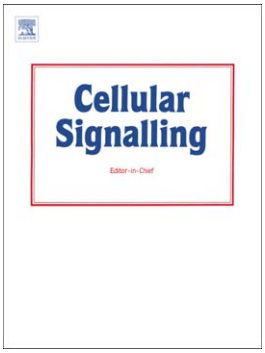


Growth-factor dependent expression of the translationally controlled tumour protein TCTP is regulated through the PI3-K/Akt/mTORC1 signalling pathway

Ulrich-Axel Bommer, Valentina Iadevaia, Jiezhong Chen, Bianca Knoch, Martin Engel, Christopher G. Proud

PII: S0898-6568(15)00145-X
DOI: doi: 10.1016/j.cellsig.2015.04.011
Reference: CLS 8461

To appear in: *Cellular Signalling*

Received date: 7 January 2015
Revised date: 21 April 2015
Accepted date: 25 April 2015

Please cite this article as: Ulrich-Axel Bommer, Valentina Iadevaia, Jiezhong Chen, Bianca Knoch, Martin Engel, Christopher G. Proud, Growth-factor dependent expression of the translationally controlled tumour protein TCTP is regulated through the PI3-K/Akt/mTORC1 signalling pathway, *Cellular Signalling* (2015), doi: 10.1016/j.cellsig.2015.04.011

Title:

Growth-factor dependent expression of the translationally controlled tumour protein TCTP is regulated through the PI3-K/Akt/mTORC1 signalling pathway

Ulrich-Axel Bommer,[1,2#] Valentina Iadevaia,[3,4] Jiezhong Chen,[1,5] Bianca Knoch[1], Martin Engel[1] and Christopher G. Proud[3,6]

Author affiliations:

[1]Illawarra Health and Medical Research Institute and [2]Graduate School of Medicine, Faculty of Science, Medicine and Health, University of Wollongong, Wollongong 2522 NSW, Australia

[3]Centre for Biological Sciences, University of Southampton, UK

Present addresses:

[4]School of Biomedical Sciences, University of Surrey, Guildford, Surrey, UK

[5]School of Biomedical Sciences, University of Queensland, St Lucia, QLD, Australia

[6]South Australian Health & Medical Research Institute (SAHMRI), Adelaide, Australia.

Running title:

TCTP mRNA is translationally regulated by mTORC1

Corresponding author:

Dr U-A Bommer, Graduate School of Medicine and
Illawarra Health & Medical Research Institute (IHMRI)
University of Wollongong, Northfields Avenue,
Wollongong, New South Wales 2522, Australia.
E-mail: ubommer@uow.edu.au

Postal address:

Dr U-A Bommer,
Mendelstrasse 23
13187 Berlin, Germany
Tel: +4930-91422415.

Abstract

Translationally controlled tumour protein TCTP (gene symbol: TPT1) is a highly-conserved, cyto-protective protein implicated in many physiological and disease processes, in particular cancer, where it is associated with poor patient outcomes. To understand the mechanisms underlying the accumulation of high TCTP levels in cancer cells, we studied the signalling pathways that control translation of TCTP mRNA, which contains a 5'-terminal oligopyrimidine tract (5'-TOP). In HT29 colon cancer cells and in HeLa cells, serum increases the expression of TCTP two- and four-fold, respectively, and this is inhibited by rapamycin or mTOR kinase inhibitors. Polysome profiling and mRNA quantification indicate that these effects occur at the level of mRNA translation. Blocking this pathway upstream of mTOR complex 1 (mTORC1) by inhibiting Akt also prevented increases in TCTP levels in both HeLa and HT29 colon cancer cells, whereas knockout of TSC2, a negative regulator of mTORC1, led to derepression of TCTP synthesis under serum starvation. Overexpression of eIF4E enhanced the polysomal association of the TCTP mRNA, although it did not protect its translation from inhibition by rapamycin. Conversely, expression of a constitutively-active mutant of the eIF4E inhibitor 4E-BP1, which is normally inactivated by mTORC1, inhibited of TCTP mRNA translation in HEK293 cells. Our results demonstrate that TCTP mRNA translation is regulated by signalling through the PI3-K/Akt/mTORC1 pathway. This explains why TCTP levels are frequently increased in cancers, since mTORC1 signalling is hyperactive in ~80% of tumours.

Keywords:

Translationally controlled tumour protein TCTP, PI 3-kinase pathway, Akt, mTOR complex 1, initiation factor eIF4E, TOP mRNA.

1. Introduction

Translationally controlled tumour protein TCTP, the product of the TPT1 gene, also referred to as histamine-releasing factor (HRF) or fortilin, is a highly-conserved, multifunctional protein, which has been implicated in a range of both cell physiological and disease processes [1-4]. One of the best-established roles of TCTP is its anti-apoptotic, or more generally cytoprotective, function in cellular responses to a wide range of stresses, such as oxidative stress, heat shock, Ca^{++}-stress, or stress induced by heavy metals or drug treatment [2]. This is underscored by the finding that gene knockout of TCTP in mice is embryonically lethal, due to excessive apoptosis early in embryogenesis [5-7]. TCTP has been found to bind to microtubules [8] and actin filaments [9], and to a range of other proteins [10]. TCTP has been implicated in the regulation of cell division [11], DNA damage repair [12], protein synthesis [13], and in the early development of both animals and plants [11, 14].

There is now substantial evidence demonstrating that TCTP is related to cell growth and to the development of cancer [1-3, 15] TCTP is overexpressed in many cancer cell lines, and its protein levels are positively related to properties of these cells relating to growth behaviour, apoptosis prevention and tumorigenicity [1, 2], and even metastatic potential [3, 15]. Increased TCTP levels are detected in a range of human tumours [2, 3, 15], and a high TCTP status has been proposed as a prognostic marker for a poor outcome in breast cancer [16], hepatocellular cancer [17], and in gliomas [18]. A solid understanding of how TCTP levels are regulated in cancer cells is therefore urgently required. Extensive work on the experimental ‘tumour reversion model’ has shed light on the importance of TCTP in cancer development [15, 19], and its anti-apoptotic activity, including the demonstrated antagonism to the tumour suppressor protein p53 [16, 20, 21], has been proposed as an underlying mechanism. Alternative mechanisms by which TCTP exerts its anti-apoptotic activity have been proposed as [2, 22]. Several reports indicated that TCTP might be involved in growth signalling pathways; for

example, overexpression of TCTP in HeLa cells resulted in activation of phospholipase C-γ, as well as the mitogen-activated protein (MAP)-kinase and phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (Akt) pathways [23]. Other investigations demonstrated that knock-out of TCTP reduces cell size and general growth in *Drosophila* [24]. These authors concluded that TCTP is an upstream regulator of mTORC1 (mammalian target of rapamycin complex 1); however, other studies obtained different results in mammalian cells [25, 26], so this point remains controversial.

The earliest observations published on TCTP, at the time designated Q23 [27], p21 [28] or p23 [29], described the rapid growth induction of its synthesis at the translational level as a main feature of the protein, supporting the notion that TCTP is important for cell growth [30]. Although it has meanwhile been established that TCTP levels may also be regulated by mechanisms other than translational control [1, 2], the cellular response to growth stimuli typically involves translational up-regulation of TCTP levels, at least in mammalian cells. However, the precise signalling pathway and mechanism involved are not yet completely characterised. We have previously shown that eukaryotic initiation factor 4E (eIF4E) is involved in regulating TCTP levels after serum stimulation of mouse fibroblasts [31]. At this time, the link between mTORC1 and the regulation of eIF4E had not yet been established, and it is now important to examine whether translation of the TCTP mRNA is indeed regulated through the PI3-K/Akt/mTORC1 signalling pathway, which is frequently hyperactive in human tumours.

mTORC1 is the hub of a signalling network that integrates a wide range of environmental signals and regulates an array of important cellular processes, in particular anabolic pathways [32]. The best characterised role of mTORC1 is in growth signalling and the regulation of protein synthesis. Growth factor binding to tyrosine kinase receptors results in activation of mTORC1 typically through the PI3-K/Akt signalling pathway [32-34], although the MAP kinase or Wnt signalling pathways can also regulate mTORC1 [35, 36]. A key intermediary player is the small

GTPase Rheb (Ras homologue enhanced in brain), a direct activator of mTORC1, which is kept in an inactive state through the tuberous sclerosis complex TSC [37], containing the proteins TSC1 and 2. Growth factor signalling via PI3-K and Akt results in phosphorylation and inactivation of TSC2, and consequently in activation of mTORC1 [33, 36, 38]. Deregulation of mTORC1 is frequently observed in cancer cells reflecting the fact that many components of the signalling pathways upstream of mTORC1 are mutated in human cancers, as exemplified by the familial cell growth syndrome tuberous sclerosis [32].

Once activated, mTORC1 stimulates protein synthesis through at least two mechanisms, i.e. (1) activation of p70-S6 kinase, which phosphorylates ribosomal protein S6 (RpS6) and inhibits eukaryotic elongation factor 2 kinase [39], and (2) inactivation of the inhibitor of translation initiation factor eIF4E, eIF4E-binding protein 1 (4E-BP1). The consequences of RpS6 phosphorylation are yet to be fully elucidated, whereas the importance of 4E-BP1 inactivation for the control of cap-dependent translation, cell proliferation and tumour development is well established [32, 40]. Activation of eIF4E through the PI3-K/Akt/mTORC1 signalling pathway is believed to enhance selectively the translation of mRNAs encoding proteins involved in important biological processes that contribute to cancer development: protein synthesis and ribosome biogenesis, energy metabolism, cell cycle activation, prevention of apoptosis, as well as promotion of angiogenesis and metastasis [32]. Translation of a particular subset of mRNAs, called TOP-mRNAs (based on the presence of a 5'-tract of oligopyrimidines, 5'-TOP), is specifically up-regulated upon mTORC1 activation, although the exact mechanism for this still remains to be elucidated [41, 42]. The mRNAs for ribosomal proteins and translation factors [43-45] are the classical representatives of this group, but recently also mRNAs for other proteins where shown to bear this signature [45]. The mRNA coding for TCTP also contains a 5'-TOP [14, 30, 46]; in fact several potential TOP sequences were identified in the vicinity of its transcription start site [45]. Therefore, we hypothesised that its translation is specifically

regulated through mTORC1, similarly to the mRNAs for other anti-apoptotic proteins, such as Mcl-1 [47, 48]. Here we provide the following evidence in support of this hypothesis. We show that the serum-stimulated induction of TCTP in HeLa cells is partially inhibited by rapamycin and completely blocked by mTOR kinase inhibitors, indicating that mTOR is indeed involved in regulating TCTP synthesis. We performed Northern blot analysis of polysome profiles to demonstrate that this regulation occurs at the translational level, and we provide evidence that Akt and TSC2 (upstream regulators of mTORC1) and eIF4E (a downstream effector) are involved in regulating TCTP expression.

2. Materials and Methods

2.1. Cell culture and treatments

HeLa cells and HT29 colon cancer cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% foetal bovine serum (FBS), glutamine and 1% penicillin/streptomycin. For growth-induction experiments, cells were seeded in 6-well plates and grown to about 70% confluence; cells were then serum-starved for a minimum of 20h. Growth-induction was performed by addition of 20% FBS for the indicated periods of time. Where indicated, the following mTOR inhibitors were added 30 min prior to serum stimulation: 50 nM rapamycin (kindly provided by Selleck Chemicals, Houston, TX USA, to UAB), 100 nM AZD8055 (purchased from Selleck), 1 μM PP242 (Sigma-Aldrich) or 5 μM Akt inhibitor VIII (Calbiochem-Merck, Kilsyth, VIC Australia; the company's datasheet states that this inhibitor targets all three isoforms of Akt).

2.2. Cell lysis

For preparation of extracts, HeLa cells were washed three times with ice-cold PBS, lysed on the plates by scraping in about 120 μl lysis buffer (50 mM Tris/HCl, 50 mM 2-glycerophosphate, 1 mM EGTA, 1 mM EDTA and 1% (v/v) Triton X-100, freshly supplemented with 2-

mercaptoethanol and protease inhibitor cocktail). HT-29 cells were lysed in NP40 cell lysis buffer (Life Technologies, Burwood, VIC Australia) with freshly added 1 mM DTT and protease inhibitor cocktail. Lysates were centrifuged for 10 minutes at 15 000 rpm and 4ºC. Supernatants were stored at –80^{o}C and protein concentrations were determined using the Bradford assay.

2.3. Western blotting

Equal amounts of cell extract (typically 15 μg total protein) were separated by SDS-gel electrophoresis on 12.5% polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. Blots were blocked in 3% (w/v) defatted milk and incubated with the indicated primary antibody overnight at 4^{o}C. For visualisation, blots were either incubated with fluorescently-tagged secondary antibodies and scanned using a LI-COR Odyssey imaging system, or alternatively, incubated with HRP-linked secondary antibodies, developed with Lumi-Glo (Cell Signalling Technology; Genesearch, Arundel, QLD Australia) and exposed to Kodak X-Omat film. After scanning (GS-800 scanner; Bio-Rad, Gladesville, NSW Australia), signals were quantified using the Multi-Analyst software (Bio-Rad).

2.4. Antibodies

The mouse monoclonal anti-TCTP antibody (AB58362) was purchased from Abcam (UK) via Sapphire Bioscience, Waterloo, NSW Australia. Phospho-RpS6 (Ser 240/244; #2215), phospho-Akt (Ser 473; #9271), eIF-4E (#9742) and secondary HRP-linked antibodies were from Cell Signalling Technology. Anti-4EBP1 (sc6024) and anti-β-tubulin were obtained from Santa Cruz Biotechnology (Heidelberg, Germany), while anti-actin was from Sigma and anti-α-tubulin from Amersham Biosciences (UK).

2.5. Polysome profiles and Northern blot analyses

To generate polysome profiles, cytoplasmic cell extracts were layered onto 20–50% (w/v) sucrose gradients and centrifuged for 150 min at 37,000 rpm (240 000 x *g*) at 4^{o}C in a Beckman

SW41 rotor. Gradients were pumped out by upward displacement and fractionated into nine fractions. The absorbance at 254 nm was continuously monitored. RNA was isolated from each gradient fraction and processed for Northern blot analysis as previously described [49]. Radioactive probes were prepared by the random primer technique using DNA fragments isolated from plasmids containing PCR-amplified cDNA sequences. Northern blots were visualised and quantified using a Typhoon phosphorimager (GE Healthcare, Hatfield, Herts, UK).

2.6. Quantification of TCTP mRNA by qPCR

HeLa cells were grown in 12-well plates to about 70% confluence. Cells were then serum-starved for 21 h. A subgroup of samples was re-stimulated for 6 h by addition of 20% FBS. Where indicated, the mTOR inhibitors rapamycin (100 nM), PP242 (1 μM) or AZD8055 (100 nM) were present for the same time period, also in the case of serum-starved cells. All treatments were performed in triplicate. RNA was extracted with Tri-reagent (TR 118, Medical Research Centre) according to the manufacturer's instructions. Genomic DNA was removed using the Ambion Turbo DNA-free kit, followed by reverse transcription using an M-MLV reverse transcriptase for 2 h at 37°C (M1705, Promega). Quantitative real time PCR was carried out in technical triplicates on a LightCycler 480 (Roche) using the SYBR select master mix (4472918, Life Technologies) according to manufacturer's instructions. Reactions for each target were optimized for an efficiency of >1.8 and specificity was confirmed via melt curve analysis. The following primers (KiCqStart SYBR green, Sigma) were used: TCTP (TPT1 gene) (sense, 5'-TACTCTTTCTGGTCTCTGTTC-3'; antisense, 5'-CAAGTTTCACAAAAGAAGCC-3'), and β2-tubulin (B2T) (sense, 5'-AAGGACTGGTCTTTCTATCTC-3'; antisense, 5'-GATCCCACTTAACTATCTTGG-3') at 400 nM in 20 μl reactions with 80 ng cDNA. Treatment effects on mRNA expression levels were assessed using one-way ANOVA for each target. Statistical significance was accepted at $P<0.05$ and data are presented as the mean ± standard error of the mean.

2.7. Overexpression studies

To overexpress initiation factor eIF4E, HeLa cells were transiently transfected with a plasmid for the expression of HA-tagged human eIF4E [50] using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The medium was changed after 5h and the cells were then maintained in medium containing 10% foetal calf serum for 48h.

HEK293 cells tetracycline-inducible for the expression of eIF4E or for the expression of a non-phosphorylatable (constitutively-active) mutant of 4E-BP1 were obtained from the lab of Dr Simon Cook (Babraham Institute, UK) [51]. Cells were induced for the expression of the respective protein by incubation with tetracycline (1 μg/ml) for 48 h prior to performing the experiment.

3. Results

3.1. Growth factor-dependent induction of TCTP synthesis in cancer cells is sensitive to treatment with rapamycin, and particularly to mTOR kinase inhibitors

TCTP is overexpressed in several human tumours, and our own preliminary data on immunohistostaining of human colon cancer samples indicate an overexpression of TCTP in adenomas and adenocarcinomas of the colon, compared to normal colon tissue (P Puri, M Radojkovic, P Colligan, A Lochhead & U Bommer, unpublished results). Since the PI3-K/Akt/mTORC1 signalling pathway is often activated in colon cancer [52, 53], it was of interest to study whether this pathway is also involved in regulating TCTP levels in colon cancer cells. The presence of a 5'-TOP in the mRNA of TCTP does indeed suggest a possible role of the PI3-K/Akt/mTORC1 pathway in the translational induction of TCTP synthesis.

Serum-stimulation of mouse fibroblasts was originally used to demonstrate the growth factor-dependent regulation of TCTP synthesis [27]. We performed serum-starvation and refeeding experiments on HT29 colon cancer cells to study the regulation of TCTP synthesis, in presence or absence of rapamycin, the classical inhibitor of mTORC1 (Fig. 1A). Serum stimulation resulted in an about two-fold increase in TCTP levels in HT29 cells, which was completely inhibited by rapamycin, indicating that mTORC1 indeed regulates TCTP expression in colon cancer cells. As an indication for mTORC1 activity, we also analysed the phosphorylation of RpS6 on serine residues 240/244, specific targets for p70-S6 kinase, which is activated by mTORC1. The observation that RpS6 phosphorylation is fully inhibited by rapamycin indicates that mTORC1 is indeed inactivated by this treatment in HT29 colon cancer cells.

Next, we chose HeLa cells to see whether the rapamycin-sensitive induction of TCTP synthesis is also observed in other cancer cell types. We found that the response to serum stimulation in terms of relative increase of cellular TCTP levels is about four-fold in these cells (Fig. 1B), which resembles the level of induction previously observed in murine cell lines [29, 54]**.** We also observed that in HeLa cells, rapamycin only partially blocked the serum-induced increase of TCTP levels. Rapamycin inhibits mTORC1 by binding together with the FKBP12 protein to a site adjacent to the kinase domain of mTOR [55], but does not inhibit all functions of mTORC1 [56, 57]. Therefore, we made use of two of the more recently-developed mTOR kinase inhibitors (mTOR-KIs), which directly block the kinase activity of mTOR, i.e. PP242 [57] and AZD8055 [58]. In contrast to rapamycin, these mTOR kinase inhibitors do completely block serum-induced TCTP accumulation in HeLa cells (Fig. 1B), which further confirms that mTOR is likely to be involved in the regulation of TCTP synthesis under these conditions. On the other hand, the incomplete effect of rapamycin in blocking the increase in TCTP levels does not reflect a partial effect of this drug, since RpS6 phosphorylation was completely inhibited by rapamycin (results

not shown). Rather, additional, rapamycin-insensitive but mTOR-dependent signalling events appear to be involved.

The accumulation of the TCTP protein after serum stimulation of cells could principally be due to protein stabilisation and/or an increased rate of synthesis. In a recent study, we applied the method of pulsed stable isotope labelling with amino acids in cell culture (pSILAC) to assess the relative rate of synthesis of individual proteins in HeLa cells over a period of 6 h, in the presence or absence of rapamycin or the mTOR-KIs PP242 or AZD8055 [59]. This method measures the *de novo* rate of accumulation of specific proteins, which are identified and quantitated by mass spectrometry. In this way, we generated a large dataset that allowed us to distinguish which proteins are specifically regulated through mTORC1, i.e., whose rates of synthesis are sensitive to inhibition by rapamycin or mTOR-KIs, from those ones that are not. Inspection of this dataset revealed that TCTP mRNA behaves like known TOP mRNAs, in that the rate of synthesis of TCTP is inhibited by rapamycin (about 50%) and more strongly (about 80%) by the mTOR-KIs PP242 or AZD8055 (Fig. 1C). In contrast, the degree of inhibition by mTOR-KIs for proteins encoded by non-TOP mRNAs, such as hnRNP-A3 (right panel), is typically much weaker (in the range of 40%), and the inhibition by rapamycin of the synthesis of this and other proteins encoded by non-TOP mRNAs is on average only about 14% [59].

3.2. Serum induction of TCTP synthesis occurs at the level of translation and is completely inhibited by mTOR kinase inhibitors

The pSILAC data demonstrate that the serum-induced increase in TCTP levels is likely due to an increase in its rate of synthesis and the results with the mTOR kinase inhibitors indicate that this is driven by mTORC1 signalling. To assess whether the observed regulation of TCTP synthesis is associated with alterations in TCTP mRNA levels, we subjected HeLa cells to the same conditions as employed for Western blot analysis (Fig. 1B), i.e. serum-starvation and 6 h

refeeding with 20% FBS, both in the presence or absence of mTOR kinase inhibitors, and performed TCTP mRNA quantification by Real-Time-qPCR (Fig. 2A). The results clearly show that TCTP mRNA levels are not affected by serum starvation/refeeding or by mTOR kinase inhibitors.

To demonstrate that the increase in TCTP synthesis is actually regulated at the level of translation of its mRNA, we monitored the polysomal distribution of TCTP mRNA in the presence and absence of mTOR inhibitors. In such experiments, translationally active polysome-associated mRNAs are resolved from non-polysomal mRNAs. This is detected by Northern blotting of the corresponding fractions from the sucrose density gradient (Fig. 2B). The top panels show the absorbance profiles of the gradients, i.e. the ribosome (rRNA) distribution, of the HeLa cell extracts after the indicated treatments. The Northern blots were probed for TCTP mRNA, the mRNA for RpS7, a known TOP mRNA [43], and also for the mRNA of the RNA binding protein hnRNP-A3, the translation of which is not regulated by mTORC1 (see Fig. 1C). Blots were quantified and the percent mRNA per fraction was plotted in the graphs in the bottom panel. The total percentage of mRNA present in the polysomal fractions (fractions 5-8), i.e. in the translationally active state, were calculated and plotted in the graph shown in Fig. 2C. The Northern blot data for the reference mRNAs, RpS7, a classical TOP mRNA, and hnRNPA3, a non-TOP mRNA, are shown only from one experiment here, since we have published similar data before [59, 60]. Our results demonstrate that, under serum-starved conditions, only a small proportion (25-30%) of TCTP mRNA is in the polysomal fraction, whereas after serum stimulation for 6h about 50% of TCTP mRNA had shifted into the polysome region of the gradient, clearly indicating that serum stimulation results in activation of the translation of a substantial proportion of this mRNA (Fig. 2C). This shift of the TCTP mRNA into polysomes is strongly inhibited by rapamycin and is completely prevented by the mTOR-KIs AZD8055 or PP242. This behaviour is very similar to that of the mRNA for RpS7, an established TOP mRNA,

although the shifts into and out of polysomes are more pronounced in the case of the ribosomal protein mRNA (from 30 to 70%; Fig. 2C). In contrast, the mRNA for hnRNP-A3 displayed a completely different behaviour; at least 60% of this mRNA remained in polysomes under all conditions tested; and none of the mTOR inhibitors substantially affected its polysomal association. These results clearly demonstrate that the TCTP mRNA behaves as a typical TOP mRNA, in that it is regulated at the level of recruitment into polysomes, i.e. at translation initiation, by mTORC1 signalling (Fig. 2B,C). Thus, mTOR signalling positively regulates the translation of the TCTP mRNA, but not its level of expression (Fig. 2A).

3.3. TCTP synthesis is regulated by the Akt pathway upstream of mTORC1

We then asked whether elements of the signalling pathway upstream of mTORC1 participate in the growth-dependent regulation of TCTP synthesis. First, we used Akt inhibitor VIII (Calbiochem), which inhibits all three Akt isoforms, to see whether it prevents serum-induced increase of TCTP levels in HeLa cells. Probing immunoblots with antibodies specific for phospho-Akt (Ser473) showed that Akt phosphorylation is indeed blocked under these conditions (Fig. 3A, right graph), in some blots even completely. This compound blocked the serum-induced accumulation of TCTP in HeLa cells, similarly to mTOR kinase inhibitors (Fig. 3A, left graph). Since we were particularly interested to probe whether the PI3-K/Akt pathway is involved in regulating TCTP levels in colon cancer cells, we also tested the effect of the Akt inhibitor on TCTP levels in HT29 cells. As shown in Fig. 3B, it fully blocked the ability of serum to increase TCTP protein levels.

Active Akt phosphorylates and inactivates the TSC complex, an important inhibitor of mTORC1, it therefore promotes mTORC1 signalling.To study the influence of the TSC complex on TCTP protein expression, we made use of mouse embryonic fibroblasts (MEFs) from TSC2-knockout and corresponding wild-type animals (Fig. 4). The results clearly demonstrate that the

expression levels of TCTP are generally two-fold higher in the TSC2-KO cells compared to wild-type cells. We also observed that TCTP levels are down-regulated during serum-starvation (the 0 h time-point) in wildtype cells, whereas this effect is not seen TSC2-KO cells (Fig. 4, left graph and top western blot). Monitoring the levels of RpS6 phosphorylation (Fig. 4, right graph and bottom western blot) confirmed that, as expected, mTORC1 signalling is constitutively activated in TSC2-KO cells, including in serum-starved cells (0 h), whereas it is very low in serum-starved wildtype cells. Taken together, the data in Figs. 3 and 4 imply that both the tuberous sclerosis complex and Akt participate in the serum-dependent regulation of TCTP levels.

3.4. The downstream targets of mTORC1, 4E-BP1, and its binding partner, initiation factor eIF4E, are involved in regulating TCTP mRNA translation.

The best-characterised output of mTORC1 activity that impinges on mRNA translation initiation is the phosphorylation and concomitant inactivation of 4E-BP1, resulting in the increased availability of eIF4E to engage in translation initiation [42, 61]. To initially assess whether eIF4E plays a role in regulating TCTP mRNA translation, we transiently transfected HeLa cells with a vector encoding HA-tagged human eIF4E and analysed the polysomal distribution of TCTP mRNA in cells with and without overexpressed eIF4E. In this system, eIF4E overexpression caused a substantial increase in the polysomal association of TCTP mRNA (Fig. 5; left panels). This effect was reversed by treatment with rapamycin, indicating that eIF4E is unable to override the inhibitory effect of this mTORC1 inhibitor. We also probed the blots for the mRNA for RpS7, another TOP-mRNA, and found that it behaved very similarly to the TCTP mRNA (Fig. 5; right panels). Similar results were also obtained for the mRNA for another ribosomal protein (TOP) mRNA, RpL11 [59].

To assess the importance of eIF4E and 4E-BP1 for the control of TCTP mRNA translation, we also made use of HEK293 cells engineered to be tetracycline (tet)-inducible for the expression

of eIF4E or a constitutively active mutant of 4E-BP1, respectively [51]. Fig. 6A demonstrates that overexpression of eIF4E in HEK293 cells resulted in an about 1.3-fold increase in the polysomal association of TCTP mRNA, which is prevented in the presence of rapamycin. Compared to the situation observed in HeLa cells (Fig. 5), the relative shift of the TCTP mRNA into polysomes and its inhibition by rapamycin was less pronounced in these cells. The mRNA for RpS6, another TOP message, displayed a similar behaviour, although the polysomal shift caused by eIF4E overexpression and the effect of rapamycin was more pronounced for this mRNA, compared to TCTP mRNA. In contrast, the (non-TOP) actin mRNA was already heavily associated with polysomes and its polysomal association was only affected to a small extent by overexpressing eIF4E or by rapamycin (Fig. 6A). Expression of a constitutively active (phosphorylation-defective) mutant of 4E-BP1 in HEK293 cells resulted in a similar (20-30%) inhibition of polysomal association of all three mRNAs (Fig.6A). This indicates that 4E-BP1 does play a role in regulating the translation of the TCTP mRNA, but that this reflects a rather general effect, not one specific for TOP mRNAs. Further data for Rp and actin mRNAs obtained with this tet-inducible HEK293 cell system have been submitted elsewhere for publication.

We also tested whether the activation of translation of the TCTP mRNA by increased eIF4E levels is reflected in TCTP protein levels. Expression of eIF4E was induced in HEK293 cells by treatment with tetracycline for 48 h. Cells were then subjected to serum-starvation and subsequent re-feeding. Under both conditions, TCTP protein levels were higher in eIF4E-overexpressing cells than in control cells (Fig. 6B), confirming that eIF4E does promote TCTP expression, relative to that of actin, even under serum-starved conditions. We also tested the effect of the interfering mutant of 4E-BP1 on TCTP expression levels by Western blotting and found that it substantially reduced TCTP levels in HEK293 cells (Fig. 6C). Taken together, these studies demonstrate that 4E-BP1 levels and eIF4E availability can both contribute markedly to regulating TCTP expression.

4. Discussion

Initial evidence for the translational regulation of TCTP synthesis came from the observation that the TCTP (P21) mRNA is stored in cytoplasmic untranslated mRNP particles [28], and from the rapid induction by serum in the rate of TCTP ('Q23' or 'P23') synthesis, which is not impaired by inhibitors of transcription, such as actinomycin D [29]. However the mechanism(s) regulating the translation of its mRNA had not previously been fully characterised. Demonstration of the importance of initiation factor eIF4E for the translational regulation of growth-regulatory mRNAs [62] prompted us to study the involvement of this factor in the control of TCTP (P23) synthesis, which provided evidence in support of this [31]. At that time, the prevailing view was that eIF4E activity is particularly required for highly structured mRNAs that are otherwise poorly translated [63], and the link between the mTORC1 pathway and control of eIF4E availability was only established later.

A characteristic feature of the TCTP mRNA is the presence of a 5'-TOP [14, 30, 45, 46], which is found in many mRNAs whose translation is controlled through mTORC1 signalling. This prompted us to undertake the present study, which was aimed at exploring the involvement of the Akt/mTORC1 signalling pathway in the translational regulation of TCTP synthesis in cancer cells. In both HT29 colon cancer cells (Fig.1A) and HeLa cells (Fig. 1B), the allosteric mTORC1 inhibitor rapamycin substantially inhibited serum-dependent TCTP accumulation, but in HeLa cells it did not do so completely. In contrast, two recently-developed mTOR kinase inhibitors, PP242 and AZD8055, each completely inhibited the induction of TCTP in HeLa cells (Fig. 1B). These results provide clear evidence for the involvement of mTOR signalling in the serum-dependent induction of TCTP synthesis. Partial inhibition of synthesis by rapamycin and strong inhibition by mTOR-KIs is observed for many proteins, including proteins encoded by TOP mRNAs [59, 64, 65]. Our own study employing the pSILAC method, which measures *de novo* rates of protein accumulation, generated a large dataset of mTORC1-regulated proteins [59].

The data obtained for TCTP by this method support the conclusion that *de novo* synthesis of TCTP is positively regulated by mTORC1 activity (Fig. 1C). Two other studies used ribosome profiling in combination with deep-sequencing to generate transcriptome-wide datasets of mTORC1-regulated mRNAs [64, 65]. Compilation of the results for the TCTP mRNA/protein from all three papers (Table 1) shows that for TCTP, both methods yield very similar results, indicating that all mTOR kinase inhibitors tested (Torin 1, PP242 and AZD8055) inhibit TCTP mRNA translation by at least 80% (more strongly than many other TOP mRNAs), whereas rapamycin resulted in only slightly more than 50% inhibition (similar to many other TOP mRNAs). Similar results were obtained in all three mammalian cell types investigated (Table 1). These data also correspond well with our findings for HeLa cells, demonstrating (1) that the serum-induced increase in TCTP levels is inhibited by around 50% by rapamycin and completely prevented by mTOR-KIs (Fig.1B) and (2) that this effect involves translational up-regulation (recruitment of the mRNA into polysomes) which is prevented by mTOR-KIs (Fig. 2B).

We also asked whether components upstream of mTORC1 are involved in growth-dependent up-regulation of TCTP synthesis. An inhibitor of Akt, a kinase which indirectly activates mTORC1 by phosphorylating TSC2, completely prevented induction of TCTP in both HeLa (Fig. 3A) and HT29 colon cancer cells (Fig. 3B). The latter finding also supports our hypothesis that the increased expression levels of TCTP observed in adenocarcinomas of the colon (our unpublished immunohistochemistry results) are likely to be caused by increased signalling through the PI3-K/Akt pathway, an effect which is frequently observed in colon cancer [52, 53]. Similar to the observations of [66], we find that MEFs from TSC2-knockout mice have lost their ability to repress both mTORC1 activity (assessed by monitoring RpS6 phosphorylation) and TCTP synthesis in response to serum starvation (Fig. 4). Overall, the results obtained with the Akt inhibitor and with the MEFs from TSC2-KO mice clearly support the view that TCTP synthesis is regulated through these upstream regulators of mTORC1.

We also studied the effect of downstream effectors of mTORC1 on TCTP mRNA translation. Since activation of mTORC1 increases the availability of eIF4E through inactivation of 4E-BP1 [42, 61], we asked whether increased levels of eIF4E contribute to activation of TCTP mRNA translation and whether it is impaired by increased activity of 4E-BP1. Our results demonstrate that increased eIF4E levels are able to drive the TCTP mRNA into heavy polysomes in both HeLa cells (Fig. 5) and HEK293 cells (Fig.6A), although the extent to which this occurs differs between different the two cell lines. Generally, we observe in our polysome profiling experiments (Figs. 2, 5 and 6) that the responses of the TCTP mRNA to alterations of cellular conditions (in terms of polysome shift) are less than that of the ribosomal protein mRNAs, the classical TOP mRNAs. However, the general pattern of behaviour (response to serum stimulation or sensitivity to mTOR inhibition) is very similar between TCTP mRNA and Rp mRNAs, which underscores the classification of TCTP mRNA as a TOP-mRNA.

The polysome profiling results obtained from HeLa cells (Fig. 5) show that, in this cell line, eIF4E overexpression cannot overcome the inhibitory effects of rapamycin, indicating that additional mechanisms are involved in the mTORC1-dependent regulation of TCTP mRNA translation. Our results obtained by Western blotting (Fig. 6B) confirm that eIF4E levels do indeed have a considerable effect on TCTP protein accumulation in HEK293 cells. They also show that eIF4E can partially overcome the inhibitory effect of serum starvation on TCTP protein levels, complementing our earlier findings obtained in murine fibroblasts overexpressing eIF4E [31].

Since 4E-BP1, rather than eIF4E itself, is the direct target of mTORC1, we also asked whether 4E-BP1 affects TCTP mRNA translation. Our results show that expression of a constitutively-active mutant of 4E-BP1 in HEK293 cells resulted in the partial release of the TCTP mRNA from heavy polysomes (Fig. 6A), and also in a significant reduction of cellular TCTP levels (Fig. 6C). These findings clearly establish the involvement of 4E-BP1 in the control of TCTP mRNA translation. The response of another TOP mRNA (for RpS6) to increased 4E-BP1 activity is

similar to that of TCTP mRNA. We also observed that the polysomal association of the (non-TOP) actin mRNA, was significantly decreased by mutant 4E-BP1 (Fig. 6A). This indicates that the effect of 4E-BP1 is a rather general one and is not restricted to TOP-mRNAs. This point requires future investigation, particularly in the light of recent data indicating that the eIF4E/4E-BP system is unlikely to be involved in the oxygen-dependent regulation of TOP mRNA translation via mTORC1 [67].

Taken together, the present data strongly support the conclusion that the TCTP mRNA belongs to the set of 5'-TOP mRNAs, the translation of which is specifically regulated through the mTORC1 pathway. This group of translationally controlled proteins includes Rps and other components of the translational apparatus of the cell [43-45]. More recently, many additional mRNAs containing a 5'-TOP, have been found to be regulated by mTORC1 [45, 59, 64, 65].

TCTP has been characterised as an anti-apoptotic protein [1, 2, 68] and this fits with its identification as a member of the set of mTORC1-regulated proteins. Other established examples of such translationally-regulated anti-apoptotic proteins include Bcl-2 and Bcl-XL [69], Mcl-1 [47, 48, 69] and survivin [32]. Interestingly, Bcl-XL and Mcl-1 have also been shown to be interaction partners for TCTP [68]. These proteins can be considered as survival factors that may be required by the cell in rapid responses to environmental stimuli, which is why fast-acting translational regulation of their synthesis is advantageous. We also found previously that *down*-regulation of TCTP may occur at the translational level, through activation of protein kinase PKR [54, 70], a mechanism similarly reported for Mcl-1 [71].

There is overwhelming evidence that the PI3-kinase/Akt/mTORC1 pathway is closely linked to tumorigenesis [32, 36, 48, 52, 53]. In particular, the 4E-BP1/eIF4E axis downstream of mTORC1 has been implicated in promoting cancer, through the translational activation of mRNAs encoding proteins involved in cell proliferation [40] and in tumour initiation and metastasis [64]. Importantly, two reports stress the importance of mTOR-dependent

translational activation of the anti-apoptotic protein Mcl-1 for cell survival [47] and for cancer initiation and progression [48]. As a further anti-apoptotic protein involved in cancer progression [1-3, 68, 72], TCTP is thus clearly part of the protein network involved in mTOR-dependent cancer promotion, as was recently demonstrated for neurofibromatosis type 1-associated tumours [73]. Antagonism to the tumour suppressor p53 has been proposed as an important mechanism of TCTP's cancer promoting activity [16, 20, 21]. Since p53 interferes with the PI3-K/Akt/mTORC1 pathway by inducing its negative regulators PTEN and TSC2 [74], our present findings imply that p53 also antagonises TCTP expression at the translational level, hence adding another layer of regulation to the complex interplay between p53 and TCTP.

5. Conclusion

Serum stimulation of HeLa and HT29 colon cancer cells results in an up to five-fold increase in cellular TCTP levels. This effect is partially inhibited by rapamycin and completely blocked by mTOR kinase inhibitors. Northern blot analyses of polysome profiles show that serum stimulation results in mTORC1-dependent activation of TCTP mRNA translation. Upstream effectors of mTORC1, Akt and TSC-2, are also involved in the regulation of TCTP synthesis, as are its downstream effectors eIF4E and 4EBP1. These results demonstrate that TCTP levels are regulated through the PI3-kinase/Akt/mTORC1 signalling pathway, which is frequently hyperactive in tumours.

6. Acknowledgements

This work was supported by a Small Grant from the Illawarra Health and Medical Research Institute, Wollongong, (to UAB and JC) and by a Study Leave Assistant Grant (to UAB) from the University of Wollongong. CGP gratefully acknowledges support from Cancer Research UK. We wish to thank members of the Proud lab and our colleagues from the Illawarra Health and Medical Research Institute for help and support throughout the project, and we appreciate the provision of rapamycin by Selleck Chemicals.

7. References

1. Acunzo, J., et al., *TCTP as therapeutic target in cancers.* Cancer Treat Rev, 2014. **40**(6): p. 760-9.
2. Bommer, U.A., *Cellular Function and Regulation of the Translationally Controlled Tumour Protein TCTP.* The Open Allergy Journal, 2012. **5**: p. 19-32.
3. Chan, T.H., L. Chen, and X.Y. Guan, *Role of translationally controlled tumor protein in cancer progression.* Biochemistry research international, 2012. **2012**: p. 369384.
4. Macdonald, S.M., *Potential role of histamine releasing factor (HRF) as a therapeutic target for treating asthma and allergy.* Journal of asthma and allergy, 2012. **5**: p. 51-9.
5. Chen, S.H., et al., *A knockout mouse approach reveals that TCTP functions as an essential factor for cell proliferation and survival in a tissue- or cell type-specific manner.* Molecular Biology of the Cell, 2007. **18**(7): p. 2525-32.
6. Susini, L., et al., *TCTP protects from apoptotic cell death by antagonizing bax function.* Cell Death Differ, 2008. **15**(8): p. 1211-20.
7. Koide, Y., et al., *Embryonic lethality of fortilin-null mutant mice by BMP-pathway overactivation.* Biochim Biophys Acta, 2009. **1790**(5): p. 326-38.
8. Gachet, Y., et al., *The growth-related, translationally controlled protein P23 has properties of a tubulin binding protein and associates transiently with microtubules during the cell cycle.* J Cell Sci, 1999. **112 (Pt 8)**: p. 1257-71.
9. Bazile, F., et al., *Complex relationship between TCTP, microtubules and actin microfilaments regulates cell shape in normal and cancer cells.* Carcinogenesis, 2009. **30**(4): p. 555-65.
10. Amson, R., et al., *TPT1/ TCTP-regulated pathways in phenotypic reprogramming.* Trends in cell biology, 2013. **23**(1): p. 37-46.

11. Brioudes, F., et al., *Translationally controlled tumor protein is a conserved mitotic growth integrator in animals and plants.* Proc Natl Acad Sci U S A, 2010. **107**(37): p. 16384-9.
12. Zhang, J., et al., *Role of the translationally controlled tumor protein in DNA damage sensing and repair.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(16): p. E926-33.
13. Cans, C., et al., *Translationally controlled tumor protein acts as a guanine nucleotide dissociation inhibitor on the translation elongation factor eEF1A.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 13892-7.
14. Berkowitz, O., et al., *Characterization of TCTP, the translationally controlled tumor protein, from Arabidopsis thaliana.* Plant Cell, 2008. **20**(12): p. 3430-47.
15. Amson, R., J.E. Karp, and A. Telerman, *Lessons from tumor reversion for cancer treatment.* Current Opinion in Oncology, 2013. **25**(1): p. 59-65.
16. Amson, R., et al., *Reciprocal repression between P53 and TCTP.* Nat Med, 2012. **18**(1): p. 91-9.
17. Chan, T.H., et al., *Translationally controlled tumor protein induces mitotic defects and chromosome missegregation in hepatocellular carcinoma development.* Hepatology, 2012. **55**(2): p. 491-505.
18. Miao, X., et al., *TCTP overexpression is associated with the development and progression of glioma.* Tumour Biology, 2013. **34**(6): p. 3357-3361.
19. Telerman, A. and R. Amson, *The molecular programme of tumour reversion: the steps beyond malignant transformation.* Nat Rev Cancer, 2009. **9**(3): p. 206-16.
20. Rho, S.B., et al., *Anti-apoptotic protein TCTP controls the stability of the tumor suppressor p53.* FEBS Lett, 2011. **585**(1): p. 29-35.
21. Chen, Y., et al., *Physical and functional antagonism between tumor suppressor protein p53 and fortilin, an anti-apoptotic protein.* J Biol Chem, 2011. **286**: p. 32575-32585.

22. Jung, J., et al., *Interaction of translationally controlled tumor protein with Apaf-1 is involved in the development of chemoresistance in HeLa cells.* BMC Cancer, 2014. **14**: p. 165.

23. Kim, M., J. Jung, and K. Lee, *Roles of ERK, PI3 kinase, and PLC-gamma pathways induced by overexpression of translationally controlled tumor protein in HeLa cells.* Arch Biochem Biophys, 2009. **485**(1): p. 82-7.

24. Hsu, Y.-C., et al., *Drosophila TCTP is essential for growth and proliferation through regulation of dRheb GTPase.* Nature, 2007. **445**(7129): p. 785-8.

25. Rehmann, H., et al., *Biochemical characterisation of TCTP questions its function as a guanine nucleotide exchange factor for Rheb.* FEBS Lett, 2008. **582**(20): p. 3005-10.

26. Wang, X., et al., *Re-evaluating the roles of proposed modulators of mammalian target of rapamycin complex 1 (mTORC1) signaling.* J Biol Chem, 2008. **283**(45): p. 30482-92.

27. Thomas, G., G. Thomas, and H. Luther, *Transcriptional and translational control of cytoplasmic proteins after serum stimulation of quiescent Swiss 3T3 cells.* Proc Natl Acad Sci U S A, 1981. **78**(9): p. 5712-6.

28. Yenofsky, R., I. Bergmann, and G. Brawerman, *Messenger RNA species partially in a repressed state in mouse sarcoma ascites cells.* Proc Natl Acad Sci U S A, 1982. **79**(19): p. 5876-80.

29. Bohm, H., et al., *The growth-related protein P23 of the Ehrlich ascites tumor: translational control, cloning and primary structure.* Biochem Int, 1989. **19**(2): p. 277-86.

30. Bommer, U.A. and B.J. Thiele, *The translationally controlled tumour protein (TCTP).* Int J Biochem Cell Biol, 2004. **36**(3): p. 379-85.

31. Bommer, U.A., et al., *Translational regulation of the mammalian growth-related protein P23: involvement of eIF-4E.* Cell Mol Biol Res, 1994. **40**(7-8): p. 633-41.

32. Laplante, M. and D.M. Sabatini, *mTOR signaling in growth control and disease.* Cell, 2012. **149**(2): p. 274-93.

33. Kim, S.G., G.R. Buel, and J. Blenis, *Nutrient regulation of the mTOR Complex 1 signaling pathway.* Molecules and cells, 2013. **35**(6): p. 463-73.

34. Zheng, X., et al., *Current models of mammalian target of rapamycin complex 1 (mTORC1) activation by growth factors and amino acids.* Int J Mol Sci, 2014. **15**(11): p. 20753-69.

35. Mendoza, M.C., E.E. Er, and J. Blenis, *The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation.* Trends in biochemical sciences, 2011. **36**(6): p. 320-8.

36. Zoncu, R., A. Efeyan, and D.M. Sabatini, *mTOR: from growth signal integration to cancer, diabetes and ageing.* Nature reviews. Molecular cell biology, 2011. **12**(1): p. 21-35.

37. Zhang, Y., et al., *Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins.* Nature cell biology, 2003. **5**(6): p. 578-81.

38. Huang, J. and B.D. Manning, *A complex interplay between Akt, TSC2 and the two mTOR complexes.* Biochem Soc Trans, 2009. **37**(Pt 1): p. 217-22.

39. Wang, X., et al., *Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase.* EMBO J, 2001. **20**(16): p. 4370-9.

40. Dowling, R.J., et al., *mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs.* Science, 2010. **328**(5982): p. 1172-6.

41. Patursky-Polischuk, I., et al., *The TSC-mTOR pathway mediates translational activation of TOP mRNAs by insulin largely in a raptor- or rictor-independent manner.* Molecular and cellular biology, 2009. **29**(3): p. 640-9.

42. Proud, C.G., *mTORC1 signalling and mRNA translation.* Biochemical Society transactions, 2009. **37**(Pt 1): p. 227-31.

43. Iadevaia, V., et al., *All translation elongation factors and the e, f, and h subunits of translation initiation factor 3 are encoded by 5'-terminal oligopyrimidine (TOP) mRNAs.* RNA, 2008. **14**(9): p. 1730-6.

44. Meyuhas, O., *Synthesis of the translational apparatus is regulated at the translational level.* European journal of biochemistry / FEBS, 2000. **267**(21): p. 6321-30.

45. Yamashita, R., et al., *Comprehensive detection of human terminal oligo-pyrimidine (TOP) genes and analysis of their characteristics.* Nucleic Acids Res, 2008. **36**(11): p. 3707-15.

46. Yubero, N., et al., *Molecular cloning, expression analysis and chromosome localization of the Tpt1 gene coding for the pig translationally controlled tumor protein (TCTP).* Mol Biol Rep, 2009. **36**(7): p. 1957-65.

47. Mills, J.R., et al., *mTORC1 promotes survival through translational control of Mcl-1.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(31): p. 10853-8.

48. Hsieh, A.C., et al., *Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E.* Cancer cell, 2010. **17**(3): p. 249-61.

49. Iadevaia, V., et al., *Evaluation of mTOR-regulated mRNA translation.* Methods Mol Biol, 2012. **821**: p. 171-85.

50. Waskiewicz, A.J., et al., *Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo.* Molecular and cellular biology, 1999. **19**(3): p. 1871-80.

51. Cope, C.L., et al., *Adaptation to mTOR kinase inhibitors by amplification of eIF4E to maintain cap-dependent translation.* J Cell Sci, 2014. **127**(Pt 4): p. 788-800.

52. Saif, M.W. and E. Chu, *Biology of colorectal cancer.* Cancer journal, 2010. **16**(3): p. 196-201.

53. Huang, X.F. and J.Z. Chen, *Obesity, the PI3K/Akt signal pathway and colon cancer.* Obesity reviews : an official journal of the International Association for the Study of Obesity, 2009. **10**(6): p. 610-6.

54. Bommer, U.A., et al., *The mRNA of the translationally controlled tumor protein P23/TCTP is a highly structured RNA, which activates the dsRNA-dependent protein kinase PKR.* RNA, 2002. **8**(4): p. 478-96.

55. Yang, H., et al., *mTOR kinase structure, mechanism and regulation.* Nature, 2013. **497**(7448): p. 217-23.

56. Thoreen, C.C., et al., *An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1.* The Journal of biological chemistry, 2009. **284**(12): p. 8023-32.

57. Feldman, M.E., et al., *Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2.* PLoS biology, 2009. **7**(2): p. e38.

58. Chresta, C.M., et al., *AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mammalian target of rapamycin kinase inhibitor with in vitro and in vivo antitumor activity.* Cancer Research, 2010. **70**(1): p. 288-98.

59. Huo, Y., et al., *Stable isotope-labelling analysis of the impact of inhibition of the mammalian target of rapamycin on protein synthesis.* The Biochemical journal, 2012. **444**(1): p. 141-51.

60. Caldarola, S., et al., *Translational regulation of terminal oligopyrimidine mRNAs induced by serum and amino acids involves distinct signaling events.* J Biol Chem, 2004. **279**(14): p. 13522-31.

61. Hay, N. and N. Sonenberg, *Upstream and downstream of mTOR.* Genes Dev, 2004. **18**(16): p. 1926-45.

62. Lazaris-Karatzas, A., K.S. Montine, and N. Sonenberg, *Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap.* Nature, 1990. **345**(6275): p. 544-7.

63. Koromilas, A.E., A. Lazaris-Karatzas, and N. Sonenberg, *mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E.* The EMBO journal, 1992. **11**(11): p. 4153-8.

64. Hsieh, A.C., et al., *The translational landscape of mTOR signalling steers cancer initiation and metastasis.* Nature, 2012. **485**(7396): p. 55-61.

65. Thoreen, C.C., et al., *A unifying model for mTORC1-mediated regulation of mRNA translation.* Nature, 2012. **485**(7396): p. 109-13.

66. Bilanges, B., et al., *Tuberous sclerosis complex proteins 1 and 2 control serum-dependent translation in a TOP-dependent and -independent manner.* Molecular and cellular biology, 2007. **27**(16): p. 5746-64.

67. Miloslavski, R., et al., *Oxygen sufficiency controls TOP mRNA translation via the TSC-Rheb-mTOR pathway in a 4E-BP-independent manner.* J Mol Cell Biol, 2014. **6**(3): p. 255-66.

68. Nagano-Ito, M. and S. Ichikawa, *Biological effects of Mammalian translationally controlled tumor protein (TCTP) on cell death, proliferation, and tumorigenesis.* Biochemistry research international, 2012. **2012**: p. 204960.

69. Robert, F. and J. Pelletier, *Translation initiation: a critical signalling node in cancer.* Expert opinion on therapeutic targets, 2009. **13**(11): p. 1279-93.

70. Bommer, U.A., et al., *Roles of the translationally controlled tumour protein (TCTP) and the double-stranded RNA-dependent protein kinase, PKR, in cellular stress responses.* Oncogene, 2010. **29**(5): p. 763-73.

71. Fritsch, R.M., et al., *Translational repression of MCL-1 couples stress-induced eIF2 alpha phosphorylation to mitochondrial apoptosis initiation.* The Journal of biological chemistry, 2007. **282**(31): p. 22551-62.

72. Baylot, V., et al., *Targeting TCTP as a new therapeutic strategy in castration-resistant prostate cancer.* Mol Ther, 2012. **20**(12): p. 2244-56.

73. Kobayashi, D., et al., *Translationally Controlled Tumor Protein is a Novel Biological Target for Neurofibromatosis Type 1 (NF1)-associated Tumors.* J Biol Chem, 2014.

74. Feng, Z., et al., *The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways.* Cancer research, 2007. **67**(7): p. 3043-53.

Figure legends

Fig. 1 The serum-induced increase in TCTP levels in cancer cells is inhibited by rapamycin and mTOR kinase inhibitors. (A) HT29 colon cancer cells were starved of serum overnight and re-stimulated with 20% FBS for the indicated periods of time. Where indicated, 50 nM rapamycin was added 30 min before serum. Relative levels of TCTP (and β-tubulin as a loading control) were assessed in cell extracts by Western blotting. As an indicator of mTORC1 activity, levels of phosphorylated RpS6 (Ser240/244) were assessed. (Open bars: without rapamycin, filled bars: with rapamycin). (B) HeLa cells were starved of serum overnight and re-stimulated with 20% FBS for the indicated periods of time. Where indicated, rapamycin or the mTOR kinase inhibitors PP242 (1 μM) or AZD8055 (100 nM) were added 30 min before stimulation with serum. Relative levels of TCTP and of β-tubulin, as a loading control, were assessed by Western blotting. Average values from three independent experiments were plotted (± SD), compared to the serum-starved control. (C) *De novo* synthesis of TCTP is blocked by mTOR kinase inhibitors. Levels of newly-synthesised proteins, over a period of 6h, were assessed in HeLa cells incubated in the absence or presence of the mTORC1 inhibitors rapamycin, PP242 or AZD8055, using the pSILAC (pulsed stable isotope labelling with amino acids in cell culture) method. The data are taken from the recently published dataset [59] and plotted relative to the untreated control. For comparison, the results for hnRNPA3, a protein whose synthesis is not regulated through mTORC1, are shown.

Fig. 2 The serum-stimulated activation of TCTP mRNA translation is blocked by mTOR kinase inhibitors. A) Cellular TCTP mRNA levels are not affected by serum starvation and by refeeding, or by mTOR inhibitors. Levels of the TCTP mRNA were assessed by qPCR in HeLa cells either serum-starved for 21 h, or subsequently re-stimulated with serum for 6h with 20% FBS, as indicated below the graph. Where indicated, rapamycin (100 nM) or the mTOR kinase inhibitors PP242 (1 μM) or AZD8055 (100 nM) were present either during the last 6 h of serum starvation

or throughout the period of serum stimulation. Ct-values for TCTP mRNA were normalised against β2-tubulin mRNA and plotted as percentage relative to the serum-starved control. The bars represent the average from three independent experiments (± SEM).

B) HeLa cells were serum-starved overnight (all parts) and then re-stimulated for 6 h with 20% FBS (except for the left-most panel). Where indicated, rapamycin or the mTOR KIs PP242 (1 μM) or AZD8055 (100 nM), were added 30 min before stimulation with serum. Cell lysates were fractionated on sucrose gradients and absorbance was monitored at 254 nm. The traces are shown at the same scale for each panel; the positions of 40S, 60S and 80S ribosomal particles are indicated in the left panel. Bars at the bottom of each panel/graph indicate the position of polysomes. Northern blots were prepared from all fractions and probed for mRNAs of TCTP, RpS7 and hnRNP-A3. Blots were quantified and the percent mRNA in each fraction is plotted in the graphs displayed below the Northerns. C) The percent of mRNA present in the polysome fractions was calculated for each blot and plotted in the graph. The data for TCTP mRNA represent the average from two independent experiments.

Fig. 3 Serum induction of TCTP levels is blocked by an inhibitor of Akt. (A) HeLa cells were starved of serum for 24 h and then re-stimulated with 20% FBS for the indicated periods of time. Where indicated, the Akt inhibitor VIII was added at 5 μM 30 min before stimulation. Relative levels of TCTP and of β-tubulin as a loading control were assessed by Western blotting. Phosphorylation of Akt (at Ser473) was also monitored. (B) HT29 colon cancer cells were treated in the same way as HeLa-cells. Relative levels for TCTP, and for β-tubulin, were assessed by Western blotting (open bars: no inhibitor; filled bars: Akt inhibitor). Graphs represent averages of three independent experiments (± SD).

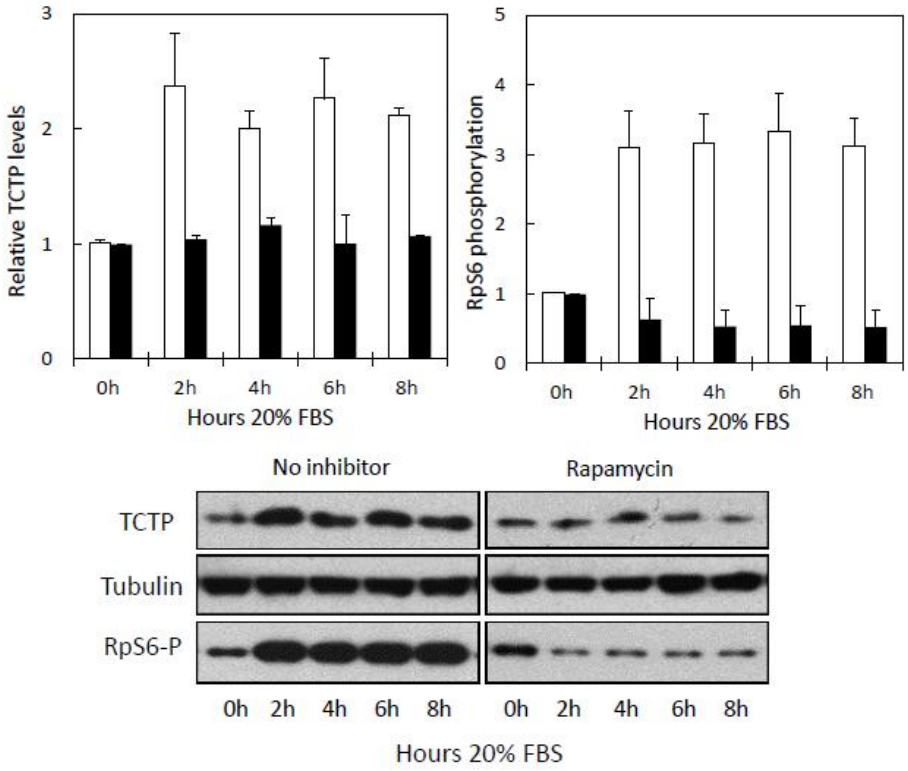
Fig. 4 Knockout of tuberous sclerosis complex 2 (TSC2) results in constitutive expression of TCTP, even during serum starvation. Mouse embryonic fibroblasts (MEFs) from TSC2-KO mice or from wild-type control cells were starved of serum for 22 h and then re-stimulated with 20%

FBS for the indicated periods of time. Relative levels of TCTP and β-tubulin were assessed by Western blotting. To monitor mTORC1 activity, levels of RpS6 phosphorylation (Ser240/244) were also determined. Graphs showing relative TCTP levels (left) and RpS6 phosphorylation (right) represent the average of two independent experiments, normalised to the tubulin loading control (open bars: wild-type MEFs; filled bars: TSC2-KO MEFs).

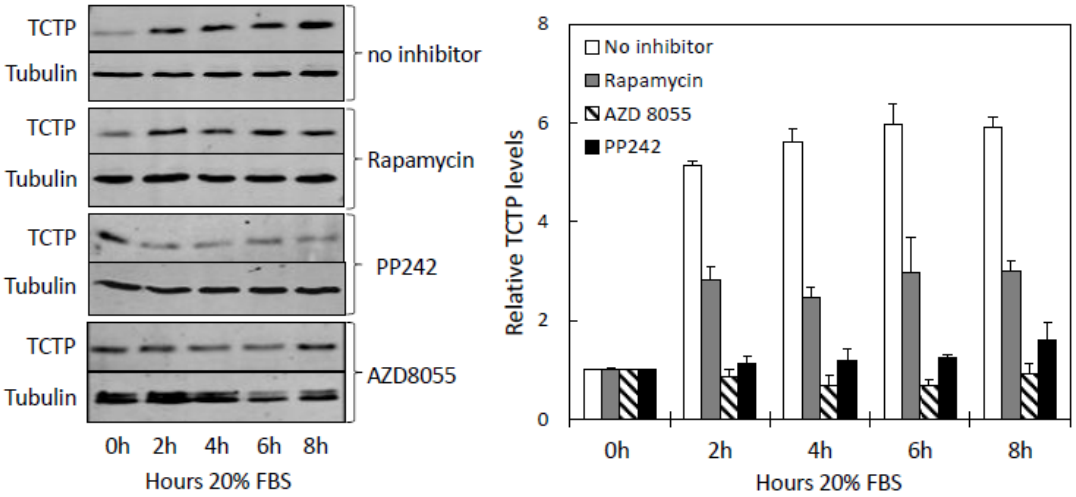
Fig. 5 Overexpression of eIF4E enhances TCTP mRNA translation in HeLa cells, but does not overcome the inhibitory effect of rapamycin. HeLa cells were transfected with an expression vector for HA-tagged eIF4E or left un-transfected, as indicated. Cells were either kept at normal growth conditions (top two panels) or treated for 6 h with rapamycin. Polysome profiles were generated from cytoplasmic cell extracts, and probed by Northern blotting for the distribution of the mRNAs of TCTP and of RpS7, as another TOP mRNA (blots at the top). Graphs show the quantified distribution of these mRNAs under all three conditions; the bottom graphs display the percentage of the given mRNA in polysomes. For the TCTP mRNA, the average of two independent experiments is shown.

Fig. 6 eIF4E and 4E-BP1 levels affect TCTP mRNA translation and protein levels in HEK293 cells. (A) HEK293 cells were either not induced, where indicated, or treated for 48h with tetracycline to induce overexpression of eIF4E or a non-phosphorylatable (constitutively-active) mutant of 4E-BP1. Where indicated, cells were subjected to an additional 6 h treatment with rapamycin. Polysome profiles were generated from cytoplasmic cell extracts, fractions collected, and probed by Northern blotting for the distribution of the following mRNAs: TCTP, RpS6 and actin (left and centre sections). Graphs on the right show the percentage of each mRNA in polysomes for all conditions and mRNAs. For TCTP mRNA the average of two independent experiments is shown. (B) Western blot showing the effect of eIF4E overexpression on TCTP protein levels in HEK293 cells. Cells were either left uninduced (control) or induced for 48 h with tetracycline (o.e. eIF4E). For both conditions, cells were either serum-starved overnight (- FBS)

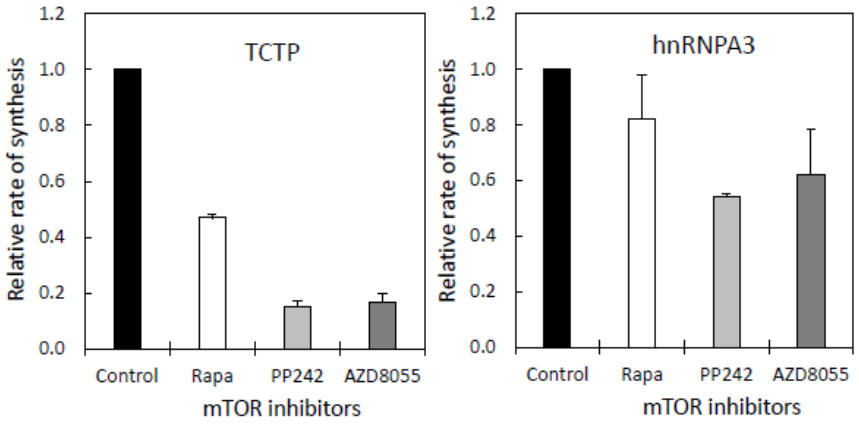
or serum-starved overnight and restimulated with 20% foetal bovine serum for 6h (+ FBS). Immunoblots were probed for TCTP, eIF4E and actin as loading control. The graph shows the relative TCTP levels normalised for actin, the ratio for control cells being set as 1. (C) Expression of a constitutively-active mutant of 4E-BP1 reduces TCTP protein levels in HEK293 cells. Cells were either not induced (-) or induced by tetracycline for 48 h (+) to induce the expression of the constitutively-active mutant of 4E-BP1 as indicated. Western blots were probed for TCTP, 4E-BP1 or actin as loading control. The graph shows relative TCTP levels normalised to actin (control = 1). The experiment was performed in duplicate.



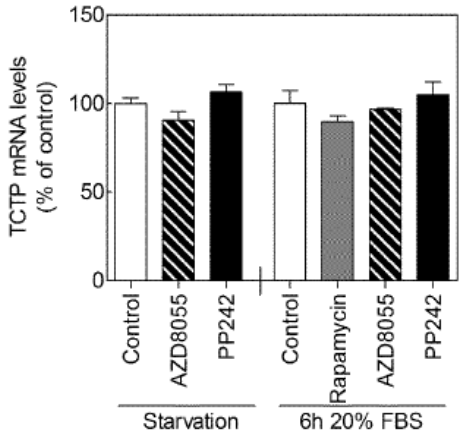
Bommer et al, Fig. 1A



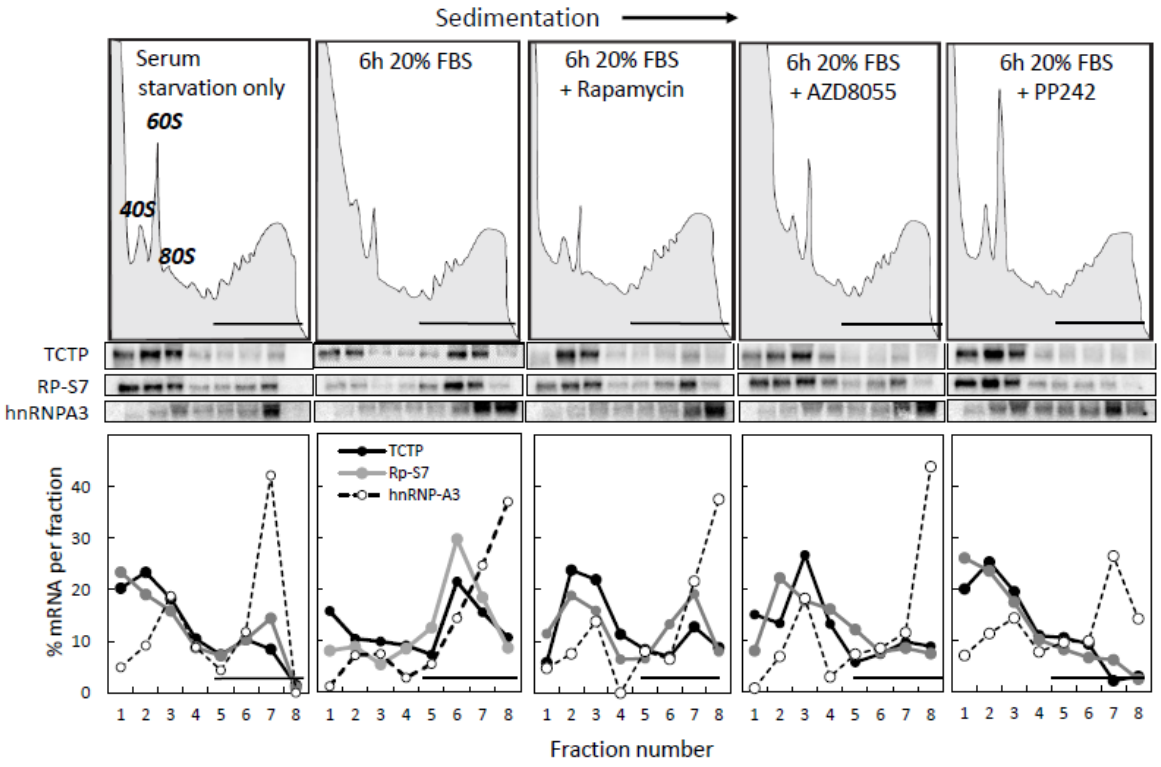
Bommer et al, Fig. 1B



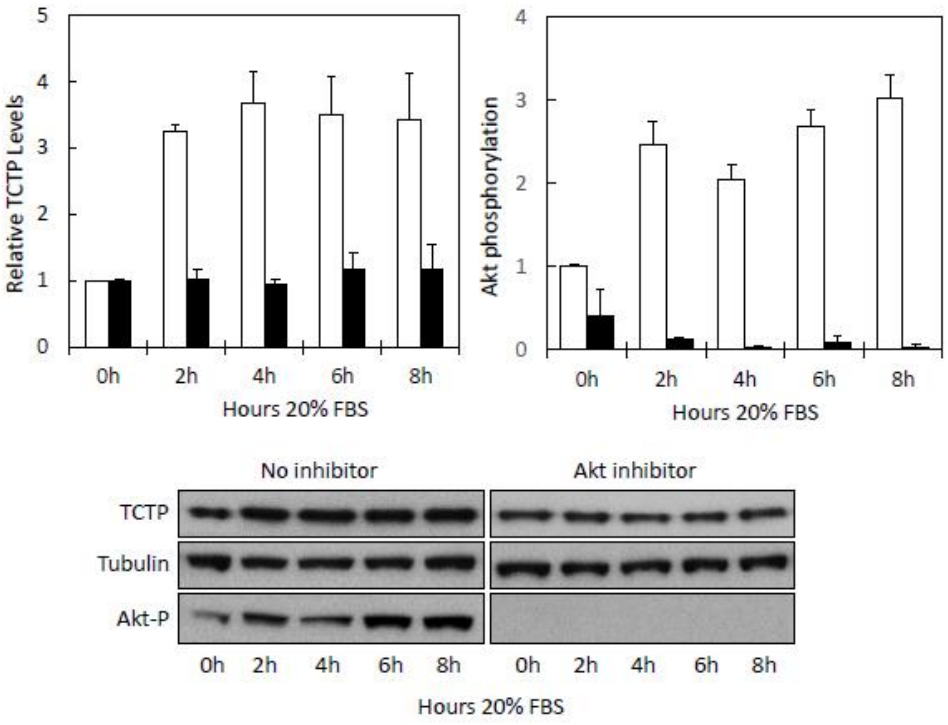
Bommer et al, Fig. 1C



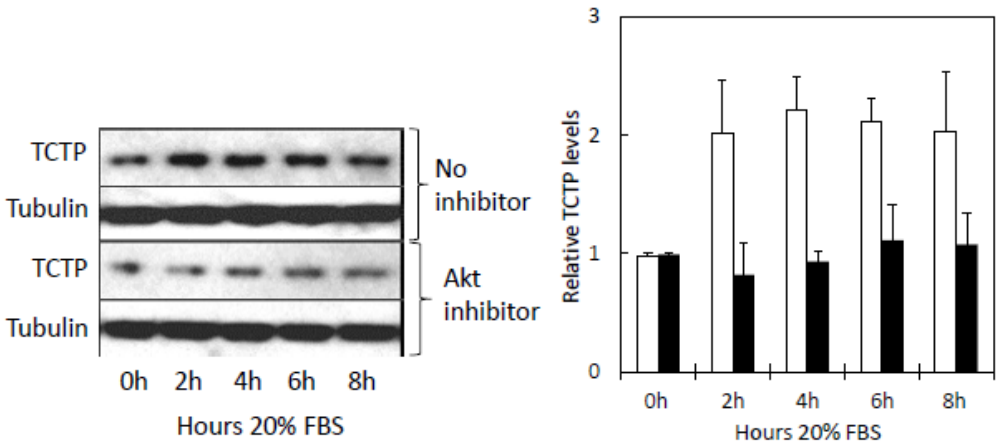
Bommer et al, Fig. 2A



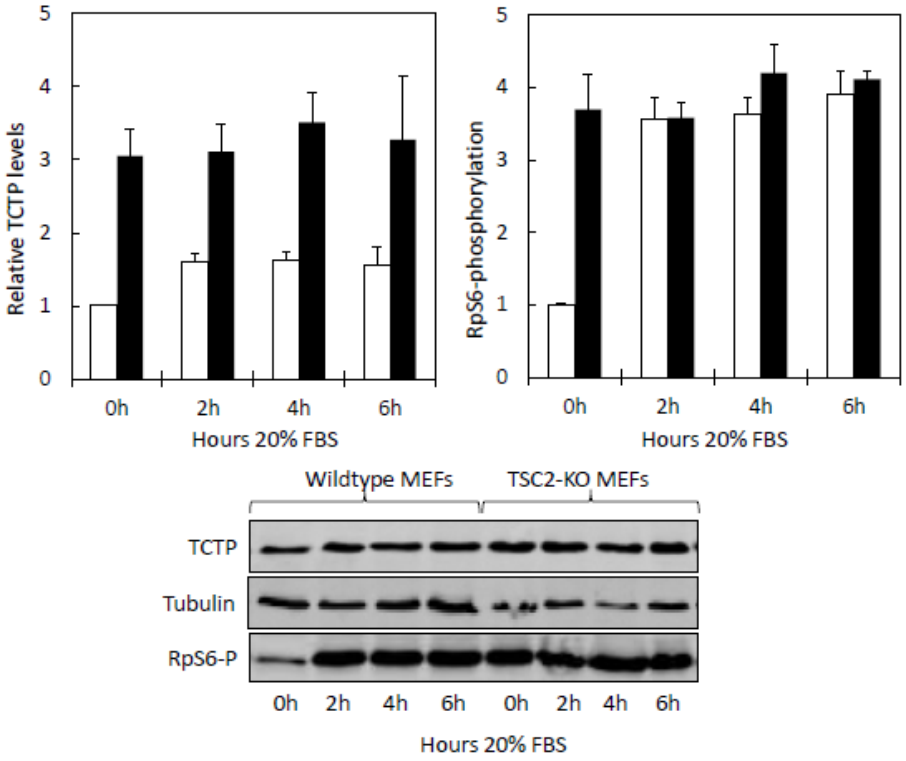
Bommer et al, Fig. 2B



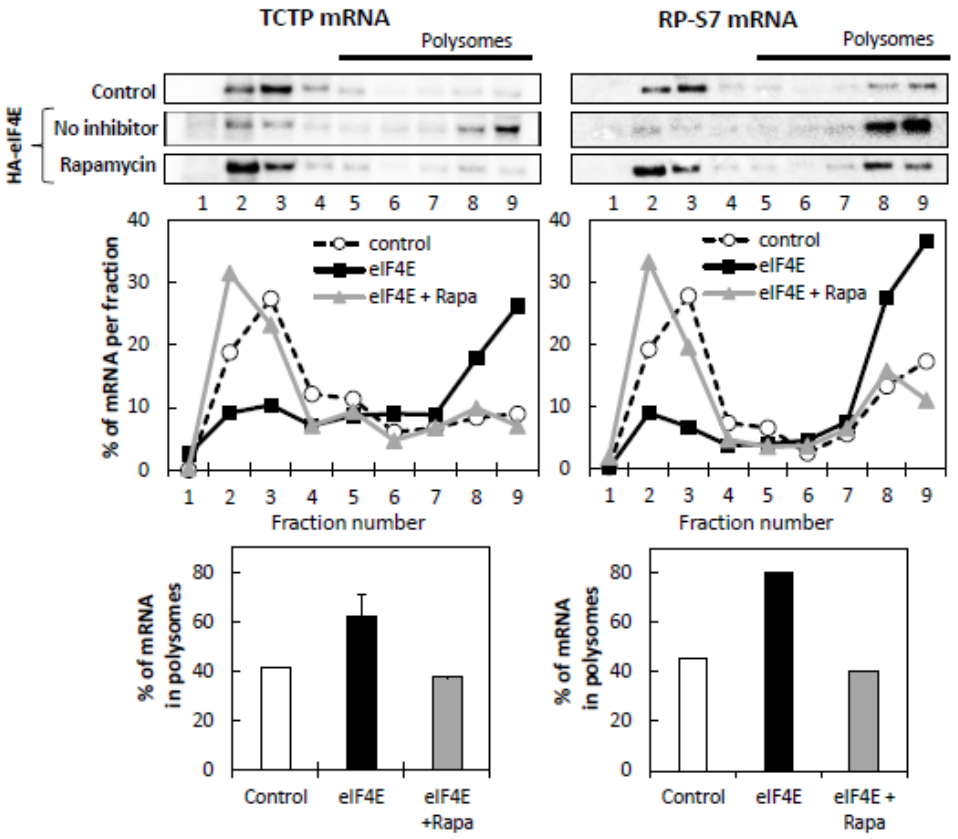
Bommer et al, Fig. 3A



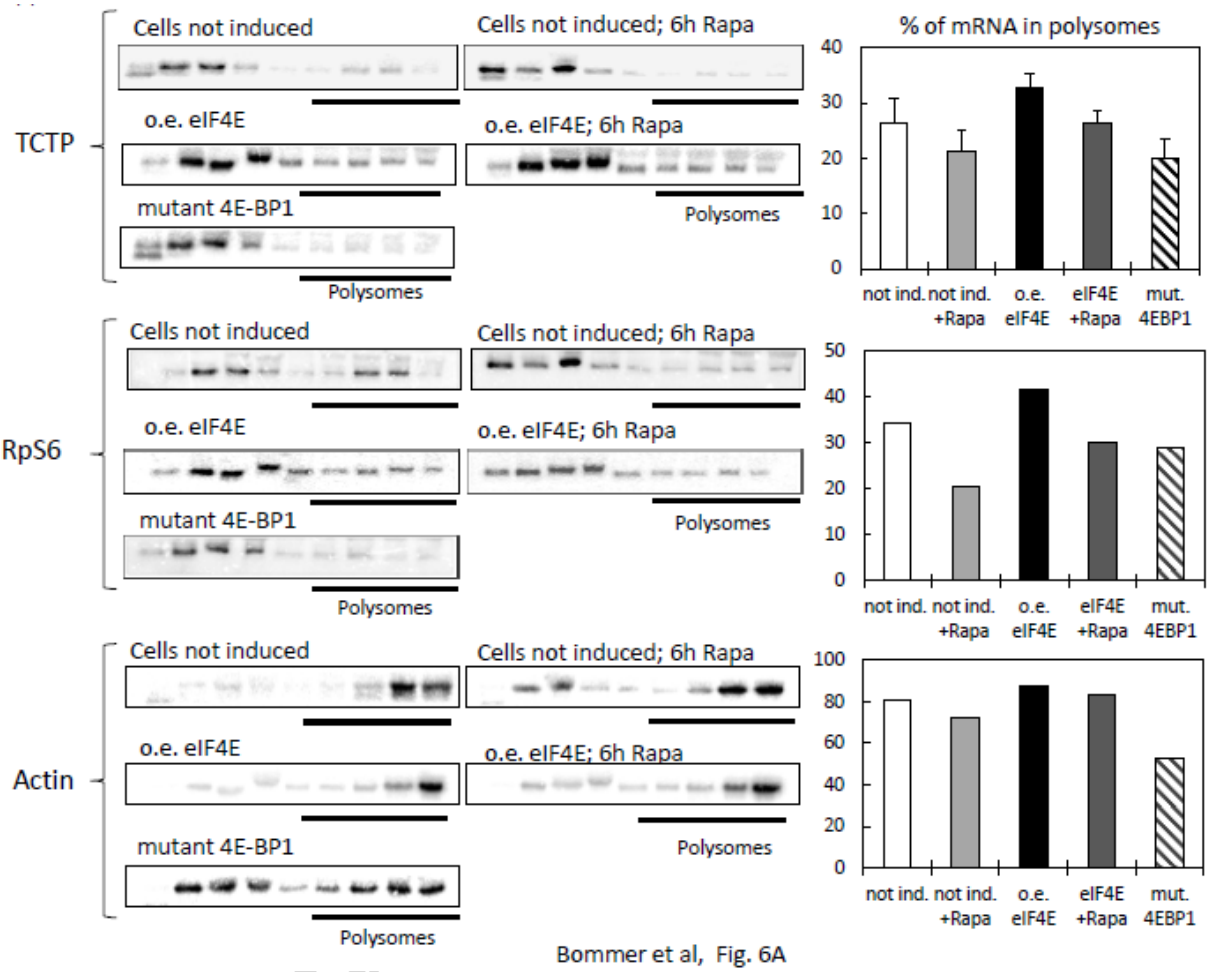
Bommer et al, Fig. 3B



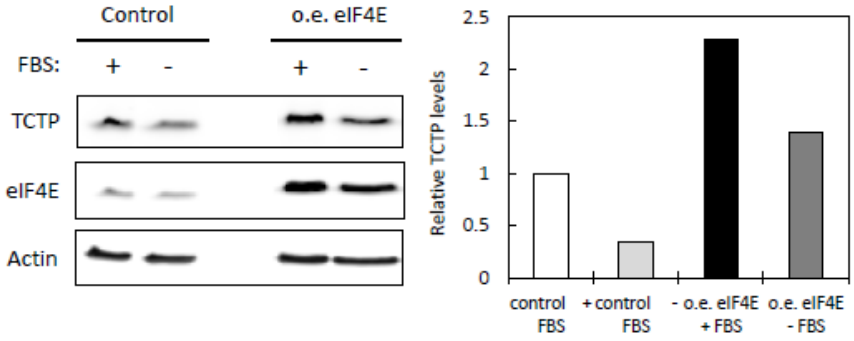
Bommer et al, Fig. 4



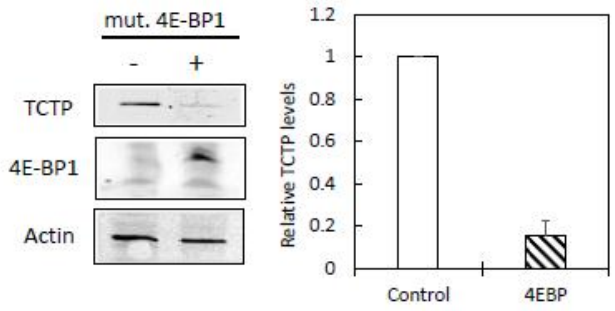
Bommer et al, Fig. 5



Bommer et al, Fig. 6A



Bommer et al, Fig. 6B



Bommer et al, Fig. 6C

Table 1. The effect of mTOR inhibitors on TCTP mRNA translation
(Summary of data from three independent datasets)

mTOR inhibitor			Cell line	Relative TCTP expression[1]	Ref.	Method
Name	**Conc n.**	**Cell treatm.**				
Torin 1	250 nM	2h	Mouse (MEFs)	0.191	[64]	Ribosome profiling with deep sequencing
Rapamycin	50 nM	3h	PC3 prostate cancer cells	0.429	[63]	
PP242	2.5 μM	3h		0.225		
Rapamycin	100 nM	6h	HeLa cells	0.469	[59]	p-SILAC (rate of de-novo protein synthesis)
PP242	1 μM	6h		0.154		
AZD8055	100 nM	6h		0.166		

[1]Relative TCTP expression refers to translational efficiency (ribosome profiling) or rate of *de-novo* TCTP protein synthesis (pSILAC), relative to untreated control. Data for the TCTP protein (Tpt1 gene) were taken from the datasets published in the supplementary materials of the relevant references.

Bommer et al, Growth-factor dependent expression of the translationally controlled tumour protein TCTP is regulated through the PI3K/Akt/mTORC1 signalling pathway.

Highlights

- TCTP is often overexpressed in cancers, where it is associated with a poor outcome.
- We studied the signalling pathway involved in serum-induction of TCTP synthesis.
- mTOR kinase inhibitors prevent growth-associated increase in TCTP mRNA translation.
- The upstream effectors of mTORC1, Akt and TSC-2, also regulate TCTP expression.
- Activation of eIF4E/inhibition of 4E-BP1 is important for efficient TCTP mRNA translation.