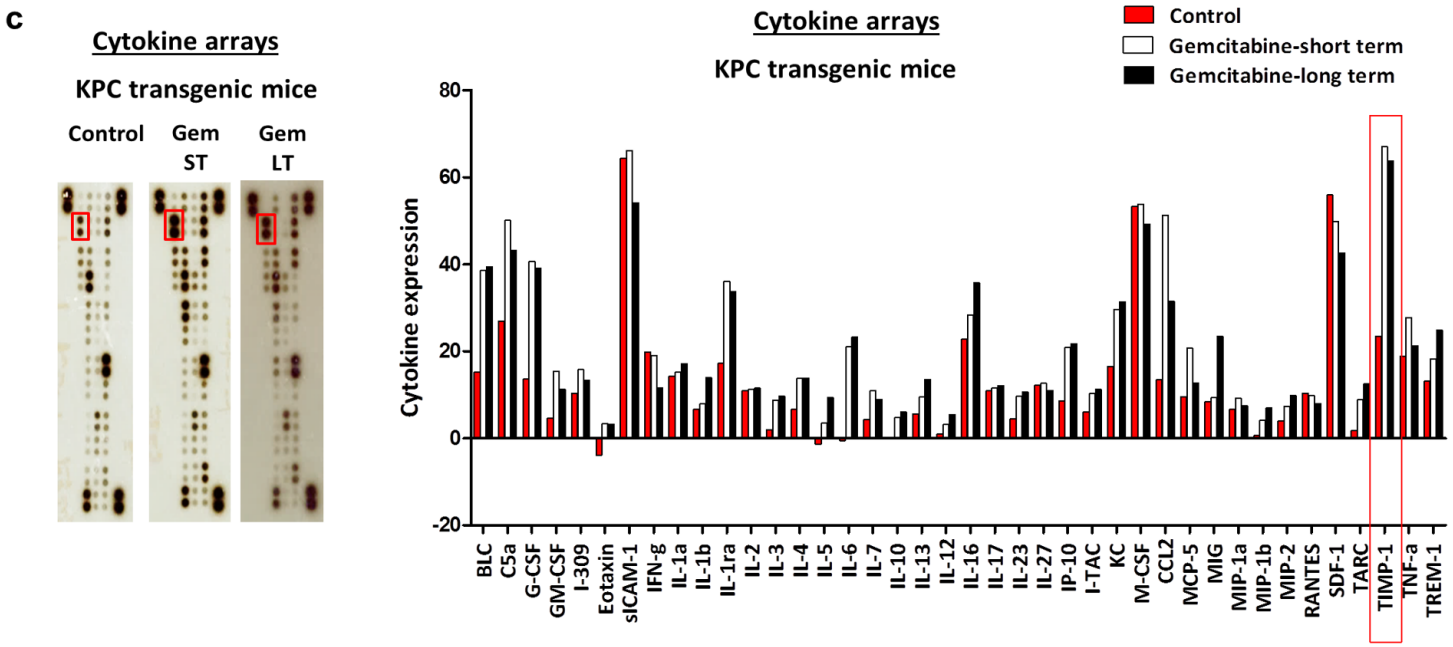
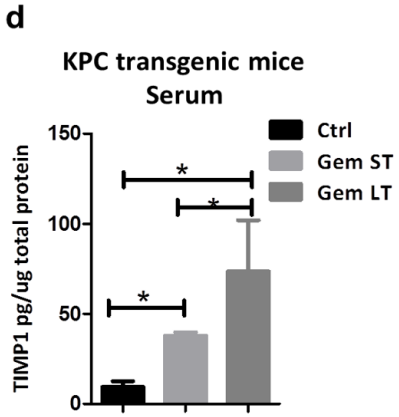
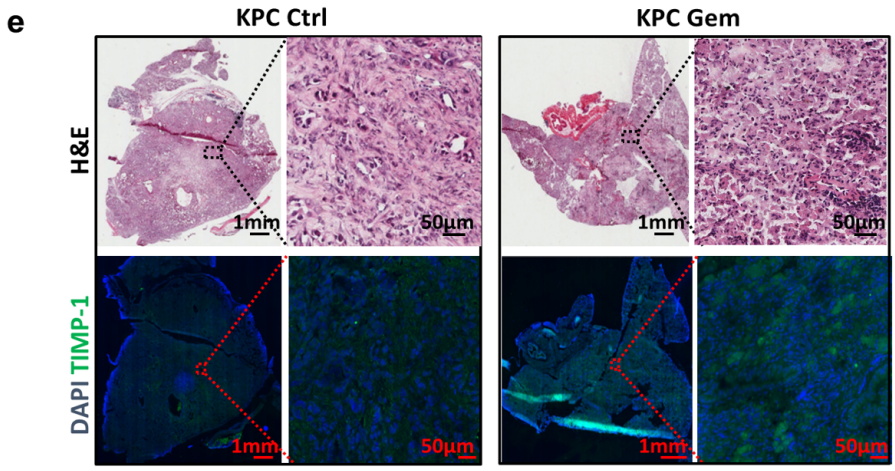
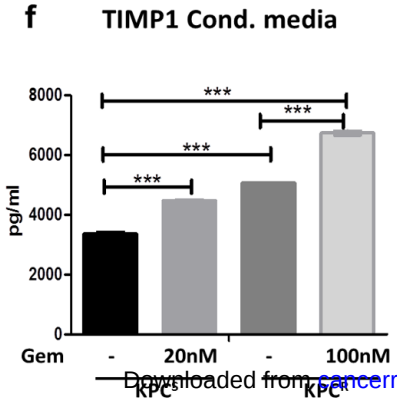
Figure 1

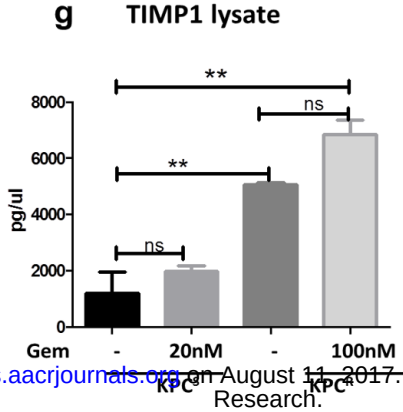












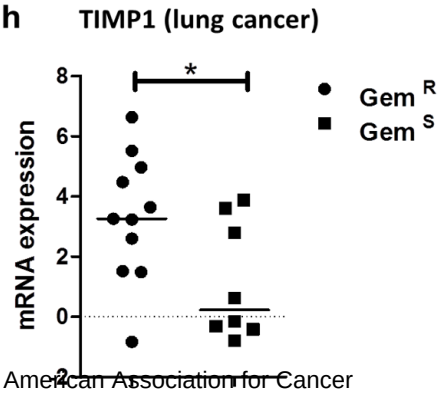


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Figure 2

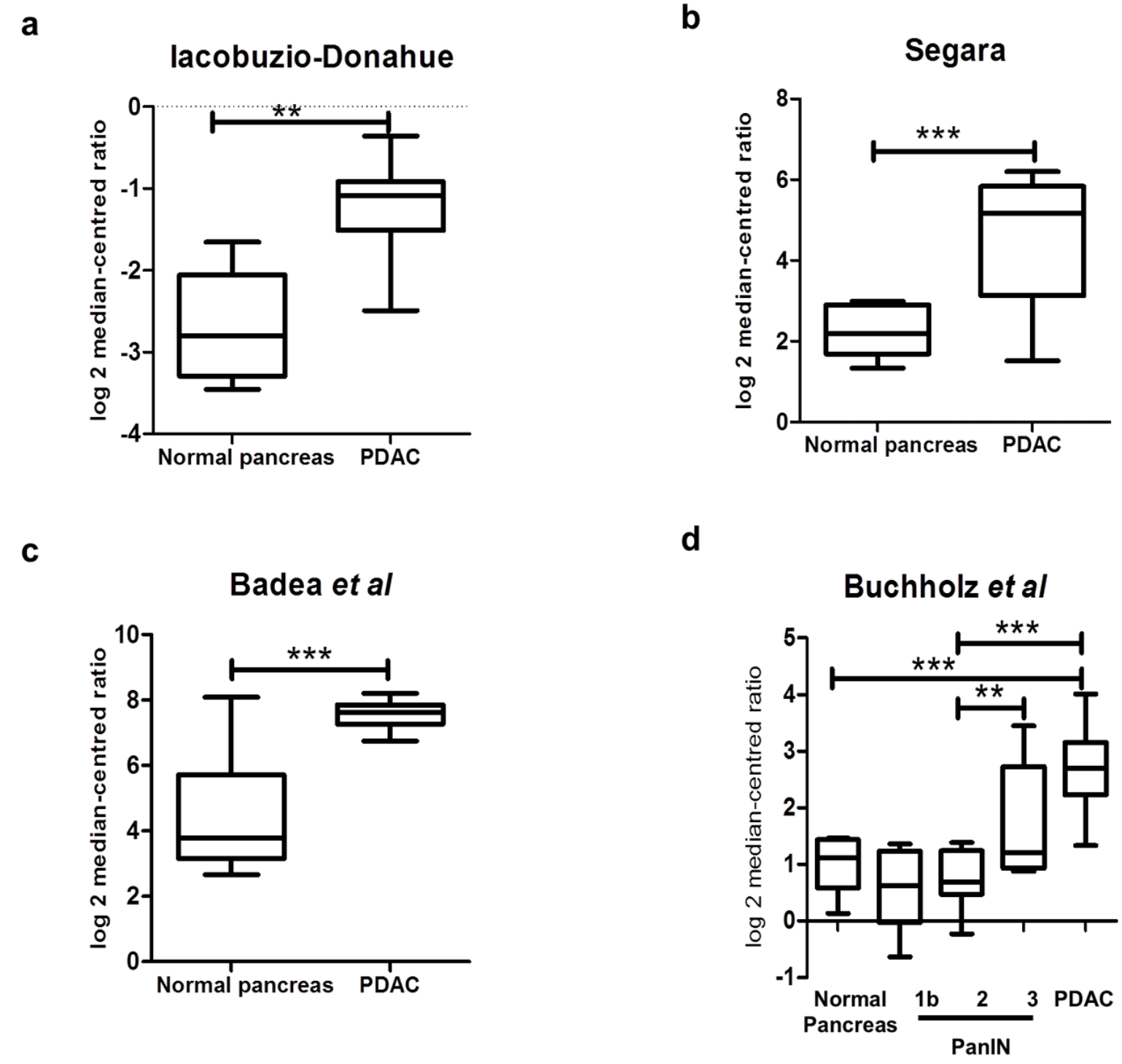

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Figure 3

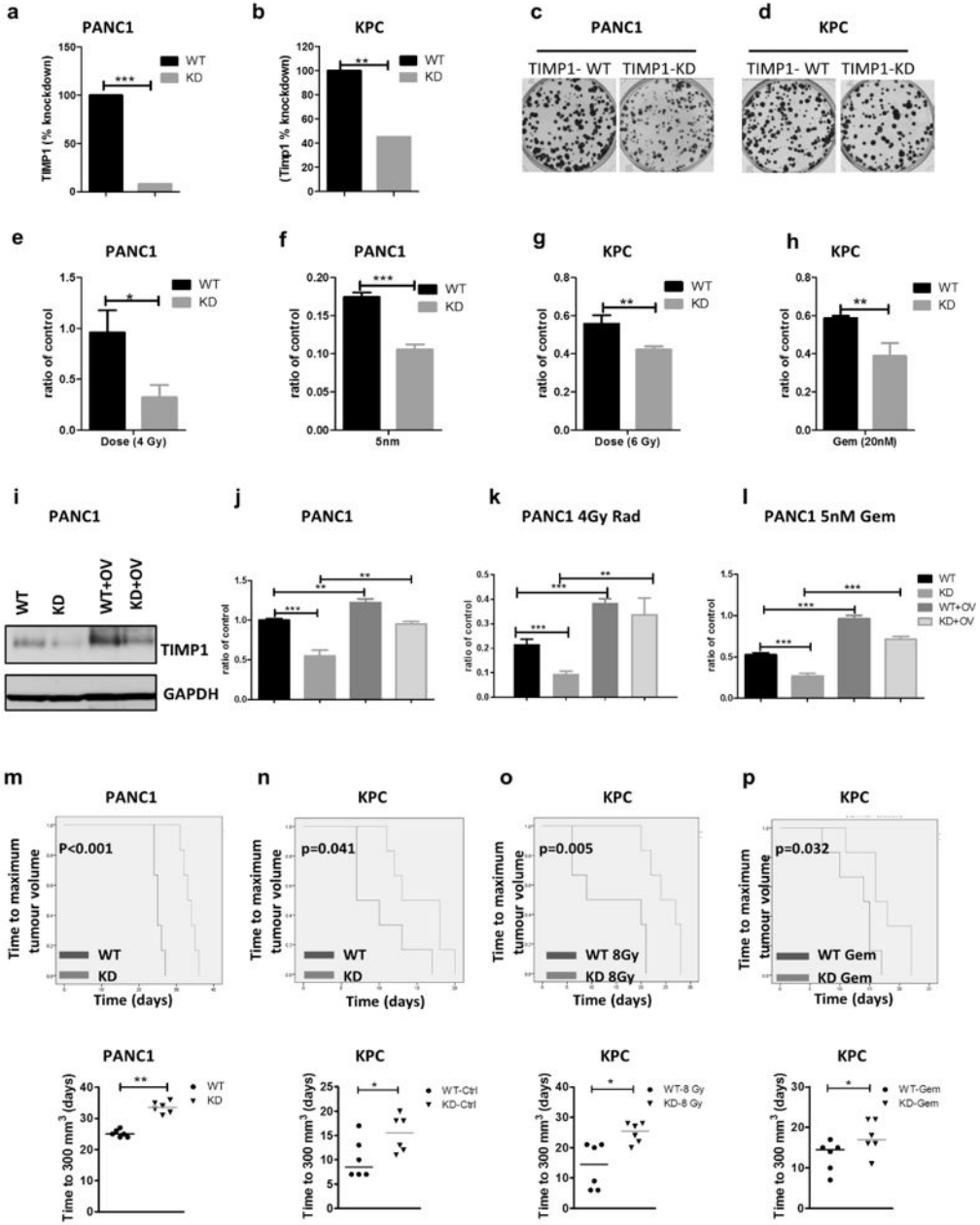

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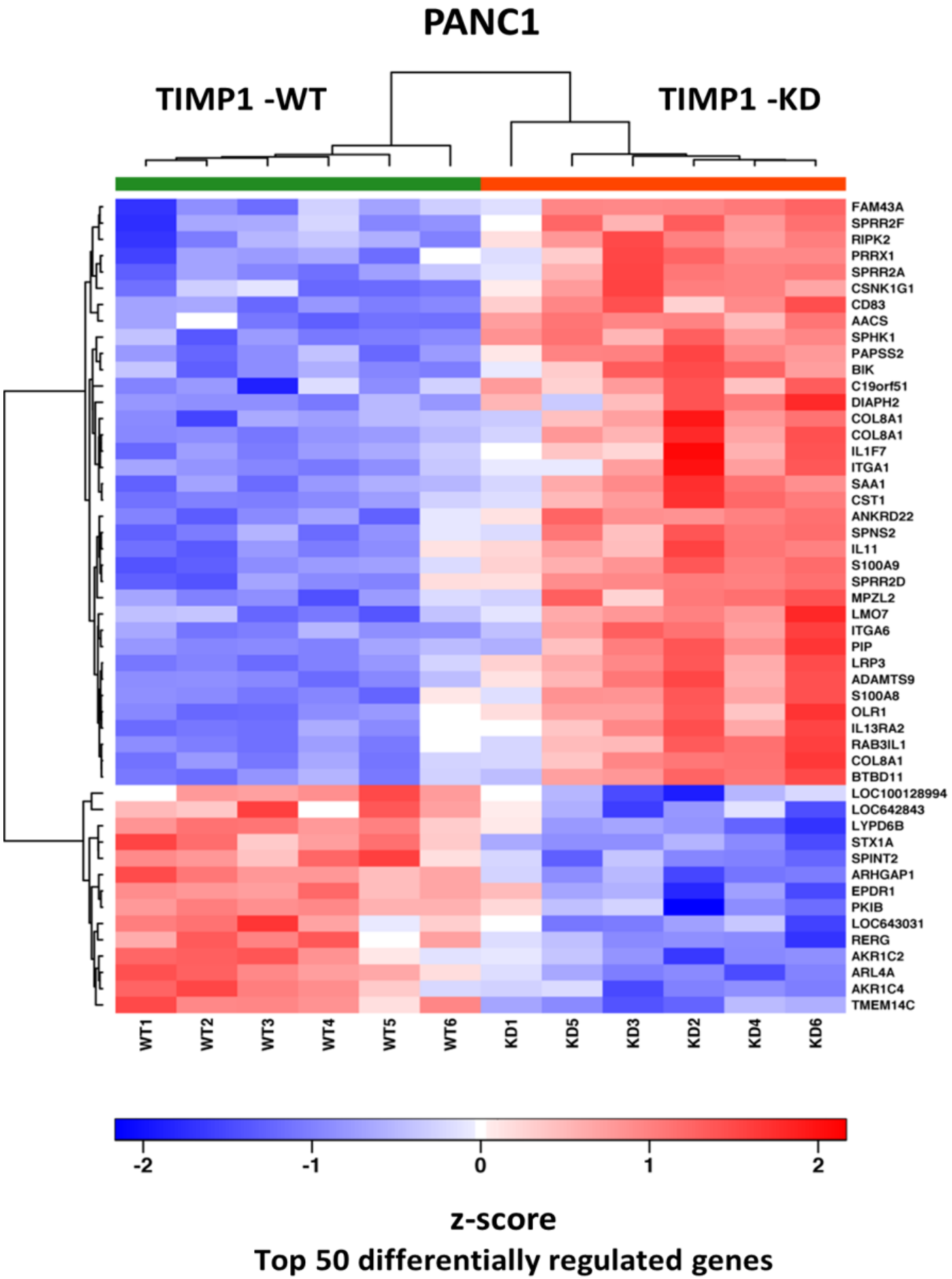
Figure 4



Top 50 differentially regulated genes

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Figure 5

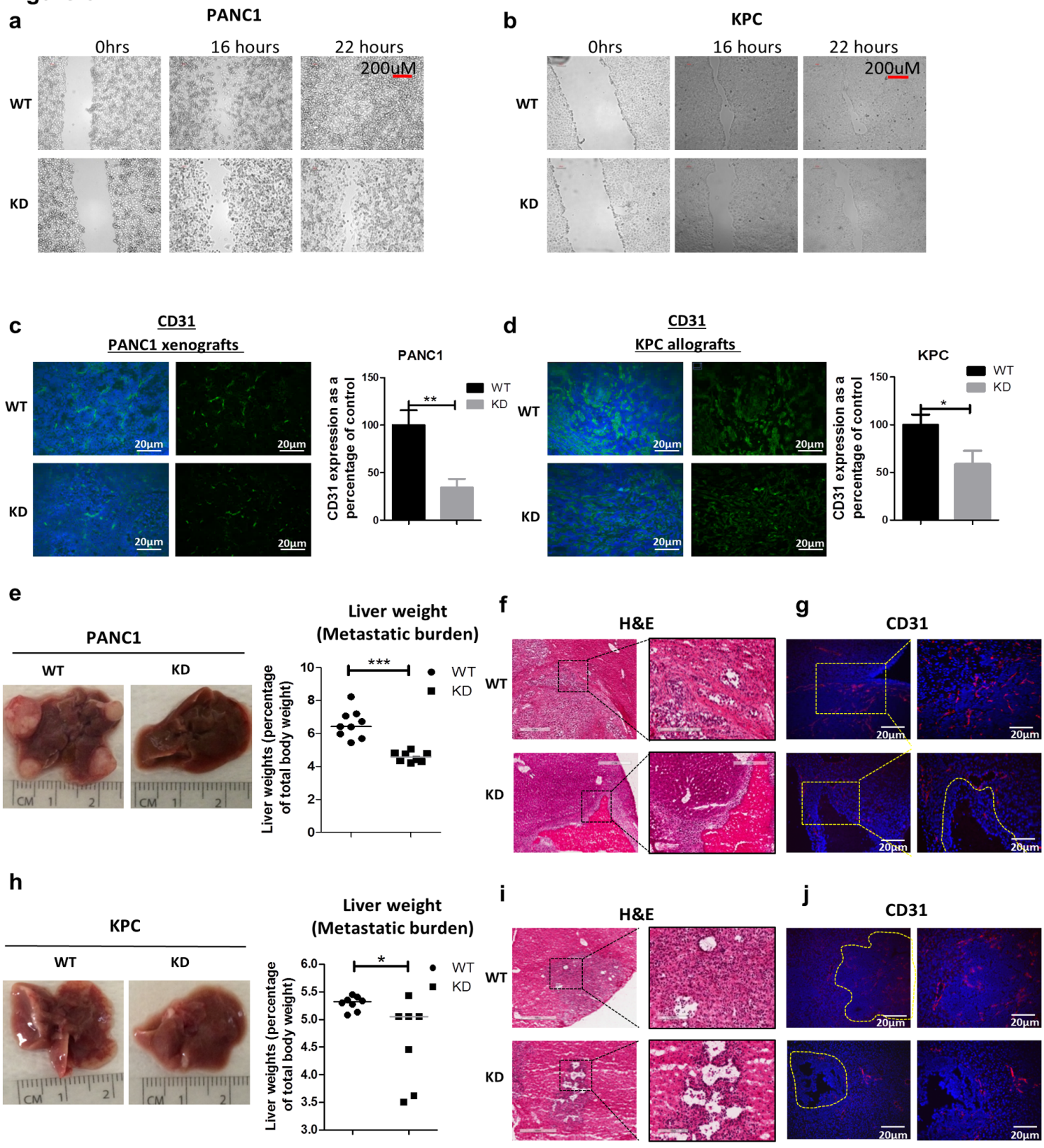

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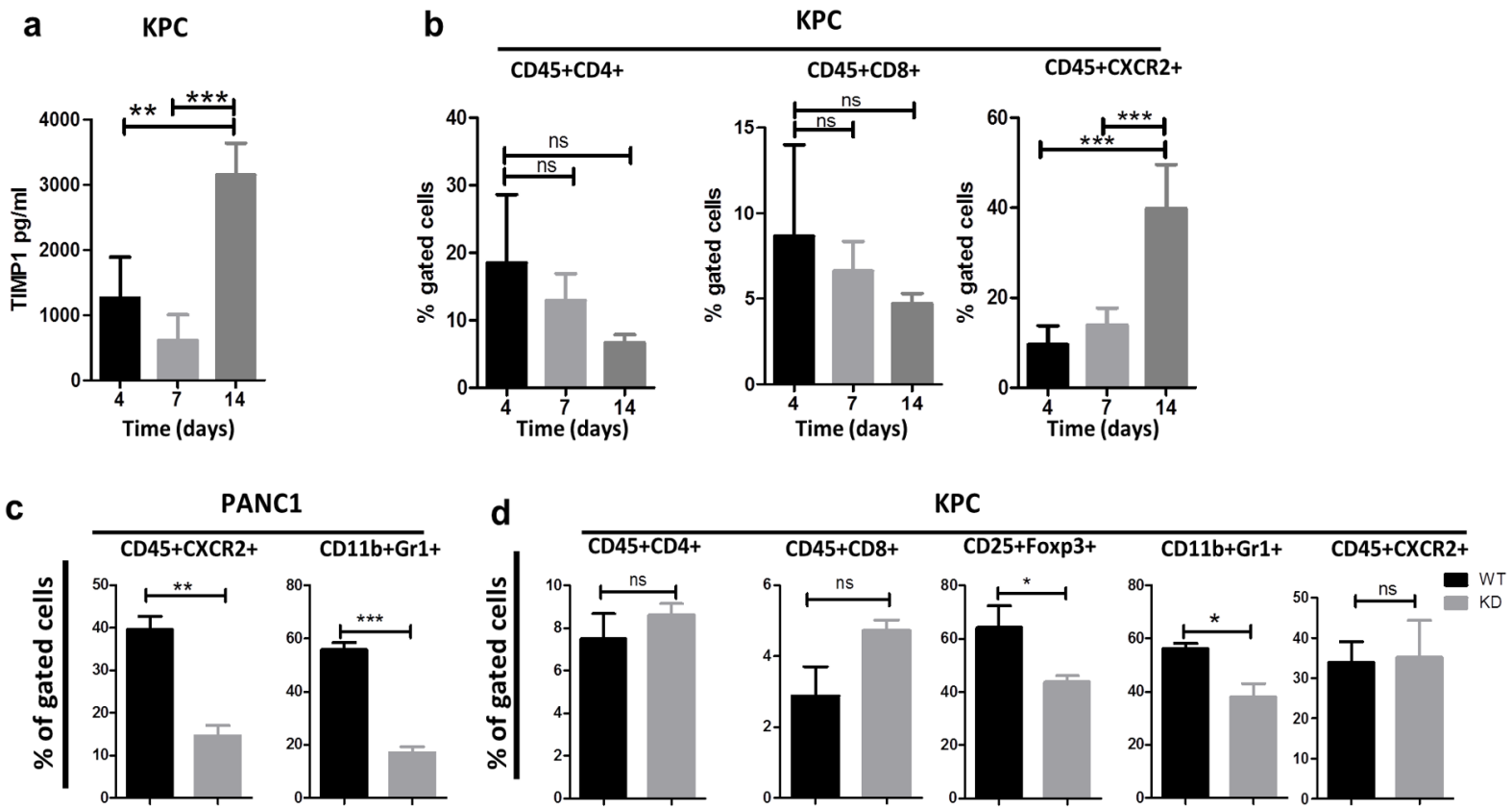
Figure 6



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Gemcitabine-induced TIMP1 attenuates therapy response and promotes tumor growth and liver metastasis in pancreatic cancer

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