1 **Implication of 4E-BP1 protein dephosphorylation and accumulation in**
2 **pancreatic cancer cell death induced by combined gemcitabine and**
3 **TRAIL**

4

5 **Androulla Elia[1,*], Ricky Henry-Grant[1], Charlotte Adiseshiah[1],**
6 **Catherine Marboeuf[2], Rebecca J Buckley[3], Michael J Clemens[1, 4]**
7 **Satvinder Mudan[5] and Stéphane Pyronnet[2]**

8

9 [1]Translational Control Group, Molecular and Clinical Sciences Research
10 Institute, St George's, University of London, Cranmer Terrace, London
11 SW17 0RE, UK; [2] INSERM UMR-1037, Cancer Research Center of
12 Toulouse (CRCT), Equipe Labellisée Ligue Contre le Cancer and
13 Laboratoire d'Excellence Toulouse Cancer (TOUCAN), 31432 Toulouse,
14 France. [3]Reproductive and Cardiovascular Disease Research Group,
15 Molecular and Clinical Sciences Research Institute, St. George's, University
16 of London, Cranmer Terrace, London SW17 0RE, UK [4]Department of
17 Biochemistry and Molecular Biology, School of Life Sciences, University
18 of Sussex, Falmer, Brighton BN1 9QG, UK. [5]Department of Surgery, Royal
19 Marsden Hospital, Fulham Road, London SW3 6JJ.

20 * To whom correspondence should be addressed (E-mail aelia@sgul.ac.uk)
21

22 **Running title:** Role of 4E-BP1 in pancreatic cancer cell death

23

Abstract

Pancreatic cancer cells show varying sensitivity to the anti-cancer effects of gemcitabine. However as a chemotherapeutic agent, gemcitabine can cause intolerably high levels of toxicity and patients often develop resistance to the beneficial effects of this drug. Combination studies show that use of gemcitabine with the pro-apoptotic cytokine TRAIL can enhance the inhibition of survival and induction of apoptosis of pancreatic cancer cells. Additionally following combination treatment there is a dramatic increase in the level of the hypophosphorylated form of the tumour suppressor protein 4E-BP1. This is associated with inhibition of mTOR activity, resulting from caspase-mediated cleavage of the Raptor and Rictor components of mTOR. Use of the pan-caspase inhibitor ZVAD-FMK indicates that the increase in level of 4E-BP1 is also caspase-mediated. ShRNA-silencing of 4E-BP1 expression renders cells more resistant to cell death induced by the combination treatment. Since the levels of 4E-BP1 are relatively low in untreated pancreatic cancer cells these results suggest that combined therapy with gemcitabine and TRAIL could improve the responsiveness of tumours to treatment by elevating the expression of 4E-BP1.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with five year survival rates that have remained at only about 5% (1, 2). The disease is often only detected at a late stage but, additionally, tumours are commonly resistant to conventional therapies (3). As a single agent, the nucleoside analogue gemcitabine, has been the standard treatment for

49 pancreatic cancer for several years and patients have been shown to have an
50 improved quality of life following therapy (4). However the development of
51 resistance to treatment presents an urgent need for novel strategies,
52 including the identification of agents that can enhance the effect of
53 gemcitabine at doses that have low toxicity (5, 6) .

54 In many cancers the protein kinase mammalian target of rapamycin
55 (mTOR) is hyper-activated, leading to an increase in the phosphorylation of
56 several downstream targets (7, 8). One such target is the tumour suppressor
57 4E-BP1. In its hypophosphorylated form 4E-BP1 functions as a binding
58 protein that regulates the availability of the oncogenic polypeptide chain
59 initiation factor eIF4E during the initiation of protein synthesis (9-11).
60 Previous studies have shown that in some pancreatic cancer cells 4E-BP1 is
61 expressed at very low levels and that the protein is highly phosphorylated
62 (12). Indeed the levels of phosphorylated 4E-BP1 have been used as a
63 prognostic indicator in a number of cancer types (13-17).

64 Many studies have established that the levels of eIF4E are elevated
65 in a number of malignancies and that excessive expression of eIF4E is
66 oncogenic due to its ability to confer resistance to apoptosis (17-19).
67 Conversely, the dephosphorylated form of 4E-BP1 has pro-apoptotic
68 effects, (20, 21). There is a correlation between the extent of
69 phosphorylation of 4E-BP1 and the state of aggressiveness of tumours (22,
70 23), and changes in the levels of the tumour suppressor can affect the ability
71 of malignant cells to undergo apoptosis (24, 25).

72 A better understanding of cancer immunotherapy has identified the
73 tumour necrosis factor-related apoptosis inducing ligand (TRAIL) as a

74 cytokine with the ability to target cancer cells whilst sparing non-malignant
75 cells. This property indicates that TRAIL has the potential to be an
76 important anticancer agent (26-28). TRAIL induces extrinsic apoptosis by
77 binding to either of two death receptors (DRs), TRAIL-R1/DR4 and
78 TRAIL-R2/DR5 (29). However recent work indicates that many cancer cell
79 lines are resistant to TRAIL treatment and this has limited its therapeutic
80 use (30, 31). In fact several clinical trials using soluble forms of TRAIL
81 such as dulanerim have proved disappointing (32, 33). With the emergence
82 of newer and more stable forms of TRAIL, coupled with more efficient
83 delivery methods, the potential for more effective therapies looks promising
84 (28, 34, 35). Relatively few studies have thus far focused on the possible use
85 of combination therapy using gemcitabine together with TRAIL (36-38).

86 We have previously investigated the role of 4E-BP1 in regulating the
87 sensitivity of pancreatic cancer cells to TRAIL-induced apoptosis (24).
88 However the possible importance of 4E-BP1 in determining the
89 effectiveness of TRAIL in combination with gemcitabine has not been
90 addressed. In this study we have used soluble recombinant human TRAIL in
91 combination with gemcitabine to investigate possible effects on the
92 regulation of apoptosis in pancreatic cancer cells. We demonstrate that the
93 use of gemcitabine and TRAIL enhances the inhibition of survival of
94 pancreatic cancer cells and provide data to show that both the extent of
95 dephosphorylation and the level of total 4E-BP1 are strongly increased as a
96 result of the combination treatment. These changes are associated with an
97 inhibition of mTOR activity and caspase-mediated cleavage of the Raptor
98 and Rictor components of mTOR. Reducing the expression of 4E-BP1 using

small hairpin (sh) RNAs impairs the induction of cell death following combination treatment of the pancreatic cancer cells. Possible mechanisms by which 4E-BP1 functions as an important determinant of the sensitivity of pancreatic cancer cells to cell death effects of gemcitabine and TRAIL are discussed.

Results

Cytotoxic effects of gemcitabine and TRAIL treatment on human pancreatic cancer cells.

As gemcitabine is widely used as a first-line chemotherapeutic drug in the treatment of pancreatic cancer, characterisation of its cytotoxic effects have been widely reported (39-41). Using the MTT assay we have extended these studies to examine the effects of gemcitabine in combination with TRAIL in three PDAC cell lines; BxPC-3, MIA PaCa-2, and PANC-1. All three cell lines exhibited relatively poor sensitivity to the cytotoxic effects of gemcitabine alone after 24 h exposure to concentrations up to 1000 μM (Figure 1a). In parallel with these assays we tested the sensitivities of the cell lines to TRAIL alone. MIA PaCa-2 cells were the most sensitive to treatment and exposure to a concentration of 10 ng/ml TRAIL significantly inhibited their survival. BxPC-3 were resistant to TRAIL at up to 100 ng/ml (4 h treatment), and there was no significant effect of TRAIL on the survival of PANC-1 cells even at a 10-fold higher concentration (Figure 1b).

We then examined whether co-treatment of the cells with both reagents could result in a more significant inhibition of survival. The MTT assays showed that a treatment using 100 μM gemcitabine in combination

124 with 100 ng/ml TRAIL significantly inhibited cell survival in all three cell
125 types (Figure 1c-e). For example, whereas 100 μM gemcitabine alone had
126 only effects of 24.3% and 4.9% on BxPC-3 and MIA PaCa-2 cells
127 respectively, in the presence of TRAIL at 100 ng/ml for 4 h the inhibitory
128 effects of gemcitabine were increased to 56.5% and 39.2%. As the PANC-1
129 cell line was less responsive to TRAIL, we extended the treatment time to 6
130 h and were able to show similar effects in these cells too (Figure 1e and
131 Supplementary Figure S1a).

132 **Gemcitabine enhances TRAIL-induced apoptosis.**

133 Since TRAIL is a well-known inducer of apoptosis we used the trypan blue
134 exclusion assay to assess the effect of co-treatment with gemcitabine on cell
135 viability. Even in the case of PANC-1 cells, the least responsive of the cell
136 types, 100 μM gemcitabine in combination with 100 ng/ml TRAIL
137 significantly inhibited viability, reducing it by 43.8%, whereas either agent
138 alone was much less effective (Figure 2a).

139 The induction of apoptosis following combination treatment of the
140 PANC-1 cells was monitored using a variety of methods. Using flow
141 cytometry we observed that combination treatment of PANC-1 cells resulted
142 in significantly enhanced externalisation of phosphatidylserine (measured
143 by annexin V binding) compared to the treatments with gemcitabine or
144 TRAIL alone (Figure 2b). Time-lapse microscopy was used to assess
145 morphological changes over time and to measure the % of cells that become
146 committed to apoptosis (Figure 2c and d). Figure 2c demonstrates that after
147 a 24 h period of treatment with 100 μM gemcitabine in combination with
148 100ng/ml TRAIL, 82.5% of PANC-1 cells had undergone complete

apoptosis, significantly much higher than with the individual treatments alone.

We further examined the ability of the combination therapy to enhance apoptosis using western blotting to determine the cleavage of caspase 8 and poly (ADP-ribose) polymerase (PARP) (Figure 3a). All three cell lines showed enhanced cleavage of both caspase substrates following the combination treatment, with PANC-1 cells exhibiting virtually complete cleavages at 6 h. Additionally we observed cleavage of BID, a BH3 domain–containing pro-apoptotic Bcl2 family member in PANC-1 cells (Figure 3b). Such cleavage releases a potent pro-apoptotic activity of BID and provides a critical link between the activation of caspase 8 and the intrinsic apoptotic pathway (42).

Both the inhibition of survival and the induction of apoptosis following combination treatment were caspase-dependent as these effects were blocked by the addition of the pan caspase inhibitor z-VAD-FMK (Figures 3c and 3b).

Effects of gemcitabine and TRAIL on the mTOR pathway

The pharmacological targeting of the mTOR/4E-BP1 pathway in pancreatic cancer has been previously reported (43). In order to investigate whether the pathway is involved in the inhibition of survival and pro-apoptotic effects of gemcitabine and TRAIL on PDAC cells, we characterised the effects of these agents on various aspects of the mTOR pathway. The effects of the gemcitabine and TRAIL combination were apparent at the level of phosphorylation of mTOR itself, which showed dephosphorylation at Ser ^{2448} (Figure 4a). In addition there was TRAIL-mediated and caspase-

dependent cleavage of the proteins Raptor and Rictor, which are associated with the mTORC1 and mTORC2 complexes respectively (Figure 4a).

We have previously shown that TRAIL can cause the dephosphorylation of the mTOR substrate 4E-BP1 in pancreatic cancer cells (24). However the effect of the cytokine when used in combination with gemcitabine on the phosphorylation state of 4E-BP1 has not previously been investigated. Extracts made from the three cell lines were analysed by western blotting using both antibodies to total 4E-BP1 and phospho-specific antibodies recognizing the phosphorylation sites Ser^{65}, $\text{Thr}^{37/46}$ and Thr^{70} (Figure 4b and 4c).

With the exception of MIA PaCa-2 cells there was very little effect on the levels of total 4E-BP1 following the individual treatments, (in PANC-1 cells very little 4E-BP1 could be detected under these conditions) (Figure 4b). TRAIL treatment alone had no significant effect on phosphorylation of 4E-BP1 at any of the sites investigated. Interestingly gemcitabine alone caused dephosphorylation of 4E-BP1 at Ser^{65} in all three cell lines (Figures 4b and c). In PANC-1 cells this dephosphorylation was observed despite negligible levels of total protein being detectable (Figure 4b and 4c). Gemcitabine treatment of PANC-1 cells resulted in a significant 57% reduction in phosphorylation of 4E-BP1 at Ser^{65} whereas gemcitabine plus TRAIL resulted in a 74.6% reduction (Figure 4d).

The most dramatic changes in the levels and phosphorylation of 4E-BP1 followed combination treatment of the cells, where a marked elevation in the levels of total 4E-BP1 was observed in all three cell lines (particularly BxPC-3 and MIA PaCa-2) (Figure 4b). Additionally, all cell types exhibited

199 strong dephosphorylation of Ser^{65} in response to gemcitabine plus TRAIL
200 (Figures 4b and 4c). Dephosphorylation at the other sites was observed but
201 is only apparent when the large increases in total levels of 4E-BP1 are taken
202 into account. The substantial increase in the level of total 4E-BP1 is of
203 considerable interest in view of the fact that 4E-BP1 expression is severely
204 repressed in a high proportion of human pancreatic tumours (12). As we did
205 not observe any changes in the levels of the potentially oncogenic factor
206 eIF-4E following treatment, (Figure 4b), the ratio of 4E-BP1 to eIF4E
207 becomes much higher after gemcitabine and TRAIL treatment and it is
208 therefore not surprising that there was a marked inhibition of protein
209 synthesis (Supplementary Figure S1b and c and data not shown).

210 Using protein synthesis assays we determined that the effects of
211 gemcitabine and TRAIL were synergistic, as revealed by Combination
212 Index (CI) studies using the well-established method of Chou and Talalay
213 (44) (Supplementary Figure S1a). For example, analysis of the data gave a
214 CI value of 0.43 for MIA PaCa-2 cells using 100 μM gemcitabine for 24 h
215 in combination with 100 ng/ml TRAIL for 4 h. CI values that are below 1
216 indicate a synergistic effect of combination treatments.

217 Consistent with the above findings, the use of m^7GTP-Sepharose
218 affinity chromatography to purify eIF4E and its associated proteins
219 demonstrated a large increase in the binding of 4E-BP1 to eIF4E in PANC-1
220 cells treated with 100 μM gemcitabine in combination with 100 ng/ml
221 TRAIL for 6h (Supplementary Figure S1b).

222 Since TRAIL enhances caspase activity in its target cells we
223 investigated the caspase-dependence of the effects of this combination
224 treatment, using the board specific caspase inhibitor z-VAD-FMK.
225 Interestingly, both the increases in levels of 4E-BP1 and the
226 dephosphorylation of 4E-BP1 and mTOR described above require caspase
227 activity as pre-treatment of the cells with the pan-caspase inhibitor Z-VAD-
228 FMK was able to prevent these effects (Figure 4a).

229 **Role of 4E-BP1 in the cytotoxic effects of gemcitabine and TRAIL**

230 Following on from the above data, we investigated whether 4E-BP1 plays a
231 required role in the regulation of survival of PDAC cells by the combination
232 of gemcitabine and TRAIL. For this purpose as the MIA PaCa-2 cell line is
233 the only cell line which expresses constitutive high levels of 4E-BP1 while
234 eIF4E is equally expressed in the three (Figure 4b) (45,46), we employed
235 two stable MIA PaCa-2 cell lines engineered to express either small hairpin
236 RNA (shRNA) directed against 4E-BP1 or scrambled shRNA as a control
237 (47).

238 In contrast to the MIA PaCA-2 cells used in our earlier work, both
239 genetically modified cell types were resistant to TRAIL alone
240 (Supplementary Figure S2a), likely due to acquired changes during the
241 process of stable cell line selection. Furthermore when we tested the
242 combination treatment using a TRAIL treatment time of 6 h it was apparent
243 that there was no difference between the extent of survival of the two cell
244 types as determined by the MTT assay (Supplementary Figure S2b).
245 However after an extended treatment time of 24 h with gemcitabine plus

246 TRAIL we did observe significant resistance of the cells in which 4E-BP1
247 expression had been silenced (Figure 5a), suggesting a role for the tumour
248 suppressor protein in the longer term effects of the combination treatment.
249 Using m^7GTP-Sepharose affinity chromatography we were able to
250 demonstrate that in the cells in which 4E-BP1 had not been silenced there
251 was an increase in the binding of dephosphorylated 4E-BP1 to eIF4E that
252 was more apparent following combination treatment of the cells (Figure 5b).

253

254 **Discussion**

255 Although various trials have investigated treatments using gemcitabine in
256 combination with a number of reagents, none of these treatments was shown
257 to be significantly more effective than gemcitabine alone (48). So despite
258 being first approved 30 years ago, gemcitabine still remains the first line
259 therapy for pancreatic cancer. In this manuscript we have investigated the
260 effect of combining gemcitabine with the cytokine TRAIL on the survival of
261 three PDAC and two genetically modified PDAC cell lines. We have
262 established that using TRAIL and gemcitabine in combination can
263 significantly inhibit survival and induce apoptosis in these cells. In
264 particular the combination treatment was effective in the survival of the
265 PANC-1 cell line that is highly resistant to gemcitabine treatment alone.
266 Although all three PDAC cell lines examined showed differing sensitivities
267 to treatment with TRAIL, as previously shown (24), it is of significance that
268 in the presence of TRAIL the cells become responsive to concentrations of
269 gemcitabine that alone are ineffective. Moreover, in the more gemcitabine-

270 sensitive cell line, BxPC-3, TRAIL renders the cells responsive to much
271 lower concentrations of gemcitabine. We used the MIA PaCa-2 cell type to
272 establish that the combined effect of 100 μM gemcitabine together with 100
273 ng/ml TRAIL was synergistic in nature, at the level of total protein
274 synthesis.

275 In analysing the induction of apoptosis in PDAC cells we have
276 shown that the combination of gemcitabine and TRAIL activates a caspase-
277 mediated mechanism that leads to the cleavage of a number of substrates,
278 namely PARP, caspsase-8 and BID. In the gemcitabine-resistant cell line
279 PANC-1 we also identified additional new caspase targets, notably the
280 Rictor and Raptor components of the mTORC-1 and mTORC-2 complexes
281 of mTOR. There is recent evidence indicating that Raptor is indeed cleaved
282 by caspases but this has never been investigated in this model (49). TRAIL-
283 induced cleavage of components of mTORC-1 and mTORC-2 during cell
284 death in PDAC cells suggests treatment options targeting this pathway (50).

285 Previous studies of the underlying mechanisms by which
286 gemcitabine and TRAIL induce cell death have implicated a number of
287 signalling molecules. We have previously shown that TRAIL can cause
288 dephosphorylation of the regulatory protein 4E-BP1 in a number of tumour
289 cell types, (24, 51, 52). However the effects of a combination treatment
290 using gemcitabine and TRAIL on the phosphorylation and levels of this
291 tumour suppressor in PDAC cell lines have been overlooked until now. Our
292 present findings suggest that gemcitabine treatment of all PDAC cell lines
293 investigated leads to dephosphorylation of 4E-BP1 at residue Ser^{65}.
294 However gemcitabine alone is not sufficient to induce cell death. Since

295 there is little or no effect of gemcitabine alone on the activity of mTOR, as
296 judged by the state of phosphorylation of residue Ser^{2448}, it is likely that the
297 effect of gemcitabine on 4E-BP1 phosphorylation is mTOR-independent.
298 Using western blotting we were able to see a dephosphorylation of 4E-BP1
299 at Ser^{65} in all cell lines following treatment with 100 μM gemcitabine and
300 100 ng/ml TRAIL, and in the PANC-1 cells the combination treatment
301 significantly reduced the phosphorylation of this residue compared to
302 untreated cells. The latter effect coincides with dephosphorylation of mTOR
303 at Ser^{2448} as well as caspase-dependent cleavages of Raptor and Rictor.
304 Overall, these observations indicate that the combination of gemcitabine and
305 TRAIL acts via both mTOR-dependent and -independent pathways.

306 In addition to the dephosphorylation of 4E-BP1 we noted very
307 marked increases in the levels of total 4E-BP1 in all cell lines following the
308 combination treatment. This is likely to be of considerable significance with
309 regards to the functional activity of the protein. In PANC-1 cells binding of
310 4E-BP1 to eIF4E, isolated on m^7GTP-Sepharose, was only observed at the
311 higher levels of 4E-BP1, namely after TRAIL treatment alone or after
312 TRAIL in combination with gemcitabine. This is likely to be of particular
313 relevance in PDAC cells where the basal levels of 4E-BP1 are very low
314 (12). Taken together, these data suggest that gemcitabine leads to a
315 dephosphorylation of 4E-BP1 but that this alone is not sufficient to induce
316 cell death. However gemcitabine potentiates the pro-apoptotic effect of
317 TRAIL by a mechanism that may involve enhanced expression of 4E-BP1.

318 To test whether changes in the levels of 4E-BP1 play a role in
319 determining the sensitivity of PDAC cells to the combination treatment we

320 used a MIA PaCa-2 cell line in which 4E-BP1 can be down-regulated (53).
321 The cell lines used for this experiment were derived from MIA PaCa-2 but
322 proved to be much more resistant to TRAIL than the MIA PaCa-2 cells used
323 in our other studies. Treatment of both the control and 4E-BP1-negative
324 cells with concentrations of TRAIL up to 1000ng/ml for 6 h had little effect
325 on the survival of these MIA PaCa-2-derived cell lines. This may be a
326 consequence of the selection of stable transfectants with puromycin during
327 the development of the cell line. However, extended treatment of these cells
328 with TRAIL for 24h enabled us to demonstrate that in the absence of 4E-
329 BP1 the cells were significantly more resistant to the combination treatment.
330 The data from these experiments further suggest that the pro-apoptotic
331 effect of TRAIL alone is not dependent on 4E-BP1 but the potentiating
332 effect of gemcitabine is dependent on expression of the tumour suppressor.

333 Although in some circumstances TRAIL has been shown to promote
334 the growth of pancreatic cancer (54) there is extensive evidence for a
335 physiological function of endogenous TRAIL as a tumour suppressor. The
336 cytokine has been shown to be an important natural effector molecule in the
337 armoury of host defences against transformed cells and it has a critical role
338 in immune surveillance (55-57). Whilst we have investigated the effect of
339 combining gemcitabine with TRAIL as a basis for an improved
340 chemotherapeutic approach, newly emerging immunotherapies targeted
341 against pancreatic cancer that increase the levels of endogenous TRAIL may
342 also benefit from the combined use of gemcitabine (58-60). Endogenously
343 expressed TRAIL is known to be several orders of magnitude more active
344 than conventional soluble trimeric TRAIL (28). Irrespective of either

345 therapeutic approach, this study shows the promising potential of using a
346 combination of gemcitabine with TRAIL as a way of re-sensitizing
347 gemcitabine-resistance PDAC cells, ultimately inducing these cells to
348 undergo apoptosis. Our data suggest that the marked upregulation and
349 dephosphorylation of 4E-BP1 is likely to play an important role in this
350 promotion of cell death.

351

352 Experimental

353 Materials

354 Tissue culture reagents were supplied by Sigma, Poole, Dorset, UK. Antibody
355 to 4E-BP1 (R113) was from Santa Cruz Biotechnology, CA, USA. Antibodies
356 against phosphorylated 4E-BP1 (anti-Ser^{65} catalogue number 9451, anti-
357 $Thr^{37/46}$ catalogue number 9459 and anti-Thr^{70} catalogue number 9455),
358 caspase-8, biotinylated gel markers and cell lysis buffer were all from Cell
359 Signalling Technology, Hitchin, Herts, UK. Mouse anti-PARP was purchased
360 from BD Pharmingen, Oxford, UK. The antibody to GAPDH was from
361 Millipore, Watford, UK. All secondary antibodies (anti-rabbit-HRP linked,
362 anti-mouse-HRP linked or anti-biotin-HRP linked) were obtained from Cell
363 Signalling Technology. PVDF membrane and rainbow markers were supplied
364 by GE Healthcare, Amersham, Bucks, UK. Immobilised m^7GTP-Sepharose
365 was from Jena Biosciences, Jena, Germany. Human TRAIL was from
366 PeproTech EC Ltd, London, UK. Thiazolyl blue tetrazolium bromide (MTT)
367 was from Sigma, Poole, Dorset UK.

368 Cell culture

369 The pancreatic cancer cell lines MIA PaCa-2, BxPC-3 and PANC-1 were all
370 ATCC-certified. MIA PaCa-2 and PANC-1 were maintained in Dulbecco's
371 modified Eagle medium (DMEM) supplemented with penicillin (50
372 units/ml), streptomycin (50 units/ml) and 10% foetal bovine serum (FBS).
373 BxPC-3 cells were maintained in RPMI 1640 supplemented with antibiotics
374 as above and 20% FBS. Cells were maintained in monolayer cultures at
375 $37^{o}C$ in humidified air with 5% CO_2. MIA PaCa-2 cells with constitutive
376 silencing of 4E-BP1 were engineered using pLKO vectors (Sigma Poole,
377 Dorset, UK), as previously described (47). shRNA vector accession
378 numbers are: 4E-BP1 TRCN0000040203 and non-target shRNA control
379 SHC002. Small interfering RNAs targeting 4E-BP1 (Applied Biosystems
380 and Life Technologies, Carlsbad, CA, USA, forward 50-
381 CAAGAACGAACCCUUC CUU-30 and reverse) were transfected using
382 the siPort NeoFx reagent (Applied Biosystems and Life Technologies),
383 according to the manufacturer's instructions.

384 **Immunoblotting**

385 Cells were harvested, washed in PBS and subjected to lysis using cell lysis
386 buffer (20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA,
387 1% Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM
388 Sodium orthovanadate (Na_3VO_4), 1µg/mL leupeptin). Cell pellets were
389 vortexed with buffer and lysed by incubating with lysis buffer on ice for 5
390 minutes. Samples were sonicated for approximately 5 pulses using a
391 sonicator (Jencons), and then centrifuged at 14,000g for 10 minutes at 4°C.
392 Equal amounts of whole cell extract were fractionated by electrophoresis on
393 sodium dodecyl sulphate (SDS) polyacrylamide gels and the proteins

394 transferred to PVDF paper and immunoblotted as described (61) . Band
395 intensities were determined by quantitative densitometry using Image J
396 (http://rsbweb.nih.gov/ij/).

397 **Tetrazolium reduction assay**

398 Cells were seeded in 96-well plates at 3 x 10^4 cells/cm^2. Following the
399 various cell treatments, 25 µl of MTT were added to each well and left for 2
400 hours in the incubator at 37^oC. The formazan crystals generated by viable
401 cells were solubilized using sodium dodecyl sulphate (SDS) reagent and
402 cells were incubated overnight in an atmosphere of 5% CO_2 in a 37^oC
403 humidified incubator. Quantitative determination of cell viability was
404 obtained by utilizing a SpectraMax® 340PC384 Microplate Reader;
405 absorbance of each sample was measured in quadruplicate at a wavelength
406 of 595 nm.

407 **Trypan blue exclusion assay**

408 Cells were seeded in triplicate in 12-well plates at 3 x 10^4 cells/cm^2.
409 Following treatment all media and cells were transferred from each well into
410 labelled Eppendorf tubes. 200µl per sample were then transferred to fresh
411 Eppendorf tubes with 200µl 0.4% Trypan Blue solution and tubes were briefly
412 vortexed. Several counts were made for each tube and percentage viability
413 was determined using the following formula: [(number of total cells –
414 number of dead (blue) cells)/ number of total (blue and white) cells] x 100 =
415 percentage cell viability].

416 **Time lapse microscopy**

417 The kinetics of the commitment of cells to apoptosis were measured by
418 time-lapse digital image microscopy as previously described, (62). Cells
419 were observed in an Olympus IX70 inverted microscope enclosed within a
420 37°C chamber in a 5% CO_2/95% air atmosphere. Images were captured
421 every 15 min using a Hamamatsu C4742-95 digital camera and, for each
422 condition, 40 cells per field of view were randomly chosen at the beginning
423 of the time-course. The images were analyzed using Image Pro Plus
424 software (Media Cybernetics, USA) with cells committed to apoptosis
425 scored according to the time at which clear changes in morphology
426 (cytoplasmic and nuclear shrinkage and a change to a phase bright
427 appearance) were first observed.

428 **Flow cytometry**

429 The cells were lifted from the plates with accutase and resuspended in 1 ml
430 cold PBS together with the supernatant media that the cells had been grown
431 in (containing any cells that may have lifted as a result of treatment). Cells
432 were pelleted and the wash repeated. Cells were resuspended in 1 x binding
433 buffer at a concentration of 1 x 10^6 cells and stained using an FITC Annexin
434 V Apoptosis Detection Kit 1 (BD Pharmingen, San Diego, USA) according
435 to the manufacturer's instructions. Flow cytometry was carried out on a
436 LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). Analysis was
437 carried out with FlowJo software (Tree Star, Ashland, OR, USA). Unstained
438 cells and cells stained only with FITC Annexin V were used as controls.

439 **Measurement of overall rates of protein synthesis**

440 Protein synthesis in intact cells was measured by the incorporation of [^{35}S]
441 methionine (2-4 μCi/ml for 1h) into trichloroacetic acid (TCA)-insoluble
442 material as described previously (63). Total cellular protein content was
443 determined and overall rates of protein synthesis were calculated as counts per
444 min incorporated per μg protein.

445 **m^{7}GTP-sepharose chromatography**

446 Initiation factor eIF4E and its associated proteins were isolated from cell
447 extracts (containing equal amounts of protein) by affinity chromatography on
448 m^{7}GTP-Sepharose beads as described (64). Bound proteins were eluted with
449 SDS gel sample buffer and analyzed by gel electrophoresis and
450 immunoblotting as described above.

451 **Statistical analysis**

452 All data are presented as the means ± SEM of at least three independent
453 measurements. Prism 5 software (GraphPad) was used for statistical
454 analysis. A 'P'value of <0.05 was considered to be statistically significant.
455 For determination of the synergistic effects of gemcitabine and TRAIL on
456 overall protein synthesis, combination index values were calculated using
457 Calcusyn software (Biosoft). ImageJ was used to analyse the density of
458 bands on Western blots (http://rsbweb.nih.gov/ij/).

459 **Acknowledgements**

460 This work was supported by grants from the Ralph Bates Pancreatic Cancer
461 Research Fund to AE and Programme Hospitalo-Universitaire en
462 Cancérologie (CAPTOR) to SP.

463 **Conflicts of interest:** There are no conflicts of interest.

464 **References**

465 1. Kamisawa T, Wood LD, Itoi T and Takaori K (2016) Pancreatic cancer.
466 Lancet 388:73-85
467

468 2. Elshaer M, Gravante G, Kosmin M, Riaz A and Al-Bahrani A (2016) A
469 systematic review of the prognostic value of lymph node ratio, number of
470 positive nodes and total nodes examined in pancreatic ductal
471 adenocarcinoma. Ann. R. Coll. Surg. Engl.:1-6
472

473 3. Cid-Arregui A and Juarez V (2015) Perspectives in the treatment of
474 pancreatic adenocarcinoma. World J. Gastroenterol. 21:9297-9316
475

476 4. Zabernigg A, Giesinger JM, Pall G, Gamper EM, Gattringer K, Wintner
477 LM *et al*., (2012) Quality of life across chemotherapy lines in patients with
478 cancers of the pancreas and biliary tract. BMC. Cancer 12:390
479

480 5. Michl P and Gress TM (2013) Current concepts and novel targets in
481 advanced pancreatic cancer. Gut 62:317-326
482

483 6. Von Hoff DD, Ramanathan RK, Borad MJ, Laheru DA, Smith LS, Wood
484 TE *et al*., (2011) Gemcitabine plus nab-paclitaxel is an active regimen in
485 patients with advanced pancreatic cancer: a phase I/II trial. J. Clin. Oncol.
486 29:4548-4554
487

488 7. Guertin DA and Sabatini DM (2005) An expanding role for mTOR in
489 cancer. Trends Mol. Med. 11:353-361
490
491 8. Zoncu R, Efeyan A and Sabatini DM (2011) mTOR: from growth signal
492 integration to cancer, diabetes and ageing. Nat. Rev. Mol. Cell Biol. 12:21-
493 35
494
495 9. Merrick WC (2015) eIF4F: a retrospective. J. Biol. Chem. 290:24091-
496 24099
497
498 10. Martineau Y, Azar R, Bousquet C and Pyronnet S (2013) Anti-
499 oncogenic potential of the eIF4E-binding proteins. Oncogene 32:671-677
500
501 11. Qin X, Jiang B and Zhang Y (2016) 4E-BP1, a multifactor regulated
502 multifunctional protein. Cell Cycle 15:781-786
503
504 12. Martineau Y, Azar R, Muller D, Lasfargues C, El KS, Anesia R *et al.*,
505 (2014) Pancreatic tumours escape from translational control through 4E-
506 BP1 loss. Oncogene 33:1367-1374
507
508 13. Martinez-Saez E, Peg V, Ortega-Aznar A, Martinez-Ricarte F, Camacho
509 J, Hernandez-Losa J *et al.*, (2016) peIF4E as an independent prognostic
510 factor and a potential therapeutic target in diffuse infiltrating astrocytomas.
511 Cancer Med. 5:2501-2512
512

513 14. Qu Y, Zhao R, Wang H, Chang K, Yang X, Zhou X *et al.*, (2016)
514 Phosphorylated 4EBP1 is associated with tumor progression and poor
515 prognosis in Xp11.2 translocation renal cell carcinoma. Sci. Rep. 6:23594
516 15. O'Reilly KE, Warycha M, Davies MA, Rodrik V, Zhou XK, Yee H *et*
517 *al.,* (2009) Phosphorylated 4E-BP1 is associated with poor survival in
518 melanoma. Clin. Cancer Res. 15:2872-2878
519

520 16. Armengol G, Rojo F, Castellvi J, Iglesias C, Cuatrecasas M, Pons B *et*
521 *al*., (2007) 4E-binding protein 1: a key molecular "funnel factor" in human
522 cancer with clinical implications 2. Cancer Res. 67:7551-7555
523

524 17. Coleman LJ, Peter MB, Teall TJ, Brannan RA, Hanby AM, Honarpisheh
525 H *et al*., (2009) Combined analysis of eIF4E and 4E-binding protein
526 expression predicts breast cancer survival and estimates eIF4E activity. Br.
527 J. Cancer 100:1393-1399
528

529 18. Siddiqui N and Sonenberg N (2015) Signalling to eIF4E in cancer.
530 Biochem. Soc. Trans. 43:763-772
531

532 19. Koromilas AE, Lazaris-Karatzas A and Sonenberg N (1992) mRNAs
533 containing extensive secondary structure in their 5' non-coding region
534 translate efficiently in cells overexpressing initiation factor eIF-4E. EMBO
535 J. 11:4153-4158
536

537 20. Li S, Sonenberg N, Gingras AC, Peterson M, Avdulov S, Polunovsky
538 VA *et al.*, (2002) Translational control of cell fate: availability of
539 phosphorylation sites on translational repressor 4E-BP1 governs its
540 proapoptotic potency. Mol. Cell Biol. 22:2853-2861
541 21. Wang J, Ye Q and She QB (2014) New insights into 4E-BP1-regulated
542 translation in cancer progression and metastasis. Cancer Cell Microenviron.
543 1
544
545 22. Castellvi J, Garcia A, Ruiz-Marcellan C, Hernandez-Losa J, Peg V,
546 Salcedo M *et al.*, (2009) Cell signaling in endometrial carcinoma:
547 phosphorylated 4E-binding protein-1 expression in endometrial cancer
548 correlates with aggressive tumors and prognosis. Hum. Pathol. 40:1418-
549 1426
550
551 23. Roh MS, Lee JH, Kang KW, Nam HY, Jung SB, Kim K *et al.*, (2015)
552 Phosphorylated 4E-binding protein 1 expression is associated with poor
553 prognosis in small-cell lung cancer. Virchows Arch. 467:667-673
554
555 24. Chakravarthy R, Clemens MJ, Pirianov G, Perdios N, Mudan S,
556 Cartwright JE *et al.*, (2013) Role of the eIF4E binding protein 4E-BP1 in
557 regulation of the sensitivity of human pancreatic cancer cells to TRAIL and
558 celastrol-induced apoptosis. Biol. Cell 105:414-429
559
560 25. Dumstorf CA, Konicek BW, McNulty AM, Parsons SH, Furic L,
561 Sonenberg N *et al.*, (2010) Modulation of 4E-BP1 function as a critical

562 determinant of enzastaurin-induced apoptosis. Mol. Cancer Ther. 9:3158-
563 3163
564
565 26. Lemke J, von KS, Zinngrebe J and Walczak H (2014) Getting TRAIL
566 back on track for cancer therapy. Cell Death Differ. 21:1350-1364
567 27. Amarante-Mendes GP and Griffith TS (2015) Therapeutic applications
568 of TRAIL receptor agonists in cancer and beyond. Pharmacol. Ther.
569 155:117-131
570
571 28. de MD, Lemke J, Anel A, Walczak H and Martinez-Lostao L (2016)
572 Onto better TRAILs for cancer treatment. Cell Death Differ. 23:733-747
573
574 29. Johnstone RW, Frew AJ and Smyth MJ (2008) The TRAIL apoptotic
575 pathway in cancer onset, progression and therapy. Nat. Rev. Cancer 8:782-
576 798
577
578 30. Zhang L and Fang B (2005) Mechanisms of resistance to TRAIL-
579 induced apoptosis in cancer. Cancer Gene Ther. 12:228-237
580
581 31. Trivedi R and Mishra DP (2015) Trailing TRAIL Resistance: Novel
582 Targets for TRAIL Sensitization in Cancer Cells. Front Oncol. 5:69
583
584 32. Hellwig CT and Rehm M (2012) TRAIL signaling and synergy
585 mechanisms used in TRAIL-based combination therapies. Mol. Cancer
586 Ther. 11:3-13

587

588 33. Soria JC, Mark Z, Zatloukal P, Szima B, Albert I, Juhasz E *et al.*, (2011)
589 Randomized phase II study of dulanermin in combination with paclitaxel,
590 carboplatin, and bevacizumab in advanced non-small-cell lung cancer. J.
591 Clin. Oncol. 29:4442-4451
592 34. Berg D, Lehne M, Muller N, Siegmund D, Munkel S, Sebald W *et al.*,
593 (2007) Enforced covalent trimerization increases the activity of the TNF
594 ligand family members TRAIL and CD95L. Cell Death Differ. 14:2021-
595 2034
596

597 35. Perlstein B, Finniss SA, Miller C, Okhrimenko H, Kazimirsky G,
598 Cazacu S et al., (2013) TRAIL conjugated to nanoparticles exhibits
599 increased anti-tumor activities in glioma cells and glioma stem cells in vitro
600 and in vivo. Neuro. Oncol. 15:29-40
601

602 36. Hylander BL, Sen A, Beachy SH, Pitoniak R, Ullas S, Gibbs JF *et al.*,
603 (2015) Tumor priming by Apo2L/TRAIL reduces interstitial fluid pressure
604 and enhances efficacy of liposomal gemcitabine in a patient derived
605 xenograft tumor model. J. Control Release 217:160-169
606

607 37. Han Z, Lee S, Je S, Eom CY, Choi HJ, Song JJ *et al.*, (2016) Survivin
608 silencing and TRAIL expression using oncolytic adenovirus increase anti-
609 tumorigenic activity in gemcitabine-resistant pancreatic cancer cells.
610 Apoptosis. 21:351-364
611

38. Zhao B, Li L, Cui K, Wang CL, Wang AL, Sun ZQ *et al.*, (2011) Mechanisms of TRAIL and gemcitabine induction of pancreatic cancer cell apoptosis. Asian Pac. J. Cancer Prev. 12:2675-2678

39. Schniewind B, Christgen M, Kurdow R, Haye S, Kremer B, Kalthoff H *et al.*, (2004) Resistance of pancreatic cancer to gemcitabine treatment is dependent on mitochondria-mediated apoptosis. Int. J. Cancer 109:182-188

40. Quint K, Tonigold M, Di FP, Montalbano R, Lingelbach S, Ruckert F *et al.*, (2012) Pancreatic cancer cells surviving gemcitabine treatment express markers of stem cell differentiation and epithelial-mesenchymal transition. Int. J. Oncol. 41:2093-2102

41. Awasthi N, Zhang C, Schwarz AM, Hinz S, Wang C, Williams NS *et al.*, (2013) Comparative benefits of Nab-paclitaxel over gemcitabine or polysorbate-based docetaxel in experimental pancreatic cancer. Carcinogenesis 34:2361-2369

42. Li H, Zhu H, Xu CJ and Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94:491-501

43. Duluc C, Moatassim-Billah S, Chalabi-Dchar M, Perraud A, Samain R, Breibach F *et al.*, (2015) Pharmacological targeting of the protein synthesis

637 mTOR/4E-BP1 pathway in cancer-associated fibroblasts abrogates
638 pancreatic tumour chemoresistance. EMBO Mol. Med. 7:735-753
639
640 44. Chou TC and Talalay P (1984) Quantitative analysis of dose-effect
641 relationships: the combined effects of multiple drugs or enzyme inhibitors.
642 Adv. Enzyme Regul. 22:27-55
643
644 45. Azar R, Alard A, Susini C, Bousquet C and Pyronnet S (2009) 4E-BP1
645 is a target of Smad4 essential for TGFbeta-mediated inhibition of cell
646 proliferation. EMBO J. 28:3514-3522
647
648 46. Mishra R, Miyamoto M, Yoshioka T, Ishikawa K, Matsumura Y, Shoji
649 Y *et al*., (2009) Adenovirus-mediated eukaryotic initiation factor 4E binding
650 protein-1 in combination with rapamycin inhibits tumor growth of
651 pancreatic ductal adenocarcinoma in vivo. Int. J. Oncol. 34:1231-1240
652
653 47. Dowling RJ, Topisirovic I, Alain T, Bidinosti M, Fonseca BD,
654 Petroulakis E *et al.,* (2010) mTORC1-mediated cell proliferation, but not
655 cell growth, controlled by the 4E-BPs. Science 328:1172-1176
656
657 48. Long J, Zhang Y, Yu X, Yang J, LeBrun DG, Chen C *et al*., (2011)
658 Overcoming drug resistance in pancreatic cancer. Expert. Opin. Ther.
659 Targets. 15:817-828
660

661 49. Martin R, Desponds C, Eren RO, Quadroni M, Thome M and Fasel N
662 (2016) Caspase-mediated cleavage of raptor participates in the inactivation
663 of mTORC1 during cell death. Cell Death Discov. 2:16024
664 50. Iriana S, Ahmed S, Gong J, Annamalai AA, Tuli R and Hendifar AE
665 (2016) Targeting mTOR in Pancreatic Ductal Adenocarcinoma. Front
666 Oncol. 6:99
667
668 51. Jeffrey IW, Bushell M, Tilleray VJ, Morley S and Clemens MJ (2002)
669 Inhibition of protein synthesis in apoptosis: Differential requirements by the
670 tumor necrosis factor alpha family and a DNA-damaging agent for caspases
671 and the double-stranded RNA-dependent protein kinase. Cancer Research
672 62:2272-2280
673
674 52. Jeffrey IW, Elia A, Bornes S, Tilleray VJ, Gengatharan K and Clemens
675 MJ (2006) Interferon-alpha induces sensitization of cells to inhibition of
676 protein synthesis by tumour necrosis factor-related apoptosis-inducing
677 ligand. Febs Journal 273:3698-3708
678
679 53. Dowling RJO, Topisirovic I, Alain T, Bidinosti M, Fonseca BD,
680 Petroulakis E *et al.*, (2010) mTORC1-Mediated Cell Proliferation, But Not
681 Cell Growth, Controlled by the 4E-BPs 15. Sci 328:1172-1176
682
683 54. Beyer K, Normann L, Sendler M, Kading A, Heidecke CD, Partecke LI
684 *et al.*, (2016) TRAIL Promotes Tumor Growth in a Syngeneic Murine

685 Orthotopic Pancreatic Cancer Model and Affects the Host Immune
686 Response. Pancreas 45:401-408
687 55. Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MR and
688 Yagita H (2003) Nature's TRAIL--on a path to cancer immunotherapy.
689 Immunity. 18:1-6
690
691 56. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta
692 S *et al*., (2001) Involvement of tumor necrosis factor-related apoptosis-
693 inducing ligand in surveillance of tumor metastasis by liver natural killer
694 cells. Nat. Med. 7:94-100
695
696 57. Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ and Smyth MJ
697 (2002) Increased susceptibility to tumor initiation and metastasis in TNF-
698 related apoptosis-inducing ligand-deficient mice. J. Immunol. 168:1356-
699 1361
700
701 58. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G *et*
702 *al*., (2007) Targeting human {gamma}delta} T cells with zoledronate and
703 interleukin-2 for immunotherapy of hormone-refractory prostate cancer.
704 Cancer Res. 67:7450-7457
705
706 59. Simons MP, O'Donnell MA and Griffith TS (2008) Role of neutrophils
707 in BCG immunotherapy for bladder cancer. Urol. Oncol. 26:341-345
708

709 60. Bremer E (2013) Targeting of the tumor necrosis factor receptor
710 superfamily for cancer immunotherapy. ISRN. Oncol. 2013:371854
711 61. Elia A, Constantinou C and Clemens MJ (2008) Effects of protein
712 phosphorylation on ubiquitination and stability of the translational inhibitor
713 protein 4E-BP1 3. Oncogene 27:811-822
714

715 62. Dash PR, McCormick J, Thomson MJ, Johnstone AP, Cartwright JE and
716 Whitley GS (2007) Fas ligand-induced apoptosis is regulated by nitric oxide
717 through the inhibition of fas receptor clustering and the nitrosylation of
718 protein kinase Cepsilon. Exp. Cell Res. 313:3421-3431
719

720 63. Jeffrey IW, Bushell M, Tilleray VJ, Morley S and Clemens MJ (2002)
721 Inhibition of protein synthesis in apoptosis: Differential requirements by the
722 tumor necrosis factor a family and a DNA-damaging agent for caspases and
723 the double-stranded RNA-dependent protein kinase. Cancer Res. 62:2272-
724 2280
725

726 64. Morley SJ (1997) Signalling through either the p38 or ERK mitogen-
727 activated protein (MAP) kinase pathway is obligatory for phorbol ester and
728 T cell receptor complex (TCR-CD3)-stimulated phosphorylation of
729 initiation factor (eIF) 4E in Jurkat T cells. FEBS Lett. 418:327-332
730

731 **Figure Legends**

732 **Figure 1** Effect of gemcitabine and/or TRAIL on PDAC survival. BxPC-3,
733 MIA PaCa-2 and PANC-1 cells were seeded in 96-well plates at a cell
734 seeding density of $3x10^4$ cells/cm^2. (**a**) Sensitivity of cells to gemcitabine

735 was assessed by MTT assay. Cells were treated with increasing amounts of
736 gemcitabine (0.001-1000 µM) for 24 h (n=4). (**b**) Sensitivity of cells to
737 TRAIL was assessed by MTT assay. Cells were treated with increasing
738 amounts of TRAIL (0.001-1000 ng/ml) for 4 h (n=4). (**c-e**) Sensitivity of
739 cells to gemcitabine and TRAIL combination treatment was assessed by
740 MTT assay. Cells were treated with increasing amounts of gemcitabine (0.1-
741 100 µM) for 24 h (n=4) and/or 10 or 100 ng/ml TRAIL for 4 h for BxPC-3
742 and MIA PaCa-2 cells and 6 h for PANC-1 cells (n=4). All experiments
743 were repeated three times and data are provided as means ± SEM (one
744 representative experiment is shown). P-values were calculated using
745 Student's t test to determine the statistical significance of the difference
746 between (**a** and **b**) untreated cells and cells treated with either 1000 µM
747 gemcitabine or 1000 ng/ml TRAIL respectively (ns: P>0.05, * P <0.05, **
748 P< 0.01) and (**c-e**) cells treated with 100 µM gemcitabine and cells treated
749 with 100 µM gemcitabine plus 100 ng/ml TRAIL, (*** P< 0.001).

750 **Figure 2** Combination treatment induces apoptosis. (**a**) PANC-1 cells were
751 seeded in triplicate in 12-well plates at a cell seeding density of 3 x 10^4
752 cells/cm^2 and left to attach overnight. Cells were treated with 100 µM
753 gemcitabine for 24 h and/or 100 ng/ml TRAIL for 6 h. The viability of the
754 cells was assessed by trypan blue exclusion assay. Quadruplicate cell counts
755 were used to calculate each cell density. These were performed for three
756 independently seeded wells and percentage viability was determined. (**b**) 1 x
757 10^6 cells PANC-1 cells were treated with 100 µM gemcitabine for 24 h
758 and/or 100ng/ml TRAIL for 6 h. Induction of early apoptosis in PANC-1
759 cells was assessed using flow cytometry following staining with FITC

760 Annexin V. The data represent means ± SEM. of three experiments
761 performed in triplicate. (**c** and **d**) PANC-1 cells were seeded in triplicate in
762 12-well plates at a cell seeding density of 3 x 10^4 cells/cm^2 and left to attach
763 overnight. Cells were treated with 100 μM gemcitabine for 24 h and/or 100
764 ng/ml TRAIL for 6 h and monitored by time lapse microscopy. (**c**) The
765 appearance of a pre-apoptotic morphology was scored and the % apoptotic
766 cells after 24 h determined. The data are the means ± SEM from three
767 independent experiments. (**d**) Phase contrast microscopy images of cells
768 treated as indicated. (**a-c**) All experiments were repeated three times and data
769 are provided as means ± SEM (one representative experiment is shown). P-
770 values were calculated using Student's t test to determine the statistical
771 significance of the difference between cells treated with 100 μM
772 gemcitabine and cells treated with 100 μM gemcitabine plus 100 ng/ml
773 TRAIL, (* $P <0.05$, ** $P< 0.01$, *** $P< 0.001$).

774 **Figure 3** Combination treatment induces caspase-dependent apoptosis.
775 BxPC-3, MIA PaCa-2 and PANC-1 cells were seeded in 96-well plates at a
776 cell seeding density of $3x10^4$ cells/cm^2. (**a** and **b**) Caspase-mediated
777 cleavage of caspase-8 and PARP was assessed by western blotting in cells
778 treated with 100 μM gemcitabine for 24 h and/or 100 ng/ml TRAIL for 4 h
779 for BxPC-3 and MIA PaCa-2 cells and 4 and 6 h for PANC-1 cells (n=3).
780 One representative experiment is shown. Lysates were prepared and equal
781 amounts (15 μg total protein) were subjected to SDS–PAGE, transferred to
782 PVDF membranes and then immunoblotted with antibodies directed against
783 (**a**) PARP (top panel), caspase-8 (middle panel) or GAPDH (bottom panel).
784 (**b**) Caspase-mediated cleavages of caspase-8, PARP and BID in the

785 presence or absence of the pan caspase inhibitor Z-VAD-FMK (10 μM)
786 were assessed by western blotting in cells treated as described above.
787 Membranes were immunoblotted with antibodies directed against caspase -
788 8, PARP, and BID. GAPDH was used as a loading control. (**c**) The
789 inhibition of cell survival following combination treatment was assessed in
790 the presence or absence of the pan caspase inhibitor Z-VAD-FMK. PANC-1
791 cells were seeded in 96-well plates at a cell seeding density of $3x10^4$
792 cells/cm^2. Cells were treated with 100 μM gemcitabine for 24 h and/or 100
793 ng/ml TRAIL for 6 h in the presence or absence of 10 μM Z-VAD-FMK.
794 Cell survival was assessed using the MTT assay. All experiments were
795 repeated three times and data are provided as means ± SEM (one
796 representative experiment is shown). P values were calculated using
797 Student's t test to determine the statistical significance of the difference
798 between cells treated with 100 μM gemcitabine and those treated with both
799 100 μM gemcitabine and 100 ng/ml TRAIL, (* P <0.05, ** P< 0.01, *** P<
800 0.001).

801 **Figure 4** Combination treatment targets the mTOR pathway and alters the
802 phosphorylation of 4E-BP1 in PDAC cells. BxPC-3, MIA PaCa-2 cells and
803 PANC-1 cells were treated with 100μM gemcitabine for 24 h and/or 100
804 ng/ml TRAIL for 4 h. 15 μg of total protein lysate was analyzed using
805 western blotting. (**a**) PANC-1 cell lysates were analyzed with antibodies
806 directed against total mTOR, mTOR Ser^{2448}, Raptor, Rictor, total 4E-BP1,
807 4E-BP1 Ser^{65} and GAPDH. (**b**) BxPC-3, MIA PaCA-2 and PANC-1 lysates
808 were analyzed to look at the effect on levels and phosphorylation of 4E-BP1
809 at residues Ser^{65}, $Thr^{37/46}$ and Thr^{70} as well as levels of eIF4E. GAPDH was

used as a loading control. (**c**) The change in phosphorylation of 4E-BP1 at Ser^{65} in PANC-1 cells following combination treatment using TRAIL treatment for either 4 h or 6h was assessed by western blotting. PVDF membranes were immunoblotted with antibodies directed against total 4E-BP1 and 4E-BP1 residue Ser^{65}. (**d**) The relative levels of phosphorylation of 4E-BP1 at Ser^{65} were quantified by scanning densitometry using ImageJ and the data are shown on the histogram as % of the values for untreated cells. All experiments were repeated three times and data are provided as means ± SEM. P values were calculated using Student's t test to determine the statistical significance of the difference between untreated cells and cells treated with either gemcitabine or gemcitabine plus TRAIL (* $P < 0.05$ and *** $P < 0.001$).

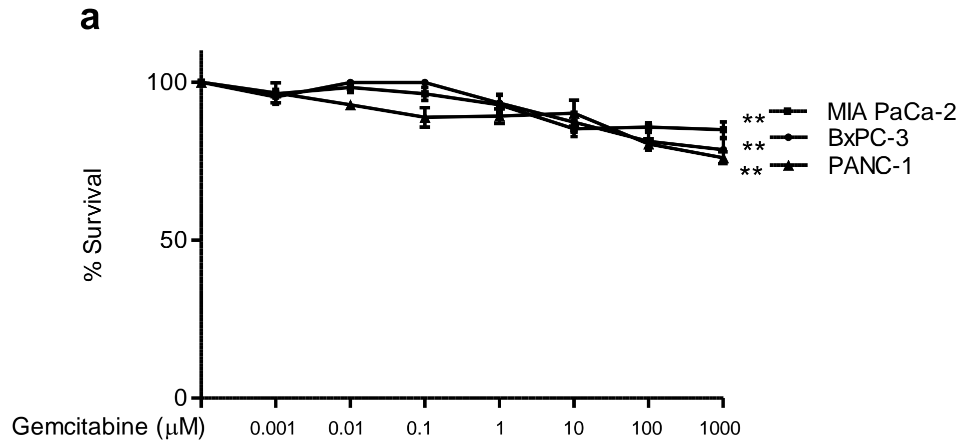
Figure 5 4E-BP1 is involved in the regulation of cell survival following gemcitabine and TRAIL treatment. (**a** and **b**) MIA PaCa-2 cells expressing a small hairpin RNA (shRNA) directed against 4E-BP1 and control cells expressing a scrambled shRNA were seeded in 96-well plates at a cell seeding density of $3x10^4$ cells/cm^2. (**a**) The sensitivity of cells to gemcitabine and TRAIL combination treatment was assessed by MTT assay. Cells were treated with increasing amounts of gemcitabine (0.1-100 μM) for 24 h (n=4) and/or 100 ng/ml TRAIL for 24 h (n=4). All experiments were repeated three times and data are provided as means ± SEM. One representative experiment is shown. P values were calculated using Student's t test to determine the statistical significance of the difference between cells expressing a scrambled shRNA and cells expressing a shRNA directed against 4E-BP1, both cell lines having been treated with 10 or 100

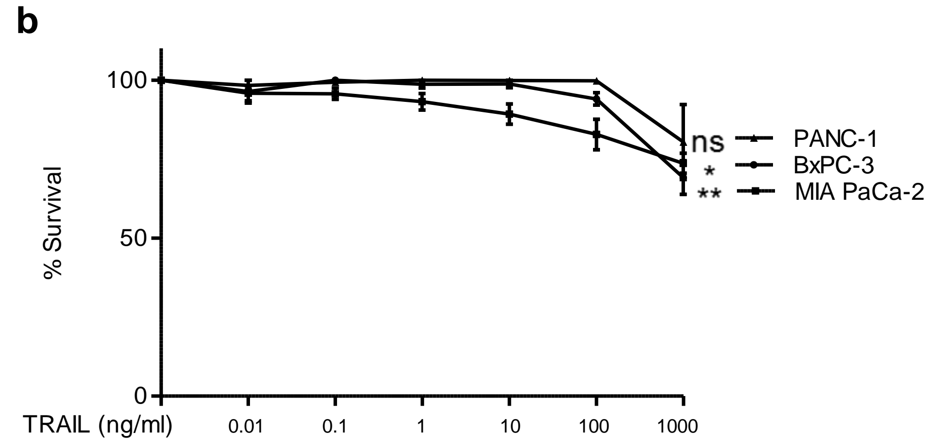
835 μM gemcitabine and 100 ng/ml TRAIL (* $P < 0.05$). (**b**) Lysates made from
836 cells treated as in (**a**) were used to purify eIF4E using chromatography on
837 m^7GTP-Sepharose beads as described in Materials and Methods. The levels of
838 eIF4E and of the 4E-BP1 associated with it were determined by SDS gel
839 electrophoresis and immunoblotting. Total cell lysates were analysed in
840 parallel. Quantification was carried out by densitometry using ImageJ and the
841 ratios of 4E-BP1 to eIF4E in the m^7GTP –purified samples (in arbitrary units)
842 are indicated.

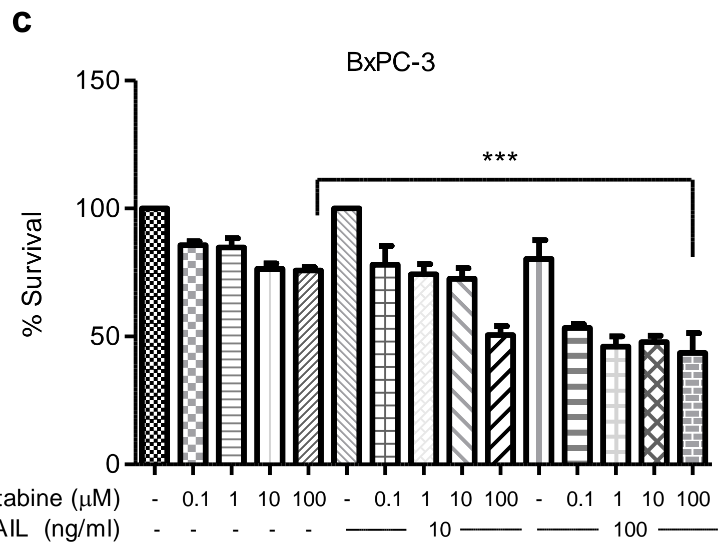
843

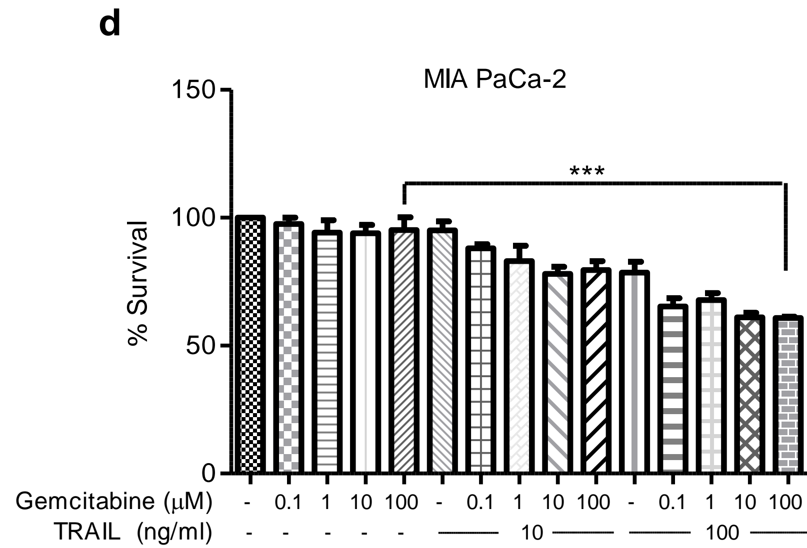
844

Figure 1









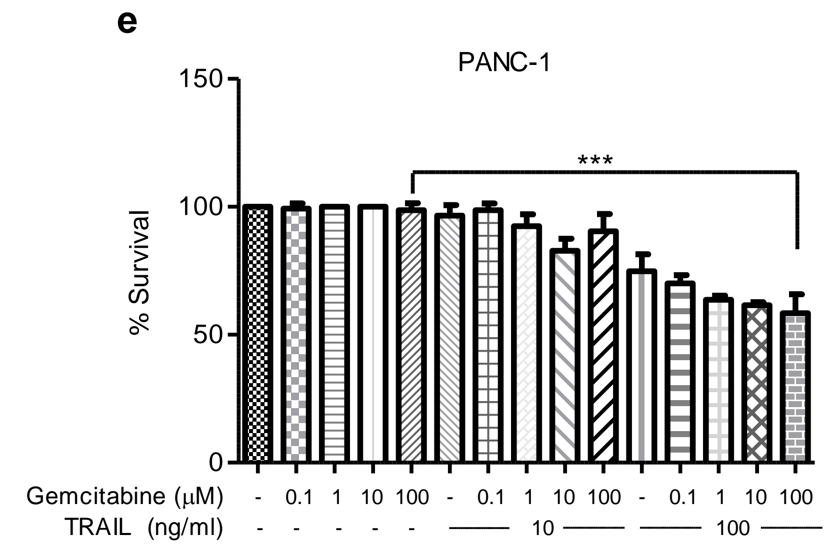
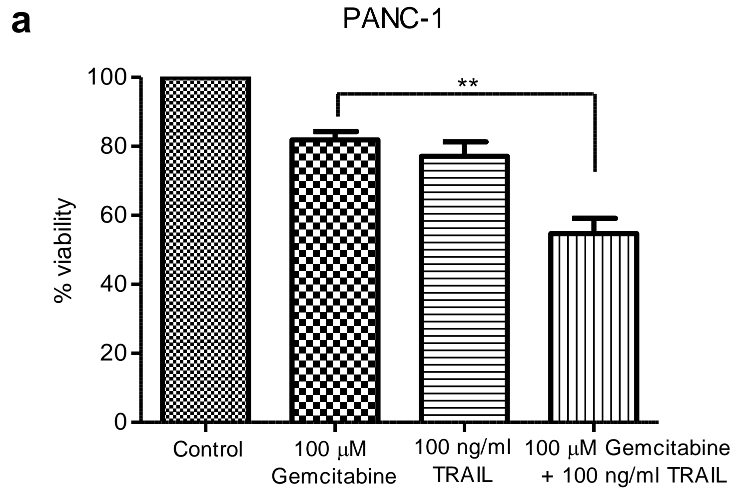
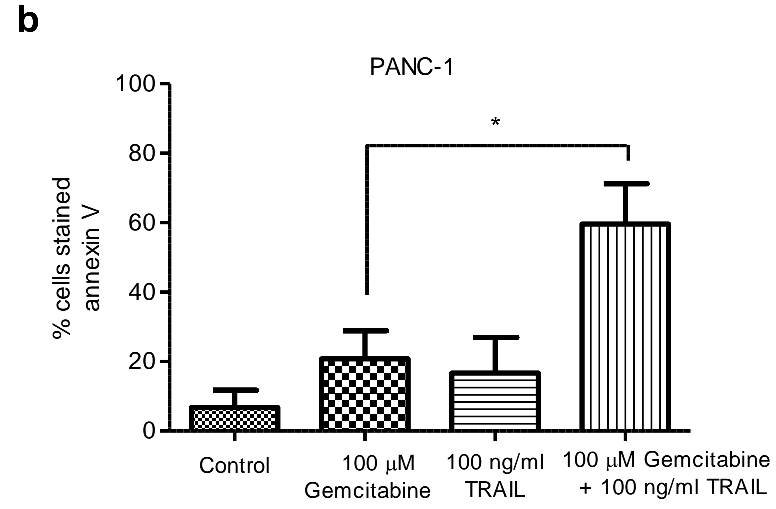
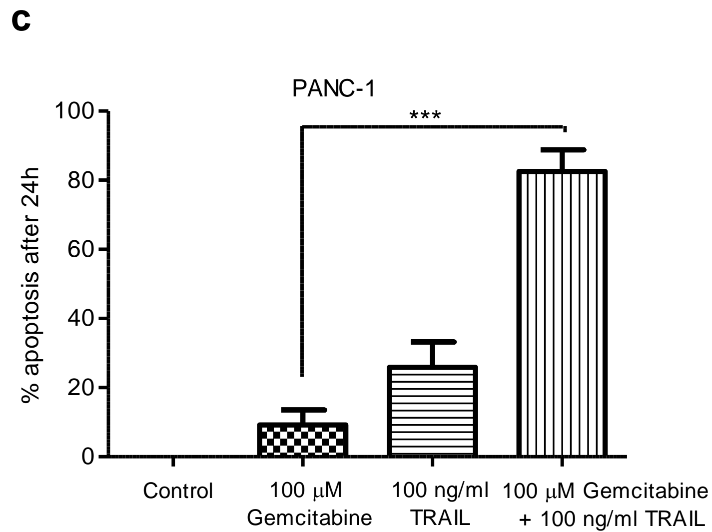


Figure 2







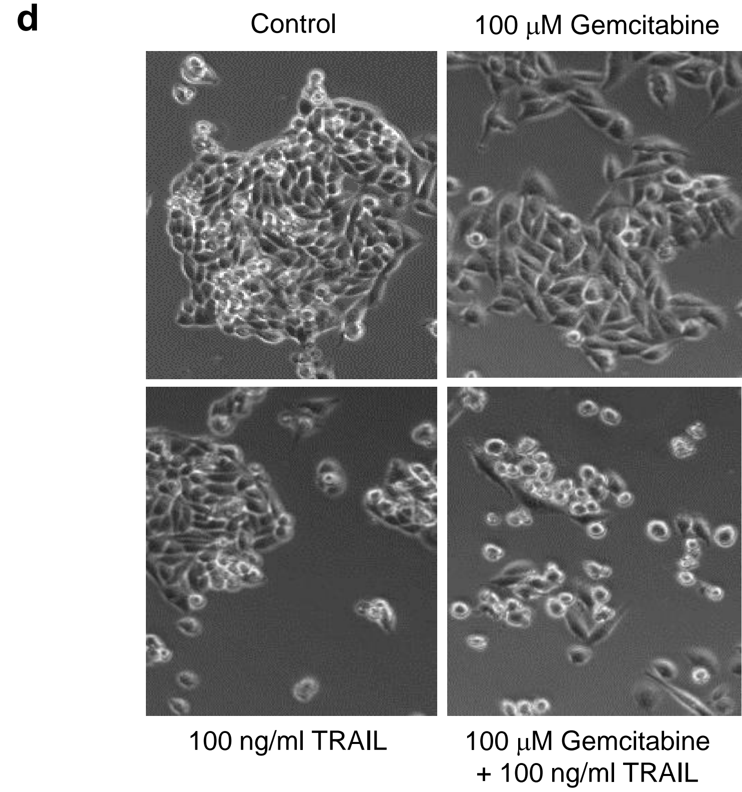
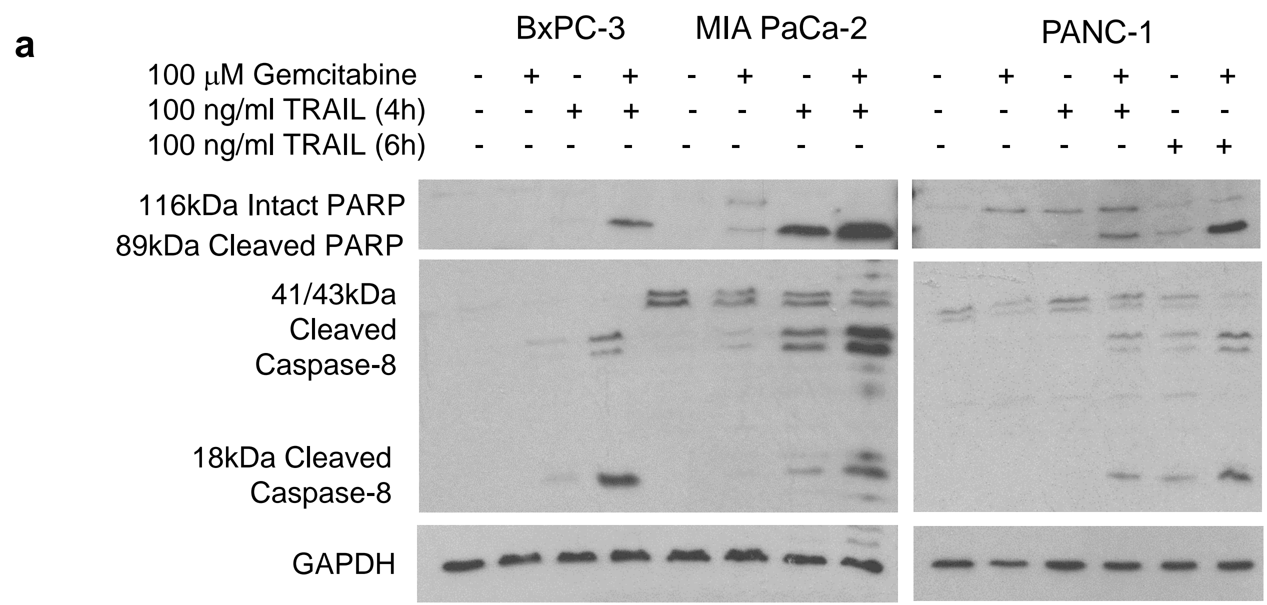
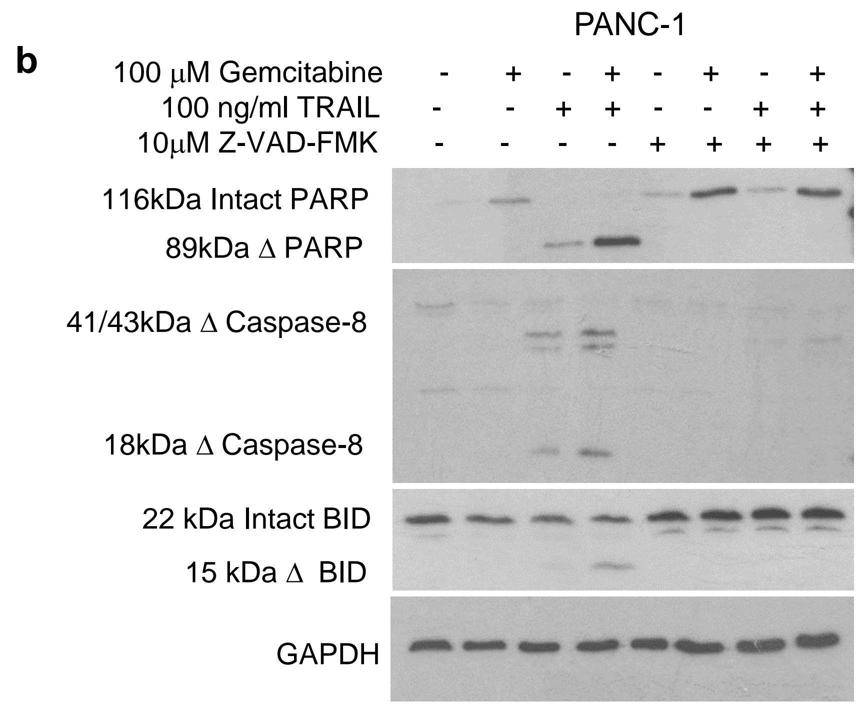


Figure 3





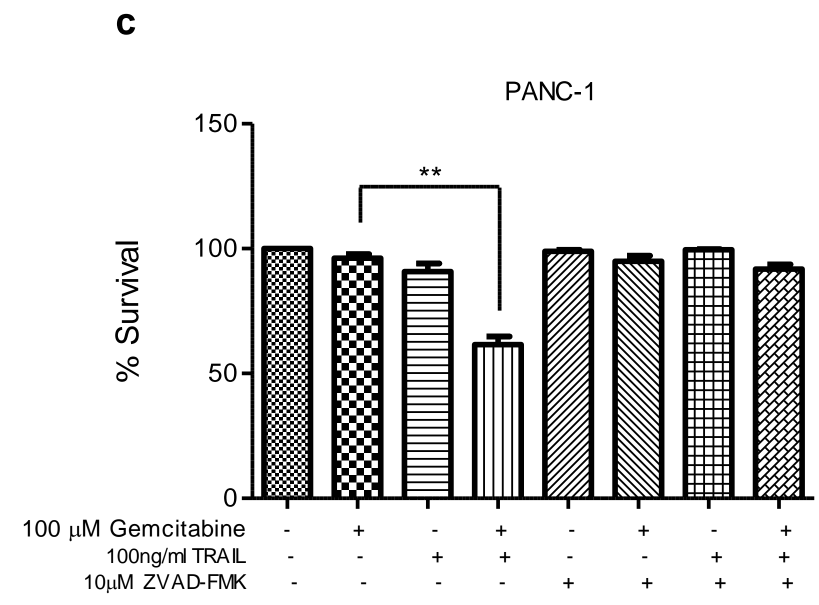
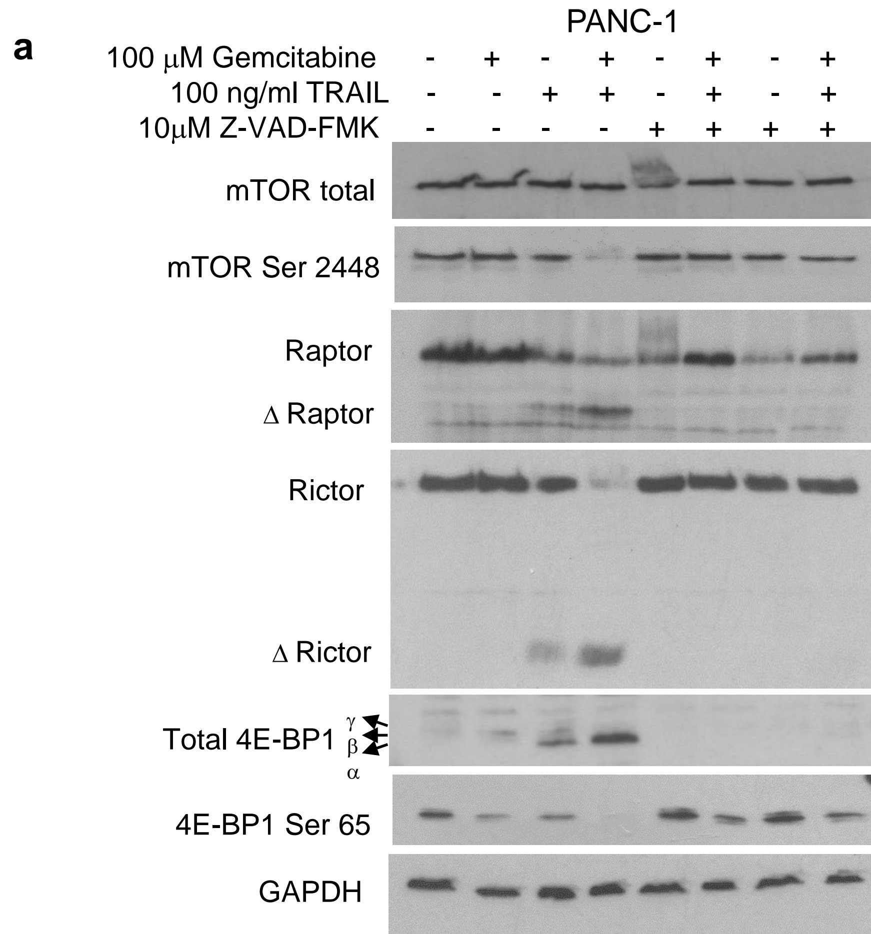
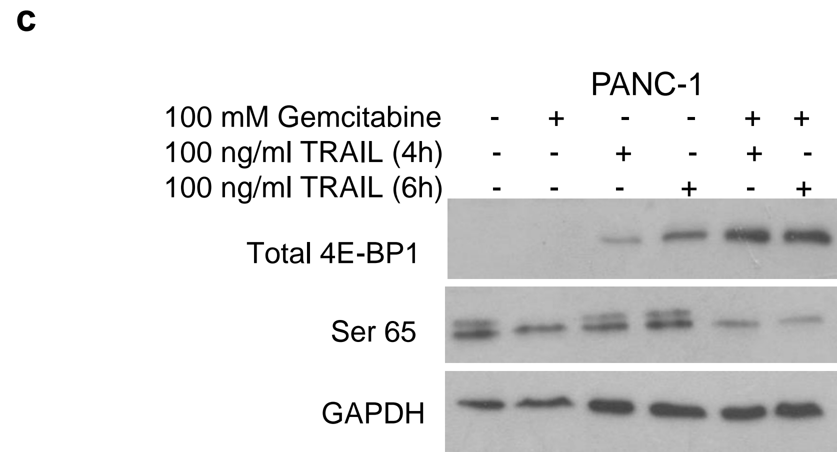


Figure 4

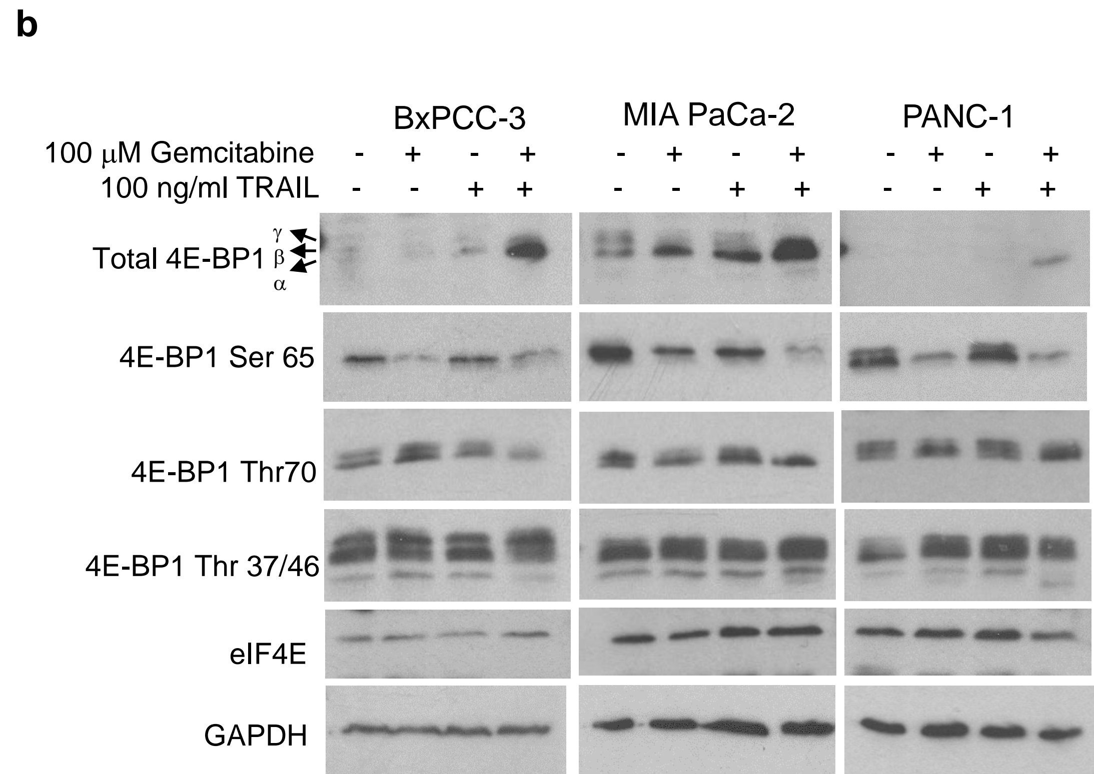
a



c



b



d

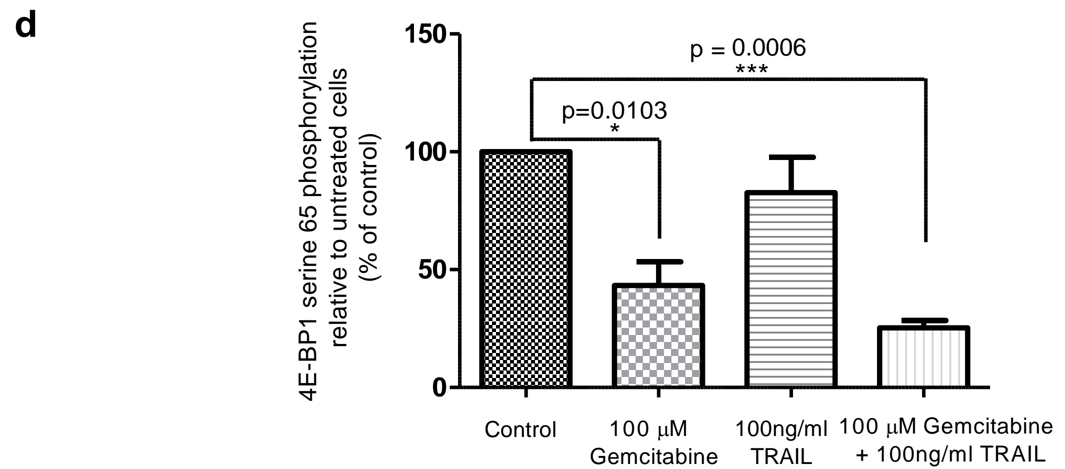


Figure 5