University of Crete
School of Medicine
Graduate program in the Molecular Basis of Human Disease

# Investigation of the role of TPL2 in colon cancer

Maria Saliakoura

Master Thesis

Supervisor: Aristides Eliopoulos, Professor, University of Crete, Medical School

Advisory committee:

Christos Tsatsanis, Associate Professor, University of Crete, Medical School

Elias Drakos, Assistant Professor, University of Crete, Medical School

HERAKLION 2016

ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΙΑΤΡΙΚΗ ΣΧΟΛΗ

# Μελέτη της επίδρασης της TPL2 στον καρκίνο του παχέος εντέρου

Μαρία Σαλιακούρα

Μεταπτυχιακή Διατριβή

Επιβλέπων: Αριστείδης Ηλιόπουλος, Καθηγητής, Πανεπιστήμιο Κρήτης, Ιατρική Σχολή

Συμβουλευτική επιτροπή:

Ηλίας Δράκος, Επίκουρος Καθηγητής, Πανεπιστήμιο Κρήτης, Ιατρική Σχολή

Χρήστος Τσατσάνης, Αναπληρωτής Καθηγητής, Πανεπιστήμιο Κρήτης, Ιατρική Σχολή

ΗΡΑΚΛΕΙΟ 2016

# Table of Contents

# ABSTRACT

Colorectal cancer is the third most common cancer worldwide and the second most common in developed countries. Its pathogenesis is attributed to both genetic and epigenetic events that take place with tumor initiation and progression. Patient prognosis and individual response to therapy varies significantly in stage-independent manner, mainly due to molecular heterogeneity. In this regard, there is an ongoing attempt to identify specific biomarkers that will allow predicting a patient's clinical outcome beyond tumor staging, and facilitate a more effective therapeutic approach. TPL2 kinase has a contradictory role in cancer, exhibiting tumor-promoting or tumor-suppressing properties in a tissue-specific manner. In the present study, we aimed to delineate the role of TPL2 in colon cancer, a field that remains, to date, poorly elucidated. Using established human colon cancer cell lines, we proceeded to TPL2 inhibition and *tpl2* gene silencing. We show that inhibition of TPL2 significantly affects the proliferative and clonogenic capacity of cell lines HT29 and HCT15. Conversely, silencing *tpl2* does not lead to similar results. Overall, our findings suggest that although TPL2 may have a role in colon cancer cell proliferation, TPL2-independent pathways also seem to be crucially involved.

# ΠΕΡΙΛΗΨΗ

Ο καρκίνος του παχέος εντέρου είναι ο τρίτος πιο συχνός καρκίνος σε παγκόσμιο επίπεδο και ο δεύτερος πιο συχνός στις ανεπτυγμένες χώρες. Η παθογένεσή του αποδίδεται τόσο σε γενετικά όσο και επιγενετικά γεγονότα τα οποία λαμβάνουν χώρα κατά την έναρξη αλλά και την εξέλιξη της νόσου. Η πρόγνωση των ασθενών, αλλά και η ατομική απόκρισή τους στη θεραπεία ποικίλει σημαντικά, κυρίως λόγω μοριακής ετερογένειας. Για το λόγο αυτό υπάρχει μια συνεχής προσπάθεια εντοπισμού συγκεκριμένων βιοδεικτών που θα επιτρέψουν την πρόβλεψη του κλινικού αποτελέσματος για κάθε ασθενή ανεξάρτητα από το στάδιο στο οποίο βρίσκονται και θα διευκολύνουν μια πιο αποδοτική θεραπευτική προσέγγιση. Ο ρόλος της TPL2 κινάσης στον καρκίνο είναι αμφίσημος, μιας και δρα τόσο με ογκογόνες όσο και με ογκοκατασταλτικές ιδιότητες ανάλογα με τον εκάστοτε ιστό. Στην παρούσα εργασία στοχεύσαμε στο ρόλο της TPL2 κινάσης στον καρκίνο του παχέος εντέρου, ο οποίος παραμένει έως και σήμερα άγνωστος. Χρησιμοποιώντας ανθρώπινες κυτταρικές σειρές καρκίνου του παχέος εντέρου προχωρήσαμε σε αναστολή και σίγαση του γονιδίου της *tpl2*. Δείχνουμε πως αναστολή της TPL2 επηρεάζει σημαντικά την ικανότητα πολλαπλασιασμού και σχηματισμού αποικιών των κυτταρικών σειρών HT29 και HCT15. Συνολικά, τα ευρήματά μας υποδηλώνουν ότι παρόλο που η TPL2 φαίνεται να έχει κάποιον ρόλο στον πολλαπλασιασμό των καρκινικών κυττάρων, στη διαδικασία εμπλέκονται και άλλα, ανεξάρτητα της TPL2, μονοπάτια.

# INTRODUCTION

## The Mitogen-Activated Protein Kinases (MAPKs) family

Mitogen-activated protein kinases (MAPKs) regulate cell proliferation, survival, and death in response to a wide range of extra-cellular signals including cytokines, growth factors and stress (Yang, Sharrocks et al. 2003, Whitmarsh 2007). Therefore, they have a crucial involvement in the pathogenesis of a plethora of inflammatory and malignant diseases. There are four main families of MAPKs in mammals: the extracellular signal regulated kinases (ERKs), Big MAP kinase-1 (BMK-1), cJun N-terminal kinases (JNKs) and p38 MAPKs. The highly conserved MAPK signaling cascade usually comprises three protein kinase tiers; the upstream MAP3K, the intermediate MAP2K and the effector downstream kinase MAPK (Burotto, Chiou et al. 2014). Each kinase family activates through phosphorylation the next kinase family (Figure 1) (Wang and Tournier 2006, Krishna and Narang 2008, Pritchard and Hayward 2013).

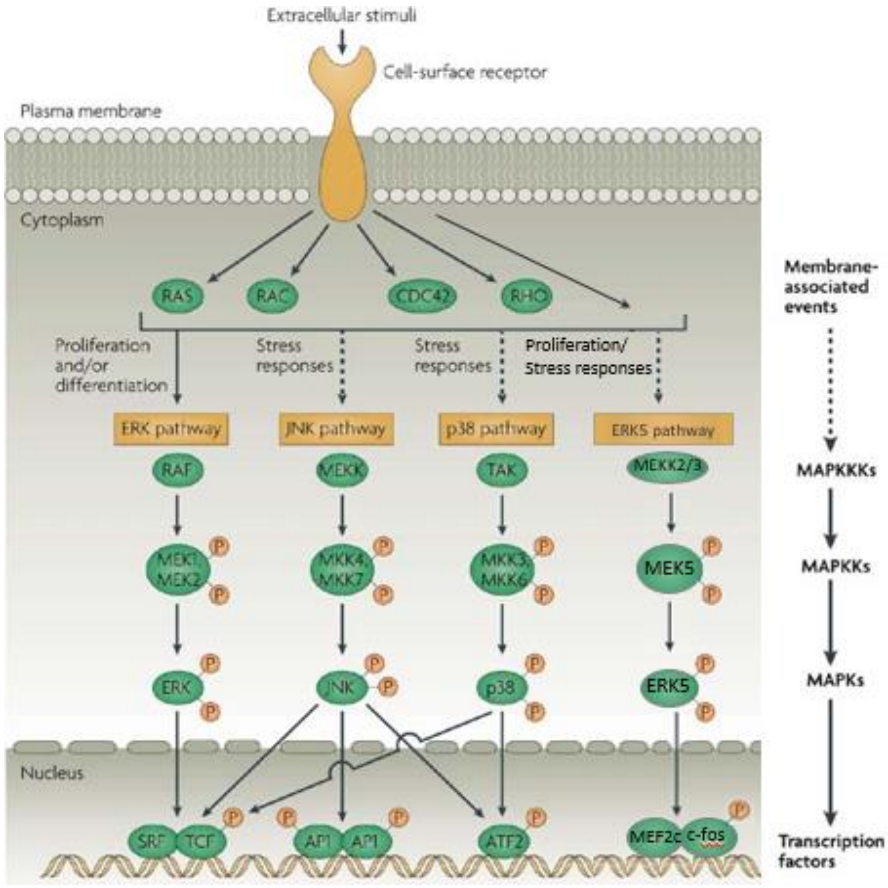



**Figure 1**: The typical MAPK signaling pathways. Extracellular stimuli activate the MAPK pathways through GTPases-mediated mechanisms (ie.RAS, RAC, CDC42 (cell-division cycle 42) and RHO (RAS homologue)). Once activated, MAP3Ks phosphorylate MAP2Ks on two serine residues. MAP2Ks in turn phosphorylate the MAPKs on both threonine and tyrosine residues, resulting in the catalytic activation of these MAPKs. Activated MAPKs can translocate to the nucleus to phosphorylate a number of transcription factors, thus altering gene transcription. (Image adapted from: Liu, Shepherd et al. 2007)

More specifically, activation of MAPKs by MAP2Ks and MAP2Ks by MAP3Ks requires a dual specific phosphorylation on serine/tyrosine residues and on serine/threonine residues respectively (Vougioukalaki, Kanellis et al. 2011). The increasing abundance of each kinase in this cascade allows for the amplification of the initial signal at each kinase tier (Sturm, Orton et al. 2010).

## Structure-Based Activation Mechanism of Tumor Progression Locus 2 (TPL2/Cot/MAP3K8)

Tumor Progression Locus 2 (TPL2), also known as COT or MAP3K8, was independently discovered by three different laboratories at the onset of the 90s. First, Miyoshi *et al* identified *Cot* (Cancer Osaka Thyroid) as a novel oncogene, through DNA isolation from a human thyroid carcinoma cell line (Miyoshi, Higashi et al. 1991). The rat homolog was subsequently cloned as a proviral integration locus of Moloney Murine Leukemia (MoMuL) virus in murine T-cell lymphoma cells (Patriotis, Makris et al. 1993). TPL2 is a member of the MAP3K family and plays a major role in the activation of the ERK MAPK through direct phosphorylation of MEK, the ERK kinase (Figure 1) (Eliopoulos, Wang et al. 2003). It conveys various intra- and extra-cellular stimuli to effector proteins of cells provoking adequate adoptive responses. The 36 Kbp long *tpl2* gene is located on chromosome 10p11.23 and comprises 13 exons. It encodes for two protein isoforms of 58 (TPL2 long; Tpl2L) and 52 kDa (TPL2 short; Tpl2S), due to alternative translational initiation at methionine 1 or methionine 30. The encoded protein contains a serine/threonine kinase domain, a 29 amino acid long N-terminal region with unknown function, and a C-terminal region that carries a "degron" sequence (amino acids 435–457), which is important for TPL2 stability and regulation of catalytic activity (Figure 2). C-terminal truncations of TPL2 (TPL-2ΔC) activate the kinase's transforming potential via two mechanisms. First, C-terminal removal elevates the specific kinase activity of TPL2. Although the exact mechanism is not fully elucidated yet, it has been suggested that the C terminus possibly modulates TPL2 catalytic activity by folding back onto the kinase domain (Ceci, Patriotis et al. 1997, Gandara, Lopez et al. 2003). Second, C-terminal truncation removes the "degron" sequence, thus reducing proteasomal degradation of TPL2 (Patriotis, Makris

et al. 1993, Watford, Hissong et al. 2008, Vougioukalaki, Kanellis et al. 2011). Consequently, TPL-2ΔC is expressed at higher levels in cells than TPL2. In comparison to wild-type TPL2, expression of TPL-2ΔC is associated with increased catalytic activity and broader signaling capacity (Ceci, Patriotis et al. 1997). Moreover, the carboxy-terminal tail of TPL2 carries phosphorylation sites critical for TPL2 activation of ERK-1/2 (Robinson, Beinke et al. 2007, Roget, Ben-Addi et al. 2012, Ben-Addi, Mambole-Dema et al. 2014) and is one of the major binding sites for the NF-κB precursor protein p105 and A20-binding inhibitor of NF-κB-2 (ABIN2) (Gantke, Sriskantharajah et al. 2011).

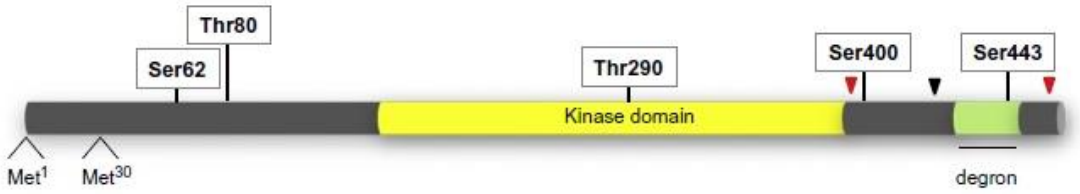



**Figure 2**: Schematic representation of the TPL2 primary structure. Two TPL2 isoforms are generated due to alternative translational initiation at methionine 1 or methionine 30. The TPL2 kinase domain, located in the center of the protein, is flanked by N-terminal and C-terminal regions. Truncation of the C-terminal results in a protein (TPL-2ΔC) with increased activity (Ceci, Patriotis et al. 1997). A "degron" sequence (435-457) found within the C terminus, confers destabilizing properties to full-length TPL2 (Gandara, Lopez et al. 2003). Consequently, TPL-2ΔC has higher expression levels and increased protein stability. Phosphorylation sites T290 and S400 regulate TPL2 MEK kinase activity in vivo. T290 phosphorylation possibly regulates TPL2 release from p105.
(Image source: Vougioukalaki, Kanellis et al. 2011)

TPL2 controls the MEK/ERK pathway in a cell-type- and stimulus-specific manner. In the absence of any stimulus, the entire pool of TPL2 is confined to a cytoplasmic complex, comprising the nuclear factor-kB subunit precursor NF-kB1/p105, and ABIN-2 (Figure 3). Apart from stabilizing TPL2 by masking the "degron" sequence, this interaction also prevents TPL2 and NF-κB from activating their downstream signaling cascades by inhibiting the kinase activity of TPL2 and the proteolysis of NF-κB precursor protein p105 (Belich, Salmeron et al. 1999, Beinke, Deka et al. 2003, Waterfield, Zhang et al. 2003). Upon stimulation, IKKβ phosphorylates p105/NF-κB1 at sites S927 and S932, leading to K48-linked ubiquitination of p105 NF-κB1 by SCF-βTrCP and triggering its complete degradation by the 26S proteasome (Beinke, Robinson et al. 2004, Waterfield, Jin et al. 2004). Although essential, IKKβ activation is not *per se* adequate to stimulate TPL2 activity. Prior to its dissociation from the

complex, TPL2 gets phosphorylated at multiple sites, two of which -threonine 290 (T290) and serine 400 (S400)- are required for full catalytic activity and subsequent MEK phosphorylation. There are two alternative scenarios regarding these phosphorylations; either they are mediated by IKKβ-dependent signals (Cho, Melnick et al. 2005), or they are an autophosphorylation event (Rousseau, Papoutsopoulou et al. 2008, Mieulet, Yan et al. 2010). The S400 phosphorylation is considered to promote kinase activation by inducing a conformational change which abolishes the inhibitory intermolecular interaction between the C-terminal tail and the kinase domain of TPL2 (Robinson, Beinke et al. 2007). S62 autophosphorylates after IL-1 stimulation and has been proposed to contribute to maximum TPL2 activation (Stafford, Morrice et al. 2006). A recent study came to the conclusion that TPL2 S400 phosphorylation by IKKβ and TPL2 S443 autophosphorylation synergize to elicit interaction of TPL2 with 14-3-3. Recruitment of 14-3-3 to the phosphorylated C-terminus stimulated TPL2 kinase activity towards MEK1 and was crucial for LPS-induced production of TNFα by macrophages, which is regulated by TPL2 independently of ERK-1/2 activation. This study gave a novel mechanistic insight into the activation of TPL2 signaling and elucidated how C-terminal deletion generates the oncogenic potential of TPL2 by rendering its kinase activity independent of 14-3-3 binding (Ben-Addi, Mambole-Dema et al. 2014). Although active towards MEK1, TPL2 is unstable and therefore undergoes rapid proteasomal degradation, thus restricting prolonged activation of ERK signaling (Waterfield, Zhang et al. 2003). As far as ABIN-2 is concerned, studies have shown its importance for stabilizing, but not activating TPL2 (Beinke, Deka et al. 2003, Papoutsopoulou, Symons et al. 2006).

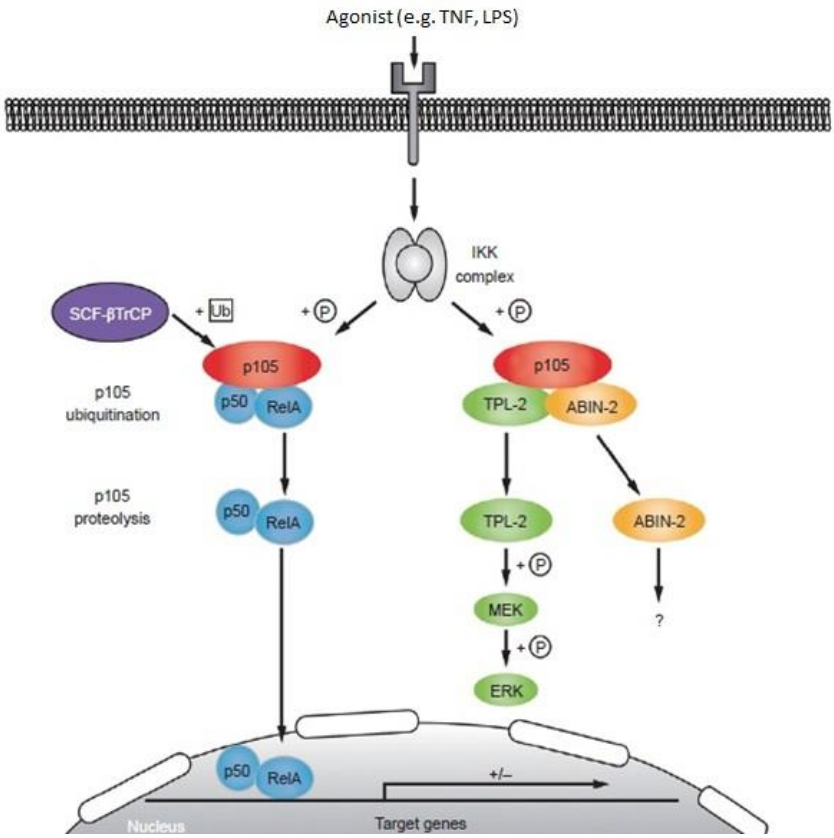



**Figure 3**: TPL2 regulation through IKK-induced p105 proteolysis. In the absence of any stimulus, TPL2 is confined to a cytoplasmic complex, comprising the nuclear factor-kB subunit precursor NF-kB1/p105, and ABIN-2, which inhibits its kinase activity. Upon stimulation, TPL2 is released from this complex and is phosphorylated at multiple sites, two of which -T290 and S400- are required for full catalytic activity and subsequent MEK phosphorylation. (Image source: Gantke et al. 2011)

## TPL2-mediated signal transduction

Apart from ERK1/2, its direct substrate, TPL2 can also activate –though to a lesser extent- JNK, p38, and ERK5 by directly phosphorylating their upstream MAP2Ks; MKK4, MKK6, and MEK5, respectively (Salmeron, Ahmad et al. 1996, Ceci, Patriotis et al. 1997, Tsatsanis, Patriotis et al. 1998, Tsatsanis, Patriotis et al. 1998, Lin, Cunningham et al. 1999). In case of UVB-induced DNA damage, TPL2 translocates to the nucleus where it then phosphorylates histone H3 at S10 increasing the transcriptional activity of c-Fos (Choi, Kang et al. 2008). When overexpressed in EGF-stimulated cells, TPL2 mediates the interaction between Protein Phosphatase 2A (PP2A) and p53, thereby inhibiting the phosphorylation of p53 at Ser15, a step of major importance for its stabilization and transcriptional activity (Khanal, Lee et al.

2009). The aforementioned signaling properties are consistent with the functional role of TPL2 in stimulating cell proliferation and transformation.

Endogenous TPL2 is inactive. However, overexpression of both wild type TPL2 and the oncogenic TPL2-ΔC renders them constitutively active in a variety of cell types (Ohara, Hirota et al. 1995, Sugimoto, Ohata et al. 2004, Kaiser, Cook et al. 2009, Khanal, Lee et al. 2009). Subsequently, TPL2 influences cell survival, proliferation, and transformation by activating multiple signaling pathways in concert with other signaling molecules, such as MAPK (Patriotis, Makris et al. 1994, Salmeron, Ahmad et al. 1996, Chiariello, Marinissen et al. 2000), nuclear factor-activated T cells (NFAT), and NF-κB (Tsatsanis, Patriotis et al. 1998, Tsatsanis, Patriotis et al. 1998, Belich, Salmeron et al. 1999, Lin, Cunningham et al. 1999). However, this widespread impact of overexpressed TPL2 may, at least in part, be due to overexpression artifacts, which needed to be further confirmed by targeted knockout studies for each downstream signaling component. The Tsichlis laboratory shed light to this possibility through the generation of a *tpl2*$^{-/-}$ mouse strain. These mice show no overt phenotype, are of normal size and weight and have a normal lifespan under pathogen-free conditions (Dumitru, Ceci et al. 2000). The development of immune cells also takes place normally. Remarkably, in contrast to the results obtained with TPL2 overexpression in T-cell lines, *tpl2*$^{-/-}$ CD4+ T cells produce similar amounts of IL-2 to WT cells after TCR stimulation, suggesting that TPL2 is not crucial for the regulation of IL-2 production (Dumitru, Ceci et al. 2000, Sriskantharajah, Belich et al. 2009). This was also the case with NF-κB activation and proteolysis of p105 after LPS treatment of *tpl2*$^{-/-}$ macrophages, indicating that TPL2 is not important for TLR4 activation of NF-κB. However, since TPL2 is only associated with a small fraction of total cellular p105, it is possible that the kinase regulates the proteolysis of this p105 pool, thus contributing to only a fraction of total NF-κB activity.

Apart from ERK, TPL2 also contributes to JNK activation in TNF-stimulated fibroblasts and is necessary for NF-κB transactivation by mediating the MSK1 dependent phosphorylation of the RelA (p65) NF-κB subunit at Ser276 (Das, Cho et al. 2005). A recent study conducted by our laboratory elegantly showed that TPL2 may also operate in the nucleus as a physical and functional partner of nucleophosmin (NPM/B23), a key nucleolar phosphoprotein that affects p53 response to DNA damage

and nucleolar stress, cellular activities that are both linked to malignancy. TPL2 mediates the phosphorylation of a fraction of NPM at Thr199, an event required for its proteasomal degradation and maintenance of steady state NPM levels underlying the TPL2 anti-tumor properties (Kanellis, Bursac et al. 2015).

## The involvement of TPL2 kinase in Cancer

The role of TPL2 in carcinogenesis remains unclear as both positive and negative attributes have been described (Table 1). It is possible that TPL2 has multiple roles (i.e. suppressing carcinogenesis, but playing a positive role in tumor growth or response to therapy) in a tissue-dependent manner.

### TPL2 as a Tumor-Promoting Oncogene

TPL2 was initially identified as a proto-oncogene activated by truncation of the C-terminal tail in murine models. Indeed, transgenic mice expressing the truncated TPL2 under the control of a T cell-specific promoter develop T-cell lymphoblastic lymphomas within 3 months whereas expression of wild-type TPL2 did not result in pathological phenotype (Ceci, Patriotis et al. 1997). Increased levels of TPL2 expression have been reported in a plethora of malignancies, including gastric colon adenocarcinomas (Ohara, Hirota et al. 1995), T-cell neoplasias (Christoforidou, Papadaki et al. 2004), Hodgkin's lymphoma, Epstein-Barr virus (EBV)-related nasopharyngeal carcinoma, and other virus-related tumors (Eliopoulos, Davies et al. 2002). Up-regulation of TPL2 expression has also been reported in approximately 40% of human breast cancers, thus indicating that increased copies of the *tpl2* gene could be a possible mechanism for TPL2 over-expression (Sourvinos, Tsatsanis et al. 1999, Krcova, Ehrmann et al. 2008). A recent study conducted by Choi's group provided robust evidence of direct interaction between TPL2 and Pin1 (Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1) which induces its phosphorylation of Ser16. This event results in cyclin D1 up-regulation and breast cancer onset (Kim, Khanal et al. 2015). Work by Kim *et al* elucidates the implication of the MEK-ERK, JNK, and STAT3 (signal transducer and activator of transcription 3) signaling pathways in breast tumor development. First, the research group revealed that TPL2 gets phosphorylated by IL-

22 through IL-22R1, activates the MEK-ERK, JNK, and STAT3 signaling pathways, and results in epithelial-mesenchymal transition (EMT) through AP-1 in breast cancer cells (Kim, Kim et al. 2014). Their second study proposes that the IL-33/ST2/TPL2 cascade induces the activation of the MEK-ERK, JNK-cJun and STAT3 signaling pathways leading to increase of AP-1 and STAT3 transcriptional activity which in turn direct IL-33-induced EMT and support cancer-associated inflammation in the tumor microenvironment (Kim, Lim et al. 2015). Recently, Gruosso *et al*, reported that pro-tumorigenic properties of TPL2 are mainly mediated by the MEK/ERK/p90RSK pathway. This group demonstrated accumulation of TPL2 in high-grade serous ovarian carcinomas (HGSCs), thus rendering it a potential prognostic marker for these tumors, as well as a reliable predictive value for the effectiveness of MEK inhibitor treatment, a promising new therapeutic approach for HGSC patients (Gruosso, Garnier et al. 2015). TPL2 overexpression has also been reported in some cases of invasive endometrioid carcinoma (Aparecida Alves, Silva et al. 2006). Pancreatic adenocarcinoma upregulated factor (PAUF), which is aTLR2 and TLR4 endogenous ligand, stimulates the TLR2-mediated TPL2-MEK-ERK signaling pathway ending up in an increase of pro-tumorigenic cytokines expression. At the same time, it blocks TLR-mediated NF-κB signaling, thereby enabling tumor growth and escape from immunosurveillance (Park, Lee et al. 2011). An association between aberrant TPL2 expression and recurrence of papillary thyroid cancer has recently been confirmed by Lee *et al*. The results obtained by the group's experiments showed a positive correlation between increased TPL2 expression levels and higher recurrence rates, as well as old age at initial diagnosis (Lee, Jeong et al. 2015).

In the field of inflammation-driven carcinogenesis, emerging data point towards a positive regulation of the inflammatory milieu of myeloma niche through a TLR2/6-dependent TPL2 pathway. The aforementioned regulation is achieved by promoting the "inflammatory switch" of macrophages in myeloma-associated monocytes/macrophages (MAM) (Hope, Ollar et al. 2014). The potential impact of TPL2 on hepatocellular carcinoma (HCC) development remains unclear. This gap was partially filled by Li *et al*, who demonstrated that TPL2 promotes diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) in mice through its pro-inflammatory effect, suggesting that TPL2 could be regarded as a molecular target for HCC

prevention (Li, Liu et al. 2015). Chronic prostatic inflammation is a major triggering event for prostate cancer. A possible involvement of the TPL2 signaling pathway in the development of prostate cancer has been proposed. This conclusion is derived from the fact that upregulation of TPL2 was found to drive androgen depletion-independent (ADI) prostate cancer growth through activation of the MEK/ERK and NF-κB signaling pathways (Jeong, Bhatia et al. 2011).

Apart from carcinogenesis, TPL2 also has an impact on tumor metastasis. The underlying mechanism seems to involve proteinase-activated receptor 1 (PAR1) signals transduced by TPL2 which then regulate the expression of matrix metalloproteinases (MMPs). These signals in turn activate Rac1 and focal adhesion kinase (FAK) -which are required for stimulation of cell migration (Nierodzik and Karpatkin 2006)- and other secreted molecules both in fibroblasts and tumor cells, thus promoting transformation, tumor cell invasion, and angiogenesis (Hatziapostolou, Polytarchou et al. 2008). In renal cell carcinoma (RCC), the most common kidney tumor with innate high metastatic ability, TPL2 mRNA levels are significantly increased in ccRCC compared with normal kidneys, strongly implying an association between the kinase and the metastasis and poor prognosis through activation of MAPK signaling and cross talk with the CXCL12-CXCR4-directed chemotaxis and chemoinvasion (Lee, Joo et al. 2013). CXCR4 and FAK upregulation by TPL2 and subsequent activation of CXCL12/CXCR4 and the FAK/Akt signaling pathway is also a fundamental event that provokes progression and metastasis of castration resistant prostate cancer (CRPC), the major lethal pathway of prostate cancer (Lee, Cho et al. 2015).

Angiogenic factors such as vascular endothelial growth factor (VEGF) and CXCL1 are implicated in the process of peritoneal dissemination of cancer cells by amplifying phosphorylation and kinase activity of TPL2 in a dose- and time-dependent manner. Hence, development of TPL2 inhibitors could potentially lead to novel anti-angiogenic and anti-metastatic treatment approaches (Lee, Lan et al. 2013).

## TPL2 as a Tumor-Suppressor Gene

Whereas the aforementioned studies strongly imply a positive association between TPL2 expression and tumor growth, under certain conditions the kinase may actually act in a tumor suppressing way. Indeed, in *tpl2*$^{-/-}$ mice bred onto a MHC Class I-restricted T-cell antigen receptor (TCR) transgenic background, a defective ERK-dependent CTLA4 induction renders CD8$^{+}$ T-lymphocytes hyperproliferative and eventually leads to development of T-cell lymphomas. This observation clearly suggests that TPL2 is of vital importance for full ERK activation in response to TCR signals (Tsatsanis, Vaporidi et al. 2008). Furthermore, in a chemically induced mouse skin carcinogenesis model, *tpl2*$^{-/-}$ mice exhibited notably higher incidence of tumors (80%) compared to WT mice (17%), as well as a reduced tumor latency and a notably higher number of total tumors. The *tpl2*$^{-/-}$ was also linked to a proinflammatory effect, due to elevated activity of the NF-kappaB pathway (Decicco-Skinner, Trovato et al. 2011). This finding is further substantiated by a study conducted in human melanomas, which associated TPL2 with de novo resistance to RAF inhibitors in B-RAF (V600E) established cell lines and acquired resistance in melanoma cells and tissue (Johannessen, Boehm et al. 2010). More recently, gene expression profiling between WT and *tpl2*$^{-/-}$ keratinocytes revealed a considerable down-regulation in the MMP inhibitor Timp3 in *tpl2*$^{-/-}$ mice, along with up-regulated activity of MMPs that stimulate the migration and invasion of cancer cells (Decicco-Skinner, Jung et al. 2013).

Another recent addition to the already existing knowledge is the suggestion that TPL2 may also influence p53-dependent functions. Indeed, a study from our lab validated that, in lung epithelial cells, TPL2 thwarts oncogene-induced cell transformation and survival through JNK dependent up-regulation of nucleophosmin (NPM), which is required for the optimal p53 response to genotoxic stress (Gkirtzimanaki, Gkouskou et al. 2013). In line with this finding, low TPL2 levels are clearly linked with meager lung cancer patient survival, earlier onset as well as a wide range of urethane-induced lung tumors in mice (Gkirtzimanaki, Gkouskou et al. 2013).

## TPL2 in Colon Cancer

Colorectal cancer (CRC) is the third most common cancer worldwide and the second most common in developed countries. According to the World Health

Organization (WHO), it accounts for roughly 1 million new cases and 600000 deaths per year.

In an attempt to address the role of TPL2 in colon tumorigenesis, *tpl2*$^{-/-}$ mice were bred into an *apc*$^{min/+}$ genetic background. Notably, TPL2 ablation was associated with a 5-fold increase in the number of intestinal adenomas. This effect was attributed to the impeded IL-10 secretion and regulatory T cells (Tregs) generation in the intestinal mucosa of *tpl2*$^{-/-}$ mice. Both of these events promote inflammation and tumorigenesis in the context of the *apc*$^{min}$ mutation. Even though adenomas and inflammation increased gradually with time in both *apc*$^{min/+}$*/tpl2*$^{+/+}$ *and apc*$^{min/+}$*/tpl2*$^{-/-}$ mice, the rate of increase was persistently higher in the *tpl2*$^{-/-}$ mice, pointing towards a plausible positive feedback mechanism through which TPL2 depletion mediates the acceleration of polyposis (Serebrennikova, Tsatsanis et al. 2012).

In order to investigate the involvement of TPL2 in intestinal homeostasis and inflammatory bowel disease (IBD) pathogenesis the DSS-induced colitis model was used. Remarkably, *tpl2*$^{-/-}$ mice were more susceptible to DSS-induced colitis and showed throughout the protocol significantly increased tissue damage histological score as reflected in loss of crypts and ulceration, but similar inflammation scores compared with WT mice. Inspection of immune and cytokine secretion pathways, in which TPL2 has been demonstrated to be involved *in vivo,* revealed no divergence in the secretion pattern in the lamina propria of *tpl2*$^{-/-}$ and WT mice. When the epithelium is injured, intestinal myofibroblasts (IMFs) sense the inflammatory signals and activate the COX-2/PGE2 signaling pathway through TPL2 (Roulis, Nikolaou et al. 2014).

Another study addressed the role of TPL2 in the azoxymethane (AOM)/dextran sodium sulfate (DSS) animal model of chronic colitis-associated cancer (CAC) using complete and tissue-specific ablation of TPL2 in mutant mice. TPL2 knock-down led to a wider multiplicity in the number and size of tumors. This finding paved the way for proposing a novel IMF-specific role for TPL2 in the upregulation of HGF production, which eventually enhances epithelial proliferation and decreases apoptosis and the prevention of epithelial tumors in CAC (Koliaraki, Roulis et al. 2012).

**Table 1**: The contradictory roles of TPL2 in cancer

| Tumor-promoting | Tumor-suppressive |
|---|---|
| 1. TPL-2ΔC expression promotes T-cell lymphoblastic lymphomas development<br>2. Elevated TPL2 expression has been demonstrated in a plethora of malignancies (i.e. gastric colon adenocarcinomas, Hodgkin's lymphoma, EBV-related nasopharyngeal carcinoma and other virus-related tumors<br>3. In breast cancer TPL2 promotes tumorigenesis and cancer progression via Pin1 phosphorylation, leading to cyclin D1 upregulation and induces EMT through IL-22/MEK/ERK and JNK/STA3/AP-1 signaling pathways<br>4. In high-grade serous ovarian carcinomas the tumorigenic properties of TPL2 are mediated by the MEK/ERK/p90RSK pathway<br>5. TPL2 facilitates tumor growth by PAUF-mediated MEK-ERK signaling power<br>6. TPL2 transduces PAR1 signals to regulate the expression of MMPs in fibroblasts and tumor cells, thus promoting cell migration and actin reorganization<br>7. TPL2 overexpression has been reported in cases of endometrioid carcinoma<br>8. Aberrant TPL2 expression has been associated with recurrence of papillary thyroid cancer<br>9. There is positive regulation of the inflammatory milieu of myeloma niche through a TLR2/TLR6-dependent TPL2 pathway<br>10. TPL2 promotes hepatocellular carcinoma through its proinflammatory effect<br>11. TPL2 upregulation drives ADI prostate cancer growth through activation of MEK/ERK and NFκB pathways<br>12. TPL2 contributes to ccRCC progression through crosstalk with CXCL12/CXCR4-directed chemotaxis and chemoinvasion<br>13. TPL2 enhances CRPC progression and metastasis via activation of FAK/Akt and CXCL12/CXCR4 signaling pathways<br>14. VEGF and CXCL1 amplify kinase activity and phosphorylation of TPL2, mediating the process of peritoneal dissemination of cancer cells | 1. Lack of TPL2 renders $CD8^+$ T-lymphocytes hyperproliferative and leads to T-cell lymphomas development via defective ERK-dependent CTLA4 induction<br>2. TPL2 ablation enhances tumor initiation and progression in a mouse skin cancer model via upregulated NF-κB signaling and MMP activity<br>3. TPL2 antagonizes cell transformation and survival via JNK-dependent upregulation of NPM required for optimal p53 response to stress in mice lung tumors<br>4. Low TPL2 levels are also linked to meager lung patient survival and earlier disease onset<br>5. TPL2 ablation is associated with a 5-fold increase in the number of intestinal adenomas, due to impeded IL-10 secretion and Tregs generation<br>6. *tpl2*$^{-/-}$ mice are more susceptible to DSS-induced colitis and show increased tissue damage histological score<br>7. In case of epithelium injury IMFs sense the inflammatory signals and activate the COX-2/PGE2 signaling pathway through TPL2 |

# MATERIALS AND METHODS

## Cell culture and treatments

HT-29 and HCT-15 cells were cultured in RPMI 1640. RKO, HCT-16 and Caco-2 cells were cultured in DMEM (4.5 g/L D-glucose). LS-174, SW403 and LoVo cells were cultured in MEM-alpha (+ fatty acids), DMEM (1 g/L D-glucose) and Ham's F-12 (+1.5 g/L sodium bicarbonate). All culture media were purchased by Gibco, Life Technologies and supplemented with 10% FBS.

Cells were treated with DMSO (Applichem) as vehicle control, 1, 5, 10 and 25μM of TPL2 kinase inhibitor (Calbiochem, Merck-Millipore) for the indicated timepoints. In another set of experiments, cells were treated with 10μM of TPL2 kinase inhibitor (Calbiochem, Merck-Millipore), or 10μM of UO126 (Calbiochem, Merck-Millipore) 30min before stimulation with 100ng/ml of LPS for various time points.

## Clonogenic Assay

### Materials:

Cell culture medium

Phosphate buffered saline (PBS)

Fetal bovine serum (FBS)

Trypsin/ EDTA (25200-056, Life Technologies, Invitrogen™)

Crystal violet (C3886, Sigma-Aldrich)

Methanol (34860, Sigma-Aldrich)

Glacial acetic acid (320099, Sigma-Aldrich)

Colony fixation solution (Glacial acetic acid/methanol 1:7 vol/vol)

Crystal violet solution (0.5%)

### Method:

#### *Treatment and plating:*

In a 12-well plate, $4x10^4$ cells were seeded per well, in triplicates and were incubated for 24 hours in a $CO_2$ incubator at 37°C to allow them to attach to the plate. The cells were then treated with Tpl2 inhibitor (5μM and 10μM) and DMSO for 72

hours. After the indicated time point, they were harvested and 500 cells were seeded per dish and cultured for 10-15 days, until cells in control plates had formed colonies that were of a substantially good size (50 cells per colony is the minimum for scoring).

*Fixation and staining:*

Cells were washed with PBS 1X and fixed with 2-3 ml of fixation solution for 5 min, RT. The fixation solution was then removed and 0.5% crystal violet solution was added for 20 minutes, RT. After careful removal of the crystal violet, the dishes were immersed in tap water to wash and let air-dry.

*Data analysis:*

To analyze the data, the number of colonies were counted under a stereomicroscope and the plating efficiency (PE) and surviving fraction (SF) were calculated according to the following formulas:

a. PE = no. of colonies formed/ no. of cells seeded x 100%
b. SF = no. of colonies formed after treatment/ no. of cells seeded x PE

## MTT Cell Proliferation Assay

### Materials:

MTT Formazan (Sigma-Aldrich, catalog number: M2003-1G)
Phosphate buffered saline (PBS)
Dimethyl Sulfoxide Reagent (Sigma-Aldrich, catalog number: D5879-1L)

### Method:

5000 cells per well were seeded in a 96 well plate, in triplicates, in 100µl final volume. Cells were incubated for 24 hours in a $CO_2$ incubator at 37°C to allow them to attach to the plate. The following day, cells were treated with Tpl2 inhibitor (1µM, 5µM & 10µM), in a final volume of 200 µl per well and incubated for up to 72hrs, at 37°C. At each timepoint, 22 µl of 5 mg/ml MTT solution were added aseptically to each well. Cells were incubated for 4 hours at 37°C in culture hood and then media was replaced by 200µl DMSO. The absorbance was read at 595 nm.

## siRNA Transfection

### Materials:

MAP3K8 siRNA (Ambion, catalog number: 4392420, ID: s3384 & s3385, stock 20µM)

Luci siRNA (Ambion, catalog number: AM16204, stock 20µM)

RIP siRNA (40µM)

Opti-MEM Reduced Serum Medium (Invitrogen, catalog number: 11058021)

### Method:

Day 0: Cells were seeded to be 60-80% confluent at transfection

Day 1: Lipofectamine® RNAiMAX Reagent and siRNAs were diluted in Opti-MEM® Medium. Diluted siRNAs were added to the diluted Lipofectamine® RNAiMAX Reagent (1:1 ratio) and mixed thoroughly. After 5 minutes of incubation, the siRNA-lipid complex was added to the cells dropwise and cells were incubated for 1-3 days at 37°C.

Day 2-4: Transfected cells were analyzed.

## Protein isolation

Following treatment, cells were lysed in RIPA buffer, containing 50mM Tris-HCl (pH 7.4), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors (Sigma- Aldrich, catalog number: S8830), 100mM $Na_3VO_4$ (Sigma-Aldrich) and 1mM NaF (Sigma-Aldrich). For Western blot analysis, levels of total proteins were determined using the BCA assay (Thermo-Scientific, catalog number: 23227).

## SDS-PAGE and Western blot analysis

### Materials:

**Separating gel (10%)**

7.9 ml $H_2O$

6.7 ml 30% Acrylamide mix

5ml 1,5M Tris-HCl (pH 8.8)

0.2 ml 10% SDS

0.2 ml APS 10%

0.008 ml TEMED

**Stacking gel**

4.1 ml $H_2O$

1 ml Acrylamide mix

0.75 ml 1M Tris-HCl (pH 6.8)

0.06 ml 10% SDS

0.06 ml APS 10%

0.006 ml TEMED

**Running buffer (TGS) 10X (pH 8.3), 1L**

30.3 gr Tris-Base

144.2 gr Glycine

10 gr SDS

**Transfer buffer, 1L**

100 ml 10X TGS

200 ml methanol

700ml $dH_2O$

**Loading buffer (5X)**

5 ml 1M Tris-HCl (pH 6.8)

4 ml Glycerol

1.6 gr SDS

8 gr bromophenol blue

1.6 ml β-mercaptoethanol

$dH_2O$ up to 20 ml

### Method:

An amount of 30μg protein per sample was subjected to SDS-PAGE. Protein samples were supplemented with the 5x loading buffer, and boiled at $100^0$C for 10 min in order to be denatured in their primary structure. Samples were centrifuged at 13000rpm for 1 min at 4ºC and loaded on 10% SDS polyacrylamide gel. The BenchMark Prestained Protein Ladder (10748010, Invitrogen) was used as size marker. Gel Electrophoresis was performed in 1X Running Buffer at 100-120Volts using Mini-

PROTEAN Tetra Cell System (Biorad). Following SDS-PAGE, proteins were transferred to nitrocellulose transfer membrane. The transfer procedure was performed using Mini-PROTEAN Tetra Cell System (Biorad) by electroblotting in 1L transfer buffer at 400mA for 90min. Membranes were blocked in 5% non-fat milk in TBS-Tween-20 (TBS-T) for 30min-1h. Incubation with the primary antibodies was performed according to the manufacturer's guidelines.

The following antibodies were used for immunoblotting: TPL2 (M-20, SC-720, Santa Cruz Biotechnology Inc.), pERK1/2 (CS4370, Cell Signaling Technology Inc.), ERK1/2 (CS4695, Cell Signaling Technology Inc.), RIP (SC-7881, Santa Cruz Biotechnology Inc.), β-actin (clone X-4, Millipore), GAPDH (G8795, Sigma Aldrich). Secondary anti-rabbit and anti-mouse HRP-conjugated antibodies were purchased by Sigma and used at a concentration of 1:15.000 in 5% non-fat milk in TBS-T. Membranes were incubated for 1h, RT. Enhanced Chemiluminesence ECL method (NEL104001EA, PerkinElmer, Life Sciences) was used for signal development. Protein bands were visualized on a ChemiDoc XRS+ imaging system (Biorad).

# RESULTS

## TPL2 protein levels of colon cancer cell lines do not correlate with their p53, BRAF and KRAS mutational status

After determining the p53, BRAF and KRAS mutational status of the available colon cancer cell lines through data mining (Table 2), we attempted to reveal a possible correlation with the TPL2 expression levels. Interestingly, although TPL2 expression levels varied significantly between the different cell lines, there was no association with their p53, BRAF or KRAS status (Figure 4).

**Table 2:** p53, BRAF and KRAS mutational status of colon cancer cell lines CaCo2, RKO, HCT116, HT29, LS174T, SW403, LoVo and HCT15.

| | p53 | BRAF | K-RAS |
|---|---|---|---|
| **Caco-2** | Mut (Glu204Xaa) | WT | WT |
| **RKO** | WT | Mut (Val600Glu) | WT |
| **HCT 116** | WT | WT | Mut (Gly13Asp) |
| **HT29** | Mut (Arg273His) | Mut (Val600Glu) | WT |
| **LS 174T** | WT | WT | Mut (Gly12Asp) |
| **SW403** | Mut (Glu51*) | WT | Mut (Gly12Val) |
| **LoVo** | WT | WT | Mut (Gly13Asp, Ala14Val) |
| **HCT15** | Mut (Ser241Phe) | WT | Mut (Gly13Asp) |

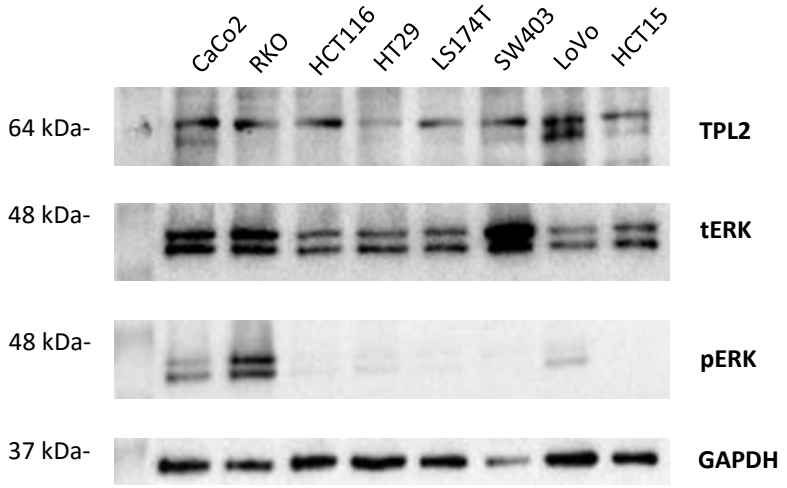



**Figure 4:** Western blot analysis, screening the TPL2, tERK and pERK protein levels of colon cancer cell lines CaCo2, RKO, HCT116, HT29, LS174T, SW403, LoVo and HCT15.

## Response of colon cancer cell lines to TPL2 inhibitor treatment

In order to address the role of TPL2 in cell proliferation, we treated the aforementioned cell lines with TPL2 inhibitor and assessed their proliferation rates at three different time points (24, 48 and 72hrs) using MTT assay (Figure 5). Notably, a significant difference in the response of each cell line was observed. Overall, the proliferation of RKO and CaCo2 was less affected (Figure 5, A and H). Conversely, TPL2 inhibitor almost completely thwarted the proliferation of HCT15 and HT29 cells (Figure 5, B and G). Therefore, they were selected for use in all future experiments. In all cases, the impact TPL2 inhibitor was evident only after the 48hr time point.

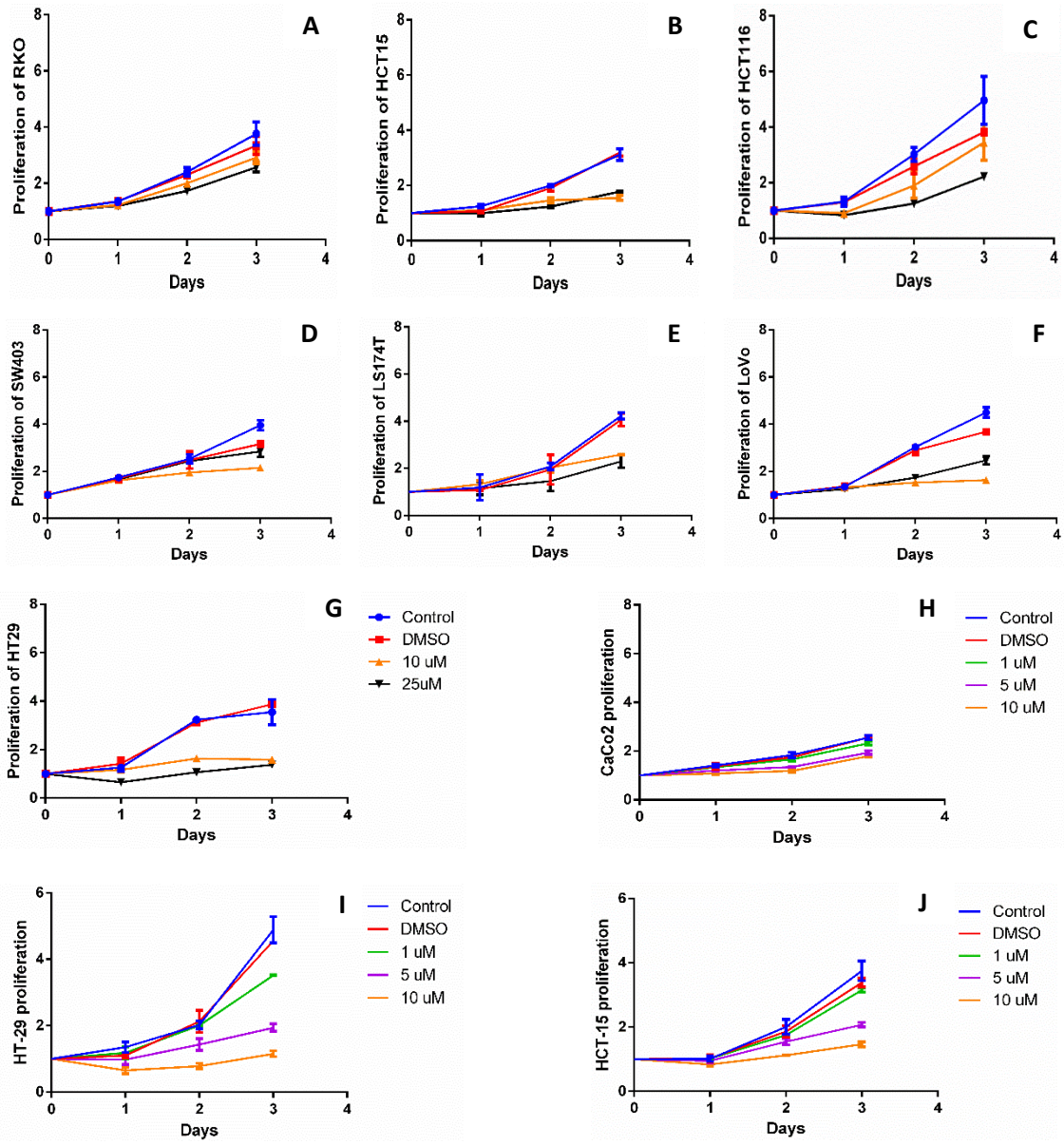


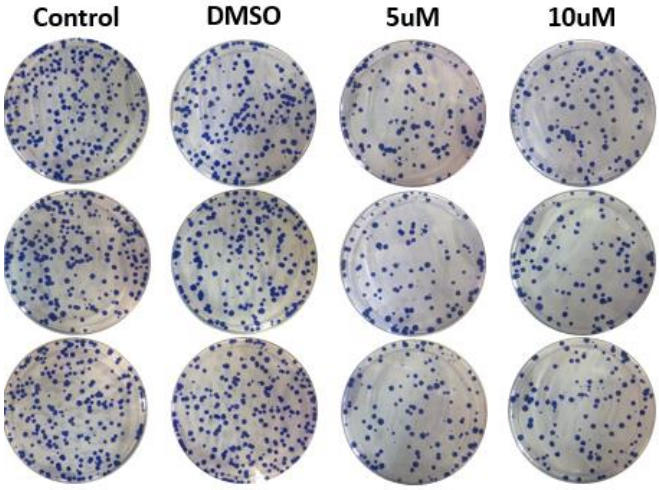

**Figure 5:** Response of colon cancer cell lines to TPL2 inhibitor treatment

As a next step, using only the HCT15 and HT29 cell lines, we evaluated the optimal concentration of TPL2 inhibitor to achieve its maximum effect and concluded that 10μM of inhibitor was able to consistently impede proliferation by roughly 80% (Figure 5, I and J).

## Treatment with TPL2 inhibitor affects the clonogenic capacity of HT29 cells

Cancer cells are generally characterized by increased clonogenicity, an attribute that has been correlated with their metastatic potential *in vivo*. We therefore decided to investigate whether cells that had undergone treatment with TPL2 inhibitor exhibited reduced clonogenic capacity.

Indeed, when treated with 5μM or 10μM of TPL2 inhibitor for 72hrs, the capacity of HT29 cells to form colonies was diminished by 50% (Figure 6).




Plating efficiency (PE) = no. of colonies formed/ no. of cells seeded x 100%

**Control: 48.6%** **DMSO: 42.2%** **5μM: 22.9%** **10μM: 22.9%**

**Figure 6:** TPL2 inhibitor treatment reduces the clonogenic capacity of colon cancer cells. HT29 cells that had undergone treatment with 5μM or 10μM of TPL2 inhibitor for 72hrs were seeded in 100mm$^2$ plates at 500 cells per well, and were stained with crystal violet 15–20 days later. Plates are representative of three independent experiments for each treatment condition.

## The effect of TPL2 inhibitor treatment as an add-on therapy to 5-FU

To date, the most widely used chemotherapeutic agent in colorectal cancer cases remains 5-FU. Despite their positive initial response, a significant percentage of patients eventually stop responding to treatment. Hence, we examined the possibility of a synergistic effect between 5-FU and TPL2 inhibitor. To address this hypothesis, we treated HT29 and HCT15 cells with various 5-FU and TPL2 concentrations, both alone and in combination, for a 72hr period and then performed MTT assay to measure their proliferation rates. Interestingly, administration of TPL2 inhibitor was not able to add to the effect already induced by 5-FU (Figure 7).

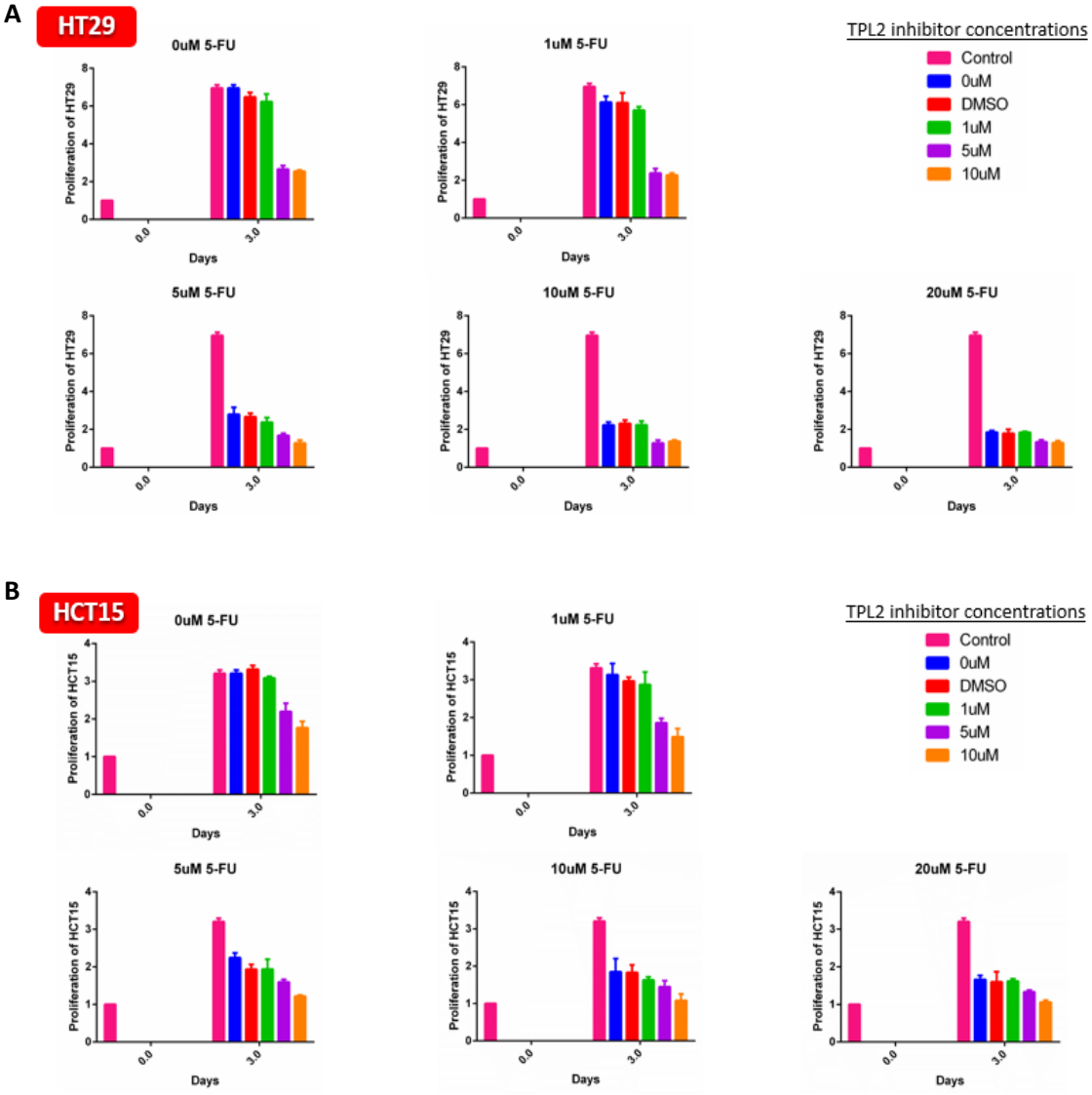



**Figure 7:** Treatment with TPL2 inhibitor has no synergistic effect with 5-FU. 0, 1, 5 and 10µM of TPL2 inhibitor were used as stand-alone or add on treatment with 1, 5, 10 and 20µM 5-FU in HT29 (A) and HCT15 (B) cells. After 72hrs, cell proliferation did not change significantly when the inhibitor was added to the standard 5-FU treatment.

## Response of colon cancer cell lines to MEK inhibitor treatment

It is well established that MEK is directly phosphorylated by TPL2, in a signaling cascade that leads to activation of ERK. To explore the impact of MEK/ERK signaling pathway in cell proliferation, cells were exposed to the MEK inhibitor UO126. Inhibition of MEK did not alter cell proliferation of neither HT29 nor HCT15 cells (Figure 8, A and B). Remarkably, cell lines that did not respond to TPL2 inhibitor treatment were not affected by MEK inhibition either (Figure 8, C and D).

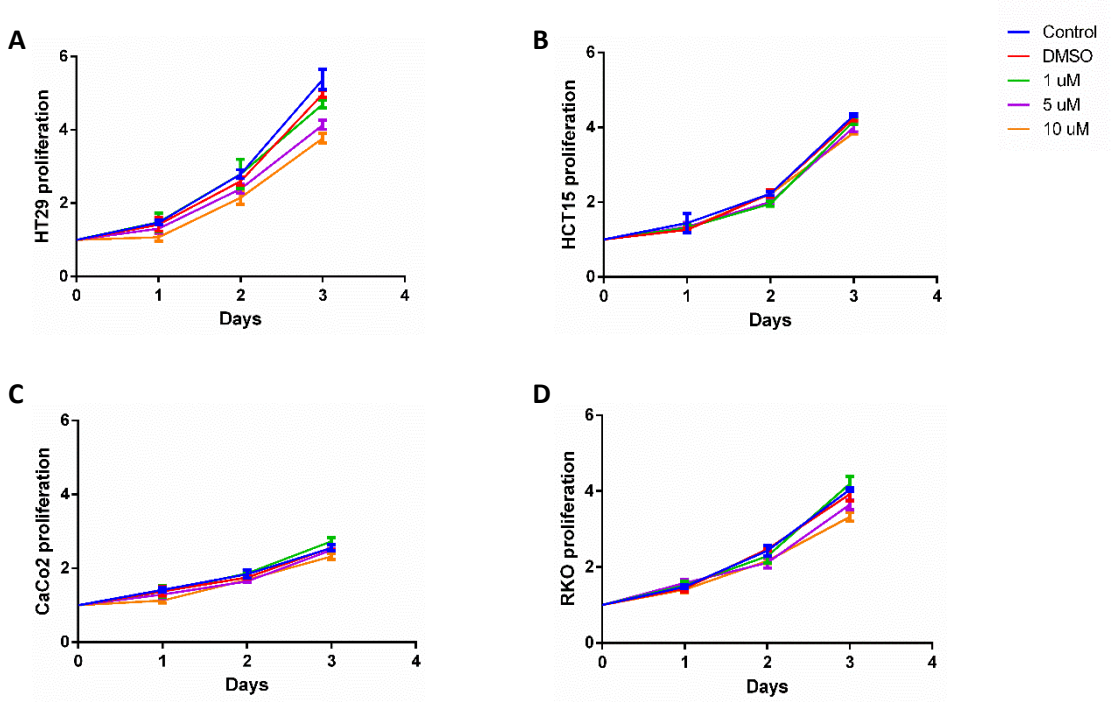



**Figure 8:** Cell proliferation is not affected by MEK inhibitor treatment. Cells were exposed to 1, 5 and 10µM of MEK inhibitor and cell proliferation was measured at three different time points (24, 48 and 72hrs) via MTT assay.

## ERK phosphorylation is regulated through a TPL2 independent mechanism

The fact that proliferation of the aforementioned cell lines was not affected by MEK inhibition, prompted us to further delineate the ERK activation mechanism. To assess this question, HT29 cells were exposed to the TPL2 inhibitor or the MEK inhibitor UO126 prior to LPS stimulation. Inhibition of MEK but not TPL2 completely impeded ERK phosphorylation compared to cells treated with LPS alone (Figure 9, A). This observation, coupled with the insignificant effect of TPL2 inhibitor on LPS-

mediated ERK phosphorylation (Figure 9, B), indicates the involvement of TPL2-independent pathways in ERK regulation.

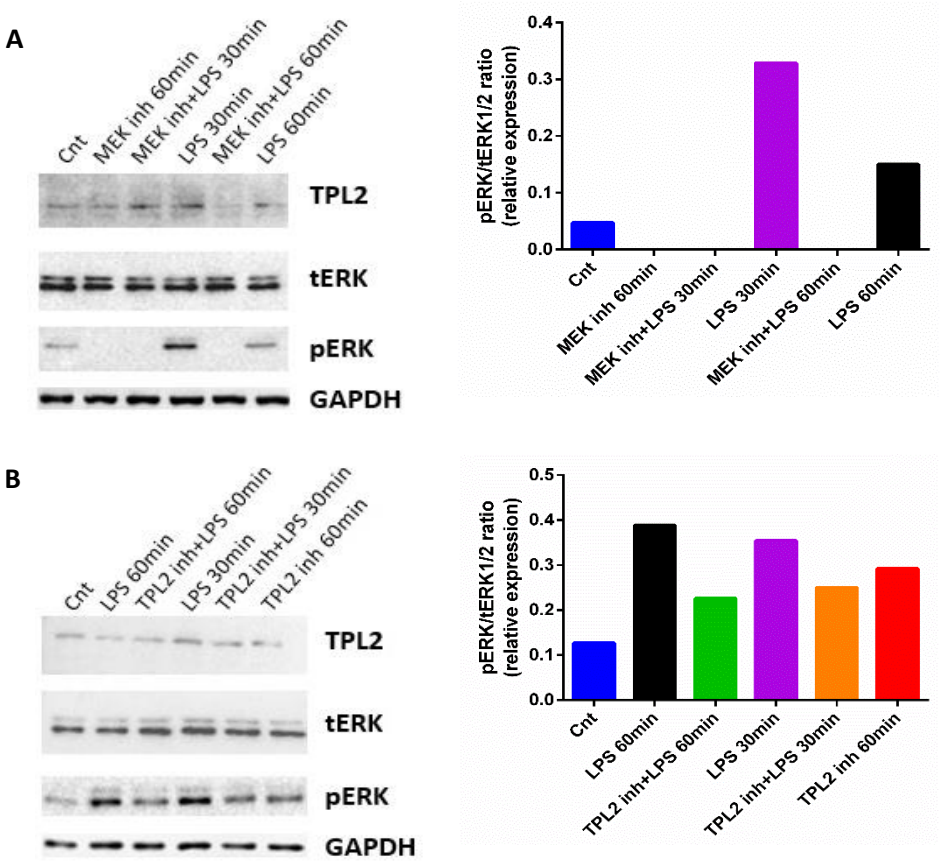



**Figure 9:** ERK phosphorylation is regulated through a TPL2 independent mechanism. HT29 cells were treated with TPL2 inhibitor, MEK inhibitor or vehicle control in the presence or absence of LPS for 30 and 60min. Protein lysates were collected and analyzed for TPL2, phosphorylated and total ERK1/2, with GAPDH serving as control for equal loading. Levels of pERK and tERK1/2 were quantified and pERK/ tERK1/2 ratio was calculated

## TPL2 knock down has no impact on the effect of TPL2 inhibitor treatment

To further substantiate the impact of TPL2 on cell proliferation, we proceeded to silencing *tpl2* gene expression in HT29 cells, which were then treated with TPL2 inhibitor. The effectiveness of the knock down was validated with western blot analysis (Figure 10, A). Contrary to our expectations, *tpl2* silencing did not alter the effect of TPL2 inhibitor treatment on cell proliferation compared to control (Figure 10, B and C). These data suggest that the TPL2 inhibitor may actually have secondary targets other that TPL2 kinase itself that are able to majorly affect cell proliferation.

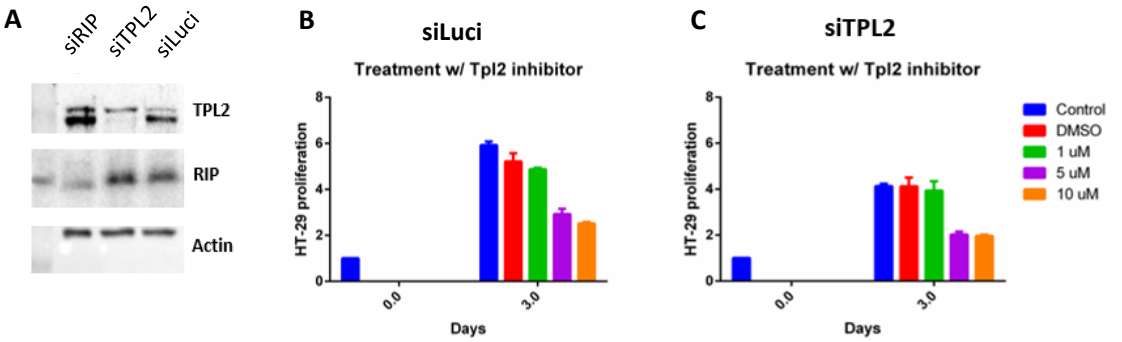



**Figure 10:** TPL2 knock down has no impact on the effect of TPL2 inhibitor treatment. siRNA was performed for the tpl2 gene (C), with siLuci serving as control (B). After validating the success of the knock down, silenced cells were treated with TPL2 inhibitor and their proliferation was measured via MTT assay.

## Generation of TPL2 inhibitor-resistant cell lines

At the prospect of elucidating the genes involved in resistance to TPL2 inhibitor treatment, we attempted to generate TPL2 inhibitor-resistant HT29 and HCT15 cell lines by submitting them to chronic treatment with increasing concentrations of TPL2 inhibitor for a two-month period of time. We then evaluate the progress made, by measuring cell proliferation after inhibitor treatment in both parental and treated cells (Figure 11). The results showed no improvement in cell proliferation of either cell line, indicating that treatment for a significantly longer period of time is probably required.

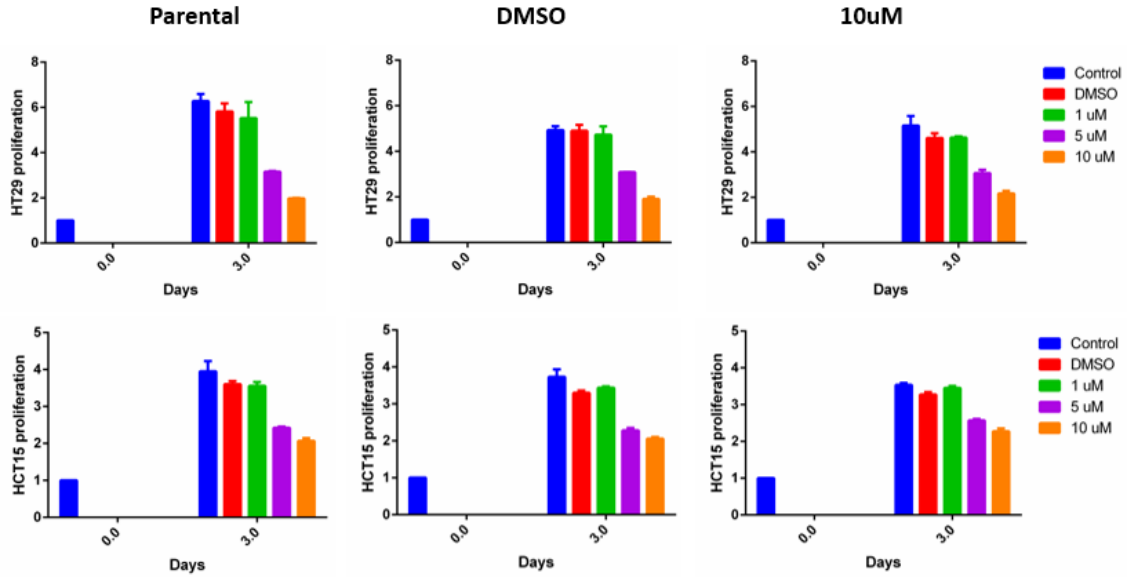



**Figure 11:** Generation of TPL2 inhibitor-resistant cell lines. HT29 and HCT15 cell lines were treated with 10μM TPL2 inhibitor over a 2-month period. Effectiveness of the treatment was evaluated with MTT assay.

# DISCUSSION

Colorectal cancer is the third most commonly diagnosed cancer in men and second most commonly diagnosed cancer in women worldwide. Its pathogenesis is the result of both genetic and epigenetic events that take place with tumor initiation and progression. Patient prognosis and individual response to therapy varies significantly in stage-independent manner, mainly due to molecular heterogeneity. In this regard, there is an ongoing attempt to identify specific biomarkers that will allow predicting a patient's clinical outcome beyond tumor staging, and facilitate a more effective therapeutic approach. Nowadays, next-generation sequencing allows for high throughput gene expression profiling that identified certain genes exhibiting mutations with important roles in colorectal cancer. TP53, APC, SMAD4, PIK3CA, BRAF and KRAS mutations were, as anticipated, among these genes (The Cancer Genome Atlas 2012). Based on these data, several groups have reported molecular classifications of colorectal cancer and in many cases these subtypes have been proven a valuable predictive (De Sousa E Melo, Wang et al. 2013, Sadanandam, Lyssiotis et al. 2013) or prognostic tool (Budinska, Popovici et al. 2013, De Sousa E Melo, Wang et al. 2013, Marisa, de Reyniès et al. 2013, Roepman, Schlicker et al. 2014).

In the setting of metastatic colorectal cancer, great progress has been made concerning personalized medicine. When used as an add on therapy to conventional cytotoxic chemotherapeutic agents, monoclonal antibodies targeting angiogenesis or the epidermal growth factor receptor (EGFR) signaling pathway have made a great impact on patient survival (Hurwitz , Fehrenbacher et al. 2004, Karapetis , Khambata-Ford et al. 2008, Douillard , Oliner et al. 2013, Heinemann, von Weikersthal et al. 2014). Conversely, the aforementioned monoclonal antibodies fail to improve outcome of non-metastatic patients (Alberts, Sargent et al. 2012, Allegra, Yothers et al. 2013, Taieb, Tabernero et al. 2014).

TPL2 kinase has a contradictory role in cancer with tumor-promoting or tumor-suppressing properties. Initially, the kinase was identified as a proto-oncogene, an attribute that has been confirmed in cases including high-grade serous ovarian carcinoma (Gruosso, Garnier et al. 2015), gastric colon adenocarcinomas (Ohara, Hirota et al. 1995), Hodgkin's lymphoma, nasopharyngeal carcinoma (Eliopoulos, Davies et al. 2002), breast cancer (Sourvinos, Tsatsanis et al. 1999), prostate cancer

(Jeong, Bhatia et al. 2011) etc. On the other hand, there is robust evidence regarding TPL2's tumor-suppressive role in T-cell lymphomas (Tsatsanis, Vaporidi et al. 2008), melanomas (Decicco-Skinner, Trovato et al. 2011), lung cancer (Gkirtzimanaki, Gkouskou et al. 2013), intestinal adenomas (Serebrennikova, Tsatsanis et al. 2012) and DSS-induced colitis (Roulis, Nikolaou et al. 2014). In the present study, we aimed to delineate the role of TPL2 in colon cancer, a field that remains, to date, poorly elucidated.

In contrast to other cancer types, in established human colon cancer cell lines there seems to be no association between neither TPL2 protein levels or their response to TPL2 inhibitor treatment and their p53, KRAS or BRAF mutation status. This observation does not exclude the possibility of TPL2 playing a role in colon carcinogenesis through its activity, rather than its protein levels. At the same time, pERK levels varied significantly among the different cell lines, thus indicating that phosphorylation of ERK is achieved through TPL2-independent pathways. The fact that cell lines were affected by the TPL2 inhibitor regardless of their p53, KRAS and BRAF status indicates that the inhibitor could be considered a possible candidate for treating colon cancer cases. Granted, further studies are required towards this direction before reaching a solid conclusion. Treatment with TPL2 inhibitor also led to a significant decrease in the clonogenic capacity of HT29 cells. This could prove of great importance, since clonogenicity is directly implicated in the metastatic ability of cancer cells.

5-fluorouracil is still the gold standard when it comes to treatment of colorectal cancer. Hence, new drugs and combination regiments have to be compared with it, despite the absence of an ideal dose-intense 5-FU monotherapy approach (Sobrero, Aschele et al. 1997). Therefore, we decided to evaluate the effect of 5-FU when used alone, or combined with TPL2 inhibitor in various concentrations. Data presented in this study show that once reaching a certain level by using each of the agents alone cell proliferation could not be further reduced by a combination of the two, suggesting a lack of synergistic effect in their mechanism of action.

MEK is directly phosphorylated by TPL2, eventually leading to ERK activation. Constitutive activation of the MEK/ERK pathway has been implicated in cancer cell resistance to chemotherapeutic agents in a plethora of human malignancies, including

pancreatic, colon, lung, ovary, breast, thyroid and kidney cancers (Seger, Ahn et al. 1992, Mansour, Matten et al. 1994). Of note, inhibition of MEK did not yield a result similar to that of TPL2 inhibition regarding cancer cell proliferation. This observation applies for both responsive and non-responsive to TPL2 inhibitor cells. The fact that in cells pretreated with the MEK inhibitor, LPS stimulation resulted in complete impediment of ERK phosphorylation indicates the involvement of TPL2-independent pathways in ERK regulation. Strikingly, knock down of TPL2 does not modify the effect observed by TPL2 inhibitor treatment. When put together, these data demonstrate that the TPL2 inhibitor probably has secondary targets, other that TPL2 kinase itself that are the main regulators of cell proliferation. This comes in accordance with the results obtained by screen profiling of TPL2 inhibitor for its inhibitory activity against human kinases, which revealed that the inhibitor actually radically decreased the activity of MAP4K4, and EGFR by 55% and 75% respectively (http://www.guidetopharmacology.org/).

The epidermal growth factor receptor (EGFR) is involved in the development and progression of many neoplasias, including colon cancer. Although the significance of EGFR protein expression in colorectal cancer remains controversial, is seems that overexpression of EGFR is linked with tumor progression and poor survival in colorectal cancer patients (McKay, Murray et al. , Goldstein and Armin 2001, Resnick, Routhier et al. 2004, Ceccarelli, Piazzi et al. 2005, Spano, Fagard et al. 2005). Increased MAP4K4 levels are associated with the processes of cell migration, invasiveness and adhesion in different types of cancers including ovarian cancer, prostate cancer and hepatocellular carcinoma. In the latter case, Han *et al* elegantly showed that knockdown of MAP4K4 in HepG2 cells inhibits the adhesion and cell growth (Han, Zhu et al. 2010). Furthermore, *in vivo* silencing of MAP4K4 resulted in a retarded tumor xenograft growth (Liu, Cai et al. 2011). It is therefore possible that the observed effect of TPL2 inhibitor could be attributed to inhibition of either EGFR or MAP4K.

According to the outcome of the experimental procedure we followed for the study of TPL2 kinase in colorectal cancer, we did not conclude to a significant role for TPL2 in colon carcinogenesis. Our ongoing studies aim to delineate the pathways affected by TPL2 inhibition, as well as elucidate the genes that are implicated in resistance to TPL2 inhibitor treatment. It will be interesting to investigate the

existence of a putative correlation between the expression of TPL2/COT and clinical, molecular and histopathological data in human colon cancer specimens. In the future, investigation of the effect of TPL2 inhibitor treatment in an animal model for colon cancer could also prove of significant value.

# REFERENCES

Alberts, S. R., D. J. Sargent, S. Nair, M. R. Mahoney, M. Mooney, S. N. Thibodeau, T. C. Smyrk, F. A. Sinicrope, E. Chan, S. Gill, M. S. Kahlenberg, A. F. Shields, J. T. Quesenberry, T. A. Webb, G. H. Farr, B. A. Pockaj, A. Grothey and R. M. Goldberg (2012). "Effect of Oxaliplatin, Fluorouracil, and Leucovorin With or Without Cetuximab on Survival Among Patients With Resected Stage III Colon Cancer: A Randomized Trial." JAMA : the journal of the American Medical Association **307**(13): 1383-1393.

Allegra, C. J., G. Yothers, M. J. O'Connell, S. Sharif, N. J. Petrelli, S. H. Lopa and N. Wolmark (2013). "Bevacizumab in stage II-III colon cancer: 5-year update of the National Surgical Adjuvant Breast and Bowel Project C-08 trial." J Clin Oncol **31**(3): 359-364.

Aparecida Alves, C., I. D. Silva, F. E. Villanova, S. M. Nicolau, M. A. Custodio, C. Bortoletto and W. J. Goncalves (2006). "Differential gene expression profile reveals overexpression of MAP3K8 in invasive endometrioid carcinoma." Eur J Gynaecol Oncol **27**(6): 589-593.

Beinke, S., J. Deka, V. Lang, M. P. Belich, P. A. Walker, S. Howell, S. J. Smerdon, S. J. Gamblin and S. C. Ley (2003). "NF-kappaB1 p105 negatively regulates TPL-2 MEK kinase activity." Mol Cell Biol **23**(14): 4739-4752.

Beinke, S., M. J. Robinson, M. Hugunin and S. C. Ley (2004). "Lipopolysaccharide activation of the TPL-2/MEK/extracellular signal-regulated kinase mitogen-activated protein kinase cascade is regulated by IkappaB kinase-induced proteolysis of NF-kappaB1 p105." Mol Cell Biol **24**(21): 9658-9667.

Belich, M. P., A. Salmeron, L. H. Johnston and S. C. Ley (1999). "TPL-2 kinase regulates the proteolysis of the NF-kappaB-inhibitory protein NF-kappaB1 p105." Nature **397**(6717): 363-368.

Ben-Addi, A., A. Mambole-Dema, C. Brender, S. R. Martin, J. Janzen, S. Kjaer, S. J. Smerdon and S. C. Ley (2014). "IkappaB kinase-induced interaction of TPL-2 kinase with 14-3-3 is essential for Toll-like receptor activation of ERK-1 and -2 MAP kinases." Proc Natl Acad Sci U S A **111**(23): E2394-2403.

Budinska, E., V. Popovici, S. Tejpar, G. D'Ario, N. Lapique, K. O. Sikora, A. F. Di Narzo, P. Yan, J. G. Hodgson, S. Weinrich, F. Bosman, A. Roth and M. Delorenzi (2013). "Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer." The Journal of Pathology **231**(1): 63-76.

Burotto, M., V. L. Chiou, J. M. Lee and E. C. Kohn (2014). "The MAPK pathway across different malignancies: a new perspective." Cancer **120**(22): 3446-3456.

Ceccarelli, C., G. Piazzi, P. Paterini, M. Pantaleo, M. Taffurelli, D. Santini, G. Martinelli and G. Biasco (2005). "Concurrent EGFr and Cox-2 expression in colorectal cancer: proliferation impact and tumour spreading." Annals of Oncology **16**(suppl 4): iv74-iv79.

Ceci, J. D., C. P. Patriotis, C. Tsatsanis, A. M. Makris, R. Kovatch, D. A. Swing, N. A. Jenkins, P. N. Tsichlis and N. G. Copeland (1997). "Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation." Genes Dev **11**(6): 688-700.

Chiariello, M., M. J. Marinissen and J. S. Gutkind (2000). "Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation." Mol Cell Biol **20**(5): 1747-1758.

Cho, J., M. Melnick, G. P. Solidakis and P. N. Tsichlis (2005). "Tpl2 (tumor progression locus 2) phosphorylation at Thr290 is induced by lipopolysaccharide via an Ikappa-B Kinase-beta-dependent pathway and is required for Tpl2 activation by external signals." J Biol Chem **280**(21): 20442-20448.

Choi, H. S., B. S. Kang, J. H. Shim, Y. Y. Cho, B. Y. Choi, A. M. Bode and Z. Dong (2008). "Cot, a novel kinase of histone H3, induces cellular transformation through up-regulation of c-fos transcriptional activity." Faseb j **22**(1): 113-126.

Christoforidou, A. V., H. A. Papadaki, A. N. Margioris, G. D. Eliopoulos and C. Tsatsanis (2004). "Expression of the Tpl2/Cot oncogene in human T-cell neoplasias." Mol Cancer **3**(1): 34.

Das, S., J. Cho, I. Lambertz, M. A. Kelliher, A. G. Eliopoulos, K. Du and P. N. Tsichlis (2005). "Tpl2/cot signals activate ERK, JNK, and NF-kappaB in a cell-type and stimulus-specific manner." J Biol Chem **280**(25): 23748-23757.

De Sousa E Melo, F., X. Wang, M. Jansen, E. Fessler, A. Trinh, L. P. M. H. de Rooij, J. H. de Jong, O. J. de Boer, R. van Leersum, M. F. Bijlsma, H. Rodermond, M. van der Heijden, C. J. M. van Noesel, J. B. Tuynman, E. Dekker, F. Markowetz, J. P. Medema and L. Vermeulen (2013). "Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions." Nat Med **19**(5): 614-618.

Decicco-Skinner, K. L., S. A. Jung, T. Tabib, J. C. Gwilliam, H. Alexander, S. E. Goodheart, A. S. Merchant, M. Shan, C. Garber and J. S. Wiest (2013). "Tpl2 knockout keratinocytes have increased biomarkers for invasion and metastasis." Carcinogenesis **34**(12): 2789-2798.

Decicco-Skinner, K. L., E. L. Trovato, J. K. Simmons, P. K. Lepage and J. S. Wiest (2011). "Loss of tumor progression locus 2 (tpl2) enhances tumorigenesis and inflammation in two-stage skin carcinogenesis." Oncogene **30**(4): 389-397.

Douillard , J.-Y., K. S. Oliner , S. Siena , J. Tabernero , R. Burkes , M. Barugel , Y. Humblet , G. Bodoky , D. Cunningham , J. Jassem , F. Rivera , I. Kocákova , P. Ruff , M. Błasińska-Morawiec , M. Šmakal , J. L. Canon , M. Rother , R. Williams , A. Rong , J. Wiezorek , R. Sidhu and S. D. Patterson (2013). "Panitumumab–FOLFOX4 Treatment and RAS Mutations in Colorectal Cancer." New England Journal of Medicine **369**(11): 1023-1034.

Dumitru, C. D., J. D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J. H. Lin, C. Patriotis, N. A. Jenkins, N. G. Copeland, G. Kollias and P. N. Tsichlis (2000). "TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway." Cell **103**(7): 1071-1083.

Eliopoulos, A. G., C. Davies, S. S. Blake, P. Murray, S. Najafipour, P. N. Tsichlis and L. S. Young (2002). "The oncogenic protein kinase Tpl-2/Cot contributes to Epstein-Barr virus-encoded latent infection membrane protein 1-induced NF-kappaB signaling downstream of TRAF2." J Virol **76**(9): 4567-4579.

Eliopoulos, A. G., C. C. Wang, C. D. Dumitru and P. N. Tsichlis (2003). "Tpl2 transduces CD40 and TNF signals that activate ERK and regulates IgE induction by CD40." Embo j **22**(15): 3855-3864.

Gandara, M. L., P. Lopez, R. Hernando, J. G. Castano and S. Alemany (2003). "The COOH-terminal domain of wild-type Cot regulates its stability and kinase specific activity." Mol Cell Biol **23**(20): 7377-7390.

Gantke, T., S. Sriskantharajah and S. C. Ley (2011). "Regulation and function of TPL-2, an IkappaB kinase-regulated MAP kinase kinase kinase." Cell Res **21**(1): 131-145.

Gkirtzimanaki, K., K. K. Gkouskou, U. Oleksiewicz, G. Nikolaidis, D. Vyrla, M. Liontos, V. Pelekanou, D. C. Kanellis, K. Evangelou, E. N. Stathopoulos, J. K. Field, P. N. Tsichlis, V. Gorgoulis, T. Liloglou and A. G. Eliopoulos (2013). "TPL2 kinase is a suppressor of lung carcinogenesis." Proc Natl Acad Sci U S A **110**(16): E1470-1479.

Goldstein, N. S. and M. Armin (2001). "Epidermal growth factor receptor immunohistochemical reactivity in patients with American Joint Committee on cancer stage IV colon adenocarcinoma." Cancer **92**(5): 1331-1346.

Gruosso, T., C. Garnier, S. Abelanet, Y. Kieffer, V. Lemesre, D. Bellanger, I. Bieche, E. Marangoni, X. Sastre-Garau, V. Mieulet and F. Mechta-Grigoriou (2015). "MAP3K8/TPL-2/COT is a potential predictive marker for MEK inhibitor treatment in high-grade serous ovarian carcinomas." Nat Commun **6**: 8583.

Han, S.-X., Q. Zhu, J.-L. Ma, J. Zhao, C. Huang, X. Jia and D. Zhang (2010). "Lowered HGK expression inhibits cell invasion and adhesion in hepatocellular carcinoma cell line HepG2." World J Gastroenterol **16**(36): 4541-4548.

Hatziapostolou, M., C. Polytarchou, D. Panutsopulos, L. Covic and P. N. Tsichlis (2008). "Proteinase-activated receptor-1-triggered activation of tumor progression locus-2 promotes actin cytoskeleton reorganization and cell migration." Cancer Res **68**(6): 1851-1861.

Heinemann, V., L. F. von Weikersthal, T. Decker, A. Kiani, U. Vehling-Kaiser, S.-E. Al-Batran, T. Heintges, C. Lerchenmüller, C. Kahl, G. Seipelt, F. Kullmann, M. Stauch, W. Scheithauer, J. Hielscher, M. Scholz, S. Müller, H. Link, N. Niederle, A. Rost, H.-G. Höffkes, M. Moehler, R. U. Lindig, D. P. Modest, L. Rossius, T. Kirchner, A. Jung and S. Stintzing (2014). "FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial." The Lancet Oncology **15**(10): 1065-1075.

Hope, C., S. J. Ollar, E. Heninger, E. Hebron, J. L. Jensen, J. Kim, I. Maroulakou, S. Miyamoto, C. Leith, D. T. Yang, N. Callander, P. Hematti, M. Chesi, P. L. Bergsagel and F. Asimakopoulos (2014). "TPL2 kinase regulates the inflammatory milieu of the myeloma niche." Blood **123**(21): 3305-3315.

Hurwitz , H., L. Fehrenbacher , W. Novotny , T. Cartwright , J. Hainsworth , W. Heim , J. Berlin , A. Baron , S. Griffing , E. Holmgren , N. Ferrara , G. Fyfe , B. Rogers , R. Ross and F. Kabbinavar (2004). "Bevacizumab plus Irinotecan, Fluorouracil, and Leucovorin for Metastatic Colorectal Cancer." New England Journal of Medicine **350**(23): 2335-2342.

Jeong, J. H., A. Bhatia, Z. Toth, S. Oh, K. S. Inn, C. P. Liao, P. Roy-Burman, J. Melamed, G. A. Coetzee and J. U. Jung (2011). "TPL2/COT/MAP3K8 (TPL2) activation promotes androgen depletion-independent (ADI) prostate cancer growth." PLoS One **6**(1): e16205.

Johannessen, C. M., J. S. Boehm, S. Y. Kim, S. R. Thomas, L. Wardwell, L. A. Johnson, C. M. Emery, N. Stransky, A. P. Cogdill, J. Barretina, G. Caponigro, H. Hieronymus, R. R. Murray, K. Salehi-Ashtiani, D. E. Hill, M. Vidal, J. J. Zhao, X. Yang, O. Alkan, S. Kim, J. L. Harris, C. J. Wilson, V. E. Myer, P. M. Finan, D. E. Root, T. M. Roberts, T. Golub, K. T. Flaherty, R. Dummer, B. L. Weber, W. R. Sellers, R. Schlegel, J. A. Wargo, W. C. Hahn and L. A. Garraway (2010). "COT drives resistance to RAF inhibition through MAP kinase pathway reactivation." Nature **468**(7326): 968-972.

Kaiser, F., D. Cook, S. Papoutsopoulou, R. Rajsbaum, X. Wu, H. T. Yang, S. Grant, P. Ricciardi-Castagnoli, P. N. Tsichlis, S. C. Ley and A. O'Garra (2009). "TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells." J Exp Med **206**(9): 1863-1871.

Kanellis, D. C., S. Bursac, P. N. Tsichlis, S. Volarevic and A. G. Eliopoulos (2015). "Physical and functional interaction of the TPL2 kinase with nucleophosmin." Oncogene **34**(19): 2516-2526.
Karapetis , C. S., S. Khambata-Ford , D. J. Jonker , C. J. O'Callaghan , D. Tu , N. C. Tebbutt , R. J. Simes , H. Chalchal , J. D. Shapiro , S. Robitaille , T. J. Price , L. Shepherd , H.-J. Au , C. Langer , M. J. Moore and J. R. Zalcberg (2008). "K-ras Mutations and Benefit from Cetuximab in Advanced Colorectal Cancer." New England Journal of Medicine **359**(17): 1757-1765.

Khanal, P., K. Y. Lee, K. W. Kang, B. S. Kang and H. S. Choi (2009). "Tpl-2 kinase downregulates the activity of p53 and enhances signaling pathways leading to activation of activator protein 1 induced by EGF." Carcinogenesis **30**(4): 682-689.

Kim, G., P. Khanal, J. Y. Kim, H. J. Yun, S. C. Lim, J. H. Shim and H. S. Choi (2015). "COT phosphorylates prolyl-isomerase Pin1 to promote tumorigenesis in breast cancer." Mol Carcinog **54**(6): 440-448.

Kim, J. Y., S. C. Lim, G. Kim, H. J. Yun, S. G. Ahn and H. S. Choi (2015). "Interleukin-33/ST2 axis promotes epithelial cell transformation and breast tumorigenesis via upregulation of COT activity." Oncogene **34**(38): 4928-4938.

Kim, K., G. Kim, J. Y. Kim, H. J. Yun, S. C. Lim and H. S. Choi (2014). "Interleukin-22 promotes epithelial cell transformation and breast tumorigenesis via MAP3K8 activation." Carcinogenesis **35**(6): 1352-1361.

Koliaraki, V., M. Roulis and G. Kollias (2012). "Tpl2 regulates intestinal myofibroblast HGF release to suppress colitis-associated tumorigenesis." J Clin Invest **122**(11): 4231-4242.

Krcova, Z., J. Ehrmann, V. Krejci, A. Eliopoulos and Z. Kolar (2008). "Tpl-2/Cot and COX-2 in breast cancer." Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub **152**(1): 21-25.

Krishna, M. and H. Narang (2008). "The complexity of mitogen-activated protein kinases (MAPKs) made simple." Cell Mol Life Sci **65**(22): 3525-3544.

Lee, H. W., H. J. Cho, S. J. Lee, H. J. Song, H. J. Cho, M. C. Park, H. J. Seol, J. I. Lee, S. Kim, H. M. Lee, H. Y. Choi, D. H. Nam and K. M. Joo (2015). "Tpl2 induces castration resistant prostate cancer progression and metastasis." Int J Cancer **136**(9): 2065-2077.

Lee, H. W., K. M. Joo, J. E. Lim, H. J. Cho, H. J. Cho, M. C. Park, H. J. Seol, S. I. Seo, J. I. Lee, S. Kim, B. C. Jeong and D. H. Nam (2013). "Tpl2 kinase impacts tumor growth and metastasis of clear cell renal cell carcinoma." Mol Cancer Res **11**(11): 1375-1386.

Lee, J., S. Jeong, J. H. Park, C. R. Lee, C. R. Ku, S. W. Kang, J. J. Jeong, K. H. Nam, D. Y. Shin, E. J. Lee, W. Y. Chung and Y. S. Jo (2015). "Aberrant expression of COT is related to recurrence of papillary thyroid cancer." Medicine (Baltimore) **94**(6): e548.

Lee, W. J., K. H. Lan, C. T. Chou, Y. C. Yi, W. C. Chen, H. C. Pan, Y. C. Peng, K. B. Wang, Y. C. Chen, T. H. Chao, H. R. Tien, W. H. Sheu and M. L. Sheu (2013). "Tpl2 inhibitors thwart endothelial cell function in angiogenesis and peritoneal dissemination." Neoplasia **15**(9): 1036-1048.

Li, X., C. Liu, B. C. Ip, K. Q. Hu, D. E. Smith, A. S. Greenberg and X. D. Wang (2015). "Tumor progression locus 2 ablation suppressed hepatocellular carcinoma development by inhibiting hepatic inflammation and steatosis in mice." J Exp Clin Cancer Res **34**: 138.

Lin, X., E. T. Cunningham, Jr., Y. Mu, R. Geleziunas and W. C. Greene (1999). "The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF-kappaB acting through the NF-kappaB-inducing kinase and IkappaB kinases." Immunity **10**(2): 271-280.

Liu, A.-W., J. Cai, X.-L. Zhao, T.-H. Jiang, T.-F. He, H.-Q. Fu, M.-H. Zhu and S. H. Zhang (2011). "ShRNA-targeted MAP4K4 inhibits hepatocellular carcinoma growth." Clinical Cancer Research **17**(4): 710-720.

Mansour, S., W. Matten, A. Hermann, J. Candia, S. Rong, K. Fukasawa, G. Vande Woude and N. Ahn (1994). "Transformation of mammalian cells by constitutively active MAP kinase kinase." Science **265**(5174): 966-970.

Marisa, L., A. de Reyniès, A. Duval, J. Selves, M. P. Gaub, L. Vescovo, M.-C. Etienne-Grimaldi, R. Schiappa, D. Guenot, M. Ayadi, S. Kirzin, M. Chazal, J.-F. Fléjou, D. Benchimol, A. Berger, A. Lagarde, E. Pencreach, F. Piard, D. Elias, Y. Parc, S. Olschwang, G. Milano, P. Laurent-Puig and V. Boige (2013). "Gene Expression Classification of Colon Cancer into Molecular Subtypes: Characterization, Validation, and Prognostic Value." PLoS Med **10**(5): e1001453.

McKay, J. A., L. J. Murray, S. Curran, V. G. Ross, C. Clark, G. I. Murray, J. Cassidy and H. L. McLeod "Evaluation of the epidermal growth factor receptor (EGFR) in colorectal tumours and lymph node metastases." European Journal of Cancer **38**(17): 2258-2264.

Mieulet, V., L. Yan, C. Choisy, K. Sully, J. Procter, A. Kouroumalis, S. Krywawych, M. Pende, S. C. Ley, C. Moinard and R. F. Lamb (2010). "TPL-2-mediated activation of MAPK downstream of TLR4 signaling is coupled to arginine availability." Sci Signal **3**(135): ra61.

Miyoshi, J., T. Higashi, H. Mukai, T. Ohuchi and T. Kakunaga (1991). "Structure and transforming potential of the human cot oncogene encoding a putative protein kinase." Mol Cell Biol **11**(8): 4088-4096.

Nierodzik, M. L. and S. Karpatkin (2006). "Thrombin induces tumor growth, metastasis, and angiogenesis: Evidence for a thrombin-regulated dormant tumor phenotype." Cancer Cell **10**(5): 355-362.

Ohara, R., S. Hirota, H. Onoue, S. Nomura, Y. Kitamura and K. Toyoshima (1995). "Identification of the cells expressing cot proto-oncogene mRNA." J Cell Sci **108 ( Pt 1)**: 97-103.

Papoutsopoulou, S., A. Symons, T. Tharmalingham, M. P. Belich, F. Kaiser, D. Kioussis, A. O'Garra, V. Tybulewicz and S. C. Ley (2006). "ABIN-2 is required for optimal activation of Erk MAP kinase in innate immune responses." Nat Immunol **7**(6): 606-615.

Park, H. D., Y. Lee, Y. K. Oh, J. G. Jung, Y. W. Park, K. Myung, K. H. Kim, S. S. Koh and D. S. Lim (2011). "Pancreatic adenocarcinoma upregulated factor promotes metastasis by regulating TLR/CXCR4 activation." Oncogene **30**(2): 201-211.

Patriotis, C., A. Makris, S. E. Bear and P. N. Tsichlis (1993). "Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation." Proc Natl Acad Sci U S A **90**(6): 2251-2255.

Patriotis, C., A. Makris, J. Chernoff and P. N. Tsichlis (1994). "Tpl-2 acts in concert with Ras and Raf-1 to activate mitogen-activated protein kinase." Proc Natl Acad Sci U S A **91**(21): 9755-9759.

Pritchard, A. L. and N. K. Hayward (2013). "Molecular pathways: mitogen-activated protein kinase pathway mutations and drug resistance." Clin Cancer Res **19**(9): 2301-2309.

Resnick, M. B., J. Routhier, T. Konkin, E. Sabo and V. E. Pricolo (2004). "Epidermal Growth Factor Receptor, c-MET, β-Catenin, and p53 Expression as Prognostic Indicators in Stage II Colon Cancer." A Tissue Microarray Study **10**(9): 3069-3075.

Robinson, M. J., S. Beinke, A. Kouroumalis, P. N. Tsichlis and S. C. Ley (2007). "Phosphorylation of TPL-2 on serine 400 is essential for lipopolysaccharide activation of extracellular signal-regulated kinase in macrophages." Mol Cell Biol **27**(21): 7355-7364.

Roepman, P., A. Schlicker, J. Tabernero, I. Majewski, S. Tian, V. Moreno, M. H. Snel, C. M. Chresta, R. Rosenberg, U. Nitsche, T. Macarulla, G. Capella, R. Salazar, G. Orphanides, L. F. A. Wessels, R. Bernards and I. M. Simon (2014). "Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition." International Journal of Cancer **134**(3): 552-562.

Roget, K., A. Ben-Addi, A. Mambole-Dema, T. Gantke, H. T. Yang, J. Janzen, N. Morrice, D. Abbott and S. C. Ley (2012). "IkappaB kinase 2 regulates TPL-2 activation of extracellular signal-regulated kinases 1 and 2 by direct phosphorylation of TPL-2 serine 400." Mol Cell Biol **32**(22): 4684-4690.

Roulis, M., C. Nikolaou, E. Kotsaki, E. Kaffe, N. Karagianni, V. Koliaraki, K. Salpea, J. Ragoussis, V. Aidinis, E. Martini, C. Becker, H. R. Herschman, S. Vetrano, S. Danese and G. Kollias (2014). "Intestinal myofibroblast-specific Tpl2-Cox-2-PGE2 pathway links innate sensing to epithelial homeostasis." Proc Natl Acad Sci U S A **111**(43): E4658-4667.

Rousseau, S., M. Papoutsopoulou, A. Symons, D. Cook, J. M. Lucocq, A. R. Prescott, A. O'Garra, S. C. Ley and P. Cohen (2008). "TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages." J Cell Sci **121**(Pt 2): 149-154.

Sadanandam, A., C. A. Lyssiotis, K. Homicsko, E. A. Collisson, W. J. Gibb, S. Wullschleger, L. C. G. Ostos, W. A. Lannon, C. Grotzinger, M. Del Rio, B. Lhermitte, A. B. Olshen, B. Wiedenmann, L. C. Cantley, J. W. Gray and D. Hanahan (2013). "A colorectal cancer classification system that associates cellular phenotype and responses to therapy." Nat Med **19**(5): 619-625.

Salmeron, A., T. B. Ahmad, G. W. Carlile, D. Pappin, R. P. Narsimhan and S. C. Ley (1996). "Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase." Embo j **15**(4): 817-826.

Seger, R., N. G. Ahn, J. Posada, E. S. Munar, A. M. Jensen, J. A. Cooper, M. H. Cobb and E. G. Krebs (1992). "Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells." Journal of Biological Chemistry **267**(20): 14373-14381.

Serebrennikova, O. B., C. Tsatsanis, C. Mao, E. Gounaris, W. Ren, L. D. Siracusa, A. G. Eliopoulos, K. Khazaie and P. N. Tsichlis (2012). "Tpl2 ablation promotes intestinal inflammation and tumorigenesis in Apcmin mice by inhibiting IL-10 secretion and regulatory T-cell generation." Proc Natl Acad Sci U S A **109**(18): E1082-1091.

Sobrero, A. F., C. Aschele and J. R. Bertino (1997). "Fluorouracil in colorectal cancer--a tale of two drugs: implications for biochemical modulation." J Clin Oncol **15**(1): 368-381.

Sourvinos, G., C. Tsatsanis and D. A. Spandidos (1999). "Overexpression of the Tpl-2/Cot oncogene in human breast cancer." Oncogene **18**(35): 4968-4973.

Spano, J., R. Fagard, J.-C. Soria, O. Rixe, D. Khayat and G. Milano (2005). "Epidermal growth factor receptor signaling in colorectal cancer: preclinical data and therapeutic perspectives." Annals of Oncology **16**(2): 189-194.

Sriskantharajah, S., M. P. Belich, S. Papoutsopoulou, J. Janzen, V. Tybulewicz, B. Seddon and S. C. Ley (2009). "Proteolysis of NF-kappaB1 p105 is essential for T cell antigen receptor-induced proliferation." Nat Immunol **10**(1): 38-47.

Stafford, M. J., N. A. Morrice, M. W. Peggie and P. Cohen (2006). "Interleukin-1 stimulated activation of the COT catalytic subunit through the phosphorylation of Thr290 and Ser62." FEBS Lett **580**(16): 4010-4014.

Sturm, O. E., R. Orton, J. Grindlay, M. Birtwistle, V. Vyshemirsky, D. Gilbert, M. Calder, A. Pitt, B. Kholodenko and W. Kolch (2010). "The Mammalian MAPK/ERK Pathway Exhibits Properties of a Negative Feedback Amplifier." Science Signaling **3**(153): ra90-ra90.

Sugimoto, K., M. Ohata, J. Miyoshi, H. Ishizaki, N. Tsuboi, A. Masuda, Y. Yoshikai, M. Takamoto, K. Sugane, S. Matsuo, Y. Shimada and T. Matsuguchi (2004). "A serine/threonine kinase, Cot/Tpl2, modulates bacterial DNA-induced IL-12 production and Th cell differentiation." J Clin Invest **114**(6): 857-866.

Taieb, J., J. Tabernero, E. Mini, F. Subtil, G. Folprecht, J.-L. Van Laethem, J. Thaler, J. Bridgewater, L. N. Petersen, H. Blons, L. Collette, E. Van Cutsem, P. Rougier, R. Salazar, L. Bedenne, J.-F. Emile, P. Laurent-Puig and C. Lepage (2014). "Oxaliplatin, fluorouracil, and leucovorin with or without cetuximab in patients with resected stage III colon cancer (PETACC-8): an open-label, randomised phase 3 trial." The Lancet Oncology **15**(8): 862-873.

The Cancer Genome Atlas, N. (2012). "Comprehensive Molecular Characterization of Human Colon and Rectal Cancer." Nature **487**(7407): 330-337.

Tsatsanis, C., C. Patriotis, S. E. Bear and P. N. Tsichlis (1998). "The Tpl-2 protooncoprotein activates the nuclear factor of activated T cells and induces interleukin 2 expression in T cell lines." Proc Natl Acad Sci U S A **95**(7): 3827-3832.

Tsatsanis, C., C. Patriotis and P. N. Tsichlis (1998). "Tpl-2 induces IL-2 expression in T-cell lines by triggering multiple signaling pathways that activate NFAT and NF-kappaB." Oncogene **17**(20): 2609-2618.

Tsatsanis, C., K. Vaporidi, V. Zacharioudaki, A. Androulidaki, Y. Sykulev, A. N. Margioris and P. N. Tsichlis (2008). "Tpl2 and ERK transduce antiproliferative T cell receptor signals and inhibit transformation of chronically stimulated T cells." Proc Natl Acad Sci U S A **105**(8): 2987-2992.
Vougioukalaki, M., D. C. Kanellis, K. Gkouskou and A. G. Eliopoulos (2011). "Tpl2 kinase signal transduction in inflammation and cancer." Cancer Lett **304**(2): 80-89.

Wang, X. and C. Tournier (2006). "Regulation of cellular functions by the ERK5 signalling pathway." Cell Signal **18**(6): 753-760.

Waterfield, M., W. Jin, W. Reiley, M. Zhang and S. C. Sun (2004). "IkappaB kinase is an essential component of the Tpl2 signaling pathway." Mol Cell Biol **24**(13): 6040-6048.

Waterfield, M. R., M. Zhang, L. P. Norman and S. C. Sun (2003). "NF-kappaB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase." Mol Cell **11**(3): 685-694.

Watford, W. T., B. D. Hissong, L. R. Durant, H. Yamane, L. M. Muul, Y. Kanno, C. M. Tato, H. L. Ramos, A. E. Berger, L. Mielke, M. Pesu, B. Solomon, D. M. Frucht, W. E. Paul, A. Sher, D. Jankovic, P. N. Tsichlis and J. J. O'Shea (2008). "Tpl2 kinase regulates T cell interferon-gamma production and host resistance to Toxoplasma gondii." J Exp Med **205**(12): 2803-2812.

Whitmarsh, A. J. (2007). "Regulation of gene transcription by mitogen-activated protein kinase signaling pathways." Biochim Biophys Acta **1773**(8): 1285-1298.

Yang, S. H., A. D. Sharrocks and A. J. Whitmarsh (2003). "Transcriptional regulation by the MAP kinase signaling cascades." Gene **320**: 3-21.