IDENTIFYING TBK1-SPECIFIC ROLES IN COLORECTAL CANCER

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By
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We certify that we have read this thesis and that in our opinion it is fully adequate,in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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M.Sc. in Molecular Biology and Genetics

Advisor: Serkan İsmail Göktuna

June 2022

Colorectal cancer (CRC) is the second most lethal cancer type, with a high incidence rate among adults. The CRC is a highly heterogenic disease with a high rate of mutations in different molecular pathways. Moreover, resistance to standard treatment options is seen frequently among CRC patients. Therefore, a better understanding of the mechanisms behind the initiation, progression, and drug resistance allows us to increase the life quality of CRC patients. TBK1 is a kinase protein with central roles in most cellular signaling pathways. This protein has been reported to be an oncogene in some cancer types. However, the role of TBK1 in colorectal cancer is not yet established. In this study, we generated stable TBK1 knockdown CRC cell lines and mainly focused on the role of TBK1 in colorectal cancer pathogenesis. Upon TBK1 depletion in CRC, we observed increased cell proliferation and migration *in vitro*. The role of TBK1 in cell proliferation is further confirmed in xenograft models *in vivo.*

Moreover, a shift in EMT and resistance to Gefitinib, an EGFR inhibitor, is indicated in TBK1 knockdown cells. TBK1 is also diminished in our Gefitinib-resistant CRC cell lines, suggesting a role for this protein in drug resistance. The findings of this study suggest a tumor-suppressive role for TBK1 in CRC. Hence, further investigations on the mechanisms behind this tumor-suppressive role of TBK1 in CRC will pave the way for developing novel therapeutic options for CRC treatment.

Keywords: Colorectal Cancer, TBK1, EMT, drug resistance, Gefitinib

ÖZET

KOLOREKTAL KANSERDE TBK1'E ÖZGÜ ROLLERİN BELİRLENMESİ

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Kolorektal kanser (KRK), yetişkinler arasında yüksek bir vaka oranına sahip ikinci en ölümcül kanser tipidir. KRK yüksek bir heterojeniteye sahip olup farklı sinyal yolaklarında görevli çeşitli proteinlerde mutasyonlar gözlemlenmektedir. Ayrıca çoğu hasta standart tedavi yöntemlerine karşı direnç geliştirmektedir. Bu yüzden, KRK'nin başlangıcı, ilerleyişi ve kemoterapi direncinde rol alan mekanizmaların anlaşılması KRK hastalarının hayat kalitesinin arttırılması için önemlidir. TBK1 hücrede birçok sinyal yolağında mühim rolleri olan bir kinaz proteini olup bazı kanser türlerinde onkogen olduğu görülmüştür. Ancak, TBK1'in KRK'daki rolü henüz tam olarak anlaşılmamıştır. Bu çalışmada stabil TBK1 knockdown KRK hücre hatları geliştirip TBK1'in KRK'nin patogenezindeki ana rolünü inceledik. TBK1'in KRK'deki delesyonu sonucunda hücre proliferasyonu ve göçünde *in vitro* artış gözlemledik. TBK1'in hücre proliferasyonundaki rolü *in vivo* ksenograft modelleriyle de doğrulanmıştır.
Bunların yanı sıra, TBK1 knockdown hücrelerde epitel-mezenkimal geçiş (EMG) ve Gefitinib (EGFR inhibitörü) direnci gösterilmiştir. Buna ek olarak, Gefitinib-dirençli KRK hücre hatlarında TBK1'in azalması da kemoterapi direncindeki rolünü işaret etmektedir. Bu çalışmadaki bulgular TBK1'in KRK'de tümör baskılayıcı bir rolü olduğunu göstermektedir. Bu yüzden, TBK1'in KRK'deki tümör baskılayıcı mekanizmalarının incelenmesi yeni terapötik stratejilerin geliştirilmesinin önünü açacaktır.

Anahtar sözcükler: Kolorektal kanser, TBK1, EMG, kemoterapi direnci, Gefitinib

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CHAPTER 1

Introduction

1.1 Colorectal Cancer

Colorectal Cancer (CRC) is one of the most aggressive cancer types with a high incidence rate. In 2020, nearly 10% of the newly diagnosed cancer types were colorectal cancer. It is also ranked as the second most lethal cancer type in 2020 [1]. It is the second most common cancer type in women and the third most common in men, with 9.4% and 10.6% incidence rates respectively [1]. Upon some genetic mutations in specific epithelial cells in the large intestine, these cells acquire some features such as abnormal high proliferation and survival. Then they change to benign adenoma, which during the time they might become malignant carcinoma [2]. Colorectal cancer generally consists of different stages. Stages I and II of the disease are curable since the invasive cancer is not yet metastasized to the other sites of the body. Surgery is a treatment option in this stage. Although cancer cells of stage III are metastasized to the proximal lymph nodes, approximately 70% of the cancers in this stage are curable by surgery and chemotherapy. Still, cure at stage IV of the disease is primarily unsuccessful [3–5].

Colorectal cancer is classified into different types according to the different kinds of mutations in the cells. Sporadic cancers are the cancers that are happening due to point mutations. These mutations are not inherited, so they can affect individual cells. This type is the most common type that consists of approximately 70% of the cases [6]. The second type of colorectal cancer is the inherited type. Inherited mutations cause these cancers. Inherited cancers also consist of two different subgroups called familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). FAP is caused by the formation of multiple malignant polyps in the colon [7]. The main reason for HNPCC is mutations and deficiencies in DNA repair system [7].

Many risk factors increase the chance of developing malignant carcinoma. Age is the most critical risk factor for this disease. In adults, after the fifth decade of life, the risk of developing colorectal cancer increases [8]. Other than age, the history of some diseases, such as ulcerative colitis [9] or Crohn's disease [10], increases the risk of the onset of colorectal cancer. Familial history

of colorectal cancer is also a significant risk factor [11]. Other risk factors are related to the lifestyle, such as obesity, low physical activity, smoking, and alcohol consumption. It is shown that obesity and unhealthy dietary habits can increase colorectal cancer risk by up to 70% [12].

The CRC is a highly heterogenic disease with a high rate of mutations in different molecular pathways. To improve the treatment and diagnosis of this disease, it is vital to understand the molecular mechanism and genetic and epigenetic risk factors leading to this disease.

1.1.1 Molecular mechanisms of colorectal cancer

Genomic instability is a cellular feature that plays a crucial role in tumor initiation and progression. There are three different pathways of genomic instability, chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP), which are required for colorectal cancer progression [13].

Chromosomal instability (CIN) is a common mechanism observed in around 65-70% of sporadic colorectal cancer cases [14]. Accumulating mutations in various oncogene and tumor suppressor genes triggering colorectal cancer initiation and progression is a common characteristic of the CIN pathway. As a result of CIN, aneuploidy and loss of heterozygosity (LOH) happen, leading to chromosomal number and structure alterations. It is shown that there is a loss of an average of 25-30% of alleles in tumors [15]. It is observed that the loss of chromosomes 1,5,8,17, and 18 frequently happens in colorectal cancer [16].

According to the multistep genetic model of colorectal carcinogenesis proposed by Fearon and Vogelstein in 1990 [6], according to the mutations in adenomatous polyposis coli (APC) tumor suppressor gene, KRAS oncogene mutagenic activation can occur [6]. Additional mutations in different pathways such as TGF-β and TP-53 lead to malignant transformations [17,18]. According to the multistep genetic model of colorectal carcinoma represented in Figure 1 [13], according to the mutations in APC, activating mutations of the Wnt signaling pathway occur in the early stages of the disease. KRAS activation is followed by mutations in TP53 and loss of heterozygosity on chromosome 18q in the late adenoma and invasive cancer stage.

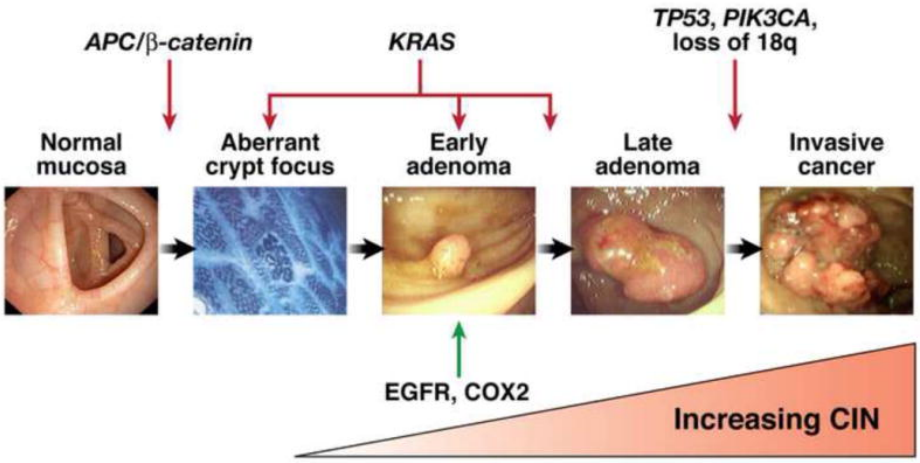


Figure 1. Steps of initiation and progression of colorectal cancer. APC mutations happen in the initial stages of colorectal cancer, while mutations follow it on different pathways such as KRAS and TP53. *Adapted from THE CHROMOSOMAL INSTABILITY PATHWAY IN COLON CANCER by Pino et al., 2010*

As mentioned above, Wnt signaling activation occurs due to APC inactivation, which can be considered the most crucial change in colorectal cancer pathogenesis. In normal cells, APC interacts with glycogen synthase kinase3-β (GSK-3β) and β-catenin, two essential components of the Wnt signaling pathway [19]. As a result, β-catenin is degraded, and the Wnt signal is suppressed. The mutation in APC prevents the complex formation. Hence, the accumulated β-catenin in the cytoplasm translocates to the nucleus where it interacts with the TCF-LEF transcription factors, which then transcription of downstream genes initiates, leading to increased tumor growth, invasion, and metastasis.

KRAS mutations have a high-rate incidence in colorectal cancers [20]. In the KRAS-mediated pathways, SOS, a guanine exchange factor, is become activated upon binding of growth factor to its receptor. SOS stimulates the exchange of GDP bond RAS with GTP, which is the active form of RAS. Active RAS leads to increased growth, differentiation, cell proliferation, and cell survival by activating multiple signaling pathways such as phosphatidylinositol 3 kinase (PI3K) and MEK-ERK pathways. Constitutively active KRAS has been detected in around 30-40% of

colorectal cancer cases [20]. NRAS mutations are also seen in colorectal cancer cases; however, their frequency rate is much less than the KRAS mutations [21].

On the other hand, TP53 mutations are also seen in up to 60% of colorectal cancer patients with poor clinical outcomes [22]. The modifications in this tumor suppressor gene are mostly AT to GC transition missense mutations that lead to the synthesis of an inactive protein.

Moreover, chromosome 18q loss has been detected in colorectal tumors, especially in the advanced stages. SMAD2 and SMAD4 are the tumor suppressor genes on 18q with cell growth and apoptosis roles. Mutations in these genes are identified in some colorectal cancers [23,24].

Other than the chromosomal instability (CIN), the microsatellite instability (MSI) phenotype has been found in some colorectal cancers. This phenotype is known for alterations in simple repetitive DNA sequences called microsatellites, caused by DNA mismatch repair mechanisms (MMR) defects.

In addition to the CIN and MSI pathways, the CpG island methylator phenotype (CIMP) is the other subset of colorectal cancer characterized by the inactivation of several tumor suppressor genes upon hypermethylation promoter CpG island sites [25].

1.1.2 Treatment of colorectal cancer

Despite advanced screening methods to detect CRC at initial stages, most cases of the CRC are detected late, when they are at advanced stages. Treatment at this stage is challenging because of metastatic tumors. The primary treatment of CRC is consisted of surgery, chemotherapy, and targeted therapy. 5-fluorouracil (5-FU) is the main chemotherapy option for CRC patients. This drug is used alone or combined with other chemotherapy agents such as oxaliplatin, irinotecan, and capecitabine [26]. Although combination therapy effectively increases the overall survival rate in CRC patients, acquired resistance to the drugs is the main problem in chemotherapy-based treatments.

Targeted therapy is a practical approach to CRC treatment, including monoclonal antibodies and small-molecule inhibitors. Several pathways have roles in the initiation, progression, and metastasis of CRC, such as EGFR, VEGF, Hedgehog, TGF-β, Notch, and Wnt/β-catenin. These pathways are potential targets for therapy against CRC [26].

EGFR is an epidermal growth factor receptor tyrosine kinase that contributes to cell survival, cell growth, and migration of tumors. Once EGFR is activated, it triggers downstream signaling pathways, including RAS/ERK, PI3K/AKT, and JAK/STAT [27]. EGFR overexpression has been detected in breast, non-small cell lung, and colorectal cancers [28–30]. EGFR activation via EGF ligands phosphorylates RAS/RAF, activating MEK/ERK kinases. Nucleus translocation of ERK causes an increase in the expression of genes related to cell survival, cell growth, and invasion [31]. PI3K pathway activation by EGFR leads to the phosphorylation and activation of AKT, which regulates cell growth and apoptosis [32].

Tyrosine kinase inhibitors (TKIs) and anti-EGFR monoclonal antibodies are the primary molecules to target EGFR in CRC treatment. Cetuximab and panitumumab are among the first FDA-approved monoclonal antibodies targeting EGFR. Cetuximab treatment alone or in combination with irinotecan has improved the overall survival of patients with irinotecan-resistant colorectal cancer [33]. In a phase III study, panitumumab is reported to increase the overall survival of metastatic colorectal cancer patients when combined with FOLFOX4 compared to FOLFOX4 alone [34].

One of the most crucial downstream signaling pathways of EGFR is the RAS/RAF pathway. Indeed, mutations of this pathway are one of the primary mediators of acquired drug resistance.

Studies show that approximately 50% of the acquired anti-EGFR-resistant cases are due to mutations of KRAS [35,36]. Bouchahda et al. reported two new mutations of KRAS in a liver metastatic and cetuximab-resistant colon cancer case. No mutations in KRAS were detected prior to cetuximab treatment [37]. In some CRC cell lines primarily sensitive to cetuximab and panitumumab, acquired resistance to these drugs was shown. In addition to KRAS mutations, mutations in BRAF and NRAS are shown in drug-resistant tumors [38].

In addition to EGFR, some growth factor receptors can activate EGFR downstream signaling pathways, such as mesenchymal-epithelial transition factor receptor (MET) and human epidermal growth factor receptor 2 (HER2). There are several studies about the overexpression of these receptors and anti-EGFR treatment resistance. Liska et al. showed a novel mechanism of cetuximab resistance by activation of the MET pathway. In their study, MET pathway activation rescued cell proliferation inhibition and cell cycle arrest induced by cetuximab. In addition, pharmacological inhibition of MET resensitized cells to cetuximab [39]. Furthermore, MET

activation is correlated with resistance to anti-EGFR therapy *in vitro* and patient-derived colorectal cancer xenografts [40].

Bertotti et al. could show the positive correlation between HER2 gene amplification and cetuximab resistance in patient-derived xenografts. This resistance was also reversed by applying HER2 inhibitors [41].

1.2 Epithelial-to-mesenchymal transition (EMT)

Epithelial-to-mesenchymal transition (EMT) is a dynamic process in which epithelial cells convert to cells with more mesenchymal features. Epithelial cells express more epithelial cell-to-cell junctions with less metastatic ability, while mesenchymal cells have gained more mobility and invasiveness by remodeling their cellular cytoskeleton [42]. In the old concept, the EMT process is defined as a unidirectional switch between two different cell states. However, the new concept suggests multiple transitional forms of cells expressing epithelial and mesenchymal genes between the endpoint epithelial or mesenchymal states [43]. This intermediates state is called partial EMT. This biological program can occur in a wide range of tissues in normal physiologic or pathologic conditions. The EMT is an essential step in embryonic morphogenesis. For instance, this process is necessary to form mesoderm from primitive streak [43,44].

Moreover, EMT plays an essential role in wound healing and cancer development. In cancers, some factors induce EMT, such as hypoxia, chemotherapy, and cytokines produced by the tumor microenvironment [45]. The importance of EMT in cancer progression is intensively studied. Mesenchymal cells induced by the EMT process are more invasive and aggressive than epithelial cells. They quickly invade the cells at the primary tumor sites, translocate through blood vessels, extravasate into the distant places and survive at those sites [46]. EMT is also reversible through the process named mesenchymal to epithelial transition (MET). It is thought that when tumor cells reach a metastatic niche, MET is necessary to facilitate the formation of a second tumor [45].

1.2.1 EMT in colorectal cancer

Increasing evidence has confirmed that EMT plays a complex and crucial role in colorectal cancer progression. The EMT process can be histologically quantified in the areas called tumor buds. Morphology change and gaining EMT profile is visible in these areas [47,48]. A tumor bud is an isolated cancer cell or a cluster of undifferentiated cells consisting of a maximum of five cancer cells from the invasion front [49]. A tumor bud detaches from the primary tumor body by losing E-cadherin. The microenvironment is critical in bud formation in CRC. TGF-β interacts with $CD10^+$ myeloid cells at the invasion front, increasing tumor budding [50].

During EMT in CRC, epithelial cell-cell adhesion molecules such as E-cadherin, claudins, occludin, and α-catenin are downregulated. In contrast, mesenchymal markers such as vimentin, Twist1, Twist2, N-cadherin, ZEB1, smooth muscle actin (SMA), and integrin αVβ6 are upregulated [47]. Downregulated E-cadherin and upregulated vimentin are associated with lymph node metastasis and poor prognosis in CRC patients [51–53].

1.2.1.1 Signaling pathways of EMT in colorectal cancer

Extracellular signals in the tumor microenvironment are essential effectors in the initiation of EMT in CRC. Various signaling pathways have been mentioned in the literature to be related to EMT and cancer progression, including CRC.

TGF-β/Smad signaling pathway is one of the significant developmental pathways which plays a role in EMT regulation. TGF-β binding to the TβRI and TβRII receptors leads to the phosphorylation and activation of Smad proteins. Smad2/3 proteins translocate to the nucleus to activate SNAI1 expression. Smad4 is an inhibitor of STAT3, which has a role in the EMT process by inducing ZEB1 expression. Smad4 loss occurs in approximately 30% of metastatic CRC and is associated with E-cadherin loss [54]. In smad4 knockout colorectal cancer cell lines, increased tumorigenesis, migration, and invasion upon TGF-β induction has been reported [55]. Moreover, smad4 loss leads to the overexpression of STAT3, associated with EMT progression and E-cadherin loss in human CRC [56].

Wnt/β-catenin signaling pathway is also one of the prominent effectors of EMT. Wnt signaling induces β-catenin by inhibiting glycogen synthase kinase-3β (GSK3β). Nucleus translocation of

β-catenin promotes EMT by activating ZEB1 and SNAI1. Activated SNAI1 then promotes Wnt signaling and interacts with β-catenin. Increased nucleus β-catenin levels and decreased E-cadherin expression are reported in invasive stages of colorectal cancer cells undergoing EMT [57]. Dickkopf-1 (DDK-1), a Wnt signaling suppressor, inhibits the EMT process and CRC progression [58].

Growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF), which are targets of receptor tyrosine kinases (RTKs), can promote EMT by activating several signaling pathways. The Ras/ERK1/2 pathway is one of the main pathways activated by RTKs. This cascade can activate AP1, Egr-1, and Fra-1 proteins, inducers of SLUG expression and the EMT process [59]. Raf-1 kinase inhibitor (RKIP) and PHLPP negatively regulate EMT by inhibiting RAF. It is observed that upon PHLPP1 or 2, knockdown causes a change in EMT markers and an increase in cell migration and invasion. Human CRC data also suggested a positive correlation between the expression of E-cadherin and PHLPP1 or 2 [60]. PI3K/AKT is the other signaling cascade activated by RTKs in response to growth factors. This pathway induces SNAI expression by activating NF-κB or inhibiting GSK3β. PTEN is a negative regulator of this pathway. Increased levels of mTORC1 and mTORC2, two effectors of this pathway, are responsible for induced EMT, motility, and metastasis of CRC by activating RhoA and Rac1 signaling [61]. Induced MET and increased sensitivity to chemotherapy drugs are reported upon knockdown of mTORC1 and mTORC2 [61].

There is a highly complex network of immune cells, inflammatory cytokines, hypoxia, epithelial and endothelial cells, and extracellular matrix components affecting the EMT process. For instance, hypoxia can induce EMT by activating the expression of TWIST1. IL-6 treatment of CRC cells leads to increased EMT, invasion, and metastasis by activating STAT3 and SNAI1 [62]. Furthermore, other inflammatory cytokines, including IL-1β, IL-8 and CXCL-20, and TGF-β can induce the EMT program and invasiveness of CRC via activating different signaling pathways in the tumor microenvironment [63–65].

1.2.1.2 EMT and drug resistance in colorectal cancer

Drug resistance is a common phenomenon happening in most cancers. Studies show that CRC cells under oxaliplatin treatment show resistance to this drug during the time. Cells in this stage

gain mesenchymal morphology and show changes in EMT markers like decreased E-cadherin and increased vimentin [66]. Studies of CRC patient samples have shown that overexpression of SNAI1 causes E-cadherin downregulation and vimentin upregulation. This overexpression also caused resistance to the 5-fluorouracil chemotherapy drug [67].

Buck et al. reported a correlation between mesenchymal characteristics such as diminished E-cadherin and enhanced vimentin levels and resistance to EGFR blockade in human colorectal tumor tissues. In their study, tissue with more mesenchymal features was correlated with advanced tumor stages and resistance to therapy [68].

Additionally, gene expression analysis showed that cetuximab treatment causes an increase in the expression levels of genes related to EMT. This study suggests that anti-EGFR treatment promotes EMT, which contributes to the resistance development to therapy [69].

Overexpression of C-terminal tensin-like (CTEN), an EMT-inducer factor, in CRC cell lines causes a reduction in levels of E-cadherin, an increase in tumor cell invasion, migration, and resistance to resistance to staurosporine-induced apoptosis [70]. Moreover, the clinical specimens from patients under chemotherapy show some molecular changes associated with EMT. For instance, it is investigated that E-cadherin loss is positively correlated with tumor growth, invasion, drug resistance, and metastasis to the liver in CRC patient samples [71].

The exact mechanism behind EMT and drug resistance is not known yet. The EMT program leads to the generation of cells with cancer stem cell (CSC) properties. These cells are self-renewing with mechanisms such as effective DNA repair, high expression of multi-drug-resistance-type membrane transporters, and protection by a hypoxic niche environment. The mentioned mechanisms make them efficiently resistant to chemotherapy regimens [72].

1.3 TBK1 gene

1.3.1 Structure and biological functions of TBK1

TBK1 is a non-canonical 84 KD serine/threonine kinase with 745 amino acids. This protein is compromised of four different domains, including a kinase domain (KD) at the N-terminal, a ubiquitin-like domain (ULD), an α-helical scaffold dimerization domain (SDD), and a C-terminal adaptor binding domain (CTD) [73]. The phosphorylation site of TBK1 is ser-172 on the kinase domain [74]. The kinase domain plays a crucial role in the phosphorylation of different substrates.

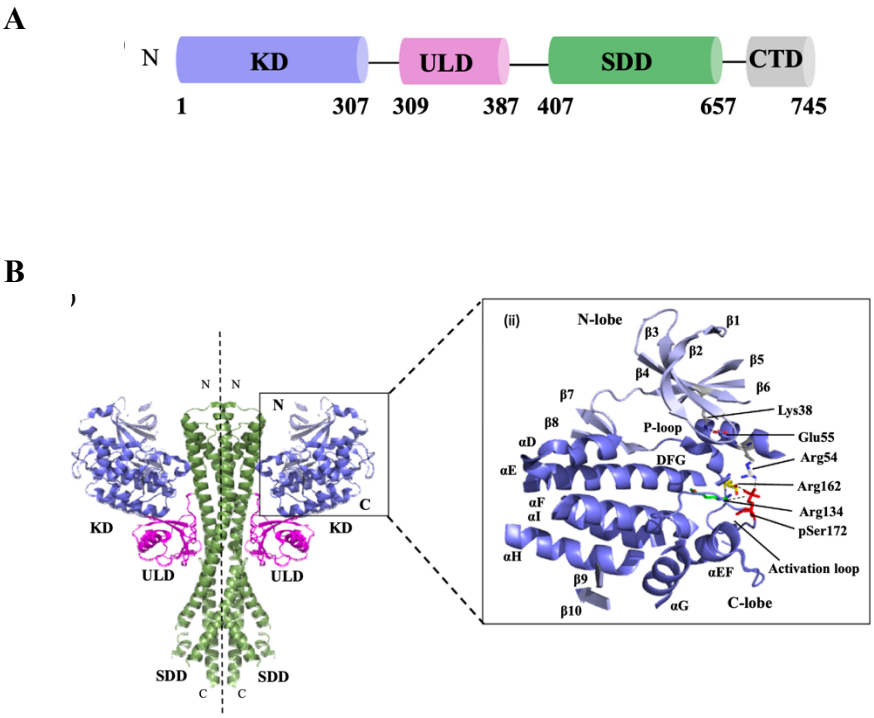


Figure 2. Structure of TBK1 protein. A) Four domains in the Structure of TBK1 including: kinase domain (KD), ubiquitin-like domain (ULD), α-helical scaffold dimerization domain (SDD), nd a C-terminal adaptor binding domain (CTD). **B)** ser172 is the phosphorylation and activation site of TBK1. *Adapted from TANK-binding kinase 1 (TBK1): An emerging therapeutic target for drug discovery by Xiang et al., 2021*

There are significant numbers of proteins that interact with TBK1. These proteins have different functions and are divided into other groups [75]. The first group of proteins is those which help TBK1 to bind to the various upstream or downstream signaling pathway proteins, including TANK, NEMO, TRIF, MYD88, TRAF3, TRAF5, TRAF6, and STING [75–79]. These proteins are adaptor proteins. The second group of the proteins is regulators of post-translation of TBK1, such as kinases [80], phosphatases [81], ubiquitin ligases [82], and histone deacetylases [83]. The last group of interacting proteins is the substrates which are phosphorylated by TBK1, including substrates in the interferon signaling pathway (IRF3, IRF7, STAT6) [80,84,85], NF-κB signaling

pathway (IKK-α, IKK-β, TANK) [86], autophagy (OPTN, CYLD, RAB7) [87,88] and proliferation (AKT, mTOR, PLK) [89–92].

1.3.2 TBK1 activation

Several factors can activate TBK1, such as bacterial infection, viral invasion, oncoproteins, and inflammatory cytokines. The innate immune system cells express various pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), RIG-1-like receptors (RLRs), and DNA receptors. With the help of these PRPs, the innate immune cells immediately recognize pathogen-associated molecules (PAMPs) such as viral DNA or RNA and lipopolysaccharide (LPS) and activate different inflammatory responses [93].

Human TLRs are divided into two different subgroups, cell surface TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) recognizing membrane-localized components of pathogens and endosomal TLRs (TLR3, TLR7, TLR8, and TLR9) identifying cytosolic RNA/DNA. Most TLRs can stimulate MyD88-IRAK4-TRAF6, NF-κB, and TBK1 signaling pathways [75].

RLRs can detect non-self RNA in the cytoplasm. After binding to these RNAs, RLRs interact with MAVs in mitochondria. MAVs then activate the TBK1 pathway by recruiting adaptor proteins TANK, NAP1, and TBKBP1 [94]. Additionally, inflammatory cytokines such as IL-1, IL-17, and oncoproteins like KRAS can activate TBK1 in cancer cells [75,95].

1.3.3 Signaling pathways of TBK1

TBK1 is a central protein regulating several signaling pathways. These pathways include immune responses and inflammation, autophagy, cell proliferation, apoptosis, and insulin metabolism [75]. TBK1 promotes innate immune responses and inflammation by inducing IRF3/IRF7, NF-κB, STAT3, and STAT6 pathways. TBK1 also regulates cellular proliferation and survival by activating AKT/mTORC1/2 and PLK1/CEP170 pathways. Moreover, it can regulate autophagy and insulin metabolism pathways [63].

1.3.4 TBK1 and innate immune responses

The innate immune system invades bacterial and viral infections immediately and regulates the production of pro-inflammatory cytokines. In this system, TBK1 plays a regulatory role in activating the IRF pathway and the production of type-I interferons. In addition, TBK1 activates

the nuclear factor κB (NF-κB) signaling pathway, which plays a crucial role in different disorders such as cancer. The NF-κB family consists of a group of transcription factors that play vital roles in regulating inflammatory and immune responses, cell proliferation, and apoptosis. Active TBK1 also promotes innate immunity and inflammation by activating the STAT6 pathway.

1.3.4.1 TBK1 and IRF pathway

Recognition of viral double-strand RNA by TLR3 or LPS recognition by TLR4 recruits different adaptors such as TIR-domain-containing adaptor-inducing IFN-β (TRIF), tumor necrosis factor receptor-associated factor 3 (TRAF3), Trif-related adaptor protein (TRAM), and stimulator of the IFN gene (STING) which causes the activation of TBK1. Once TBk1 is activated, it phosphorylates and activates IFN-regulatory factors 3 and 7 (IRF3 and IRF7). These activated proteins form homodimers, translocate to the nucleus, and trigger type-I IFN production and pro-inflammatory cytokines [96–98]. These signals are essential for immediate antiviral responses during infections. However, these signals might cause autoimmune diseases in the case of being constitutively active [99].

1.3.4.2 TBK1 and NF-κB signaling pathway

The nuclear factor κB (NF-κB) signaling pathway plays a crucial role in different disorders such as cancer. The NF-κB family consists of transcription factors that play vital roles in regulating inflammatory and immune responses, cell proliferation, and apoptosis.

NF-κB family consists of five members, including RelA (p65), Rel B, c-Rel, NF-κB1 (p50), and NF-κB2 (p52) [100]. In non-activated form, the NF-κB proteins are bound to members of the inhibitor-κB family (IκBα, IκBβ, IκBε) and are localized in the cytoplasm. These inhibitor proteins block the nuclear localization of NF-κB. Upon stimulation by different stimuli such as growth factors, phosphorylation of the IκB family by IκB kinases happen, which then leads to the degradation of IκB and nuclear localization of NF-κB where it regulates transcription of genes involved in inflammation, cell growth, cell survival, and apoptosis.

There are two main groups of canonical kinases: IKKα, IKKβ, and non-canonical kinases, including IKKε and TANK-binding kinase 1 (TBK1) in the inhibitor κB kinases family. The

kinase domain of TBK1 shares 65% similarity with the kinase domain of Ikkε; they also have similar biological functions [79,101]. TBK1 is expressed in all tissue, while IKKε is observed in high levels in peripheral blood lymphocytes and lymphoid tissues [102]. TBK1 targets different members of NF-κB family members. RelA and c-Rel are phosphorylated by TBK1, independently of the classical IκB/Ikk pathway. Phosphorylation of c-Rel leads to the nuclear translocation of this transcription factor [103]. Therefore, IKK-related kinases play crucial roles in modulating inflammatory responses by regulating the NF-κB signaling pathway.

1.3.4.3 TBK1 and STAT3/6 pathway

In addition to the roles of TBK1 in IRF and NF-κB pathways, it can also regulate inflammatory responses via STAT3/6 pathways. Chen et al. showed that in responses to viral infections, STAT6 is phosphorylated by TBK1 on Ser407. Activated STAT6 translocates to the nucleus and induces chemokines such as CCL2/20/26, essential chemokines for recruiting immune cells to fight against viral infections [104]. Therefore, TBK1 is a critical protein in innate immune system responses against invading pathogens by regulating inflammation.

TBK1 also has roles in maintaining immune homeostasis by regulating functions of dendritic cells (DCs) and T-cell activation. TBK1 phosphorylates STAT3 in DCs and inhibits type I interferon responses. This inhibition prevents aberrant activation of T cells and autoimmunity [105].

1.3.5 TBK1 and autophagy

Autophagy is a homeostatic process in which a cell degrades damaged or dysfunctional cellular organelles, components, and proteins. There are different physiological stimuli of autophagy, such as innate immune signals, hypoxia, and nutrient depletion [106]. Deregulated expression of autophagy is observed in various diseases, including cancer; however, the role of autophagy in cancer is complex. In the early stages of cancer, autophagy functions as a tumor-suppressor by limiting cell injury and inflammation, which are critical for tumor development [107]. It is also reported that autophagy is essential for tumor growth by helping tumor cells to tolerate metabolic stress caused by chemotherapy [108].

There are defined many forms of autophagy, including mitophagy and xenopaghy. Mitophagy is the process of removing dysfunctional mitochondria, while xenophagy is defined as the removal of pathogens [109,110]. Studies have shown that TBK1 has a role in xenophagy. OPTN and p62 are

adaptor proteins that bind to the pathogens for rapid autophagic removal of them. TBK1 phosphorylates these proteins on their ubiquitin-binding domains [111–113]. This activation enhances its binding to the ubiquitin chain and leads to the elimination of invading pathogen by recruiting autophagic machinery [113,114]. Therefore, TBK1 plays an essential role in combating pathogens by linking inflammation and xenophagy.

1.3.6 TBK1 in the pathogenesis of human cancers

TBK1overexpression is observed in many types of cancers, including pancreatic ductal adenocarcinoma (PDA), non-small cell lung cancer (NSCLC), clear cell renal cell carcinoma (ccRCC), melanoma, hepatocellular carcinoma, and breast cancer [115–118]. TBK1 activation in these cancers is positively correlated with poor prognosis and disease development, especially in tumors with mutated KRAS/NRAS, which suggests that TBK1 plays a role as an oncogene in these tumors [117,119]. TBK1 can induce tumor generation and progression via different mechanisms, including activating intrinsic signaling pathways, inducing the production of tumorigenic cytokines, and generating an immunosuppressive tumor microenvironment by inhibition of antitumor activities of the immune system [120].

1.3.6.1 TBK1 activates intrinsic signaling pathways in tumor cells

TBK1 promotes cell survival and growth in cancer cells by activating different signaling pathways. Some of those pathways are AKT-mTOR1 [121,122], NF-κB [123,124], Autophagy [125,126], Myc [127], and JAK-STAT [128,129].

It is observed that TBK1 is required for AKT oncogene-dependant signaling by directly phosphorylating and activating AKT on its canonical activation loop. Upon TBK1 ablation via pharmacologic inhibition or RNAi-mediated depletion, AKT activity, and cancer cell survival are impaired [121].

A recent study shows that TBK1 activation regulates mTORC1 activation and oncogenesis in lung cancer cells. Also, they showed that depletion of TBK1 causes tumorigenesis inhibition in the lung cancer mouse model. This study suggests a crucial role for TBK1 in tumor growth [129].

KRAS oncogene is mutated in a wide range of human cancers, primarily aggressive and non-responsive to the therapeutic approaches. Barbie et al. performed a comprehensive RNA interference on a wide range of human cancer cell lines and detected that TBK1 was an essential

gene in cells harboring KRAS mutations. Upon TBK1 inhibition, cell death and cell apoptosis were increased in KRAS mutant cells. They could find that TBK1 activates c-Rel and BCL-XL in these cells, which are NF-κB anti-apoptotic proteins. So, this study suggested TBK1 and NF-κB signaling as a critical pathway for cell survival [119].

The expression level of TBK1 is detected more in acute myeloid leukemia (AML) than the healthy hematopoietic cells. Increased apoptosis of AML cells was seen upon TBK1 pharmacological inhibition or shRNA knockdown, suggesting that these cells rely on TBK1 for survival. Studies indicated that TBK1 activates oncogenic gene regulator YB1 in AML cells, then YB1 binds to the Myc oncogene promoter in these cells and enhances its expression. TBK1 inhibition leads to reduction of Myc expression, viability, and tumorigenicity of AML cells. This suggests TBK1 as a potential therapeutic target for AML disease [127].

Studies demonstrated a role for TBK1 in Ras-induced oncogenic transformation. RalB phosphorylates TBK1 in the Ras-like (Ral)-guanine nucleotide exchange factor (GEF) pathway, which is necessary for Ras-induced transformation [130,131]. Upon activation, TBK1 assembles with exocyst, a multisubunit complex consisting of different secretory proteins, sec3, sec5, sec6, sec8, sec10, Exo70, and Exo84. TBK1 becomes a part of the exocyst complex via interaction with sec5. The exocyst is required to maintain the polarity of the cell membrane. Studies have shown the importance of membrane polarity in cell survival [132]. Therefore, Ral proteins contribute to tumorigenesis via exocyst-dependant maintenance. [133].

1.3.6.2 TBK1 promotes tumorigenic cytokine production

In addition to the role of TBK1 in the mentioned pathways, TBK1 can also promote the production of cytokines which play essential roles in tumorigenicity. In a study on KRAS-driven lung cancer (NSCLC), cytokines CCL5 and IL-6 are reported to be highly active. In this study, it is detected that TBK1 regulates CCL5 production. CCL5 could rescue TBK1 depleted mouse embryonic fibroblasts (MEF) clonogenic ability. These cytokines can activate the JAK-STAT signaling pathway, which induces IL-6 production and lung cancer cell proliferation [134]. Korher et al. suggested that TBK1 is expressed in many solid tumors under hypoxia conditions. They indicated that TBK1 induces the production of some factors such as RANTES or IL-8, which are essential for the proliferation of epithelial cells. The production of these factors resembles the angiogenesis mechanism in tumor cells. Impaired angiogenesis mechanism in these cells is also

observed upon TBK1 inhibition. Thus, this study makes TBK1 a promising target for cancer treatment [135].

1.3.6.3 TBK1 generates an immunosuppressive tumor microenvironment

The induction of intrinsic signaling pathways and production of tumorigenic immunosuppressive cytokines are not the only mechanisms of TBK1 for tumor promotion. TBK1 increases tumor proliferation and survival by attenuating antitumor functions of the immune system in the tumor microenvironment. Upregulation of immune checkpoint ligands like programmed cell death-ligand 1 (PD-L1) and preserving immunosuppressive tumor microenvironment are the mechanisms of TBK1 to help tumor progression [129].

Interferon-inducible 16 (IFI16) is expressed in high levels in papilloma-positive cervical cancer cells, promoting cancer development. High levels of IFI16 activate the STING-TBK1 pathway and subsequently activate NF-κB pathway, which induces PD-L1 expression [136]. Jiang et al. reported that high levels of TBK1 in hepatocellular carcinoma (HCC) are associated with poor prognostic tumors. They could also show that high levels of TBK1 are correlated with fewer tumor-infiltrating CD8+ T cells. Moreover, TBK1 inhibition has increased the number of tumor-infiltrating CD8+ T cells [116].

1.3.6.4 TBK1 is a tumor-suppressive gene in intestinal tumors

Although it is shown that TBK1 has an oncogenic role in most cancer types, there are studies suggesting tumor-suppressive functions for this kinase protein. A recent study reported that TBK1 depletion in intestinal epithelial cells (IECs) increases the production of NF-κB-mediated metallothionein 1 (MT1). MT1 is an immune modulator that induces IL-1β secretion, facilitating TH17 cell differentiation. These cells produce IL-17 which is an essential cytokine for tumor growth promotion. In the same study, they could show a positive correlation between high expression levels of TBK1 and improved overall survival in colorectal cancer patients. Moreover, increased expression of MT1 and IL-17 is correlated with poor survival in colorectal cancer patients. Therefore, this study suggests TBK1 as a regulator of the immune system and tumor formation in colon [137].

1.4 Aim of the study

Considering the roles of TBK1 in different cancer types, we aimed to study the roles of TBK1 in colorectal cancer and drug resistance development. To address this question, TBK1 loss of function cell lines were generated via lentivirus system, and the effect of TBK1 loss on colorectal cancer and drug resistance development were characterized *in vitro* and *in vivo*. Moreover, our group previously showed that IKKε depletion in some colorectal cancer cell lines leads to increased cell proliferation. Since IKKε and TBK1 are members of the same family (non-canonical IκB kinase), we also wondered if there are any redundant or additive roles for TBK1 and IKKε in colorectal cancer development.

CHAPTER 2

Materials and Methods

2.1 Materials

The materials used in this study are listed below.

2.1.1 Chemicals

Table 1. Chemicals used in this study.

Product Name	Catalog Number	Company
Ethanol	32221	Sigma Aldrich
Methanol	24229	Sigma Aldrich
2-propanol	100995	Sigma Aldrich
Glycine	GLN001.1	Bioshop Canada
Glycerol	15524	Sigma Aldrich
Tween20	777	Ambresco
EDTA	E-5134	Sigma Aldrich
Trizma base	T1503	Sigma
Sodium dodecyl sulfate	822050	Merck
Bovine Serum Albumin	sc-2323	Santa Cruz
TEMED	1610801	BioRad
Sodium chloride	S9888	Sigma Aldrich
Ammonium Persulfate (APS)	7727-54-0	Sigma Aldrich
DMSO	67-68-5	Applicam
Trichloroactic acid	SE3691003	Serva

2-Mercaptoethanol	M3148	Sigma Aldrich
40% Acrylamide/Bis solution	1610148	BioRad
Hydrochloric acid	100317	Merck
NaOH	106.462	Merck
Page Ruler Prestained Protein Ladder	26616	Thermo Scientific
Bromophenol Blue	B0126	Sigma Aldrich
Sodium Azide	S2002-5G	Sigma Aldrich
Triton x-100	T8787	Sigma Aldrich
Propodium iodide (PI)	P4170	Sigma Aldrich
Ribonuclease A	R6513	Sigma Aldrich

2.1.2 Reagents

Table 2. Reagents used in this study.

Product Name	**Catalog Number**	**Company**
cOmplete Protease Inhibitor Cocktail	11697498001	Roche
CellTiter-Glo 3D Cell Viability Assay	9682	Promega
Sulforhodamine B (SRB) sodium salt	A14769.14	Alfa Aesar
Transfectin™ lipid Reagent	1703351	Bio-Rad
Lipofectamine™ 2000 transfection reagent	11668027	Invitrogen
Pierce ECL Western Blotting Substrate	LSG-32106	Thermo Scientific

SuperSignal West Femto Maximum Sensitivity Substrate	34094	Thermo Scientific
Pierce Western Blotting Substrate-Super Signal West pico Plus	LSG-34577	Thermo Scientific

2.1.3 Cell culture media, chemicals, and solutions

Table 3. Cell culture media, chemicals, and solutions used in this study.

Product Name	**Catalog Number**	**Company**
RPMI 1640 w/stable L-Glutamine	L0498-500	Biowest
Mccoy´s 5A w/ L-Glutamine	L0210-500	Biowest
DMEM Low Glucose	L0064-500	Biowest
DMEM High Glucose	B.L0106-500	Biowest
Fetal Bovine Serum Heat Inactivated	S181H-500	Biowest
Phosphate Buffered Saline 10X	P04-53500	PAN_Biotech
L-Glutamine	G7513-100ml	Sigma Aldrich
Pen-Strep	DE17-602E	Lonza
Trypsine-EDTA 10X	X0930-100	Biowest
Puromycine	Ant-pr-1	Invivogen
Polybrene (10mg/ml)	Sc-134220	SantaCruz
Opti-MEM I	31985-070	Gibco
Gefitinib	sc-202166	SantaCruz

2.1.4 Buffers

Table 4. Buffers used in this study.

Buffer Name	Contents
Cell Lysis Buffer 2X	25mL 1M Hepes, 30 mL 5M NaCl, 5mL Triton-X100, 100mL Glycerol, 340 mL ddH_2O
Cell Lysis Buffer 1X	5mL cell lysis Buffer 2X, 500 µL NaF, 500 µL Na_3VO_4, 500 µL β-Glycerophosphate, 500 µL cOmplete protease inhibitor, up to 10 mL ddH_2O
10X Running Buffer	144g Glycine, 10g SDS, 30.2g Tris-Base, up to 1L ddH_2O
10X Transfer Buffer	144g Glycine, 30.2g Tris-Base, up to 1L ddH_2O
10X TBS_T	24.2g Tris-Base, 80g NaCl, pH=7.6, 20mL Tween, up to 1L ddH_2O
Stacking Gel	0.6mL Acrylamide/Bis solution, 3mL ddH_2O, 625µL 1M Tris pH=6.8, 125µL 0.25M EDTA, 100µL 10% SDS, 60µL 10% APS, 10µL TEMED
Separating Gel 8%	3.2mL Acrylamide/Bis solution, 5.8mL ddH_2O, 1.5mL Glycerol, 3.75mL 1.5M Tris pH=8.8, 375µL 0.25M EDTA, 300µL 10% SDS, 125µL 10% APS, 15µL TEMED
4X Protein Loading Dye	12.5mL 1M Tris-HCl pH=6.8, 5g SDS, 5mL β-mercaptoethanol, 20mL Glycerol, 0.008% Bromophenol Blue
Ponceu S	0.4g ponceu S, 20mL Acetic Acid, up to 400mL ddH_2O
Mild Stripping Buffer	3g Glycine, 0.2g SDS, pH=2.5, 20mL Tween, up to 200mL ddH_2O
0.5% Crystal Violet	0.5g Crystal Violet, 25mL methanol, 75mL ddH_2O
10X Phosphate Buffer Saline (PBS)	17.02g Na_2HPO_4, 80g NaCl, 2g KCl, 2g KH_2PO_4, up to 1L ddH_2O, pH= 6.87
50X TAE Buffer	242g Tris-Base, 57.1mL Acetic Acid, 100mL 0.5M EDTA, ut to 1L ddH_2O

LB Broth	10g NaCl, 5g Yeast Extract, 10g Tryptone
2X HBSS	0.1081g 12mM Dextrose (D-glucose), 2.8mL 5M NaCl, 5mL 10X Na_2HPO_4(10X: 0.1335g in 50mL), 2mL 250mM KCl, up to 50mL ddH_2O, pH= 7.01

2.1.5 Kits

Table 5. Kits used in this study.

Product Name	Catalog Number	Company
Pierce BCA Protein Assay Kit	LSG-2322	Thermo Scientific
E.Z.N.A Plasmid DNA Mini Kit	D6922-02	Omega Bio-Tek
Mycoplasma PCR Detection Kit	G238	ABM

2.1.6 Antibodies

Table 6. Antibodies used in this study.

Product Name	Catalog Number	Company
E-cadherin	Sc-8426	Santa Cruz
N-cadherin	14215	Cell Signaling
Vimentin	5741	Cell Signaling
Alpha smoot muscle actin (α-SMA)	Ab5684	Abcam
Integrin-α5	4705	Cell Signaling
Zo-1	13663	Cell Signaling
TBK1/NAK	3013	Cell Signaling
Ikkε	3416	Cell Signaling
Phospho-AKT	4060	Cell Signaling

Total-AKT	9272	Cell Signaling
Phospho-ERK	4370	Cell Signaling
Total-ERK	9102	Cell Signaling
Cleaved Caspase-3	9664	Cell Signaling
GAPDH	Sc-47724	Santa Cruz
Monoclonal anti-α Tubulin	T5168	Sigma Aldrich
Anti-rabbit IgG HRP-linked	7074	Cell Signaling
Anti-mouse IgG HRP-linked	7079	Cell Signaling

2.1.7 Consumables

Table 7. Consumables used in this study.

Product Name	**Catalog Number**	**Company**
1000uL tips	GR740290	Greiner
200uL tips	GR739290	Greiner
10uL tips	4840	Corning
1000uL filter tips	740288	Greiner
200uL filter tips	739288	Greiner
20uL filter tips	774288	Greiner
10uL filter tips	F161630	Greiner
Gel-loading tips	14-222-809	Axygen
5mL serological pipette	4487	Corning
10mL serological pipette	4488	Corning
25mL serological pipette	4489	Corning
1.5mL Reaction Tube	3621	Corning
1.5mL SafeLock Tube	30120086	Eppendorf
2mL Reaction Tube	430659	Corning
15mL falcon tube	188271	Greiner
15mL falcon tube	62.554.502-500	Sarstedt
50mL falcon tube	227261	Greiner

50mL falcon tube	62.547.254-300	Sarstedt
145mm tissue culture dish	639160	Greiner
100mm tissue culture dish	664160	Greiner
60mm tissue culture dish	628160	Greiner
175cm tissue culture flask	660175	Corning
75cm tissue culture flask	658175	Corning
25cm tissue culture flask	690175	Corning
6-well plate	657160	Greiner
12-well plate	665180	Greiner
24-well plate	662160	Greiner
96-well plate	655180	Greiner
Cryovials	121263	Greiner
PVDF membrane, 0.45	L-08025-001	Advansta
0.45μm filter	16555	Sartorious
0.20μm filter	16534	Sartorious

2.2 Methods

2.2.1 Cell Maintenance

DLD1 and HCT116 cells were kept in Mccoy´s 5A growth medium supplemented with 5% and 8% Fetal Bovine Serum (FBS), respectively, 1% L-Glutamine, and 1% Penicillin/Streptomycin. HT29 cells were maintained in DMEM low Glucose with 1% L-Glutamine and 1% Penicillin/Streptomycin. The growth medium was changed every 2-3 days, and they were passaged when they reached 80-90% confluency with a 1:5 ratio. All of the cell lines were kept at 37^0C, 5%CO_2 incubators.

2.2.2 Cryopreservation of Cells

After removing the old medium, cells were washed with 1X PBS solution. Then, the cells were trypsinized and incubated in 37ºC incubators for 2-3 minutes. After collecting the cells, they were centrifuged at 300 g for 5 minutes at +4ºC. The medium was removed, and the pellet was resuspended in a freezing medium consisting of 90% FBS and 10% DMSO with a maximum density of 1-2 million cells. 1.5mL of the freezing medium was added to each cryovial and placed in Mr. Frosty Cell Freezing Container and put at -80ºC for short-term storage. For long-term storage, the cells were placed in liquid nitrogen.

2.2.3 Thawing Cells

The Cryovial of the cells was thawed rapidly at 37 ºC water bath. The cryovial was opened before the cells completely thaw, and a fresh growth medium was added to the drop-by-drop cells. Then the medium containing cells were transferred to a 15mL falcon tube and centrifuged at 300g for 5 minutes. Then the supernatant was removed, and the pellet was resuspended in the complete growth medium and placed at the appropriate cell culture flask or dish.

2.2.4 PCR-based Mycoplasma test

The cells were kept in cell culture at least 48-72 hours before the test. 500µL of cell culture medium was taken and centrifuged at 13000rpm for 5 minutes to pellet the cells. The supernatant was transferred to a fresh tube and centrifuged at 13000rpm for 20 minutes. After centrifugation, 450µL of the supernatant was removed, and the pellet was resuspended in the remaining 50µL, which was considered as a test sample. The PCR Reaction was set as shown in the table below.

Table 8. Reaction mixture of PCR-based mycoplasma test.

Component	Test sample	Positive control	Negative control
2X PCR Taq MasterMix	12.5µL	12.5µL	12.5µL
Primer Mix	1µL	1µL	1µL
Test Sample	2.5µL	-	-

Positive Control	-	1µL	-
Nuclease-free H_2O	9µL	10.5µL	11.5µL
Final Volume	25µL	25µL	25µL

The Reaction tubes were placed at the thermocycler and Polymerase Chain
The reaction program was performed as follows:

Table 9. Conditions of PCR test for mycoplasma

Step	**Tempreature**	**Duration**	**Cycles**
Enzyme Activation	95°C	5 mins	-
Denaturation	95°C	30 secs	30-40
Annealing	55°C	30 secs	30-40
Extension	72°C	60 secs	30-40
Final Extension	72°C	10 mins	1
Holding	4°C	-	-

The amplified product was resolved by running at 1% agarose gel electrophoresis (120 V, 30 minutes).

2.2.5 Enzyme-based Mycoplasma test

For this test, 500µL of the medium was taken from the cell plate and then centrifuged at 13000rpm for 5 minutes. Then 100µL was taken from the supernatant and added to a 96-well white plate. 100µL reactant from the kit was added on top of the medium, and luminescence was read.
By Synergy HT Microplate Reader (Read A) after 5 minutes incubation. Then 100µL substrate from the same kit was added on top. After 10 minutes of incubation, the luminescence was read again (Read B). The results were interpreted as below:
Read A/ Read B: If: < 0.9: Negative
If: 0.9-1.1: Measure again
If: > 1.2: Positive

2.2.6 Lentiviral shRNA Transfection and Transduction

Approximately $1X10^6$ of HEK293T cells were seeded in a 6-cm cell culture dish in DMEM High Glucose medium supplemented with 8% FBS. The cells were transfected the next day when the confluency was 70-80%. Plasmids containing shRNA targeting the gene of interest and packaging plasmids for the generation of lentiviral particles were mixed with $CaCl_2$ and ddH_2O. Then this mixture was added dropwise to 2X HBSS while vortexing. Then the mixture was incubated at room temperature for 10 minutes and added dropwise on top of the HEK293T cells, and then the dish was shaken gently. Sixteen hours post-transfection, the medium of HEK293T cells was removed and fresh growth medium was added to the cells, and they were incubated at 37°C incubator.

Table 10. Components used in lentiviral transfection

Component	Amount
shRNA plasmids	9µg
VSVG packaging plasmid	3µg
R891 packaging plasmid	6µg
2M $CaCl_2$	38µL
2X HBSS	300µL
ddH_2O	Up to 600µL

Medium of the transfected HEK293T cells containing the virus was collected 48 hours post-transfection. The collected media was centrifuged at maximum speed for 10 minutes to remove cell debris, filtered through a 0.45µm filter, and added to the recipient cells with 5g/mL Polybrene to increase transduction efficiency. Cells were incubated for 48 hours, and then the medium was changed. Transduced cells were gone under selection by keeping them in a medium containing puromycine.

2.2.7 siRNA Transfection

Approximately $25x10^4$ cells per well of a 6-well dish were seeded. The following day a mixture of 100μM siRNA and OptiMEM was prepared, and HiPerfect reagent was added to the mixture. The mixture was added to the seeded cells. Six hours post-transfection, 800μL of the growth medium was added on top of the cells. Twenty-four hours post-transfection, cells were collected to isolate protein and RNA samples.

2.2.8 Generation of Gefitinib-Resistant DLD1 Cells

To generate Gefitinib resistant cell line, first, the IC_{50} value of wild-type DLD1 cells was determined (IC_{50}=10.36μM). Then generation of Gefitinib-resistant DLD1 cells was started by adding 9μM of Gefitinib to the wild-type cells. Then these cells were grown in a complete cell growth medium and exposed continuously to increasing doses of Gefitinib. The Gefitinib concentration was increased by eighter 0.5μM or 1μM every three passages. Once their IC_{50} value became at least two times more than wild-type parental cells' IC_{50} value, they were considered resistant.

2.2.9 Determination of Gefitinib IC_{50}

DLD1, HT29, and HCT116 cells were seeded at a 96-well plate in a number that reached 60-70% confluency the next day. Cells were treated with different concentrations of Gefitinib the following day and incubated at 37°C incubator for 72 hours. At the end of 72 hours, cell viability was measured by the SRB method. The IC_{50} value, which is the amount of the drug that kills 50% of the cells, was determined using GraphPad Prism Program.

2.2.10 Cell-Titer Glo Luminescent Cell Viability Assay

Cell-Titer Glo Luminescent cell Viability Assay aimed to determine the proliferation rate of the cells. The cells were seeded at a 96-well plate and grown with a complete growth medium for 3-4 days. Cells were lysed every day by adding CTG reagent to each well and shaking for 15 minutes at RT. Cell lysates were transferred to a white opaque 96-well plate. Then luminescence reading was performed by Synergy HT Microplate Reader.

2.2.11 Sulforhadamine B (SRB) assay

Sulforhadamine B (SRB) Assay was performed to assess cell growth upon Gefitinib treatment. 72 hours after the drug treatment, the old medium was removed, and 120µL fresh growth medium was added to each well. Then 80µL of 25% TCA was added on top of the cells to fix them, and the plate was incubated at +4°C for at least 1 hour. The TCA was removed after the incubation time, and the wells were washed five times with water. After completely removing water, 50µL of SRB was added to each well and incubated for 30 minutes at dark to stain the cells. Then the wells were washed with 1% Acetic Acid solution, and the plate was left to be dried under a fume hood. When it was dried entirely, 150µL 10mM tris-base was added to each well to destain. The absorbance was read by Synergy HT Microplate Reader.

2.2.12 Colony Formation Assay

The cells were seeded in 6-well or 12-well plates in a minimal number. The plate was kept at 37°C incubator for approximately 10-14 days. When the single cells formed colonies before the colonies merged, the experiment was finalized by Crystal Violet staining. After removing the old medium, the cells were washed with 1X PBS and then fixed on ice-cold 100% methanol at -20°C for 10 minutes. After incubation time, methanol was discarded, and the cells were incubated with 0.5% Crystal Violet at RT to be stained. Then Crystal Violet was discarded, and the plates were washed five times with water to remove the excess dye. Then the plates were dried at RT. The colony numbers were quantified by ImageJ software.

2.2.13 Wound Healing Assay

DLD1 and HT29 cells were seeded at a 24-well plate to reach 90% confluency the following day. The next day, the scratch was made using a 200µL tip. The plate was washed with 1X PBS 3-4 times to remove all dead cells. Then fresh growing medium containing 0.5% or 5% FBS was added to each well of DLD1 or HT29 cells, respectively, to slow down cell growth and let the cells migrate and fill the gap. At different time points (0h, 48h for DLD1 and 0h, 72h for HT29), the pictures were taken by Leica MC120 microscope. Analysis of the images was performed using TScratch software.

2.2.14 Transwell Migration Assay

A Transwell migration assay was performed to assess the migration ability of HCT116 cells. For this purpose, HCT116 cells were starved 24 hours before seeding. The next day, cells were seeded in the upper chamber in a medium with 0% FBS, and a medium with 20% FBS was added to the lower chamber. 48 hours post-seeding cells in the upper chamber were fixed with methanol and stained with Giemsa. The number of migrated cells through the chamber was quantified by ImageJ software.

2.2.15 Transwell Invasion Assay

Transwell invasion assay was conducted to assess the invasion ability of the cells. Starved DLD1 and HT29 cells were seeded with 0% FBS medium in the upper chamber of the transwell plate precoated with matrigel. Medium containing 20% FBS was added to the lower chamber. The chambers were stained with Giemsa 72 hours after the seeding. The number of invaded cells through the chamber was quantified by ImageJ software.

2.2.16 Protein Extraction from Cultured Cells

Cells at their 70% confluent and healthy status were taken out from the incubator and put on ice. After removing the old medium, cells were washed with 1X PBS. Then the cells were collected in 1X PBS by a scrapper and transferred to a 1.5mL Eppendorf tube. The tubes were centrifuged at 13000rpm for 10 minutes at +4°C. The supernatant was removed, and the pellet was resuspended in RIPA Cell Lysis Buffer for protein samples. The samples were incubated on ice for 30 minutes by vortexing them every 10 minutes. Then they were centrifuged at 13000rpm for 15 minutes. The supernatant was transferred to a new fresh Eppendorf tube and kept at -80°C.

2.2.17 Protein Quantification

Protein quantification was performed using the Pierce BCA protein assay kit. 2mg/ml BSA protein was diluted 20X with ddH_2O. The standards were prepared with different concentrations, as shown in Table 11.

Table 11. Protein quantification standards.

Concentration(μg/μL)	BSA(μL)	ddH_2O(μL)
0	0	400
1	40	260
2	80	320
3	120	280
4	160	240
5	200	200
7	280	120
10	400	0

100μL of each standard was added to each well of a 96-well plate as triplicates. Protein samples to be measured were diluted as 1:100 by ddH_2O (4μL samples + 396 μL ddH_2O) and loaded to each well. BCA reagent mixture was prepared according to the kit protocol (Reagent A+ Reagent B 1:50 ratio). 100 μL of the reagent was added on top of each well. Then the plate was incubated at 60ºC for 50 minutes. The absorbance reading was performed by Synergy HT Microplate Reader at 562nm. Then the samples were mixed with 4X Loading dye and boiled at 75ºC for 10 minutes.

2.2.18 Western Blotting

The Stacking and Separating gels were prepared according to the protocol mentioned in the Materials section. Equal amounts of boiled proteins were loaded onto the stacking gel and run at 120V for 110 minutes. Then the proteins were transferred to the PVDF membrane by a wet transfer system. After transfer, the membrane was blocked in 10% milk for 30 minutes while shaking and washed with 1X TBST twice for 10 minutes. Then the membranes were incubated with primary antibody overnight at +4ºC and washed with 1X TBST three times for 10 minutes. Next, they were incubated at HRP labeled secondary antibody and washed with 1X TBST for 30 minutes. Pierce ECL Western Blotting Substrates were used to reveal the membrane in Amersham Imager 600.

2.2.19 Generating Xenograft Mice Models

The cells were collected by trypsin and washed with 1X cold PBS. Then they were counted and prepared as $4x10^6$ cells/200µL in 1X cold PBS. Then a subcutaneous injection of the cells was performed in two flanks of 8 weeks old athymic nude mice provided by the animal facility of the Bilkent University, Department of Molecular Biology and Genetics. Ethical permission from the local ethics committee was taken before the injections. Once the subcutaneous tumor volumes reached 4-5 $mm^{3,}$ the volumes were measured by a caliper and calculated as LxW^2 (L: length, W: width). When tumor volumes reached 1000 $mm^{3,}$ the mice were sacrificed. The tumors were collected, RNA and protein samples were taken and kept at -80°C and the remaining tumors were fixed in 4% PFA and sent for histopathological analysis.

CHAPTER 3

Results

3.1 The expression level of TBK1 was detected in CRC cell lines.

To determine the cell lines used in the experiments, we first analyzed the expression level of TBK1 of CRC cell lines available in our laboratory. HT29, DLD1, and HCT116 cell lines were selected for the experiments since TBK1 has the highest expression level in these cell lines (Figure 3). TBK1 protein was depleted in the mentioned cell lines by lentiviral shRNA transfection.

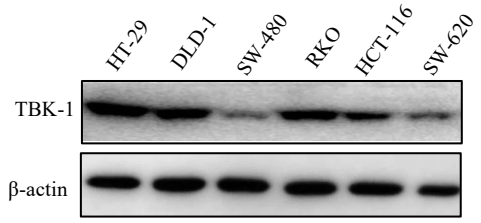


Figure 3. The expression level of TBK1 in CRC cell lines. The expression level of TBK1 was measured in six different CRC cell lines by Western Blotting. β-Actin is used as a loading control.

3.2 Cell proliferation and colony formation are enhanced upon TBK1 depletion in colorectal cancer cell lines.

The cell proliferation of shRNA TBK1 depleted CRC cell lines is measured by Cell-Titer Glo Luminescent cell viability over 3-4 days by measuring the concentration of ATP in viable cells. We could show that cell growth is significantly enhanced upon TBK1 depletion in DLD1 (Figure 4A), HT29 (Figure 4B), and HCT116 (Figure 4C).

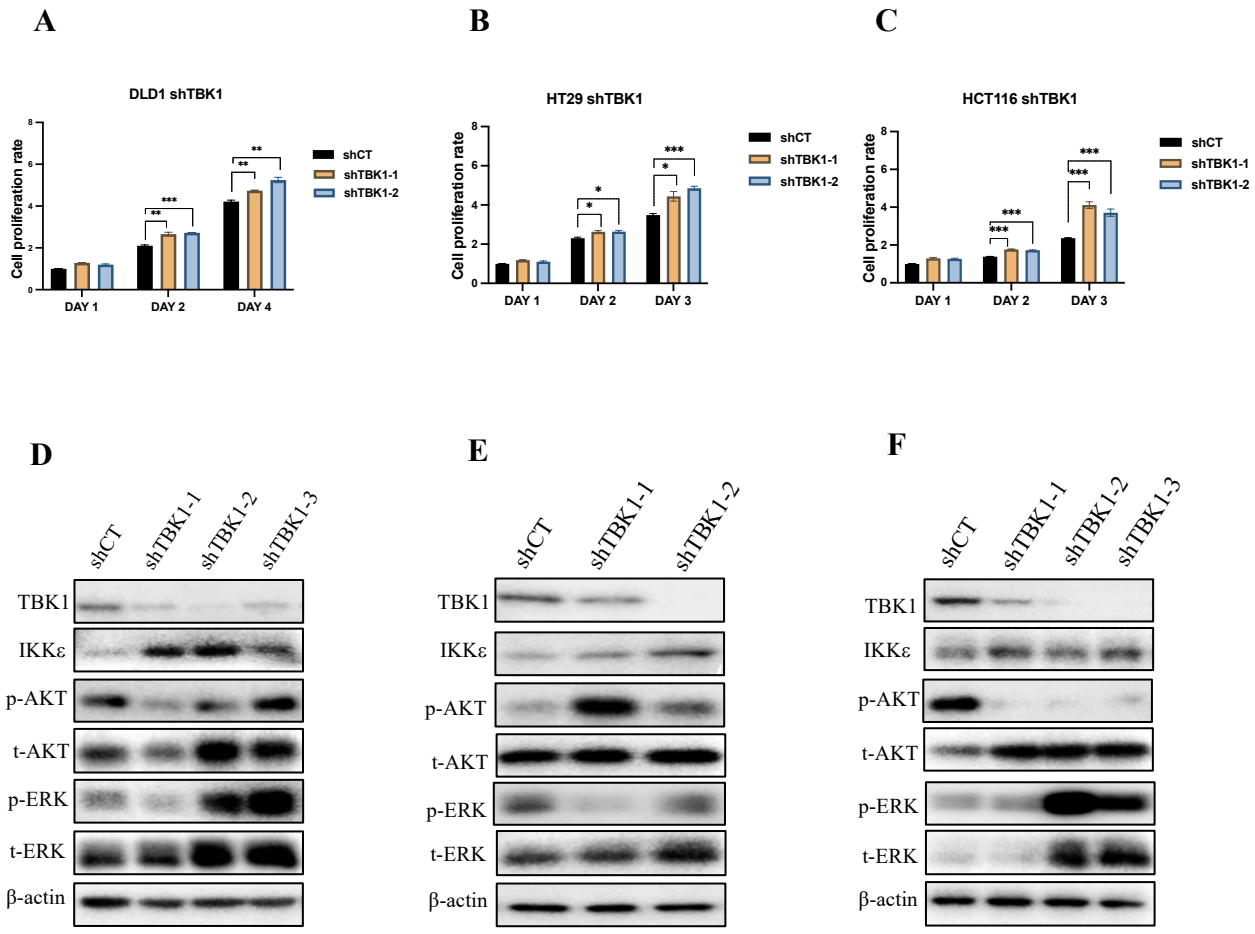
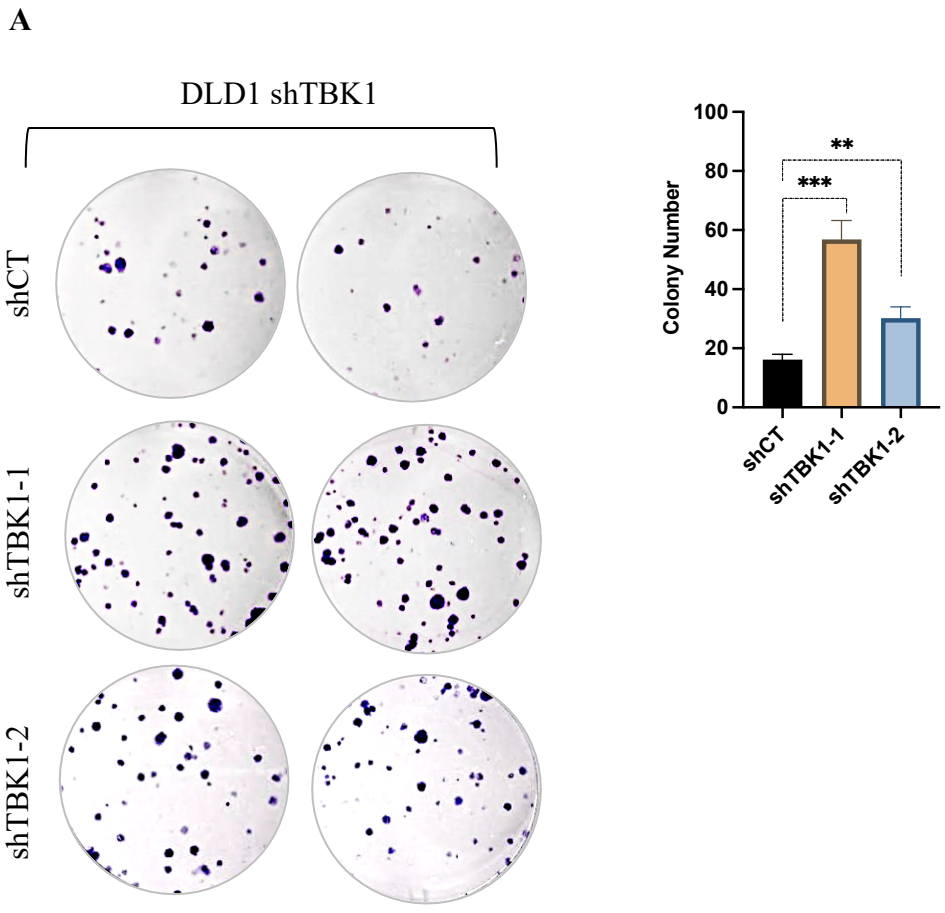


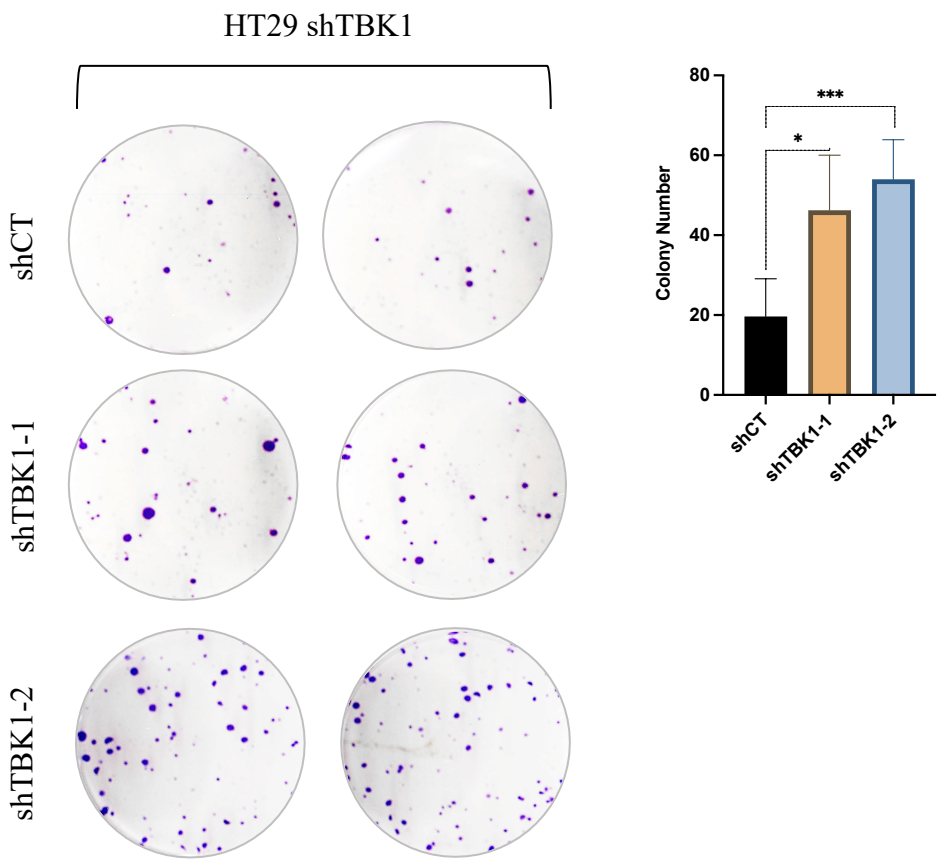
Figure 4. Cell proliferation is enhanced upon TBK1 depletion in CRC cell lines. Cell proliferation is measured in shRNA TBK1 cells by Cell-Titer Glo Luminescent cell viability assay over 4 days. **A**) DLD1 shTBK1. **B)** HT29 shTBK1. **C**) HCT116 shTBK1. Expression levels of pAKT and pERK are analyzed by western Blotting in **D)** DLD1 shTBK1, **E)** HT29 shTBK1, **F)** HCT116 shTBK1 cell lines. Data shown here are the mean ± SEM of biological triplicates for each construct. Each experiment is repeated twice. (unpaired t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.)

Additionally, AKT and ERK phosphorylation levels which are markers of cell viability, are also increased upon TBK1 depletion in the mentioned cell lines (Figure 4D, E,F).

Moreover, the colony-forming ability of CRC cell lines is measured upon TBK1 depletion. For this purpose, the shTBK1 CRC cells were seeded in low numbers in 12-well or 6-well plates to grow as single cells and make colonies. The growth of seeded cells was followed for approximately 10-14 days. Once the colonies were formed and reached a specific size, the wells were stained with 0.5% crystal violet, and the experiment was finalized. Colony numbers are quantified by ImageJ software. In this experiment, we could show that TBK1 depleted DLD1 (Figure 5A), HT29 (Figure 5B), and HCT116 (Figure 5C) can form more and larger colonies than the control cells. Finally, we concluded that TBK1 depletion increases cell growth in CRC cell lines *in vitro*.



B


HT29 shTBK1
shCT
shTBK1-1
shTBK1-2
Colony Number
80
60
40
20
0
***
*
shCT
shTBK1-1
shTBK1-2

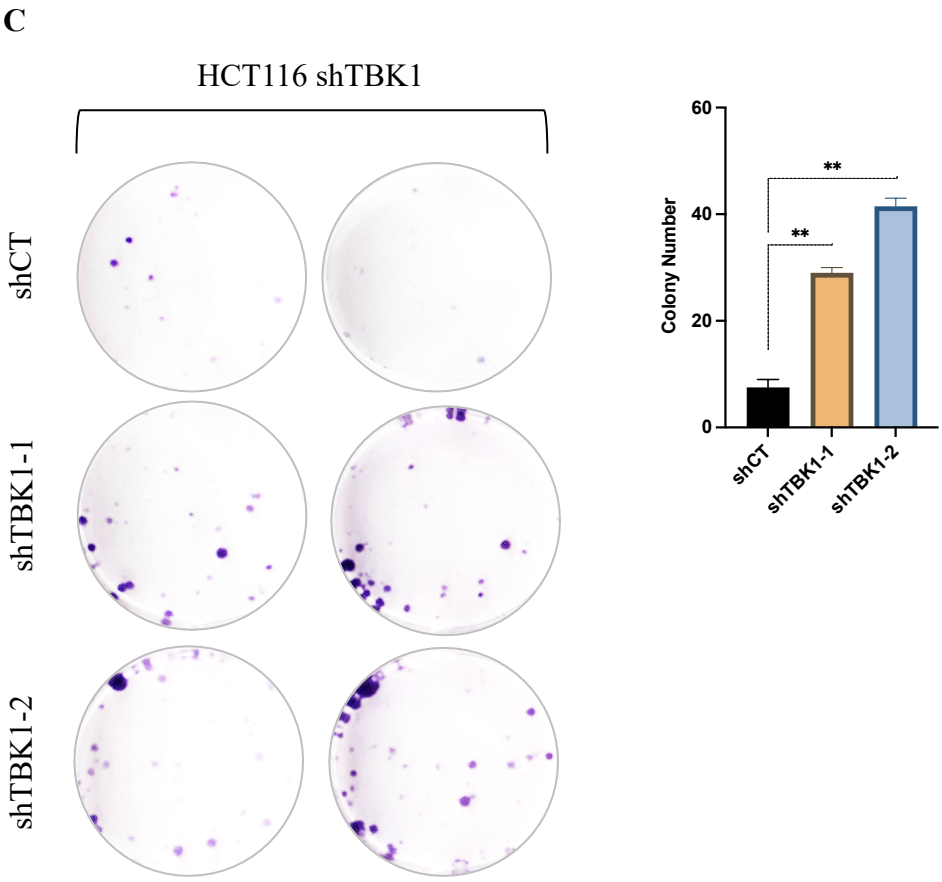


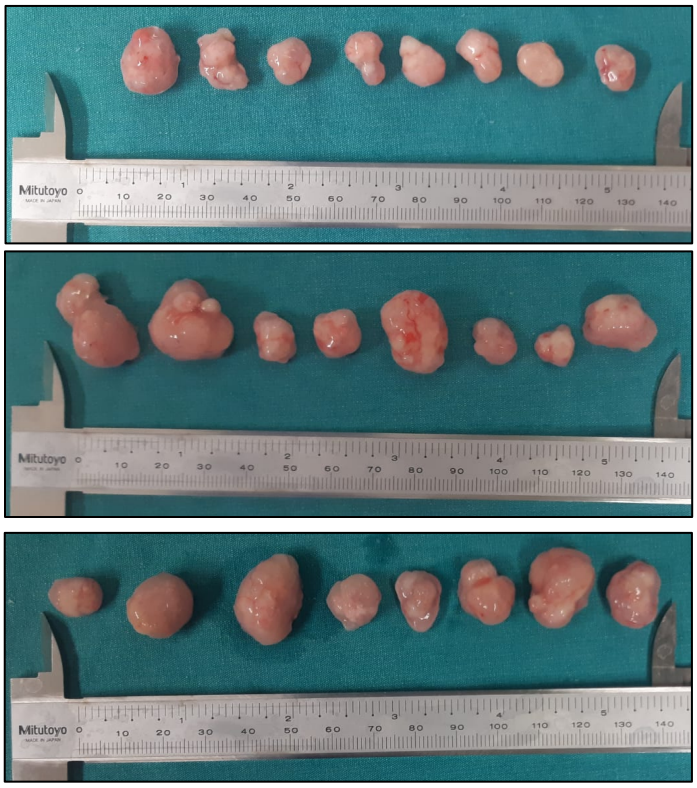
Figure 5. TBK1 inhibits single cells from forming colonies in CRC cell lines. Colony formation assay is conducted to assess the colony formation ability of TBK1 depleted cells compared to the control cells. **A)** DLD1 shTBK1 **B)** HT29 shTBK1 **C)** HCT116 shTBK1.The growth of colonies from single cells was followed for approximately 10-14 days. Once the colonies reached a certain size, the experiment was finalized by staining the plates with 0.5% Crystal Violet dye. Data shown here are the mean ± SEM of biological triplicates for each construct. Each experiment is repeated twice. (unpaired t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.)

3.3 Xenograft models of shRNA TBK1 DLD1 cell line showed promoted tumor growth.

We injected shRNA TBK1 DLD1 cell lines subcutaneously to the flanks of athymic nude mice to show the role of TBK1 in cell growth *in vivo.* Once the tumors reached a certain size after the injection, we started to measure tumor size. We followed the tumor growth for approximately 25 days after the injection. When the tumor volumes reached around 1000mm^{3,} we finalized the experiment (Figure 6A, B). Moreover, weight of the tumors were measured (Figure 6 C). The expression level of TBK1 was measured by western blotting at the starting and the end point of the experiment (Figure 6D).

In this experiment, we could show that TBK1 depletion leads to an increase in the growth of tumors *in vivo* since xenografts of TBK1 depleted cell line could form more extensive tumors compared to the control mice.

A



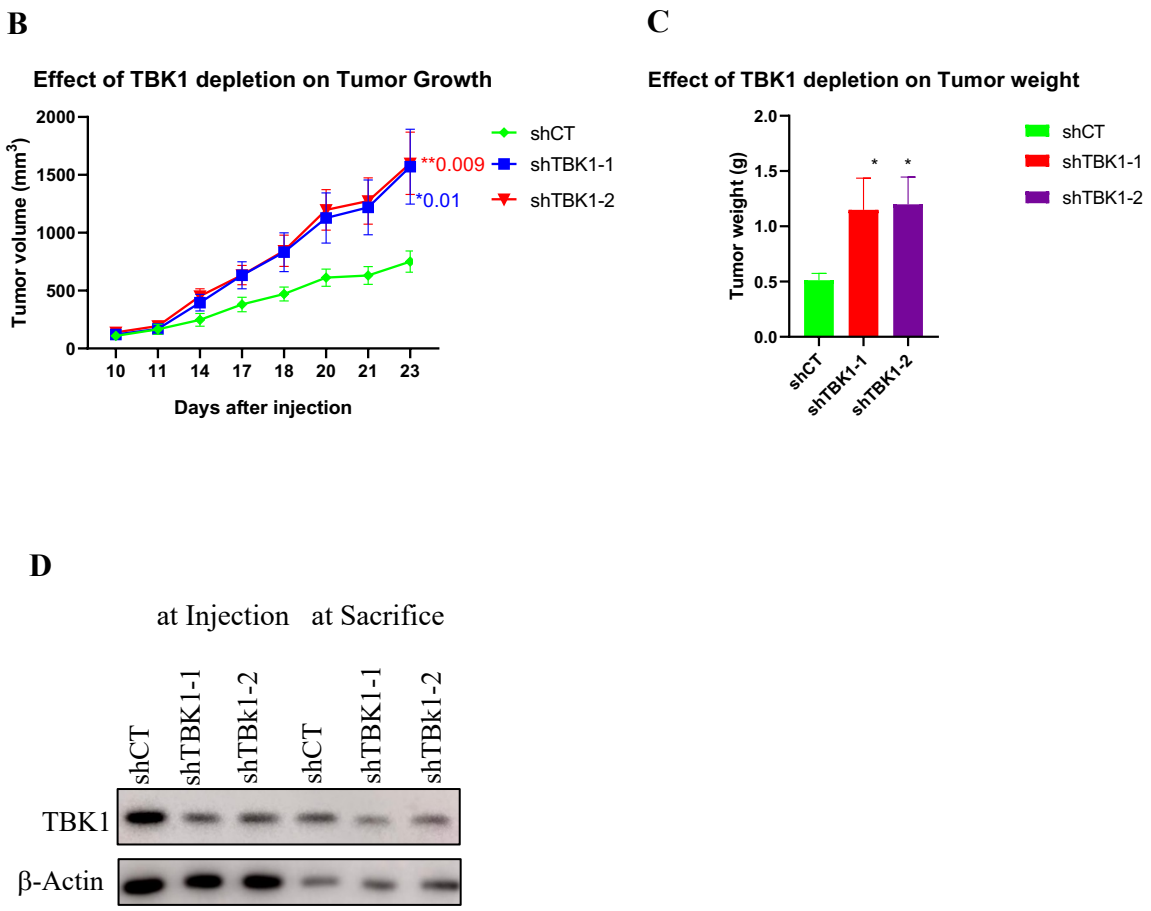
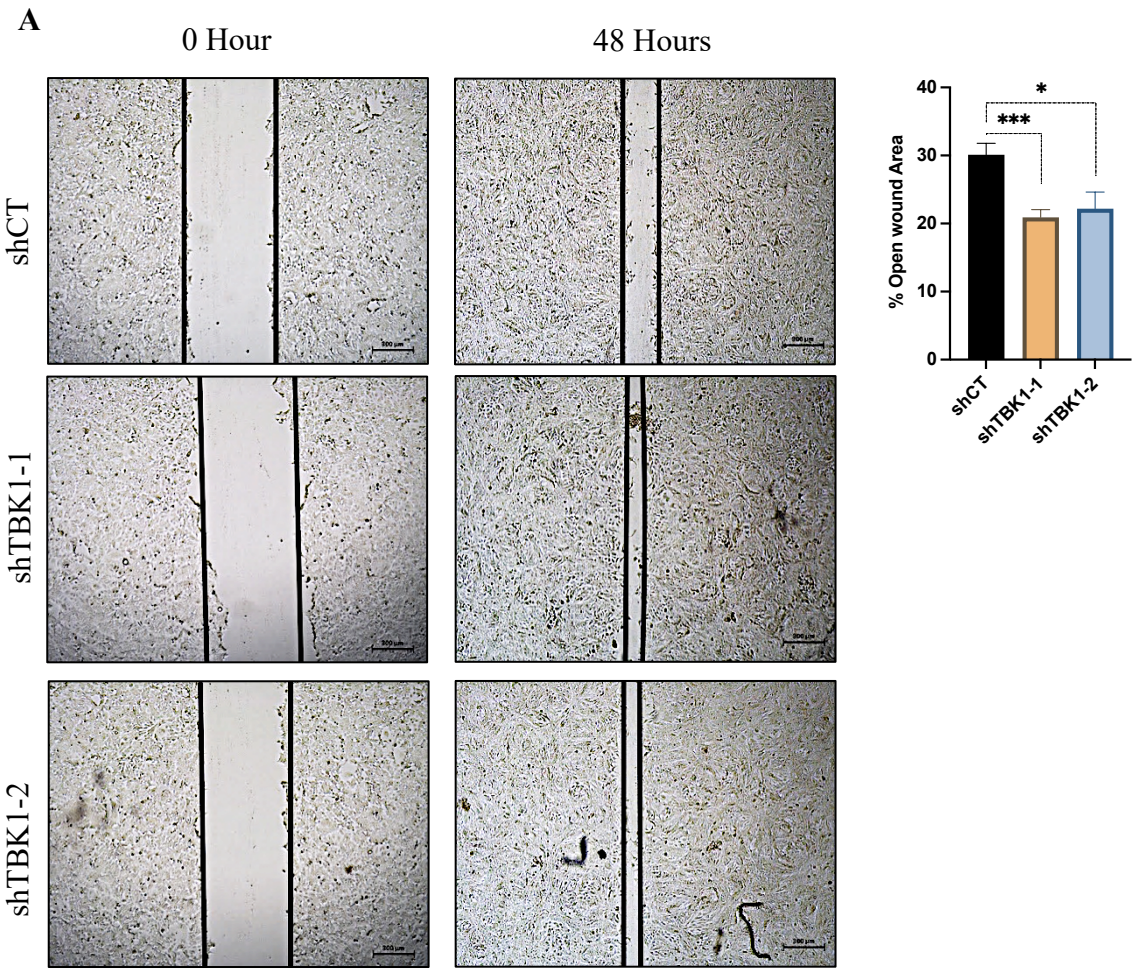


Figure 6. Promoted tumor growth was seen in shRNA TBK1 cell line-derived xenografts. **A)** Tumor growth was followed for approximately 25 days after shRNA TBK1 DLD1 cell line injection. **B)** Tumor volume was calculated as LxW^2 measured (L: length, W: width). When tumor volumes reached 1000mm3, the experiment was finalized. **C)** Effect of TBK1 depletion on tumor weight. **D)** expression level of TBK1 was measured by western blotting at the beginning and the end of the experiment. (unpaired t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.)

3.4 Loss of TBK1 has increased the migration of colorectal cancer cell lines.

To assess the role of TBK1 in the migration of CRC cell lines, we firstly performed a Wound healing assay on the DLD1 shTBK1 cell line. For this experiment, we seeded the cells, and when they reached confluency around 90%, we generated a gap between the cells. The gap pictures were taken at the beginning (0 h). Then the photos were also taken 48 hours after the generation

of the gap before it closes. The percentage of the open area was quantified by T-scratch software, and the migratory ability of TBK1 depleted cells was compared to the control cells. Representative pictures are shown in Figures 7A and B. As shown in the figures, the loss of TBK1 in DLD1 and HT29 cell lines has led to a promotion in the migration of the cells. Transwell migration assay is also performed for shRNA TBK1 HCT116 cell line to evaluate the role of TBK1 in the migration of CRC *in vitro.* Cells were incubated in 0% FBS medium overnight. The following day cells were seeded on the 0.8µm chambers in serum-free medium. Medium with 20% serum was used as an attractant. 24 hours after seeding, the experiment was finalized by staining the outer surface of chamber with Giemsa dye. Pictures of the migrated cells on the outer surface of the chambers were taken with Leica microscope (Figure 7C). As shown in Figure 7, TBK1 depletion has enhanced the migration ability of HCT116 cells.



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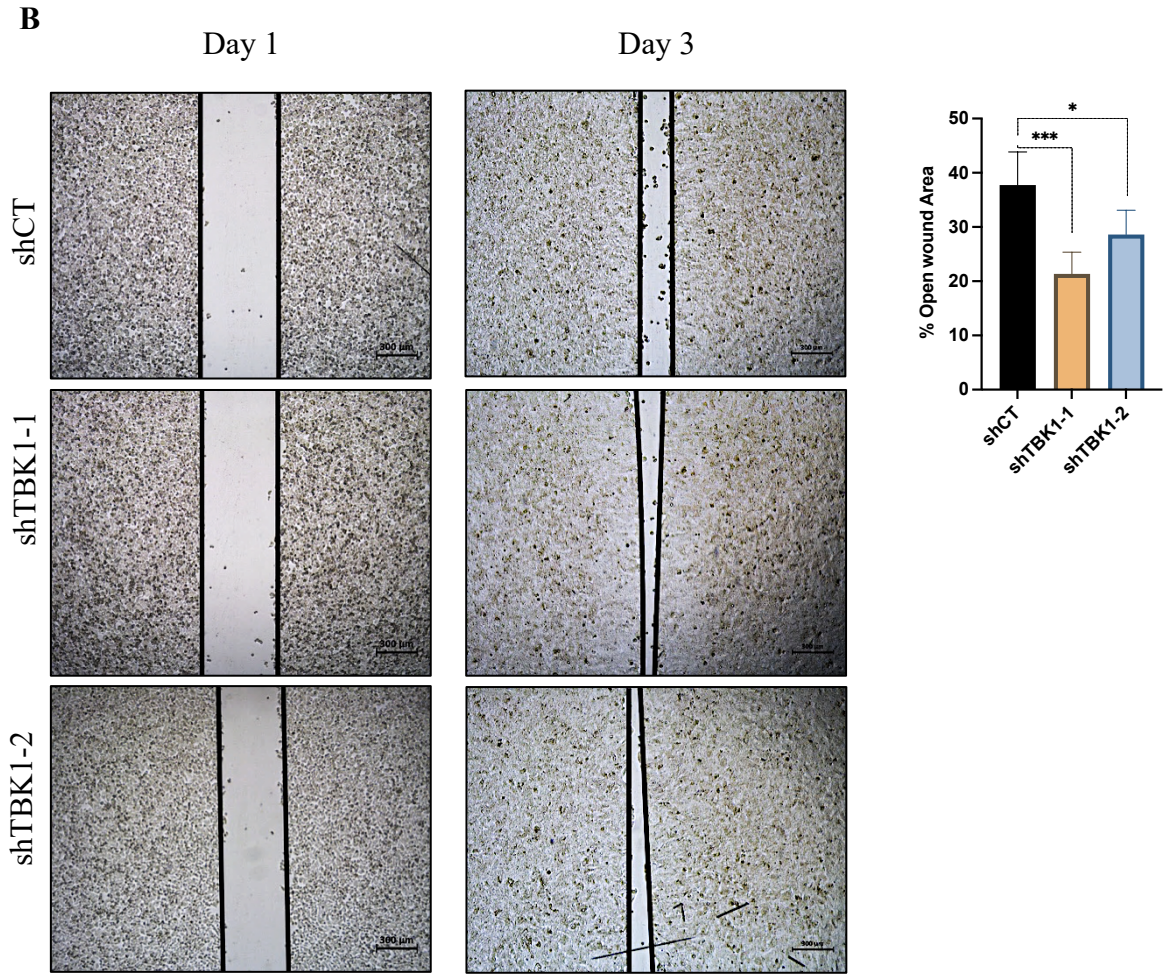

Day 1
Day 3
shCT
shTBK1-1
shTBK1-2
% Open wound Area
50
40
30
20
10
0
***
*
shCT
shTBK1-1
shTBK1-2

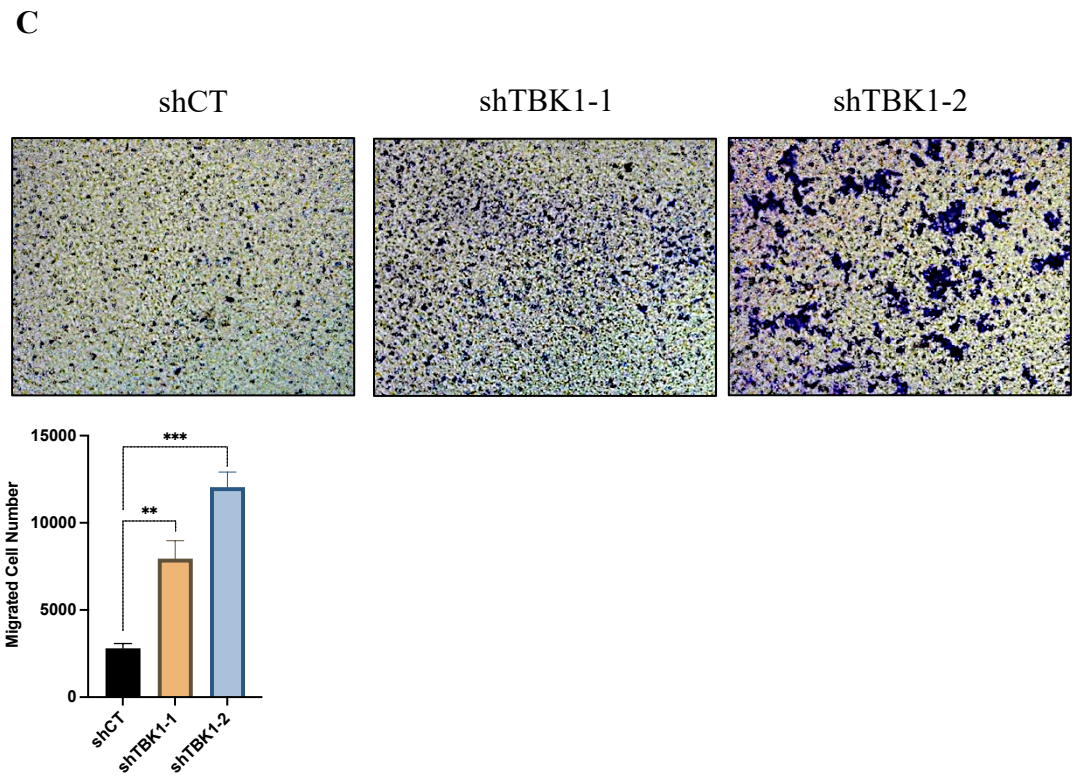


Figure 7. Migration of CRC cell lines is negatively correlated with TBK1. Wound healing assay of **A)** shRNA TBK1 DLD1 cell line **B)** shRNA TBK1 HT29 cell line . **C)** Transwell migration assay of shRNA TBK1 HCT116 cell line. TBK1 depletion led to an increase in the migration of CRC cell lines. Data shown here are mean ± SEM of biological triplicates for each construct. Each experiment is repeated twice. (unpaired t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.)

3.5 Invasion of colorectal cancer cell lines is promoted upon TBK1 depletion.

Since we could show TBK1 is negatively correlating the migration of colorectal cancer cell lines, we decided to evaluate the effect of TBK1 on the invasion of colorectal cancer cell lines. For this experiment, the starved shRNA TBK1 DLD1 and HCT116 cells were seeded on matrigel-coated transwell chambers with medium containing 0% FBS. Medium with 20% FBS was added to the lower chamber as an attractant. 48 hours later, the chambers were stained with Giemsa dye.

Pictures of the invaded DLD1 (Figure 8A) and HCT116 (Figure 8B) cells on the membrane's outer surface were taken with Leica microscope.

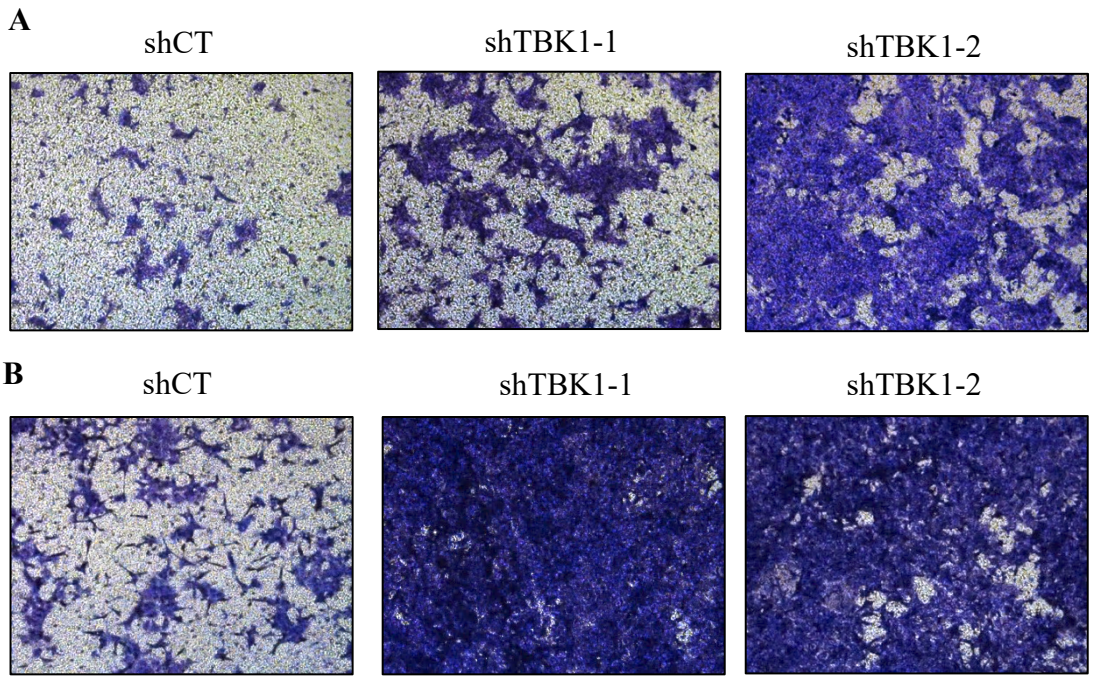
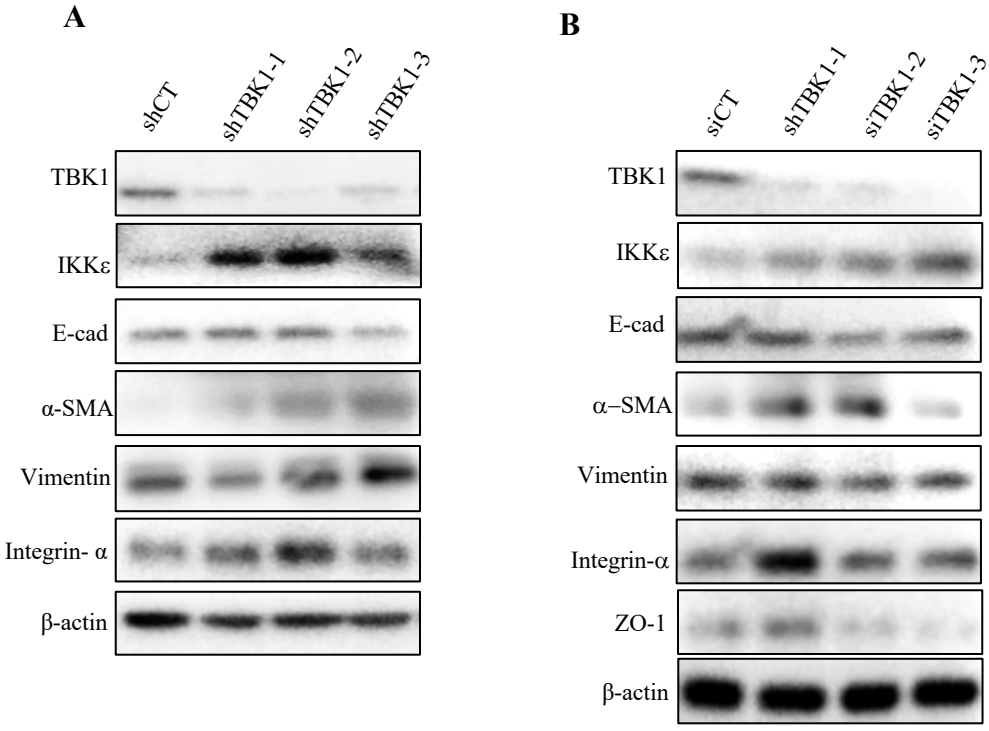


Figure 8. TBK1 depleted colorectal cancer cell lines invaded more compared to the control cells. Transwell invasion assay was performed to asses the invasion of **A)** shRNA TBK1 DLD1 **B)** shRNA TBK1 HCT116 cell lines. Data shown here are mean ± SEM of biological triplicates for each construct. Each experiment is repeated twice.

3.6 Absence of TBK1 induced epithelial to mesenchymal (EMT) in colorectal cancer cell lines.

After evaluating the role of TBK1 in cell growth and migration of CRC, we wondered if TBK1 has a role in the EMT process in colorectal cancer. In the DLD1 CRC cell line, upon TBK1 depletion, an increase was seen in some mesenchymal markers such as vimentin, integrin-α, and α-SMA, while some epithelial markers were shown to be decreased, including E-cadherin at protein level (Figure 9A). Similarly, in the siRNA TBK1 DLD1 cell line EMT process was induced by upregulation of integrin-α, and α-SMA and downregulation of E-cadherin (Figure 9B). An increase in some mesenchymal markers such as vimentin and a decrease in E-cadherin was also seen in shRNA TBK1 HT29 cells (Figure 9C). Additionally, in shRNA TBK1 HCT116 cell line expression of mesenchymal markers, integrin-α, and α-SMA was induced. In this cell line, a decrease in ZO-1, an epithelial marker, was observed (Figure 9D).



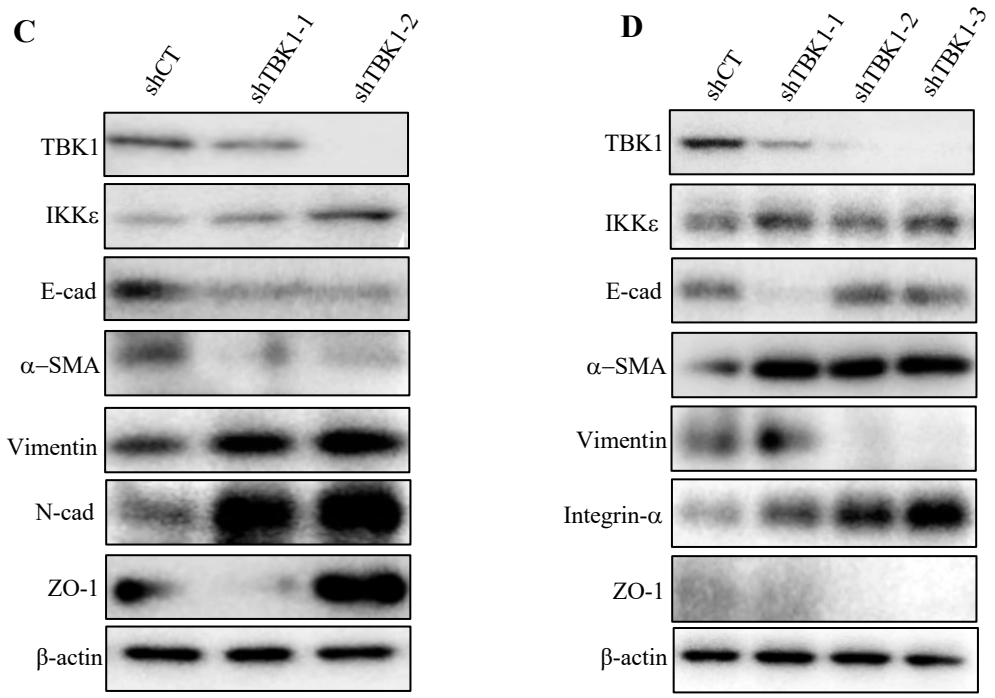
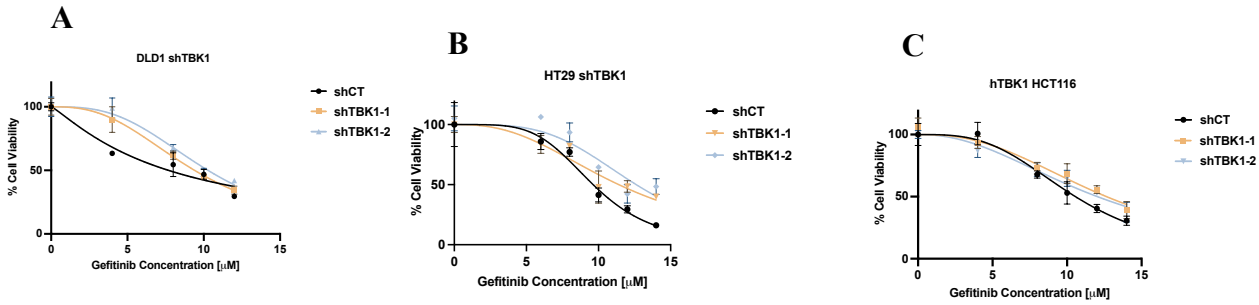


Figure 9. Epithelial to mesenchymal is induced upon TBK1 depletion in colorectal cancer cell lines. EMT markers were analysed at protein level on **A)** shRNA TBK1 DLD1, **B)** siRNA TBK1 DLD1, **C)** shRNA TBK1 HT29, and **D)** shRNA TBK1 HCT116 cells by Western Blotting. β-Actin is used as loading control.

3.7 TBK1 depletion makes colorectal cancer cell lines resistant to Gefitinib.

Considering the role of TBK1 in the proliferation and EMT of the colorectal cancer cell line, we questioned if it has any role in Gefitinib resistance. For this purpose, we treated colorectal cancer cell lines with wide ranges of Gefitinib for 72 hours. Then we measured the cell viability by Sulforhadamine B (SRB) assay. The IC_{50} value was determined by GraphPad prism. A trend of resistance to Gefitinib was seen in shRNA TBK1 DLD1 (Figure 10A), HT29 (Figure 10B), and HCT116 (Figure 10C) cell lines.



Colorectal Cancer Cell Line	shTBK1 DLD1			shTBK1 HT29			shTBK1 HCT116		
Constructs	shCT	shTBK1-1	shTBK1-2	shCT	shTBK1-1	shTBK1-2	shCT	shTBK1-1	shTBK1-2
IC_{50} value	7,68	9,39	10,28	9,63	11,41	12,62	10,56	12,65	12,04
p-value	0,0016			0,0038			0,0311		

Figure 10. TBK1 depleted colorectal cancer cell lines are more resistant to Gefitinib than control cells. A) shRNA TBk1 DLD1 **B)** shRNA TBK1 HT29 **C)** shRNA TBK1 HCT116 cells are treated with different concentrations of Gefitinib for 72 hours. Cell viability is measured by Sulforhadamine B (SRB) assay. Data shown here are the mean ± SEM of biological triplicates for each construct. Each experiment is repeated twice. (F-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.)

3.8 Generation of Gefitinib resistant DLD1 colorectal cancer cell line.

To generate a Gefitinib-resistant DLD1 cell line, the Gefitinib IC_{50} value of naive DLD1 cells was measured. As shown in Figure 11A, the Gefitinib IC_{50} value of naive DLD1 is approximately 10-11μM. Then generation of Gefitinib resistant DLD1 cells was started by exposing the cells to increasing doses of Gefitinib. The starting dose was 9μM, slightly less than the Gefitinib IC_{50} value of naive DLD1 cells. During this process, the cells were maintained in a specific concentration of Gefitinib and passaged three times. After the third passage, the cells surviving in that particular concentration of Gefitinib were exposed to an increased dose of

Gefitinib by either 0.5µM or 1µM. At the end of this process, a Gefitinib-resistant DLD1 cell line was generated.

Additionally, control cells were kept in DMSO. The amount of DMSO was equal to the Gefitinib for resistant cells. A schematic representation of the generation of Gef-DLD1 cells is shown in Figure 11B.

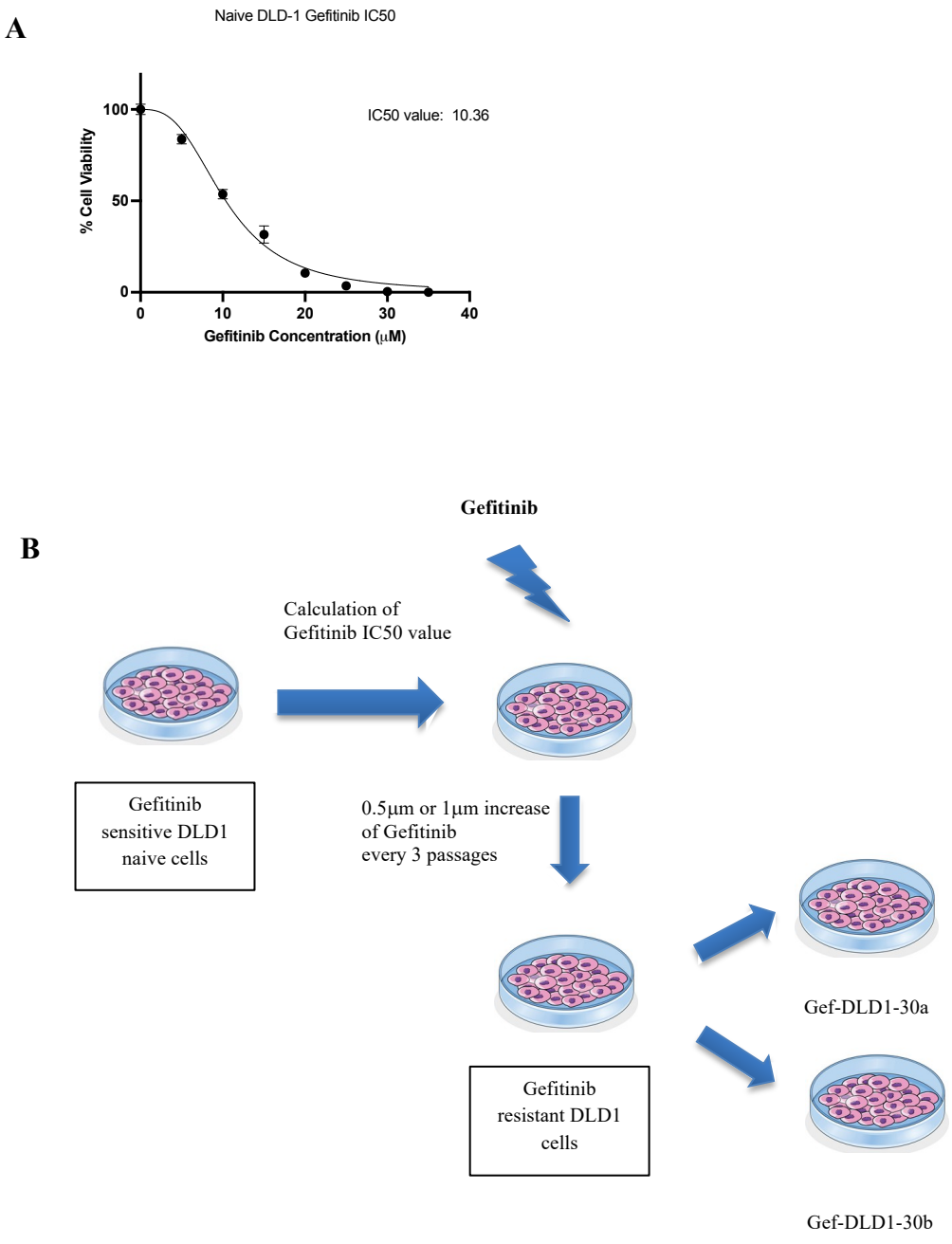
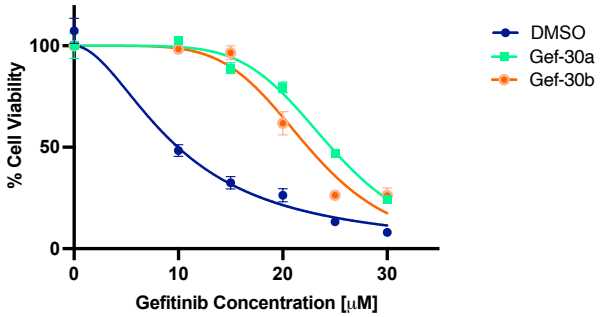


Figure 11. Generation of Gefitinib Resistant DLD1 cells. A) Gefitinib IC_{50} value of naive DLD1 cells. **B)** Generation of Gefitinib resistant DLD1 cells was started by exposing naive DLD1 cells with a concentration of Gefitinib less than the Gefitinib IC_{50} value of those cells. Cells were exposed to increasing concentrations of Gefitinib every three passages. At the end of this process, Gef-DLD1 cells were generated.

After approximately 14 months of Gefitinib treatment, a significant increase in the IC_{50} value of Gef-DLD1 cells was observed compared to the DMSO-DLD1 cells (Figure 12). Since our Gef-DLD1 cells could not survive in the higher concentrations of Gefitinib, we decided to perform our experiments on the cells growing in the medium containing 30µM Gefitinib.



DLD1	IC_{50} Value
DMSO	9.94
Gef-30a	24.60
Gef-30b	22.42

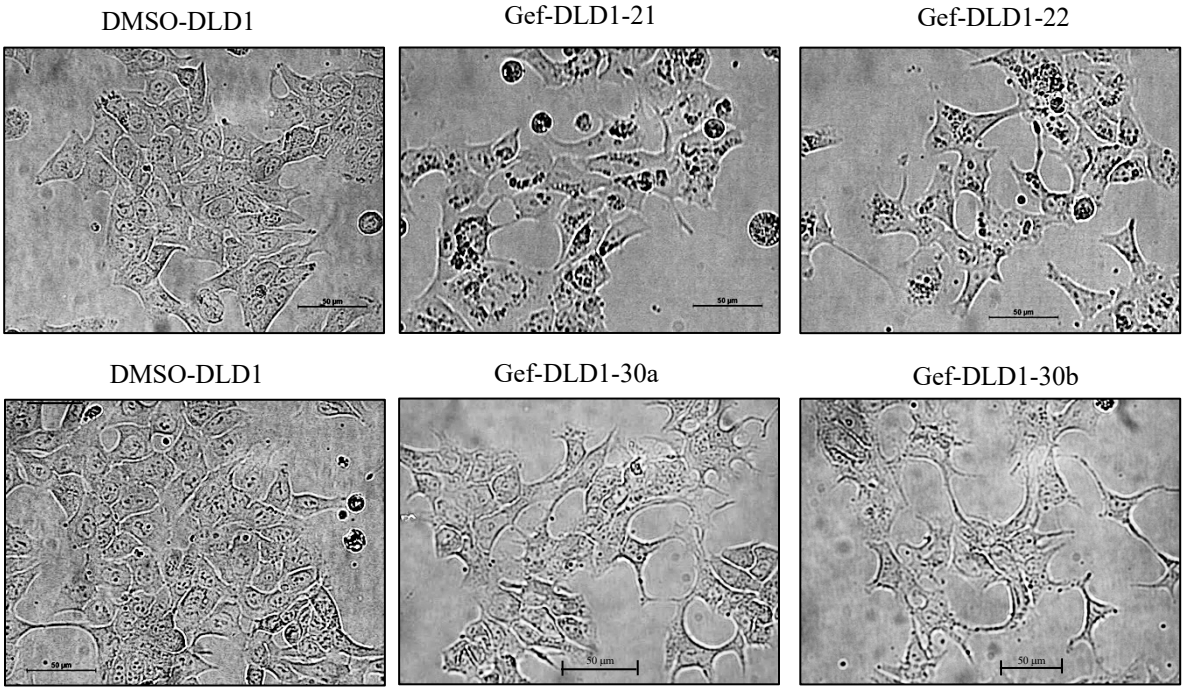
P-value: <0.0001

Figure 12. Determination of Gefitinib IC_{50} value of Gef-DLD1 and control cells. Gefitinib IC_{50} value of Gef-DLD1 cells is measured 14 months after starting the process. (F-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.)

3.9 During the time, morphology and expression levels of EMT markers of the Gefitinib resistant DLD1 cells were changed compared to the control cells.

Gef-DLD1 cell morphology was observed and recorded during the time. The morphology of the DMSO-DLD1 cells was similar to the naive DLD1 cells, while the Gef-DLD1 cells had different morphology. When our resistant cells were kept in 21 and 22μM of Gefitinib, the morphology of the cells started to change. When they reach 30μM concentration of Gefitinib their morphology was completely different from control cells. The Gef-DLD1 cells had mesenchymal morphology with long cell branches. They had a fibroblast-like shape. Moreover, the Gef-DLD1 cells grew entirely separately from each other, while the DMSO-DLD1 cells became packed when they were confluent. Pictures of Gef-DLD1 and DMSO-DLD1 cells in different concentrations are shown in Figure 13A.

A



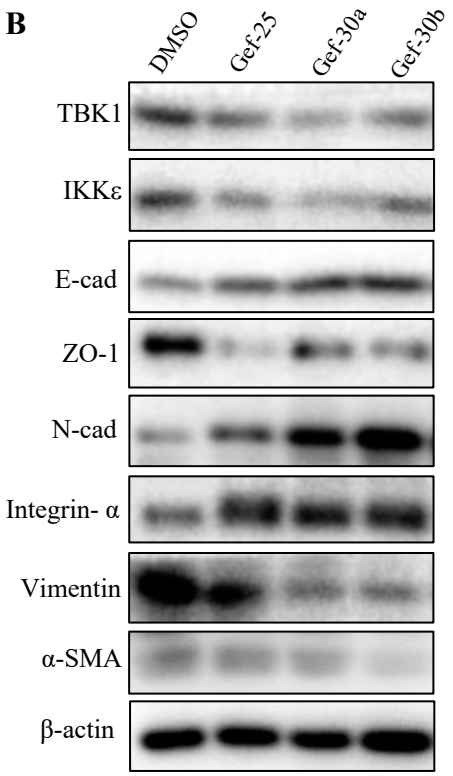


Figure 13. Morphology and expression of EMT markers were changed in Gefitinib-resistant cells compared to the control cells. A) Gef-DLD1 cells were morphologically different from DMSO-DLD1 cells. **B)** Expression levels of EMT markers were checked by Western Blotting.

Once the Gefitinib resistant cells were generated, the expression levels of some proteins were analyzed by Western Blotting. Differences were observed in the expression levels of different proteins between Gef-DLD1 and DMSO-DLD1 cells. Firstly a decrease in the levels of TBK1 protein and also IKKε was observed in Gef-DLD1 cells. Additionally, the protein expression levels of EMT markers were evaluated in generated two different Gef-DLD1 cells. The expression levels of some mesenchymal markers, including N-cadherin and Integrin-α were upregulated in the Gef-DLD1 cells compared to the control cells. At the same time, the level of ZO-1 is decreased in Gef-DLD1 cells, which is an epithelial marker (Figure 13B).

CHAPTER 4

Discussion

TBK1 is a serine/threonine kinase protein with critical roles in regulating different cell signaling pathways, including innate immune responses, autophagy, NF-κB, and cell survival [120]. By promoting various pathways, this protein involves in the pathogenesis of different diseases such as autoimmune diseases or cancer. Some studies suggest an oncogenic role for this protein in different cancer types such as breast cancer, hepatocellular carcinoma, non-small cell lung cancer (NSCLC), and melanoma [115,117,138]. NF-κB is considered a bridge between inflammation and cancer since it is a critical regulator of inflammatory responses, cell survival, cell growth, and invasion. TBK1 and IKKε are the members of non-canonical IκB kinase family, which regulates NF-κB signaling pathway to promote tumorigenesis [103]. Moreover, TBK1 involves in tumorigenesis of some cancer types by directly phosphorylating AKT and inducing cell proliferation [121].

In this study, the specific roles of TBK1 in colorectal cancer tumorigenesis and chemotherapeutic drug resistance have been investigated. In this context, we generated TBK1 loss of function cell line models via the shRNA lentiviral system to study the roles of TBK1 in colorectal cancer. The cell proliferation data obtained from our TBK1 depleted colorectal cancer cell line models indicate a suppressive role for TBK1 in tumor growth. Furthermore, the colony-forming ability of these cells is enhanced upon TBK1 depletion. Moreover, promoted tumor growth in our shTBK1 DLD1 xenograft nude mice models compared to the control mice confirmed our observation of increased cell proliferation upon TBK1 depletion *in vitro*. Consistent with this, TBK1 loss in Estrogen-receptor (ER) α positive has led to increased tumor growth by regulating ERα expression [139]. On the other hand, in some cancer types, TBK1 is observed to promote cell growth and survival by regulating intrinsic signaling pathways. In lung cancer, the requirement of TBK1 for tumorigenesis is reported by Zhu et al. [129]. In addition, a high expression level of TBK1 is detected in acute myeloid leukemia cells. TBK1 ablation in

these cells is manifested to enhance apoptosis suggesting the importance of this protein in the survival of AML cells [127]. Also, TBK1 is reported to enhance tumor proliferation by regulating the activation of the mTOR pathway [140,141]. In addition, knockdown or pharmacological inhibition of TBK1 inhibits tumor growth in renal cancer cells [142]. In some KRAS mutant cancer types, TBK1 is observed to promote cell proliferation and survival by activating NF-κB and mTOR pathways [119]. On the other hand, there are some studies showing abbarant expression levels of TBK1 in some cancer types such as KRAS mutant non-small cell lung cancer and pancreatic ductal adenocarcinoma [143,144]. However, in these cancers, there is no evidence that TBK1 is necessary for tumor growth and proliferation. For instance, knockdown of TBK1 in two different non-small cell lung cancer cell lines did not have any effect on cell viability [143]. In general, more studies need to be done to elucidate TBK1 roles in tumor growth in different cancer types.

In different studies, the direct or indirect phosphorylation of AKT by TBK1 is reported. Ou et al. showed that AKT is a natural substrate of TBK1, and TBK1 ablation has impaired the phosphorylation of AKT protein [121]. Moreover, it is reported that TBK1 is required for AKT phosphorylation, and TBK1 depletion impairs cancer cell viability [121]. In this study, we could show that the phosphorylation of AKT and ERK are upregulated in some of the TBK1 depleted cells, which explains the increase in cell proliferation. Similarly, Yang et al. showed increased phosphorylation of AKT upon TBK1 loss in Estrogen receptor (ER)-α positive breast cancer, however, the mechanism under this increase remained to be elucidated [139]. Interestingly, an increase in the expression level of IKKε is observed upon TBK1 depletion in our colorectal cancer cell lines. This increase in the IKKε expression level suggests a regulatory role of IKKε in AKT phosphorylation in colorectal cancer.

On the other hand, Göktuna et al. reported the direct regulation of ERK phosphorylation by IKKε [145,146]. Hence, the upregulation in phosphorylation of ERK can be explained by the upregulated levels of IKKε in our cells.

AKT is a central mediator of the PI3K signaling pathway. Phosphorylation of AKT activates other proteins in the PI3K pathway which ultimately controls survival [147]. There are some phosphatases like the PHLPP family that regulate the phosphorylation of AKT protein [148]. Although an increase in phosphorylation of AKT is reported in our TBK1 depleted cells, in some

shRNA TBK1 constructs no increase in pAKT levels was detected (Figure 4). Interestingly in that specific construct, there is a low expression level of tAKT. This observation can be explained by the off-target effects of that specific shRNA construct on the regulators of AKT.

In a previous project in our lab, tumor-suppressive roles of IKKε in colorectal cancer tumorigenicity are indicated. In the mentioned study, enhanced cell proliferation and viability are shown upon IKKε depletion in colorectal cancer cells. Considering the results of these two studies we can conclude that in the absence of TBK1, IKKε tries to compensate for this absence. However, this compensation is not efficient since we still observe the increase in tumor growth upon TBK1 depletion. These observations suggest a tumor-suppressive role for both IKKε and TBK1 via distinct pathways in colorectal cancer development. This is also confirmed by patient recurrent free survival (RFS) data analyzed in another project in our lab. In the mentioned analysis, patients with high levels of both TBK1 and IKKε have the highest RFS. While reduced RFS is seen in patients with low TBK1 or low IKKε levels. Additionally, this survival is much less in patients with low levels of both TBK1 and IKKε. Hence, we can consider additive but not redundant roles for TBK1 and IKKε in colorectal cancer development.
Similarly, Yang et al. showed a positive correlation between TBK1 levels and the survival of colorectal cancer patients [137]. They didn't report a role for TBK1 in intrinsic signaling pathways. However, the role of TBK1 on colorectal cancer tumorigenesis by regulating TH_{17} cell differentiation and IL_{17} production, a crucial interleukin in tumorigenesis, is emphasized in this study. The authors reported a positive association between IL_{17} production and adenoma formation in small and large intestines [137], also indicated previously in some studies [149,150]. Consistent with this, Tosolini et al. reported a poor prognosis in colorectal cancer patients with a high level of genes associated with TH_{17} [151]. The level of IL_{17} is reported to be increased in the tumor microenvironment of human colorectal carcinoma, which provided evidence that TH_{17} and IL_{17} are essential in the pathogenesis of colorectal cancer [152]. Moreover, IL_{17} in colorectal cancer is associated with angiogenesis [153]. Therefore, we can conclude the importance of the tumor microenvironment (TME) and the interactions of TBK1 with different cells in the initiation of colorectal cancer. To further understand the role of TBK1 in colorectal cancer initiation, co-culture system or *in vivo* settings should also be considered. Mentioned approaches allow us to study the interactions of TBK1 with various cell types present in the TME and the effect of

TBK1 on the production of different factors such as inflammatory cytokines on colorectal cancer onset.

Overall, the epithelial to mesenchymal process is promoted upon TBK1 ablation in colorectal cancer cells. TBK1 depletion via both shRNA and siRNA in the colorectal cancer cell lines leads to promotion in some mesenchymal markers such as integrin-α and α-SMA. Additionally, a slight decline in epithelial markers is detected in these cells. Integrins are receptors for some molecules in the extracellular matrix (ECM) like laminin and collagen. The binding of integrins to the ECM triggers cellular functions like migration [154]. Hence, this shift in EMT can explain the progression in migration/invasion upon TBK1 depletion in colorectal cancer cell lines shown by wound healing and transwell migration/invasion assay. In conclusion, we can state that loss of TBK1 leads to partial EMT in colorectal cancer. Similarly, Loss of TBK1 in Estrogen receptor (ER)-α positive breast cancer has led to induced EMT and increased tumor growth and lung metastasis [139]. However, Cruz et al. showed induced epithelial plasticity in the mouse model of pancreatic ductal adenocarcinoma upon loss of TBK1 function which leads to reduced tumor growth and metastasis [155]. These studies are confirming the regulatory role of TBK1 in EMT in cancer which is also shown in this study. The mechanisms regulating this shift in EMT remain to be elucidated.

Drug resistance is a very complex concept that has been studied in various diseases. This happens when pharmaceutical treatment becomes ineffective in the disease treatment process. There are many factors involved in this process, including EMT. The association between EMT and drug resistance is reported in some cancer types, such as lung, bladder, and breast cancer [156–158]. In colorectal cancer cell lines, TBK1 ablation improves the resistance of the cells to the Gefitinib, which is shown by an increase in the Gefitinib IC_{50} value in the TBK1 depleted cells. Moreover, in the generated Gefitinib-resistant DLD1 cell model, a reduction in TBK1 expression level is observed compared to the control cells. The regulatory role of TBK1 in drug resistance in breast cancer is pointed out previously [159]. The authors suggest that TBK1 increases resistance to tamoxifen by modifying the phosphorylation of estrogen receptor-α [159]. In addition, in melanoma, TBK1 loss has led to inhibition of resistance to MEK inhibitors [160]. In our drug-resistant DLD1 cells EMT is induced by a significant upregulation in mesenchymal marker

levels such as N-cadherin and integrin-α. Consistent with this, chronic resistance to chemotherapeutic drugs is shown to promote epithelial to mesenchymal transition in colorectal cancer [67,71]. Chronic resistance to oxaliplatin is associated with increased mesenchymal markers and morphology in the resistant cells [66]. Also in these cells increased levels of Snail, an EMT-regulatory transcription factor is observed [66].

Considering the diminished level of TBK1 and induced EMT in resistant cells, we can hypothesize that induction of EMT as a result of a decrease in TBK1 level may contribute to the acquired drug resistance in colorectal cancer. Therefore, the association between EMT and drug resistance is demonstrated in this study which is confirming the previous studies. In general, we can consider a tumor-suppressive role for TBK1 in both the early and late stages of colorectal cancer. However, this hypothesis should be further investigated by reexpressing TBK1 in the resistant cells to further understand the mechanisms of this drug resistance.

CHAPTER 5

Conclusion and future perspectives

TBK1 kinase protein regulates various cellular mechanisms such as innate immune responses, autophagy, cell proliferation, and survival. Some studies reported abbarant expression of TBK1 in different cancers such as breast, lung, and hepatocellular. The role of TBK1 in colorectal cancer is not yet well established. In this study, we mainly focused on the role of TBK1 in colorectal cancer tumorigenesis. By generating shRNA TBK1 knockdown cell models, we could indicate tumor-suppressive roles for this kinase protein in colorectal cancer. A progression in cell proliferation, migration, and invasion upon TBK1 depletion was observed in our cells. This increase in cell proliferation was confirmed further with an increase in the survival of the shRNA TBK1 cells under Gefitinib treatment, an EGFR inhibitor. We could show an increase in the level of IKKε in TBK1 depleted cells that indicates a compensatory role for IKKε. However, this compensation is not entirely efficient. These observations suggest tumor-suppressive roles for IKKε and TBK1 via distinct pathways. The role of TBK1 in cell-intrinsic signaling pathways in colorectal cancer needs to be further investigated.

We could also show that TBK1 inhibits EMT leading to a reduction in the migration of colorectal cancer cells. For better understating of TBK-specific roles in colorectal cancer development, generating TBK1 overexpressed colorectal cancer cell line models and studying the colorectal cancer tumorigenesis in this model might be a good idea.

Moreover, in this study, a suppressive role for TBK1 in the growth of drug-resistant models is indicated. By increasing TBK1 expression levels in drug-resistant cells and analysis of cell viability in this model, the effect of TBK1 in the late stages of colorectal cancer can be further proved.

Additionally, this study is done in an *in vitro* setting, not always representing the tumor microenvironment. We should consider the role of TBK1 in the immune system and inflammation, a crucial factor of the tumor microenvironment, and the importance of the tumor microenvironment in colorectal cancer initiation and progression. Although the tumor growth in xenograft models is analyzed in this study, the conclusion is challenging since xenograft models

lacking the immune system are not the best models for studying the tumor microenvironment. Hence, the co-culture or in vivo settings are suggested to be explored in the following steps.

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