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TNF receptor-2 signals clear-cell renal carcinoma proliferation via phosphorylated-4EBP1 and mitochondrial gene translation

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Running Title: TNFR2 signaling in ccRCC

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nonstandard abbreviations

ccRCC- Clear cel renal carcinoma
NK- non tumor kidney
ccRCCoC- ccRCC organ culture
NKoC- non tumor organ culture
TNF- tumor necrosis factor
TNFR1- TNF receptor 1
TNFR2- TNF receptor 2
R2TNF- TNFR2 mutein
COX- cytochrome c oxidase
Etk- Epithelial/endothelial tyrosine kinase
VEGFR2- Vascular endothelial growth factor receptor 2
PI3- phosphatidylinositol-3- kinase
mTOR- Mammalian target of rapamycin
mTORC1 - Mammalian target of rapamycin complex 1
mTORC2 - Mammalian target of rapamycin complex 2
Akt - protein kinase B
pStat3Ser727 - signal transducer and activator of transcription 3 serine 727 phosphorylated
pSer65-4EBP1 - 4E binding protein-1 serine 65 phosphorylated
IF - immunofluorescence
TUNEL- TdT-dUTP nick end labelling technique
Tom20 - Translocase of outer mitochondrial membrane subunit 20
IGEM - immunogold electron microscopy
BvECs - vascular endothelial cells
ICs - isolated interstitial cells (ICs)
Abstract

Clear cell renal cell carcinoma (ccRCC), a tubular epithelial malignancy, secretes tumor necrosis factor (TNF) which signals ccRCC cells in an autocrine manner via two cell surface receptors, TNFR1 and TNFR2, to activate shared and distinct signalling pathways. Selective ligation of TNFR2 was shown to drive cell cycle entry of malignant cells via a signalling pathway involving Etk, VEGFR2, PI3K, Akt, pSer727-Stat3 and mTOR. In this study, phosphorylated-4EBP1 serine 65 (pSer65-4EBP1) is identified as a downstream target of this TNFR2 signalling pathway. pSer65-4EBP1 expression is significantly elevated relative to total 4EBP1 in ccRCC tissue compared to normal kidneys, signal intensity increasing with malignant grade. Selective ligation of TNFR2 with the mutein R2TNF increases pSer65-4EBP1 expression in organ cultures that co-localises with internalised TNFR2 in mitochondria and increases expression of mitochondrially-encoded COX (cytochrome c oxidase subunit) Cox1, as well as nuclear-encoded Cox4/5b subunits. Pharmacological inhibition of mTOR reduces both R2TNF-mediated phosphorylation of 4EBP1 and cell cycle activation in tumor cells while increasing cell death. These results signify the importance of pSer65-4EBP1 in mediating TNFR2-driven cell-cycle entry in tumor cells in ccRCC and implicate a novel relationship between the TNFR2/pSer65-4EBP1/COX axis and mitochondrial function.
**Introduction**

Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults, accounting for approximately 90% of all adult renal malignancies and 2-3% of all cancers. The clear cell renal cell carcinoma (ccRCC) represents up to 75% of all RCC cases and originates from tubular epithelial cells. ccRCC tumor cells secrete tumor necrosis factor (TNF) which acts as an autocrine growth factor. TNF signals via two cell surface receptors; TNFR1 and TNFR2, which mediate both shared and distinct signalling pathways. Selective activation of TNFR2 was shown to drive ccRCC tumor cell proliferation by a signalling pathway involving cytosolic endothelial/epithelial tyrosine kinase (Etk)-mediated activation of vascular endothelial cell growth factor receptor type 2 (VEGFR2), phosphatidylinositol-3- kinase (PI3K), Akt, and the mammalian target of rapamycin (mTOR). Subsequent studies showed that TNFR2 also promoted the survival of RCC specifically through serine 727 phosphorylation of signal transducer and activator of transcription 3 (pStat3S727) which, devoid of tyrosine phosphorylation, translocates to mitochondria rather than the cell nucleus, where it co-localises with internalised TNFR2. Inhibition of any of the involved kinases or siRNA knockdown of TNFR2 or STAT3 promoted cell death associated with mitochondrial morphological changes, cytochrome c release and generation of reactive oxygen species.

The present study further explores the role of mTOR in the TNF pro-tumor response. mTOR is a major driver of many cancers including RCC and is an emerging therapeutic target. mTOR is a conserved serine/threonine protein kinase that exists in two different complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 promotes protein synthesis, partly through cap-dependent translation mediated by 4E binding protein-1 (4EBP1), whose mTORC1-dependent phosphorylation has been shown to enhance mitochondrial function by
increasing translation of nuclear-encoded mitochondrial-related mRNAs. Aberrant activation of the mTOR protein translational pathway presents as either a gain of growth-promoting function or a loss of inhibitory function and may affect the cell dynamics facilitating neoplastic transformation.

The present study further investigates TNFR2-mediated mTOR-dependent signalling pathways that impinge on protein synthesis by focusing on 4EBP1, prompted by the identification of 4EBP1 phosphorylated at serine-65 (pSer65-4EBP1) as a responder to selective TNFR2 activation using a phospho-proteome array. In ccRCC tissue, significantly higher expression of pSer65-4EBP1 occurred in tumor cells than in non-tumor tissue (NK) cells, the extent of phosphorylation increasing with malignant grade. In organ cultures of ccRCC, TNFR2 stimulation increased pSer65-4EBP that co-localised with TNFR2 in tumor cells, which was mainly confined to the cytoplasm and mitochondria. Moreover, 4EBP1 phosphorylation was correlated with increased expression of the cytochrome c oxidase (COX) subunits Cox1, Cox4 and Cox5b. Pre-treatment of organ cultures with mTORC1/2 inhibitors Torin 2 or Ku63794 prior to TNFR2 stimulation showed a significant reduction of pSer65-4EBP1 expression that correlated with cell cycle inhibition. Thus, pSer65-4EBP1 is likely to be an important mediator of TNFR2-driven cell-cycle activation in ccRCC tumor cells, possibly by providing an increased mitochondrial energy reserve.

Materials and Methods

General Reagents

Primary antibodies used in this study are listed in Table 1. Species-specific fluorescent secondary antibodies were either conjugated with NorthernLights (R&D Systems, Abingdon, UK) or with AlexaFluor (Invitrogen, Paisley, UK). Goat anti-rabbit HRP conjugated antibody (cat-P0448, Dakocytomation, Ely, UK); Vectashield anti-fade
mounting media (cat-H-1000, 2BScientific, Oxfordshire, UK); Clarity Western ECL Substrate (cat-170-5060, BioRad Laboratories Ltd, Hertfordshire, UK), or SuperSignal West Dura ECL substrate (cat-34075, ThermoFisher Scientific, Paisley, UK), Terminal transferase enzyme (TdT) (cat-0333566001, Roche Diagnostics, Mannheim, Germany); Hoechst-33342 (cat-62249, Thermo Fisher Scientific), TNFR2-specific 'mutein' (R2TNF) containing the point mutation D143N \(^18\) was a generous gift from Professor Peter Vandenabeele (VIB, Ghent, Belgium). R2TNF is a recombinant mutation of the TNF sequence which enables the mutated protein to bind selectively to TNFR2. Inhibitors were dissolved in DMSO and stored at -20°C or -80°C.

**Collection of tissue samples**

Samples of ccRCC and corresponding non-tumor kidney tissue (NK) (categorised as histologically normal kidney cortex in sites remote from the tumor) from radical nephrectomies removed for tumor resection were collected through Cambridge University Hospital Tissue Bank. Written informed consent was obtained from all patients in accordance with the local Ethics Committee. ccRCC samples were scored and graded on hematoxylin and eosin-stained sections as Fuhrman-grade 1 \((n = 4)\), -grade 2 \((n = 20)\), -grade 3 \((n = 16)\), and -grade 4 \((n = 20)\) by a pathologist according to the World Health Organisation/International Society of Urological Pathology (ISUP) \(^19\). High-grade tumors with extensive areas of necrosis were excluded from the analysis. All samples were either fixed overnight in 4% formaldehyde in 0.1M phosphate buffer pH 7.6 at 4°C and paraffin wax-embedded for immunofluorescence, or snap-frozen in isopentane-cooled liquid nitrogen and stored at -80°C until use. Parallel unfixed fresh tissue were processed for organ culture experiments. Sections (5 \(\mu\)m-thick) were prepared for subsequent analysis.

**ccRCC and NK organ cultures**
Organ culture protocols were performed as previously described \(^6,^{20, 21}\). In brief, duplicate <1 mm\(^3\) fragments of fresh tissue from ccRCC (grades 1-2) and NK (n=5 per study group) were immersed in M199 medium containing 10% heat-inactivated fetal calf serum (FCS), antibiotics and 2.2 mM glutamine. Cultures were either left in media alone (untreated controls; UT) or treated with R2TNF (1 μg/mL), for 3 h at 37°C \(^5, 6, 18, 21\). Parallel cultures were pretreated with either cycloheximide (CHX, 20 μg/mL), or the mTOR inhibitors; Torin 2, Ku63794 or Rapamycin (50 μM) for 1 h prior to R2TNF addition. Samples were fixed, paraffin-embedded, and sections (5 μm-thick) were prepared for subsequent analysis.

**Immunofluorescence (IF)**

Sections of cRCC, NK, and corresponding organ cultures were immunostained using previous protocols \(^5, 6, 21\) following 2 min high-pressure antigen retrieval with acid buffer (0.01 mol/L sodium citrate buffer pH 6.0). Samples were incubated with primary antibodies (1:100) for 1 h at room temperature (RT) followed by NorthernLight or AlexaFluor-conjugated secondary antibodies (1:200) at RT containing Hoescht 33342 (1 μg/ml) for nuclei detection. For negative controls, the primary antibody was replaced with an isotype-specific serum. Sections were mounted in Vectashield and viewed in a blinded manner by two observers under a Leica TCS-SPE confocal microscopy (CLSM) (Leica Microsystems, Milton Keynes, UK) or a Zeiss LSM 880 Airyscan for super-resolution imaging. Images for each fluorophore were acquired sequentially using the same constant acquisition time and settings and processed using Adobe Photoshop PS version 24.4.1. As previously described \(^22\) the intensity of fluorescence per cell was calculated as the corrected total cell fluorescence (CTCF = integrated density - (area of selected cell × mean fluorescence of background readings)) using ImageJ software version 1.53k (NIH, Bethesda, MD;
For each set of antibodies, 3 slides from the same patient sample were processed and the numbers quantified were combined into a single value. N represents the number of independent patient samples out of a pool of 4 cases for primary tissue and 5 cases for tissue organ cultures.

**Immunoblot Analysis**

ccRCC and NK tissues were lysed in RIPA buffer, cleared by centrifugation at 18,000x g for 20 min at 4 °C and 30-50 μg per sample were separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, blocked with 4% skim milk for 1 h at RT, and probed overnight at 4 °C with pSer65-4EBP1 or pThr36/47-4EBP1 (1:1000) followed by secondary HRP-conjugated antibodies. Protein bands were visualised using a ChemDoc Universal Hood III Imaging System (Bio-Rad). Relative protein levels were quantified by normalising to β-actin and Ponceau S staining.

**Immunogold electron microscopy (IGEM)**

As previously described, Formavar-coated nickel grids containing 50 nm sections of untreated (UT) and R2TNF-treated ccRCC organ cultures were incubated with antibodies to pSer65-4EBP1 or TNFR2 and HSP60 or pSer65-4EBP1 and TNFR2 (1:5 dilution) overnight at RT and further incubated with secondary antibodies-conjugated to 5 nm and 15 nm-colloidal gold particles (British Biocell, Maine, USA; 1:100 dilution) then stained with uranyl acetate and lead citrate before viewing in a Hitachi Capital PLC at an accelerating voltage of 80 kV.

**TdT dUTP nick-end labelling (TUNEL)**

Cell death was detected as previously described. In brief, sections pretreated with protease were labelled with TUNEL label mix and TdT enzyme according to the manufacturer’s instructions (Roche Diagnostics Corporation, Indianapolis, USA). This
was followed by staining with Hoechst 33342 for 10 min at RT and mounted in Vectashield before viewing on CLSM.

**Analysis of Cell Death and Proliferation**

The average number of TUNEL-positive tumor cells in ccRCC were counted in 10 random fields of view at x40 magnification from each treatment divided by the total cell numbers to generate the % positive cells. Similarly, the number of pSer65-4EBP1-positive and pH3S10-positive tumor cells were counted and divided by the total cell numbers to generate the % of positive cells, calculated as the proliferative index (PI) for each treatment.

**Statistical Analysis**

All data are expressed as mean ± standard deviation (SD). All statistical tests were performed using Microsoft Excel for Mac version 16.81 and GraphPad Prism version 10.1.0 (La Jolla, CA, USA). Statistical significance was calculated by an analysis of variance (ANOVA), followed by post-hoc multiple comparison test assuming a p-value of <0.05 as statistically significant.

**Results**

*pSer65-4EBP1 is induced in ccRCC tumor-derived tissues and co-localises with TNFR2***

To investigate whether TNFR2-dependent mTOR signalling responses depend on 4EBP1 expression and phosphorylation, patient-derived ccRCC tissue and corresponding NK from the same biopsies were analysed 6, 7. While both regions showed a similar fluorescence signal intensity for total 4EBP1 protein, NK displayed a minimal signal for pSer65-4EBP1 (<1% of cells) but sections of ccRCC showed a significantly higher signal in tumor cells (~3-fold increase in grade-2 tumors) which further increased as tumor grade progressed (~8-fold in grade-4 tumors). Some signal
was also detected in nuclei of tumor cells (~15% of positive cells, imaged using high-resolution microscopy) (Figure 1A, fluorescence intensity quantified as CTCF in 1B). Representative H&E stained sections of NK and grades 1-4 tumors are presented in Supplementary Figure 1. Immunoblotting of grade 4 tumor extracts also demonstrated an increase in pSer65-4EBP1 expression above that of NK tissue (Figure 1C). 4EBP1 phosphorylation on several sites is necessary for its dissociation from EIF4E, thereby enabling EIF4E to initiate protein translation. As phosphorylation of 4EBP1 at Ser65 has been reported to be dependent on prior phosphorylation of Thr37/46, grade 4 tumors were examined for expression of phospho-Thr37/46-4EBP1. A significant positive fluorescent signal for phospho-pThr37/46-4EBP1 was also found in tumor cells (positive for RCC-MA, a tumor marker) and corroborated by immunoblotting (Supplementary Figures 2A & 2B), thus indicating a state of 4EBP1 phosphorylation capable of initiating protein synthesis downstream of TNFR2 activation. Increased TNFR2 expression in tumor cells in ccRCC was previously reported. To investigate if pSer65-4EBP1 might be associated with elevated TNFR2, grade 4 ccRCC sections were costained for TNFR2 and pSer65-4EBP1. Cytoplasmic costaining was observed in ~80% of tumor cells as well as in vascular endothelial cells (BvECs) and in isolated interstitial cells (ICs) (Figure 1D). Hence, increased pSer65-4EBP1 is associated with increased TNFR2 expression in ccRCC.

**TNFR2 stimulation induces upregulation and co-localization of TNFR2 and pSer65-4EBP1 in ccRCC organ cultures.**

To establish whether stimulation of TNFR2 is sufficient to drive phosphorylation of 4EBP1, organ cultures from low-grade tumor tumor tissue were used (low grade was chosen to avoid possible stimulation of TNFR2 by endogenous sources of TNF.}

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*Journal Pre-proof*
TNFR2 was activated selectively using an engineered TNFR2 ‘mutein’ (labelled R2TNF)\textsuperscript{18}. Total 4EBP1 was expressed in ccRCC tumor cells (positive for tumor marker RCC-MA \textsuperscript{27}) irrespective of TNFR2 stimulation by R2TNF (% cell expression, UT, 45.0±0.8, R2TNF-treated 46.5±1.2) (Figure 2A). In contrast, R2TNF induced a significant increase in pSer\textsuperscript{65}-4EBP1 expression mainly within the cytoplasm in tumor cells (% cell expression, UT, 6.4±0.9, R2TNF-treated 48.0±0.7). Similar to advanced-grade ccRCC tissue, some staining was also found in BvECs and isolated ICs (Figure 2B; Table 2). The increase in pSer\textsuperscript{65}-4EBP1 expression in response to R2TNF was commensurate with that of TNFR2 in tumor cells, as well as in BVEC and ICs (Figure 2C), similar to the increase in both proteins in ccRCC tumor tissue. These data confirm that TNFR2-mediated signals induce phosphorylation of 4EBP1 in ccRCC tumor cells. 

**ccRCC organ cultures treated with TNFR2 stimuli show co-localization of TNFR2 or pSer\textsuperscript{65}-4EBP1 in mitochondria and induction of nuclear- and mitochondrially-encoded genes.**

As TNFR2 has been previously identified in mitochondria, it was investigated whether TNFR2-mediated expression of pSer\textsuperscript{65}-4EBP1 was also observed together with TNFR2 in mitochondria. Co-localization of the two proteins in mitochondria in tumor cells was confirmed by co-staining for HSP60 (a mitochondrial matrix protein) by IF (Figures 3A-D) and IGEM (Figures 3E-F).

The mTORC1/4EBP1 pathway has been shown to augment cellular energy homeostasis via the translation of nucleus-encoded mitochondria-related mRNAs \textsuperscript{17}. To examine whether mitochondrial-related protein translation may also be implicated in the mitochondrial localization of pSer\textsuperscript{65}-4EBP1, the effect of R2TNF-mediated signalling on expression of Cox1, a mitochondrially-encoded subunit of cytochrome c oxidase (complex IV), as well as the nuclear-encoded subunits Cox4 and Cox\textsubscript{5b} was examined. In organ cultures of ccRCC, stimulation by R2TNF caused a significant
increase in Cox1, Cox4, and Cox5b expression compared to UT cultures (~3.2-fold, ~3.7-fold, and ~2.7-fold, respectively) that colocalised in cells with elevated TNFR2, and a similarly significant increase in Cox1, Cox4 or Cox5b expression (~3.7-fold, ~6.7-fold and ~4.2-fold) that colocalised in cells with elevated pSer65-4EBP1 (Figures 4A-B, quantified as CTCF in C). R2TNF induced a smaller change of (<2.5 fold) in expression and co-localization of the COX subunits in NK, but this occurred on a signal intensity background that was about 1000 fold less intense than that of ccRCC tissue (maximum CTCF intensity in NK ~2.5x10^3 as compared to ~4x10^6 in ccRCC (Supplementary Figures 3A-B). Thus, TNFR2-mediated signalling may be involved in the translational regulation of mitochondrial as well as in nuclear-encoded mitochondrial genes in ccRCC, the former, perhaps, being mediated by mitochondrial-targeted TNFR2/pSer65-4EBP1.

Cycloheximide inhibits expression of nuclear-encoded subunits Cox4/5b but not that of mitochondrially-encoded Cox1

Cox1 expression may be modified by the cytoplasmic expression of nuclear-encoded Cox subunits 28. To further examine whether elevated expression of Cox1 is controlled by cytoplasmic translation, the cytoplasmic protein synthesis inhibitor cycloheximide was applied to RCC organ cultures 29. Immunostaining revealed that expression of Cox4/5b in mitochondria (confirmed by Tom20 co-localization) was profoundly inhibited by CHX (R2TNF ± CHX, Cox 4- ~7-fold reduction ***p<0.0001; Cox5b- ~6-fold reduction **p<0.001) whereas the expression of Cox1 was not significantly reduced (Figure 5A, quantified in 5B). Thus, the increase in Cox1 expression is likely to be controlled either by stable cytoplasmic factors or by mitochondrially-encoded mechanisms that are independent of cytoplasmic control.

Elevated pSer65-4EBP1 in ccRCC tumor cells depends on mTORC1
Inhibition of the mTORC pathway inhibits TNFR2-induced cell cycle activation in cultured ccRCC\textsuperscript{CD133+} CSCs \textsuperscript{6,7}. To examine whether the phosphorylation of 4EBP1 also correlates with TNFR2-induced cell cycle entry via mTORC signalling, organ cultures were co-stained for the proliferative marker pH3\textsuperscript{S10} and p\textsuperscript{Ser65}-4EBP1. R2TNF treatment resulted in a more pronounced dual signal for p\textsuperscript{Ser65}-4EBP1 and pH3\textsuperscript{S10} in tumor cells as compared to untreated cultures (~43.2% by R2TNF vs ~3.75% in UT cultures) (Figure 6A, quantified in 6C). R2TNF also induced a dual signal in normal tubular cells in organ cultures of NK but the effect was significantly less than in organ cultures of ccRCC (~17.6% by R2TNF vs ~<1% in UT cultures, (Supplementary Figure 4A, quantified in Figure 6C). To test whether cell cycle entry depends on p\textsuperscript{Ser65}-4EBP1, organ cultures were pre-treated with the mTORC inhibitors Torin 2, Ku63794, or Rapamycin. Torin 2 and ku63794 significantly decreased the number of R2TNF-induced p\textsuperscript{Ser65}-4EBP1+/pH3\textsuperscript{S10+} double-positive tumor cells in ccRCC (Figure 6B, quantified in 6D) with a potency of ~8.4-fold for Torin 2, ~5.25-fold for Ku63794, in keeping with their pharmacological potency \textsuperscript{30} while Rapamycin did not inhibit cell cycle significantly, as expected as it blocks the S6K1 pathway but not the 4EBP1 arm of mTORC1 signalling \textsuperscript{30}. The inhibitors caused a similar but less effective inhibition of proliferation in organ cultures of NK (~3.3-fold by Torin 2, ~2.2-fold by Ku63794, non-significantly by Rapamycin) but on a much lower signal intensity background (Supplementary Figure 4B, quantified in 6C). These results strengthen the notion that p\textsuperscript{Ser65}-4EBP1 is necessary for cell cycle entry induced by R2TNF signalling in ccRCC. In keeping with previous observations \textsuperscript{7}, the decrease in cell proliferation in the presence of mTOR inhibitors was accompanied by an analogous increase in cell death measured using TUNEL staining (TUNEL\textsuperscript{+} cell increase of ~5.0-fold induced by Torin, ~3.0-fold by Ku63794 and ~2-fold by...
Rapamycin although the latter was not significantly different from R2TNF treated alone (Supplementary Figure 5; Table 3).

Discussion

Previous studies have revealed that an important step in the TNFR2 signalling pathway which drives cell cycle entry in ccRCC involves crosstalk between phosphorylation of VEGFR2, Etk, PI-3K, Akt, mTORC and STAT3 on serine 727 (pStat3Ser727). This signalling is associated with the co-localization of pStat3Ser727 and TNFR2 within the mitochondria 5–7. In the present study, identified 4EBP1 phospho-serine 65 (pSer65-4EBP1) is identified as a significant mediator of mTORC signalling downstream of TNFR2. This conclusion is based on the following observations: 1. In ccRCC tissue, pSer65-4EBP1 expression relative to total 4EBP1 was significantly elevated in tumor cells compared to NK, signal intensity increasing with malignant grade. ccRCC extracts also displayed enhanced 4EBP1 Thr37/46 phosphorylation. 2. In organ cultures, selective activation of TNFR2 by R2TNF, a specific TNFR2 ligand, increased pSer65-4EBP1 expression to a greater extent in ccRCC compared to NK tissue. 3. mTORC1/2 inhibitors suppressed R2TNF-mediated induction of pSer65-4EBP1 expression in correlation with their potency at inhibiting cell cycle entry, and increased tumor cell death.

The role of mTORC1 in the phosphorylation of 4EBP1 is well established and contributes to the increased level of protein synthesis mediated in responses to mTORC1 activation. What is unexpected is that some pSer65-4EBP1 was colocalised with mitochondria (alongside TNFR2) and is associated with the expression of the mitochondrially-encoded COX subunit Cox1, as well as the nuclear-encoded Cox4/5ab subunits. It is unclear how these phenomena are linked mechanistically as mitochondrial ribosomes differ from cytoplasmic ribosomes and are not thought to be
affected by cytosolic elongation factors. In keeping with this notion, CHX inhibited
2RTNF-induced expression of nuclear-encoded Cox4 and Cox5b, whereas the
increased expression of mitochondria-encoded Cox1 was not significantly reduced.
Morita et al 17 showed that mTORC-mediated 4EBP1 phosphorylation was essential for
increasing ATP production by increasing the expression of nuclear-encoded
mitochondrial genes in MCF7 cells, albeit not Cox4. Thus it is possible that 4EBP1 can
upregulate mitochondrial function in ccRCC via increasing COX subunit expression.
It remains to be seen whether the expression of other components of the mitochondrial
respiratory machinery is also mediated by TNFR2 signalling via translational control.
Taken together, results herein implicate a novel relationship between the
TNFR2/\textsuperscript{pSer65}-4EBP1/COX axis and mitochondrial function related to increased cell
cycle activation in ccRCC.
Findings herein of increased TNFR2 expression in ccRCC are consistent with earlier
report 5,7, while the enhancement of tumor growth via TNFR2 is in line with findings
by other groups 31-33 and supports the notion that it is a promising target for cancer
therapy 34-35. Although observation of TNFR2 localization with mitochondria in the
present study is supported by previous reports 36-38, whether its effects on driving cell
cycle entry are mediated via its mitochondrial localization requires further
investigation. However, consistent with this hypothesis, previous studies have
indicated a role for TNFR2 in mediating mitochondrial fusion, alongside increased
mitochondrial oxidative phosphorylation (OXPHOS) activity 39, 40 and increased
mitochondrial membrane potential, intracellular ATP levels, and oxygen consumption
capacity 41.
In many cancers, including ccRCC, the PI3K/Akt/mTORC1 pathway is aberrantly
activated and contributes to oncogenesis, proliferation, invasion, and metastasis 42.
mTORC1-dependent 4EBP1 phosphorylation alleviates the inhibition of eIF4E,
releasing it to initiate cap-dependent mRNA translation. Augmented expression of phosphorylated 4EBP1 has been demonstrated in various human cancers including RCC, suggesting it plays a pivotal role in tumorigenesis and is associated with adverse prognosis in several malignancies. Findings in the present study implicating 4EBP1 in mediating TNFR2-dependent tumor growth in ccRCC are that i) TNFR2 signalling increased the amount of phosphorylated 4EBP1 in ccRCC, in particular that of pSer65-4EBP1 – a final key phosphorylation site that enables the complete dissociation of 4EBP1 from eIF4E and that ii) it is associated with TNFR2 localization in mitochondria. The role of pSer65-4EBP1 in mediating cell cycle induction by TNFR2 in ccRCC is not completely resolved, but observations in the present study of increased amount of mitochondrially-encoded Cox1 in ccRCC organ cultures after TNFR2 stimulation, in addition to nuclear-encoded Cox4 and Cox5b, suggests that TNFR2-induced cell cycle entry in ccRCC is dependent on an increased energy demand that is met in part by mobilisation of cytoplasmic and mitochondrial translational initiation complexes. That both nuclear- and mitochondrially-encoded COX protein subunits are elevated together is consistent with the observation that translation of mitochondrial Cox1 adapts to nuclear-encoded Cox4 availability, which may not necessitate mitochondrial localization of 4EBP1. The importance of COX subunits in driving mitochondrial energy production is well documented (reviewed by Timón-Gómez A et al.,). For example, depletion of Cox5b was shown to induce mitochondrial dysfunction by increasing ROS production, decreasing mitochondrial membrane potential and intracellular ATP generation. In addition, Cox4 isoform switches enabled a cell to fine-tune COX function by altering ATP production, respiration, and mitochondrial ROS production in response to tissue type, energetic status, and hypoxia. Singh RK et al. have reported mitochondrial DNA-encoded Cox1 overexpression promotes anchorage-dependent and independent proliferation.
potential in vitro experiments and somatic mutations within Cox1 and its
perturbations from orchestrated expression influence carcinogenesis.

Organ culture experiments here demonstrate that the ECs in ccRCC are more
sensitive to TNFR2 signalling than the ECs in the nontransformed parts of the kidney.
While the specific mechanisms responsible for this difference are unclear, an indirect
mechanism that could support tumor growth would be by increasing angiogenesis. In
ECs, the TNFα/TNFR2 pathway supports angiogenesis by activating the cytosolic
tyrosine kinase Etk that, in turn, phosphorylates and activates VEGFR2 in the absence
of VEGF ligand, thereby promoting cell proliferation \(^{56-58}\). Since phosphorylation of
4EBP1 plays a critical role in augmenting the cell’s capacity for protein translation and
angiogenesis \(^{59}\), it is reasonable to suppose that TNFR2/\(\text{p}^{\text{Ser65}}\)-4EBP1 axis plays a
critical role in neo-vascularisation of ccRCC in addition to its direct contribution to
tumor cell growth.

Torin 2 and ku63794 were found to significantly reduce TNFR2-triggered cell
cycle activation with a noticeable reduction in expression of \(\text{p}^{\text{Ser65}}\)-4EBP1 in tumor
cells. Rapamycin’s effects were not significant, in keeping with it being an inhibitor
primarily of the S6K1 leg of the mTOR signalling pathway rather than the 4EBP1 leg
\(^{30, 60, 61}\). Interestingly, 4EBP1 phosphorylation has been implicated in Rapamycin
resistance in certain cancer cells \(^{62, 63}\) and may explain the limited success of
Rapamycin as an anti-cancer drug \(^{64}\). The residual activity observed in the presence
of Torin 2 and Ku63794 may explain the resistance frequently seen with mTORC
inhibitors used for the treatment of advanced RCC \(^{65}\). One possibility is that TNFR2
mobilises TORC2 as well as TORC1 \(^{66, 67}\) and this pathway involves phosphorylation of
Akt \(^{68}\), which has previously been shown to be involved in driving TNFR2-mediated
survival of \(\text{CD}^{133+}\) CSCs together with VEGFR2 and PI3K \(^{7}\). Moreover, enhanced PI3K
activity has been reported to promote protein synthesis and increase mTORC2
association with ribosomes, and 4EBP1 phosphorylation has been implicated in much of the activation of translation by PI3K/AKT, which plays an important part in mediating the effects of these pathways in tumors \textsuperscript{69}. In keeping with the possibility that 4EBP1 also mediates the AKT leg of the mTOR signaling pathway, a kinase activity specific to Ser65 and Thr70 on 4EBP1 was reported to be liberated from an mTOR immunoprecipitate, suggesting the presence of a different kinase \textsuperscript{70}. This observation suggests that an alternative TNFR2-4EBP1 kinase(s) is activated in response to mTORC inhibition, which may cause translational de-repression from 4EBP1 in an mTORC-independent manner.

**Conclusions**

In summary, this study has highlighted the importance of pSer65-4EBP1 in TNFR2-driven cell-cycle entry in tumor cells in ccRCC and implicate mTOR-dependent and independent pathways in this process, schematised in Figure 7. The significant novel findings reported in the present study are the mitochondria-targeted pSer65-4EBP1 and its association with TNFR2 in ccRCC, and the coordination between nuclear and mitochondrial expression of genes that enable increased mitochondrial performance.

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References


Figure Legends

Figure 1. pSer65-4EBP1 is abundantly expressed in ccRCC tumor and co-localises with TNFR2. Representative confocal images of single-IF staining of ccRCC tumor grades (G1-G4) and corresponding non-tumor tissue (NK). A. Top row: Staining for total 4EBP1 (t4EBP1). A marked signal was detected in NK, confined to normal tubular cells (t), some isolated cells within glomeruli (Glom) and the interstitium (red arrowheads). A similar pattern and intensity was found in tumor cells in all ccRCC grades. Bottom row: Staining for pSer65-4EBP1. Signal was undetectable in NK and infrequently present in G1 ccRCC tumor cells but accumulated with increasing intensity in ccRCC according to malignant grade (G2-G4). Signal was mainly cytoplasmic but also found infrequently in the nucleus of some tumor cells (inset; low power-image arrowhead - the panel labelled super-res shows a high power image of 4E-BP1 staining in the nuclei taken by super-resolution microscopy). B. Signal intensity per cell was quantified as corrected total cell fluorescence (CTCF) Data are expressed as mean ± SD, n = 3 cases, 2-way ANOVA, ns= non-significant, ***p<0.001 vs NK and G1; ****p<0.0001 vs NK, G1 and G2). C. Immunoblot of lysates from 3 independent ccRCC G4 tumor resections (T) alongside corresponding non-tumor kidney (N) from the same tissue, showing an increased abundance of pSer65-4EBP1 in all 3 tumors compared to normal tissue. β-Actin and Ponceau S stain show protein loading. Bar graph shows the relative intensity of pSer65-4EBP1 band normalised to N for each pair. D. Representative confocal images of ccRCC-G4 staining showing co-localization of signal for pSer65-4EBP1 and TNFR2 in tumor cells and in infiltrating cells within the interstitium (inset; arrowheads). n=3 per group with similar results (A, B, D). Original magnification, ×40 and x63. Scale bar = A,D-50 μm; D-inset=25 μm.
**Figure 2.** Activation of TNFR2 by R2TNF mutein induces expression of $\text{pSer}^{65}\text{-4EBP1}$ and its co-localization with TNFR2 in tumor cells in organ cultures of ccRCC. **A.** Representative confocal images of combined-IF in low-grade ccRCC organ culture sections stained for total (t)4EBP1. A similar signal intensity was found in tumor cells (identified using the tumor marker RCC-MA) in control (UT) and R2TNF-treated cultures. **B.** In comparison, R2TNF induced expression of $\text{pSer}^{65}\text{-4EBP1}$ in tumor cells with a weak infrequent signal in controls. A signal for $\text{pSer}^{65}\text{-4EBP1}$ is also seen in endothelial cells in blood vessels (Bv, white arrowheads), negative for RCC-MA. Numbers indicate % co-localization in tumor cells (mean ± SD, n=3 per group with similar results). **C.** $\text{pSer}^{65}\text{-4EBP1}$ and TNFR2 co-localization in tumor cells, in vascular endothelial cells (Bv-white arrowheads) and in infiltrating cells (yellow arrows). Inset- shows co-signal in a bilobed tumor cell in another area on the same section. Original magnification, ×40 and x63. Scale bar = A,B,C -50 μm; C-inset= 10 μm.

**Figure 3.** R2TNF induces co-localization of $\text{pSer}^{65}\text{-4EBP1}$ and TNFR2 in mitochondria. Representative panels of high-power confocal images of combined-IF. **A.** $\text{pSer}^{65}\text{-4EBP1}$ and HSP60 (mitochondrial matrix protein) or **B.** TNFR2 and HSP60 in low-grade ccRCC organ culture sections. Images show R2TNF-induced co-localization of the two proteins in mitochondria in tumor cells (arrowheads), **C.** super-resolution images (super-res) of tumor cells show mitochondrial co-localization of the two proteins (white arrowsheads), confirmed by **D.** Triple-IF which show $\text{pSer}^{65}\text{-4EBP1}$/TNFR2/HSP60 + (arrows) within a tumor cell (white dotted lines). **E.** Representative immunogold electron micrographs show presence of $\text{pSer}^{65}\text{-4EBP1}$ and TNFR2 (15 nm-colloidal gold) in mitochondria, labelled with HSP60 (5 nm-colloidal gold) on R2TNF-treated low-grade ccRCC organ sections (white arrows) and **F.** the
close proximity of pSer65-4EBP1 (red arrow) and TNFR2 (blue arrow) in mitochondria and the presence of TNFR2 on the cell surface as compared to UT cultures which show a rare signal. m-mitochondria. n-nucleus. Original magnifications, ×40 and x63. n=3 per group with similar results. Scale bar =-A,B,-25 μm; C-D-75 μm. Electron micrographs- E-200 nm, F-left, right panels-200 nm, middle panel-500 nm.

**Figure 4.** R2TNF induces co-localization of pSer65-4EBP1 and TNFR2 with cytochrome c oxidase subunits. A. Representative confocal images of combined-IF of pSer65-4EBP1 or TNFR2 with Cox1, or Cox4, or Cox5b in low-grade ccRCC organ culture sections show R2TNF-induced co-signal of Ser65-4EBP1 or TNFR2 and Cox1, Cox4 and Cox5 (×40 magnification). B. High-power images show R2TNF-induced co-signal of Ser65-4EBP1 or TNFR2 and Cox1 (x63 magnification). C. Quantification of signal intensity of R2TNF-induced co-signal compared to UT presented as CTCF, means ± SD, n=3 per group with similar results, 2-way ANOVA, ***p<0.0001- and **p<0.01 versus controls. Scale bar = A-50 μm; B-top panel-25 μm; lower panel-10 μm.

**Figure 5.** Cycloheximide (CHX) inhibits expression of nuclear-encoded Cox4/5b but not the mitochondrially-encoded Cox1. A. Cultures pretreated with CHX (20 μg/mL) showed negligible levels of Cox1, Cox4 and Cox5b comparable to untreated cultures (UT). In comparison with UT, R2TNF alone induced increased levels of Cox1, Cox4 and Cox5b in tumor cells significantly reduced by CHX of Cox4 and Cox5b but no significant reduction of Cox1. Mitochondrial marker (Tom20, Translocase of outer mitochondrial membrane). Original magnification, ×40. B. The positive signal in tumor cells is presented as corrected mean cell fluorescence (CTCF), mean ± SD, n=3 per group with similar results, 2-way ANOVA. ***p<0.0001 vs UT or
CHX or vs R2TNF (Cox4); ***p<0.001 vs R2TNF (Cox5b) ns-not significant. Scale bars=A-25 μm.

**Figure 6. R2TNF-induced cell proliferation and pSer65-4EBP1 expression in tumor cells are reduced by mTOR inhibitors.** A. Representative confocal images of combined-IF for pSer65-4EBP1 (green) and proliferative marker phosphorylated-histone S10 (pH3S10) (red) in low-grade ccRCC organ culture either left untreated (UT) or treated with R2TNF for 3 h at 37°C show a significant increase in proliferative tumor cells (pH3S10+) also positive for pSer65-4EBP1 in R2TNF-treated cultures as compared to UT. B. Similar cultures pre-treated with mTOR inhibitors Torin 2, Ku63794 or Rapamycin for 1 h prior to R2TNF addition show a significant reduction in pSer65-4EBP1+/pH3S10+ tumor cells, more pronounced in cultures pre-treated with Torin 2 and Ku63794 than Rapamycin. C. Quantification of tumor cell proliferation as a proliferative index (% +ve of total cells) in UT or R2TNF-treated organ cultures of ccRCC (RCCoC) versus staining in normal tubular cells in organ cultures of NK (NKoC) and - in the absence or presence of the mTOR inhibitors. Original magnification ×40. D. Quantification of double-stained pSer65-4EBP1+/pH3S10 positive tumor cells in UT and R2TNF-treated cultures ± mTOR inhibitors (mean ± SD, n = 3 per group with similar results, 2-way ANOVA. C- ***p<0.0001 vs UT, *p<0.05 or ***p<0.001 vs R2TNF, ns-not significant. B-zoomed 2.3x. Scale bars=A-50 μM; B-75 μM.

**Figure 7. A model of TNFR2-driven cell cycle entry in tumor cells in ccRCC.** Selective ligation of TNFR2 by R2TNF induces an increased expression of TNFR2 and phosphorylation of 4EBP1 at serine 65 (pSer65-4EBP1) and facilitates co-localization of the two proteins in cytoplasm and mitochondria alongside increased expression of
nuclear- and mitochondrial-encoded COX subunits. pSer65-4EBP1 is also detected in the nucleus in some tumor cells. mTOR inhibitors cause a significant reduction in R2TNF-mediated cell cycle activation and pSer65-4EBP1 expression, accompanied by an increased cell death with the relative potency of Torin 2>Ku63794>Rapamycin.
**Table 1:** List of primary antibodies used in this study

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Table 2. Quantification of the percentage of total (t)4EBP1 and pSer65-4EBP1 expression in ccRCCoC and corresponding NKoC either left UT or treated with R2TNF. t4EBP1 expression is present in all cultures. In comparison, R2TNF induced a marked pSer65-4EBP1 expression in TECs, BVECs, and ICs, with a less pronounced effect in R2TNF-treated NKoC.

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<tr>
<td></td>
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<td>BvEC</td>
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<tr>
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<tr>
<td>R2TNF</td>
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Abbreviations: NKoC-organ cultures of non-tumor kidney, ccRCCoC-organ cultures of ccRCC, nTEC, normal tubular epithelial cells; mTEC, malignant tubular epithelial cells; BVECs, vascular endothelial cells; ICs, isolated interstitial cells; UT, untreated; R2TNF, TNFR2 mutein. Values are mean ± SD from n=3 independent patient samples. ccRCCoC-**** p<0.0001 vs UT; NKoC-*** p<0.001 vs UT.
Table 3. Quantification of the percentage of TUNEL\(^+\) tumor cells in low-grade ccRCCoC and of tubular cells in corresponding NKoC calculated as TUNEL\(^+\)/total cells (x100) at x40 magnification. Cells were either left UT or treated with R2TNF alone (1 \(\mu\)g/ml for 3 h at 37\(^\circ\)C) or pre-treated with mTOR inhibitors (1 h prior to R2TNF) or pre-treated with inhibitors alone.

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<th>NKoC</th>
<th>ccRCCoC</th>
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<td>R2TNF</td>
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<td>18.37±0.25%***</td>
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<td>Rapamycin</td>
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Abbreviations: NKoC-organ cultures of non-tumor kidney, ccRCCoC-organ cultures of ccRCC, UT-untreated, R2TNF-TNFR2 mutein, mTOR-mammalian Target of Rapamycin inhibitors (all used at 50 \(\mu\)m). (mean ± SD; n=3 independent patient samples, NKoC and ccRCCoC - *\(p<0.05\) vs UT; ****\(p<0.0001\); ***\(p<0.001\) vs R2TNF.