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Roles of TTK Kinase in Breast Cancer Tumorigenesis

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Roles of TTK Kinase in Breast Cancer Tumorigenesis

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An abstract of
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Abstract

Roles of TTK Kinase in Breast Cancer Tumorigenesis

By Jamie Leigh King

Cancer formation is enabled by genetic changes that control cell division, which alters how genetic information is transferred and contributes to malignant cell transformation. TTK is a mitotic kinase that is overexpressed in several cancer types, including breast cancer. While the roles of TTK in centrosome duplication and the spindle assembly checkpoint have been well characterized, the complete functional roles of TTK in cancer have not been studied. The research in this dissertation describes diverse roles of TTK in breast cancer tumorigenesis.

One well characterized role of TTK is in the control of the centrosome duplication cycle and formation of the mitotic spindle. Previous studies have shown centrosome amplification (greater than 2 centrosomes) to be associated with genomic instability and worse patient outcomes in breast cancer. We found a positive correlation between TTK expression and centrosome amplification (CA) in breast cancer cells, as well as a correlation between TTK overexpression and worse prognosis in breast cancer patients. We also determined that targeting TTK could attenuate proliferation and CA in cell lines that have high frequencies of CA.

More recently, mitotic kinases like Aurora A and PLK4 have been shown to have roles in supporting cell phenotypes that promote cancer cell invasion and metastasis. In addition to establishing a role of TTK in promoting CA, we also found that targeting TTK can attenuate the mesenchymal phenotype of aggressive triple negative breast cancer cells through various mechanisms, including TGF-β signaling, KLF5 and micro-RNAs.

In further studies, we hypothesized that TTK could also mediate radiosensitivity in breast cancer, since it has been reported that substrates in the DNA repair signaling pathway and targeting some downstream TTK substrates has shown effectiveness in radiosensitization. From this line of investigation, we also found that targeting TTK could sensitize radioresistant breast cancer cells by enhancing apoptotic signaling and minimizing active DNA repair.

Overall, this work highlights the diverse cellular processes through which TTK overexpression promotes breast cancer tumorigenesis. This provides rationale to further develop targeted inhibitors against TTK in breast cancer and other types of cancer.

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Chapter 1: Introduction

1.1.1 Cancer Overview

In the most classical definition, cancer is defined as the uncontrolled growth of cells. The uncontrolled growth of cells is caused by a multitude of complex and moderately understood factors, such as genetic and environmental as well as metabolic changes in the cells. As human understanding of cancer as a disease has expanded in modern times, we have come to understand cellular complexities underlying the various types of cancer affecting different parts of the human body. Although modern technology has advanced our understanding of so many facets of cancer and allowed us to improve treatments, it remains a devastating global health problem. In 2018, the American Cancer Society estimates there will be ~1.7 million new cases of cancer in the United States, with about 600,000 Americans expected to die of cancer (1).

1.1.2 Cancer Hallmarks

Eighteen years ago, the "Hallmarks of Cancer" were introduced by Hanahan and Weinberg (2) as a set of "rules" that transform normal cells into malignant cancer cells. These hallmarks represent acquired capabilities shared by all types of cancer cells in which molecular programs that regulate cell growth, differentiation and death are altered to enable cancer formation. These original six rules included cancer cells acquiring self-sufficiency in growth signaling, insensitivity to anti-growth signals, evading programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue and invasion and metastasis. Since cancer formation is considered a multistep process, changes in each of these six branches ultimately allows cancer cells to expand with necessary growth signals and materials, as well as move away from the primary site of origin. In 2011, Hanahan and Weinberg updated the

original six hallmarks (3) to include enabling characteristics and emerging hallmarks, which included four new features acquired by cancer cells to support malignant behavior. These revised hallmarks included tumor promoting inflammation, deregulated cellular energetics, avoiding immune destruction and genomic instability and mutation. Of these enabling characteristics, genomic instability and mutation is a central cause of changes in cell signaling pathways that supports acquisition of all the other hallmarks. For example, mutations in oncogenes like Ras (4) and tumor suppressors like p53 and Rb (5) are necessary alterations that lead to self sufficient growth signaling and insensitivity to growth inhibitory signals. These oncogenes and tumor suppressors may also contribute to cancer evolution by inducing CIN (6, 7). While these are large scale changes in cell signaling pathways, genomic instability and mutation orchestrated by overexpression of genes involved in cell division is also a proponent of genomic instability.

1.1.3 Breast Cancer

Within the scope of this dissertation research, breast cancer was used as a model system to study genetic alterations in cancer. Breast cancer remains a critical public health problem for women globally. In the United States, breast cancer has the highest incidence rate among women and is the second leading cause of cancer related deaths after lung cancer (8). In 2017, the American Cancer Society estimated there would be about 200,000 new cases of breast cancer diagnosed in the United States and 40,610 deaths associated with breast cancer (8). Currently, the lifetime risk for a woman in the US to develop breast cancer is 1 in 8 (8), but breast cancer has disproportionate effects in different ethnic groups. The incidence rates of breast cancer are relatively equal

between non-Hispanic white women and non-Hispanic black women (128.7 vs. 125.5 cases per 100,000) (Fig. 1.1) with slightly smaller incidence rates in American Indian/Alaskan Native women, Hispanic women and Asian/Pacific Island women. However, the mortality rates from breast cancer remain higher among non-Hispanic black women (29.5 vs. 20.8 per 100,000).

Similar to other cancer types, molecular profiling has allowed scientists and clinicians to classify breast cancer into various subtypes to understand the clinical presentation, treatment response and prognosis (9-11). This basic molecular profiling for breast cancer is based on expression of hormone receptors and the HER2+ receptor on breast cancer cells. Estrogen receptor positive or Progesterone receptor positive patients are considered "hormone receptor positive" and are traditionally treated with endocrine based therapies such as Tamoxifen (12-14), while Her2+ patients are treated with HER2+ blockade therapies such as Herceptin or Lapatinib (15-17). Patients whose tumors do not overexpress either hormone receptor or the Her2+ receptor are categorized as triple negative. At present, there are no available targeted therapies specifically for triple negative breast cancer (TNBC) patients since they lack overexpression of three classical therapeutic targets. As a result, TNBC patients are primarily treated with chemotherapy and radiation (18). The luminal A subtype (HR+/Her2-) is the most prevalent subtype in the US (71%), luminal B (HR+, Her2 + or -) and triple negative account for 24% of cases and Her2+ accounts for 5% (Figure 1.1). Clinically, breast tumors are staged based on pathological grade and the TNM (tumor-node-metastasis) system. This system accounts for the size of the tumor, involvement of lymph nodes and spread to sites of metastases. At the cellular level, genetic changes corresponding to each subtype can result in very different tumor biologies, further

contributing to differences in tumor behavior and response to treatment. Other factors that vary by patient but have impacts on breast tumor biology include factors that influence hormonal changes such as parity and breast density (19). Socioeconomic factors that impact access to care may also influence clinical responses in breast cancer between ethnic groups (20). However, the influence of socioeconomic and lifestyle factors is complicated and confounds underlying differences in tumor biology between ethnic groups.

When examining incidence rates of different breast cancer subtypes, the incidence of hormone receptor positive/Her2- breast cancers is highest in non-Hispanic black women, while incidence of hormone receptor positive/Her2+ and hormone receptor negative/Her2+ breast cancers is equal between ethnic groups. However, the incidence of aggressive triple negative breast cancer is dramatically higher in non-Hispanic black women (24 vs. 12 cases per 100,000) (21, 22). Black women are also likely to be diagnosed with TNBC at younger ages and more likely to succumb to breast cancer across all age groups (23). Although disparities in access to care which impact diagnosis and treatment have begun to stabilize, the racial disparities in breast cancer are still very complex and impacted by biologic factors with differential influences (24). Therefore, it is of particular clinical relevance to study genetic alterations that may be contributing to aggressive breast cancer that could also be involved in racial disparities in breast cancer.

1.2 Chromosome instability, aneuploidy and deregulated mitosis in cancer

1.2.1 Overview

In the revised cancer hallmarks described by Hanahan and Weinberg in 2011, genomic instability and mutation was included as an enabling characteristic of cancer formation (3) . Genomic instability is defined as changes in the genomic code that leads to changes in cell behavior and signaling pathways associated with cancer formation. More specifically, these changes can include mutations in the DNA, alterations in epigenetic regulators and post-translational modifications.

To ensure the accurate passage of genetic information that is carried on chromosomes in the cell, the cell cycle must be tightly regulated. The cell cycle includes four major stages, including G1, S, G2 and M. During the S phase, DNA is replicated prior to G2 and the mitotic phase. In mitosis, chromosomes are equally separated before the cell divides into two new daughter cells to pass along genetic information (25). Throughout a normal cell cycle, there are necessary checkpoints that allow for the cycle to pause if errors are detected. At these checkpoints, signals can be sent to repair errors or allow a cell to undergo apoptosis (26). Multiple mechanisms that change timing of the cell cycle give rise to genomic instability in cancer cells. Overexpression of cyclin dependent kinases, decreased expression of cell cycle inhibitors like the P16/ARF family of proteins, and loss of function of tumors suppressors such as Rb and P53 contribute to accelerated G1/S transition and G2 phases (27, 28) which can bypass required checkpoints and allow a cell to remain viable with damaged genetic information.

In mitosis, overexpression of genes that control mitotic spindle formation and the spindle assembly checkpoint also contribute to genomic instability. At the level of cellular mechanics, defective cell mitosis can result in chromosome instability and aneuploidy (incorrect chromosome number) if chromosomes do not align properly and accurately segregate during mitosis. The mitotic spindle is considered the "molecular motor" that controls how chromosomes are moved during mitosis. This "motor" consists of centrosomes that control organization of microtubules, which are fibers that attach to chromosomes during mitosis. Chromosome instability (CIN) is a dynamic process underlying aneuploidy that is associated with defective cell cycle checkpoints in which errors in chromosome segregation, mediated by the mitotic spindle can go undetected. CIN and aneuploidy are detected in a broad range of tumor types including breast cancer (29, 30).

1.2.2 Critical factors of centrosome amplification (CA)

The centrosome is the main organelle in eukaryotic cells that is responsible for organizing and directing the bipolarity of the mitotic spindle. Theodor Boveri originally described it in the early 1900's through his studies of sea urchin embryos, where he described the centrosome as "the special organ of cell division" (31). Boveri's early postulations were the foundations for modern centrosome biology since he noted that the missegregation of chromosomes could give rise to diseases such as cancer. Since Boveri's seminal contributions to the field of cell biology, the centrosome has been extensively studied at the structural and functional level. The organelle itself is comprised of two centrioles surrounded by pericentriolar material, from which the

mitotic spindle can be assembled. During mitosis, the mitotic spindle functions to pull apart chromosomes prior to cell division (32). Essential centrosome proteins include Centrin, gamma tubulin and pericentrin, which are found in the pericentiolar material (33). The presence of these proteins can be used to study various attributes of the centrosome, including gamma tubulin and pericentrin staining to observe centrosome number. Other methods can be used to observe other qualities of the centrosome, such as electron microscopy to visualize the centrosome structure and microtubule nucleation approaches to measure the functional capacity of centrosomes.

Since centrosomes have such a critical function in the cell, centrosome duplication must be tightly regulated in conjunction with the cell cycle (34). Similar to how chromosomes are separated between daughter cells, the same thing must happen to centrosomes. In mammalian cells, centrosome duplication occurs during the S phase of the cell cycle. This process is initiated by Cdk2 activation between G1/S to start duplication of existing centrioles. Following the S phase, procentrioles are formed and mature centrioles are formed by the end of the G2 phase (35). These “mother” and “daughter” centrioles are separated during mitosis. Defects in the centrosome duplication cycle can lead to incorrect duplication of the centrioles, such as overproduction of centrioles. This can eventually lead to defective mitotic spindle formation and CIN, as the chromosomes do not faithfully attach to the mitotic spindle and separate properly. The overproduction of centrosomes was coined centrosome amplification (CA), which is defined as the acquisition of three or more centrosomes. This is a particularly critical issue that can be the source of aneuploidy since it can result in genomic instability by inducing CIN (36-39).

In 2004, Salisbury et. Al summarized the "hallmarks of CA" which include increased centrosome number and volume, excess pericentriolar material, supernumerary centrioles and inappropriate phosphorylation of centrosome proteins (40). Various genetic changes can lead to defects in centrosome duplication. Cyclin dependent kinases, core regulators of the cell and centrosome cycles, are temporally regulated and are generally hyperactivated in cancer cells (41-45). Other genetic changes, such as loss of tumor suppressor genes, also contribute to CA. For example, in mouse embryonic fibroblasts that lack p53, multiple centrosomes can be generated in one cell cycle, causing unequal chromosome segregation (46). In comparison, normal mice or Rb deficient mice do not exhibit CA. Another example is the inactivation of the E2F3 transcription factor which also causes CA in MEF's, because it causes unregulated cyclin E activity and defective association between nucleophosmin B and the centrosome (47). Transcription factors aside from the E2F's also regulate CA, such as YB-1. When YB-1 was overexpressed in a murine breast cancer model, this led to altered mammary gland morphology, abnormal cell proliferation and CA (48).

1.2.3 Role of CA in Cancer

The prevalence of CA in cancer cells has also been well characterized (49) and shown to initiate tumorigenesis in Drosophila models, via ectopic expression of centrosome associated molecules (50, 51). A number of solid and hematologic cancers harbor CA and increased CA correlates with increased aggressiveness and poor survival (52). Additionally, approximately 80% of breast cancers exhibit CA and CA appears to be dependent on Her2 overexpression (53). There is further value in studying CA as a prognostic marker, since it has also been shown to induce (54) and be associated with high grade tumors (55). Higher frequencies of CA are also detected in advanced tumors

and in tumors of higher histological grade (56). Furthermore, there appears to be additional translational value in studying CA in disparate breast cancer populations, since higher frequencies of CA are observed in patients with TNBC (57, 58).

More specifically in breast cancer, there are several structural and functional defects associated with centrosomes. In 1998, excess pericentriolar material, centrioles and centrosomes and inappropriate phosphorylation of centrosome proteins were observed in breast adenocarcinoma cells (59). Following those studies, CA was also detected in in situ ductal carcinoma, indicating that CA may be an early event in breast tumorigenesis (60). Previous work in the Saavedra laboratory explored cell cycle and centrosome regulator molecules in the Her2 signaling pathway that could contribute to CA. The Ras oncogene was found to signal CA in mouse mammary lesions and human mammary epithelial cells through Cdk4 and Nek2 (41, 61). Likewise, CA in Her2+ cells is dependent on Cdk4 and Nek2 (62).

One interesting question to address with CA in cancer is how cells are able to divide at all with such disordered mitotic spindles, since it is known that most cells with CA or CIN do not survive (63, 64). One strategy employed by cancer cells to persist with CA is to "cluster" centrosomes. This allows the formation of pseudobipolar spindles which can resemble normal spindles and make mitosis tolerable to CIN and aneuploidy (65). More recently, other cellular processes that contribute to aggressive breast cancer phenotypes, such as genetic changes in genes controlling cell migration and invasion, have been tied to CA. Interestingly, supernumerary centrosomes were shown to contribute to directional cell migration (66) and oncogene like induction of cell invasion from CA was observed in breast cancer and tied to increased Rac1 activity (67). Tying together the phenotype of centrosome clustering and loss of E-Cadherin, it was recently

shown that cells with CA can downregulate E-Cadherin to alter cell contractility and promote the efficiency of centrosome clustering (68). These observations support theories that alterations in centrosome molecules contribute to cancer hallmarks beyond genomic instability.

1.3 Invasion and metastasis in cancer

1.3.1 Overview

Another critical hallmark of cancer that is accounts for a large majority of cancer related deaths is metastasis. The spread of cancerous cells to tissues away from the primary site of origin has been a large focus of cancer research, but it is a highly complex process that has many debatable points.

The metastatic cascade includes a series of steps that allows malignant cells to expand in the primary tumor and eventually colonize at a secondary organ site (69, 70). First, tumor cells must expand in the primary tumor and form invasive subclones. These invasive cells must then move through the basement membrane around the primary tumor and surrounding microenvironment to reach the bloodstream. In order to move through the basement membrane, invasive cells must also secrete enzymes to degrade the extracellular matrix that surrounds the tumor. After traveling through the bloodstream, tumor cells reach a secondary organ site where they can colonize and form micrometastases, and larger micromets. These malignant growths in secondary sites are responsible for the majority of cancer deaths, since they can sometimes be undetectable and also resistant to treatment.

1.3.2 Epithelial to mesenchymal transition (EMT)

The epithelial to mesenchymal transition (EMT) is in important process in cellular differentiation that is rewired by cancer cells to facilitate invasion and metastasis. EMT is thought to be an early cellular change involved in metastasis since it allows cells to lose apical-basal polarity and move. Fundamentally, this cellular process involves cellular changes that allow cells to change their shape in order to move to other sites of origin (71). In normal cell functions, EMT is employed by implantation, embryo formation, and organ development as well as wound healing and tissue regeneration. In these types of EMT, the cells ultimately reach a final fate and the transformation is complete.

1.3.3 EMT signaling

In cancer cells, activation of the EMT "program" involves changes in several molecular signals (72), such as transcription factor activation or repression (73), expression of cell surface proteins, reorganization of the cellular cytoskeleton and the extracellular matrix and changes in micro-RNA (74) expression. Changes in expression of molecules associated with epithelial or mesenchymal cell states can be used by researchers to characterize which status a cell belongs to. Cells that are in an epithelial state generally express E-Cadherin and ZO-1 at their cellular junctions and express micro-RNA's such as the miR-200 (75) family of microRNA's (71).

As cells transition to a mesenchymal status, they lose expression of cellular junction proteins and micro-RNA's that control expression of epithelial genes and change shape. Cells that have transitioned to a mesenchymal status express intermediate filaments like vimentin (76) and N-cadherin, micro-RNAs associated with

oncogenic functions such as miR-21 (77) and transcription factors associated with inducing expression of mesenchymal genes such as Twist, Zeb1, Snail and Slug (78, 79). There are also signaling feedback loops that contribute to the EMT program. For example, there is a feedback loop between Zeb1/2 and miR-200 in which the Zeb transcription factors suppress miR-200 expression (80). MicroRNA's have also been shown to regulate expression of cytoskeletal signaling (81). Mesenchymal cells also have increased expression of enzymes that degrade the extracellular matrix, such as MMP-2 and MMP-9. The full mechanisms for how cancer cells undergo EMT are not clear, but it is known that signaling from the tumor stroma, such as sustained TGF-β signaling (82), can induce EMT.

1.3.4 KLF5 in EMT

Kruppel-like transcription factor 5 (KLF5) is a molecule that has been demonstrated to have bidirectional roles in regulating cell proliferation, differentiation and tumorigenesis. Loss of KLF5 expression on the 13q21 tumor suppressor locus can result from chromosomal deletion (83). Specifically related to EMT, KLF5 maintains epithelial status by regulating the expression of the miR-200 family of microRNAs (84). To achieve this, KLF5 binds GC boxes in promoter regions of the miR-200 family members. In TNBC cells, specifically mesenchymal TNBC's, KLF5 is expressed at low levels. We previously showed that ectopic expression of KLF5 in these cells could upregulate miR-200 family microRNA's (84).

Another critical regulatory switch for KLF5 is acetylation at K369. This modification determines whether KLF5 has a tumor promoting or suppressive role (85). In normal cells, acetylated KLF5 promotes differentiation and maintains normal

proliferation. However, unacetylated KLF5 can be upregulated in cancer cells to promote proliferation and prevent cellular differentiation. Interestingly, unpublished data suggests that acetylated KLF5 is associated with the mesenchymal phenotype in prostate and some breast cancer cells, suggesting this modification has differential regulation in EMT.

Additionally, KLF5 is a functional effector of TGF-β signaling. KLF5 interacts with Smads-2-4, which impacts the acetylation of KLF5 (86). In normal cells, TGF-β receptor phosphorylates Smad2/3 at the C-terminal region, then this Smad complex translocates to the nucleus where KLF5 is acetylated. However, in cancer cells with active Ras signaling, Smad2/3 is instead phosphorylated at the linker region, which disrupts assembly of the acetylated-KLF5 transcriptional complex. In summary of this regulation, aberrant phosphorylation of Smad members can impact KLF5 acetylation and altered interaction between p300/KLF5 and Smad3.

Specifically in breast cancer, regulation of KLF5 levels has not fully been characterized. In some contexts of breast cancer, high KLF5 expression is correlated with increased cell proliferation and malignant behavior (87, 88). In the context of aggressive, mesenchymal TNBC, which exhibits defined EMT, KLF5 is present at low levels (83). The exact mechanism (ex. suppressed transcription or increased degradation) for why it is present at low levels in mesenchymal TNBC cells is not clear.

Since other transcription factors and EMT phenotypes are impacted by changes in centrosome and mitotic molecules, it is plausible to suspect that KLF5 regulation and functions could be impacted by centrosome signaling as well. For example, signaling pathways such as the Ras and TGF-β pathways mediate centrosome signaling and impact the activities of centrosome kinases. These pathways also overlap with the

regulation of KLF5 and its transcriptional activities. Other members of the Kruppel-like factor family, such as KLF4 and KLF14, has been shown to contribute to genomic instability and CA in cancer. KLF4 was shown to be correlated with aneuploidy and induced CA, but the exact mechanisms were not determined (89). However, KLF14 was shown to transcriptionally repress PLK4 and deletion of KLF14 led to CA and upregulated PLK4 (90). Although all members of the Kruppel-like family do not have the exact same functions, these studies provide evidence for crosstalk between activities of the Kruppel-like factors and centrosome signaling, which provide rationale to study correlations between the activities of KLF5 in EMT and molecules associated with CA in cancer.

1.4 Links between altered mitosis and EMT

Recently, changes in mitotic signaling have emerged as mediators of various levels of EMT phenotypes. At the transcriptional level, Mad1 was shown to regulate E-cadherin expression by binding the E-cadherin promoter to prevent cell migration (91). The Aurora kinases and polo-kinases are two predominant mitotic kinase families that appear to be involved in EMT. Aurora was shown to promote cell migration and invasion in head and neck cancers through regulation of FAK signaling (92) as well as phosphorylating LKB1 in lung cancer, which compromises signaling between LKB1 and AMPK to facilitate cell migration. As for the polo-like kinases, PLK1 was shown to phosphorylate vimentin to contribute to a mesenchymal status (93) and PLK4 can promote cell invasion through controlling the ARP 2/3 complex in actin filament formation (94). Since mitotic kinases appear to control various levels of EMT signaling,

it is of interest to study how alterations in mitotic signaling contribute to the "EMT" program.

1.5 Prognosis and treatment responses of patients harboring changes in centrosome/mitotic genes and exhibiting CA

Direct changes in genome stability induced by changes in centrosome and mitotic genes can also alter response to chemotherapy and are correlated to worse patient prognosis in breast cancer. In 2010, (95) a sixteen kinase gene signature including centrosome and mitotic regulators that are associated with poor prognosis in breast cancer was characterized. TTK was a part of this gene signature, as well as Bub1 and Mad1. A meta-analysis of TNBC mRNA expression revealed a prognostic signature that could be used for treatment of breast cancer (96). In addition, other projects within the Saavedra laboratory demonstrated that high expression of centrosome kinases NEK2 and PLK4 is correlated to reduced relapse free survival and metastasis in breast cancer patients (97). In this study of NEK2 and PLK4, we found the most significant changes in patient survival within the Her2+ and basal subtypes of breast cancer. In addition, CA is observed in breast cancer cell lines that are used to model cells that are intrinsically resistant to therapeutic approaches, such as the JIMT-1 cell line that is Herceptin resistant or radioresistant cell lines HCC1954 and MDA-MB-231. This provides evidence that overexpressed centrosome genes may also be involved in the responses of different breast cancer subtypes to chemotherapy and radiation.

1.6 TTK kinase

1.6.1 Background

In general, changes and/or loss of regulation of the expression and kinase activities of protein kinases can cause oncogenic activities. One such kinase that is related to mitosis and CA in breast cancer is the TTK kinase. TTK, also known as MPS1 (monopolar spindle 1) is a serine/threonine kinase whose roles have been most comprehensively studied in the spindle assembly complex, but TTK is also largely involved in the centriole duplication cycle and DNA damage response. The TTK protein is 856 amino acids long and the important functional regions within the protein include the D-box, the MDS (MPS1 degradation signal) and the classical kinase domain (Fig 1.2).

Seminal studies describing TTK's functions in yeast models are the origin of the alternative name for TTK, monopolar spindle-1, as deletion of this gene resulted in a monopolar spindle phenotype in yeast cells (98). In *S. cerevisiae*, spindle pole bodies execute centrosomal functions similar to the classical centrosome in mammalian cells. TTK was first identified by using temperature sensitive allele studies in yeast, where aberrant spindle formation was observed at nonpermissive temperatures, leading to defective chromosome segregation. Another seminal study identified TTK as a close relative of SPK1 that is present in thymus and testes (99), which are both tissues with rapid proliferation, as well as being present in malignant cells. These early studies also revealed that TTK expression could be induced in T cells by IL-2 induced cell proliferation and TTK expression peaked as cells entered G2 (100).

1.6.2 Normal TTK functions and structure

The top three normal functions for TTK and mitosis are in spindle assembly, centrosome duplication, and cytokinesis (101). From a regulatory viewpoint, TTK is localized at specific cellular locations by other molecules involved in mitotic signaling. The TPR domain, located in the N-terminus, is the important region for localizing TTK to the centrosome and kinetochore region of chromosomes (102, 103).

To maintain normal centrosome numbers during mitosis and correct chromosome number, the centriole duplication cycle must occur only once during S phase of the cell cycle (104) and TTK is temporally regulated during this process. To properly execute roles in the centriole duplication cycle, the MDS region on TTK (MPS1 degradation signal) is phosphorylated by Cdk2 to degrade TTK from centrosomes and prevent centriole re-duplication (105). In addition, the kinase domain of TTK has an affinity for microtubules and is required for centrin-2 recruitment to pro-centrioles to form mature centrosomes (106). TTK also ensures precise centriole duplication and maturation by interacting with gamma-tubulin, centrin-2 and TACC2 (figure 1.3) (107). If TTK is not degraded after G1/S, this can result in centriole reduplication, eventually leading to centrosome amplification. Overexpression of CDC25B or hyperactivity of CDK2 at the centrosome stabilizes TTK and centrin-2, which results in centriole overduplication (108). The E2F transcription factors have also been shown to maintain genomic integrity through controlling multiple mitotic regulators, including TTK (109).

TTK also localizes to kinetochores to execute functions in the spindle assembly checkpoint and is required for mitotic arrest in response to microtubule insults before mitosis (110). Chk2 also controls the kinetochore localization of TTK by phosphorylating Thr288 (111). Other mitotic kinases, including Aurora B, Polo-like kinase 1 and MAPK

phosphorylate TTK to localize it to the kinetochore prior to mitosis and ensure spindle assembly checkpoint activation (112-115). Within its role in the spindle assembly complex, TTK phosphorylates Mad1/2 and Bub1 (116, 117), two other components of the spindle assembly complex, to ensure all chromosomes are accurately segregated prior to mitosis. Upstream of this signal, TTK must be phosphorylated at S283 to allow TTK to interact with MAD1/2 (105). TTK also interacts with MAD3 to inhibit CDC20 and maintain SAC arrest if needed prior to mitosis (118). The attachment of microtubules to the kinetochore is facilitated by TTK phosphorylating Dam1 (119) and TACC2 phosphorylation by TTK is necessary for TACC2 to detect lagging or misaligned chromosomes prior to mitosis (120). Substrates of TTK are summarized in Fig 1.3.

1.6.3 Non-canonical roles of TTK

Substrates of TTK that are unrelated to functions in classical mitosis include Chk2 and Smad3 (Fig 1.3). TTK can phosphorylate Chk2 (121, 122), which is a transducer of the DNA repair response. The kinase activity of TTK is necessary to induce G2/M arrest in response to DNA damage insults such as ultraviolet irradiation. Relating to the TGF-β signaling pathway, TTK can also phosphorylate SMAD3, to induce TGF-β independent signaling (123, 124). Very recently, TTK was shown to also promote AKT signaling and migration of hepatocellular cancer cells (125), suggesting TTK may have roles in controlling EMT associated phenotypes, similar to other mitotic kinases like PLK4 and Aurora A.

1.6.4 TTK in cancer

In general, stably aneuploid tumors are more sensitive to TTK inhibition than tumors with fluctuating CIN (126), indicating there is a delicate balance that exists between signaling pathways such as the SAC that control chromosome dysfunction and the maintenance of aneuploidy in cancer cells. Across several cancer types such as breast, hepatocellular, pancreatic and brain, colon (127-131), TTK is overexpressed at the mRNA and protein level. Functionally, preventing TTK degradation at centrosomes can lead to accumulated TTK and overduplication of centrosomes. This degradation of TTK has been shown to be deregulated in cancer cells (132). High levels of TTK have also been shown to be protective of aneuploidy in breast cancer cells (133). In colon cancer, TTK overexpression attenuates normal functions of the SAC and leads to increased aneuploidy (134).

The taxane and vinca alkaloid derived therapeutics are staple mitotic therapies that are used to treat metastatic breast cancer. However, since these therapeutics are not 100% specific for cancer cells, toxicity in normal cells is an issue, as well as resistance (135). Combinations of targeted cell cycle based therapies, such as CDK4 or AURK inhibitors, has shown effectiveness in breast and other cancer types (136-138). As a result of these observations, TTK has emerged as a notable molecule for investigation as a therapeutic target, similar to other cell cycle/mitotic therapeutic targets. Several chemical inhibitors of TTK have been developed and investigated in preclinical cancer models (139-142). The approach of combining TTK inhibitors with existing antimitotic drugs has shown efficacy in some cancer models. One TTK inhibitor, BAY1161909, is currently in a clinical trial and being tested in combination with Paclitaxel treatment (143) while another TTK inhibitor, CFI-402257, was effective

in combination with PD-1 immune therapy and is planned to advance to a phase II clinical trial (144). In experimental models, combination of TTK inhibitors with Taxol in glioblastoma was shown to sensitize brain cancer cells to Taxol (130) and enhance the effects of Docetaxel in breast cancer (145). In lung cancer, treatment with a targeted TTK inhibitor abrogated the SAC and induced cell death by mitotic catastrophe (146) and induced lethal CIN in a model of pancreatic cancer (147). In addition to mediating TTK's effects in the SAC (148) and chromosome stability, TTK inhibitors have also shown effects on other cellular processes associated with tumor progression and treatment response. An example of this is TTK's role in miR-21 and miR-132 signaling to regulate the radiosensitivity of glioblastoma cells (149, 150).

1.7 Rationale for studying TTK in breast cancer and goals of dissertation

At the start of this dissertation research, one integral goal within the centrosome biology field was to understand how alterations in modulators of centrosome amplification influence disease development and cancer cell transformation. At that time, it was known that CA is associated with aggressive tumor types and metastasis, as well as responses to treatment. However, the molecular changes and comprehensive roles of CA contributing to tumorigenesis were poorly understood. Since CA is observed in breast tumors and associated with metastasis to lymph nodes, this suggested that changes in CA modulators could also be involved in the metastatic cascade.

Through early microarray analysis in this dissertation, TTK was detected as a centrosome molecule that is overexpressed in Her2+ breast cells. We also observed TTK overexpression in triple negative breast cancer cells early in this research. The initial

hypothesis was that TTK overexpression leads to CA and inhibition of TTK could suppress tumorigenic properties and further cancer evolution associated with TTK overexpression. Through several studies and patient cohorts, TTK has been shown to be highly expressed in breast cancer, most notably in Her2+ and TNBC, suggesting it could be developed as a biomarker or therapeutic target. Preclinical studies of TTK inhibitors in breast cancer have shown efficacy in TNBC (145, 151, 152). However, the oncogenic processes that could be modified by TTK inhibition have not been elucidated. There is some evidence that TTK inhibition can induce apoptosis in breast cancer cells, as well as prevent invasive and/or migratory patterns. Chapter two aims to address the impact of targeting TTK on CA, while chapter three aims to address mechanisms for how TTK overexpression promotes invasive signaling pathways in breast cancer. Chapter four focuses on the impact of targeting TTK on therapeutic responses in breast cancer cells, specifically relating to radiation treatment.

Based on several lines of evidence of the roles of centrosome regulatory molecules in promoting CIN, aneuploidy and other oncogenic processes, the end goal of studying TTK could lead to discerning how deregulated centrosome regulatory molecules can influence several aspects of mammary tumorigenesis (Fig 1.4), so that they could be further understood to create new targeted therapies or biomarkers for breast and other cancer types.

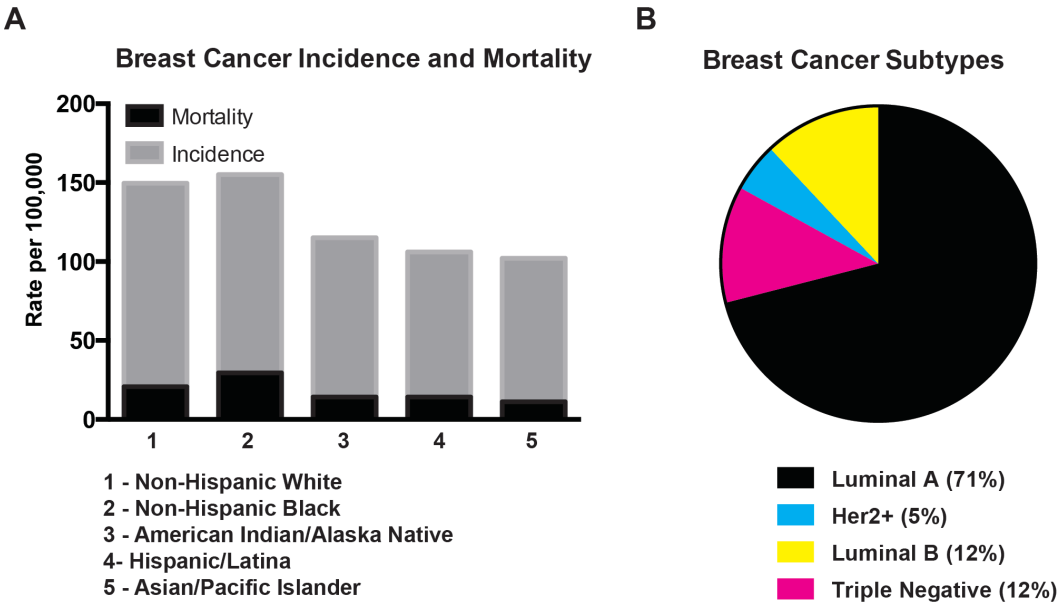


Figure 1.1 Estimated breast cancer incidence, mortality and subtype distribution in the U.S. in 2017. Adapted from American Cancer Society (ACS), *Cancer Facts and Figures 2017*. (A) Distribution of breast cancer incidence and mortality rates across ethnic groups in the United States. (B) Distribution of breast cancer subtypes among diagnosed cases in the United States.

Figure 1.2 The structure of TTK.

TTK is 856 amino acids long and contains four regions that are essential for adequate functions of the kinase. The CLD domain localizes TTK to the centrosomes, while the D-Box and MPS1 degradation signal function to degrade TTK at necessary times during centrosome duplication. The kinase domain functions for TTK to phosphorylate targeted substrates. The TPR domain, (located within the N-terminal region) is necessary for kinetochore localization of TTK.

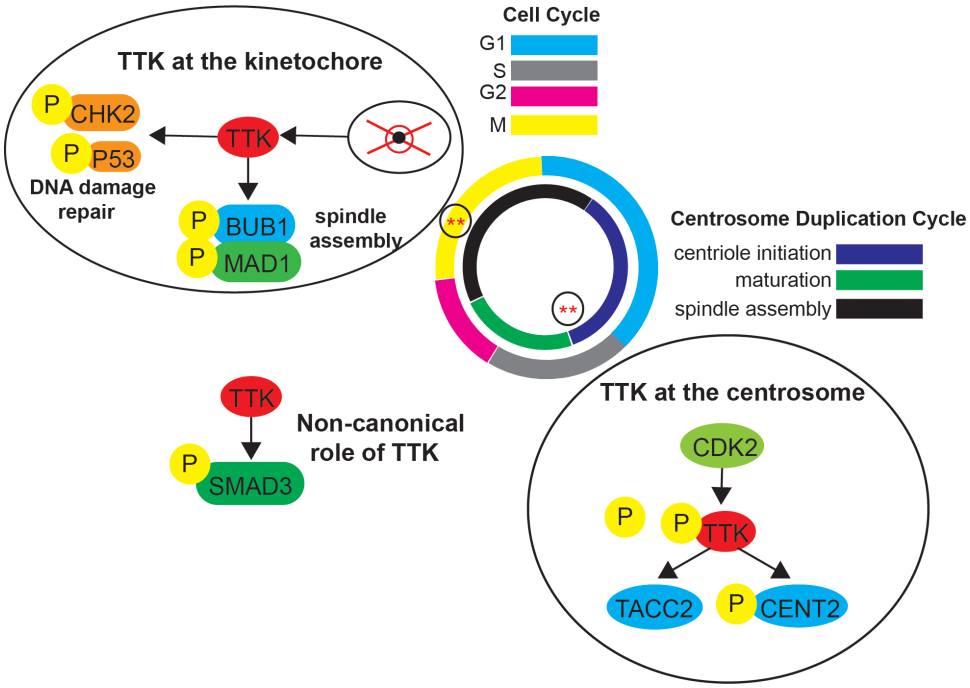


Figure 1.3 - Summary of TTK substrates and reported roles in the cell.

TTK contributes to the activity of diverse cellular processes in parallel with the cell and centrosome cycles, by phosphorylating downstream substrates.

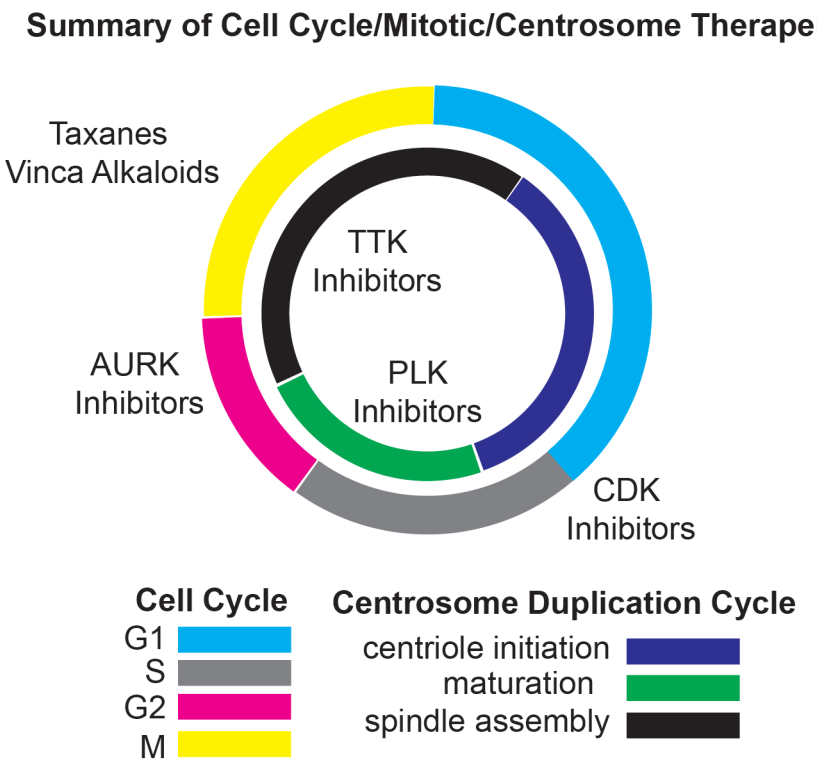


Figure 1.4 Summary of cell cycle, mitotic and centrosome targeted therapies.

Presently, taxane and vinca alkaloid based therapies are approved for metastatic breast cancer, as well as recently approved CDK inhibitors and AURK inhibitors in other solid tumors. TTK and PLK inhibitors are currently in clinical trials.

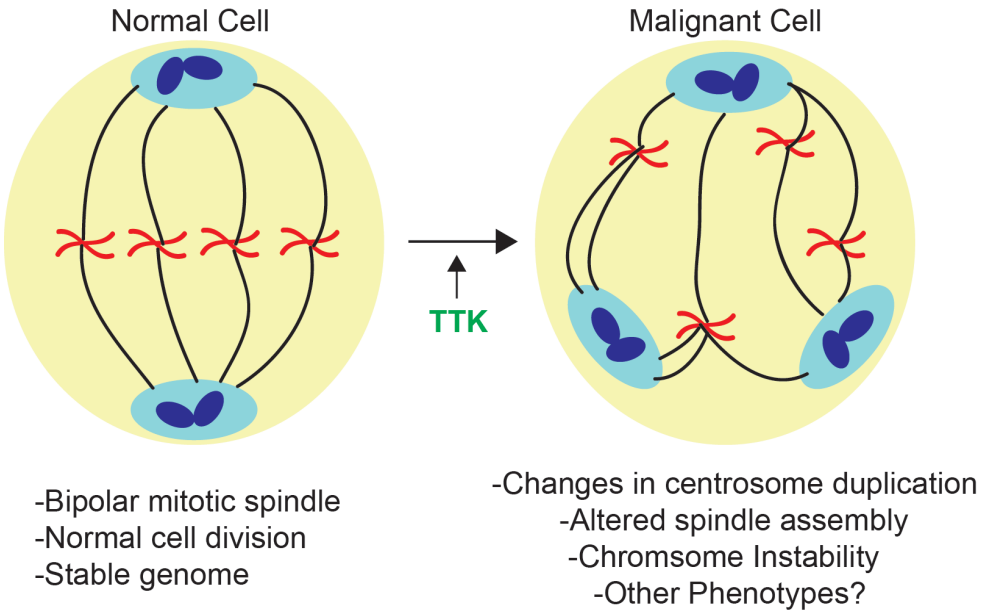


Figure 1.5 Summary of TTK functions in promoting genomic instability

As outlined in chapter one, TTK has reported roles in altering centrosome duplication, spindle assembly and chromosome instability in cancer cells. These findings provide rationale to study these changes and other cancer associated phenotypes in breast cancer

Chapter 2

Differential expression of TTK in breast cancer cells supports centrosome amplification.

Portions of this chapter have been published in Lee MY, Marina M, King JL, Saavedra H. Cell Division 2014 9:3. PMID: 25278993. JLK completed all sections and experiments pertaining to TTK.

2.1 Introduction

Chromosome instability (CIN), the active gain or loss of whole fragments of chromosomes during cell division, and aneuploidy, the state of having abnormal chromosome numbers, are sources of genetic instability and are associated with aggressive breast cancer biology (153). Centrosome amplification (CA) is a prevalent driver of genomic instability (6, 61) in specific subtypes, such as in Her2+ breast cancer (59, 154-156). Changes in specific signaling pathways associated with centrosome biology have not been fully explored in subtype specific models, including Her2+.

Molecular alterations in genes such as oncogenes and genes that control the centrosome cycle or the spindle assembly checkpoint eventually lead to CA. CA results in defective mitotic spindle formation, which can support CIN and aneuploidy in tumors. This cellular phenotype has been detected in pre-malignant lesions, DCIS (ductal carcinoma in situ) and invasive breast tumors (154, 156). Furthermore, CA is observed in more aggressive breast tumors to a greater extent, which supports the idea that this phenomenon is associated with aggressive tumor biology and progression. All of this evidence supports the pursuit of targeting molecules that mediate CA to prevent cancer initiation and tumor progression.

In this study, we utilized a model of Her2+ breast cancer to elucidate centrosome regulatory genes that are specifically altered in this breast cancer subtype. In previous screening, we observed that CA is present in a subset of Her2+ breast cancer cell lines. More specifically, we found that E2F activators, Cdk4 and Nek2 kinase are overexpressed and required to maintain CA and binucleation in Her2+ cells (62, 157). However, since these molecules regulate diverse biological processes, their clinical

utility is limited. To determine other altered genes in Her2+ cells that could be viable therapeutic targets, we carried out a gene microarray experiment comparing gene expression between two breast cell lines. We used MCF10A (normal mammary epithelial cells that do not display CA) and HCC1954 (Her2+ cells that display CA) to detect altered centrosome genes. We validated differentially expressed genes by real-time PCR and Western blotting, and then pursued a panel of upregulated and downregulated genes based on their novelty and relevance to centrosome duplication. We also conducted functional experiments to measure CA and BrdU incorporation after genetic manipulation of altered genes. From this screen, TTK emerged as a highly relevant centrosomal modulator in Her2+ breast cancer, since it was upregulated in the HCC1954 cells.

2.2 Materials and Methods

Cell Culture

All cell lines were obtained from the ATCC or from collaborators. Culture conditions for MCF10A, HCC1954, SKBR3 and JIMT-1 cells have been described.

Microarray analysis

Total RNA was isolated using RNAeasy mini kit (Qiagen) and subjected to quality control. Affymetrix Gene Expression microarrays were used according to manufacturer's instructions. Raw intensities of the arrays (two for MCF10A-pLKO.1 and two for HCC1954-pLKO.1 samples) were normalized using quantile normalization and log2 transformed in the Affymetrix Human U133 platform before analyses. Sample distribution was calculated and the top 20% of differentially expressed genes were

selected for comparative analysis. Averaged data was uploaded into Metacore where gene expression probe names were identified and differentially expressed genes (fold threshold > 1.5) for centrosome and cell cycle GO process were displayed and further analyzed.

RNA extraction and real-time PCR analysis

RNA was isolated from cells using the RNAeasy mini kit (Qiagen). Two μg of RNA was used to synthesize cDNA per manufacturer's instructions (Promega). The cDNA was diluted 1:10 for real-time PCR with iQ SYBR Green supermix (Bio-Rad). Actin was used as internal control. Primer sequences are listed here: Actin-F 5'-CgAggCCCAgAgCAAgAg-3', Actin-R 5'-CgTCCCAgTTggTAACAATgC-3', TTK-F 5'-CgCAgCTTTCTgTAgAAATggA-3', TTK-R 5'-gAgCATCACTTAGCGGAACAC-3'

siRNA transfection

Cells were seeded overnight in either 60 mm culture dishes for RNA or protein isolation or in four well chamber slides (Thermo Scientific) for microscopy. Lipofectamine RNAimax (Life Technologies) along with 200 pmol of TTK siRNA or 5μL of silencer negative control siRNA (50μM, Life Technologies) were transfected for 48 hours. SiRNA sequences are listed here: TTK_1 Sense 5′-rGrGrArGrGrUrUrCrArArGrCrArArGrGrUrArUrUrUrCrAGG-3′, TTK_1 Anti-Sense 5′rCrCrUrGrArArArUrArCrCrUrUrGrCrUrUrGrArArCrCrUrCrCrArC-3′ TTK_2 Sense 5′-rCrCrArGrArArUrCrCrUrGrCrUrGrCrArUrCrUrUrCrArAAT-3′

TTK_2 Anti-Sense 5′-rArUrUrUrGrArArGrArUrGrCrArGrCrArGrGrArUrUrCrUrGrGrUrU-3′

Immunofluorescence for Bromodeoxyuridine (BrdU) incorporation and centrosome amplification

BrdU staining was performed as described in a previous publication (41). Forty-eight hours post transfection, BrdU was incubated in the media of cells grown in four well chamber slides at a final concentration of 10 μM for 30 minutes prior to fixing the cells in 4% paraformaldehyde for 10 minutes. DNA was denatured in 2 N HCl for 20 minutes at room temperature, and then neutralized in 0.1 M sodium borate (pH 8.5) for 2 minutes. Then, cells were permeabilized in 0.1% NP-40 solution for 10 minutes after three washes with PBS. Slides were blocked in 10% normal goat serum (Life Technologies) for 1 hour before incubation with anti-BrdU antibody (NA61, Calbiochem) at 4°C overnight. DAPI (1mg/mL) counterstain was used. Two hundred cells were counted and the percentages of BrdU+ cells were calculated using fluorescent microscopy.

Centrosome amplification in transiently transfected cells was measured using four well chamber slides. Forty-eight hours post transfection, cells were fixed in 4% paraformaldehyde for 10 minutes, washed three times with PBS, permeabilized in 0.1% NP-40 for 10 minutes and blocked in 10% normal goat serum (Life Technologies) for 1 hour, followed by overnight incubation with anti-Pericentrin antibody (Abcam ab4448). Alexa Fluor-conjugated antibodies (A11008, A11002 or A21069, Life Technologies) were used as secondary antibodies and incubated on the slides for 1 hour at room temperature. DAPI (1 mg/mL) was also used as a counterstain. Two hundred cells were counted per group and cells with ≥3 pericentrin spots were counted as having CA. Percentages of cells with ≥3 pericentrin spots were calculated for each group.

Western blotting

Western blotting was performed according to previously published protocols (41, 62, 158). The TTK antibody (3255S, Cell Signaling) and secondary antibody for goat anti-rabbit (sc-2004) was used and Beta-actin (4970, Cell signaling) was used as a loading control. Signals were detected with Lumigen TMA-6 reagent.

CCK-8 Assay for Cell Proliferation

CCK-8 cell counting kit was purchased from Dojindo. Prior to NMS-P715 treatment, 1500-2000 cells were plated in 96 well plates and allowed to adhere overnight. To measure cell proliferation each day, 10 μL of CCK-8 solution was added to each well of the plate and incubated for 1-4 hours at 37 degrees Celsius. Then, the absorbance was measured at 450 nm using a microplate reader. Calculations for cell densities were normalized to media only controls to control for background readings from the microplate reader.

Statistical analysis

Student's t-test was applied to compare the significance between control and siRNA transfected groups. P-values ≤ 0.05 were considered statistically significant.

2.3 Results

Analysis of microarray targets

HCC1954 is a Her2+ breast cancer cell line that displays approximately 10% CA in unsynchronized populations, which is significantly higher than MCF10A non-transformed mammary epithelial cells (62, 97, 157). In a parallel microarray assay, we aimed to identify genes differentially expressed between HCC1954 cells silenced for E2F3 and cells expressing empty vector control (HCC1954-pLKO.1), which is why MCF10A-pLKO.1 cells were used in this study as a basis for comparison. We first selected the top 20% of genes that were differentially distributed across the microarray samples and performed Metacore gene enrichment analysis. Selected targets fell into various categories and TTK fell into the category of cell cycle/mitosis genes. The microarray data identified 2135 genes underexpressed in HCC1954 cells and 2635 genes upregulated in HCC1954 cells compared to MCF10A. We refined our analysis to genes with ≥1.5 fold change between the groups and TTK was noted as an upregulated gene in HCC1954 cells compared to MCF10A cells. All findings from the microarray were validated by real-time PCR. After analyzing genes that were upregulated in HCC1954 cells compared to MCF10A cells, we selected TTK for further analysis since the centrosome roles of this gene is not fully understood in cancer models.

TTK is overexpressed in a panel of breast cancer cell lines compared to normal mammary epithelial cells.

To validate the findings from the microarray, we analyzed TTK protein expression across a panel of breast cancer cells compared to MCF10A normal mammary epithelial cells. Increased TTK expression was observed in three Her2+ breast cancer cell lines (HCC1954, SKRB3, and JIMT-1) and two TNBC cell lines (MDA-MB-231 and

MDA-MB-468) (Fig 2.1). We also validated overexpression of TTK substrate centrin-2 in the 5 breast cancer cell lines compared to MCF10A.

Transient TTK knockdown decreases centrosome amplification in Her2+ cells.

We hypothesized that expression of TTK is required for CA and therefore proceeded to silence TTK expression via RNAi to address this question. To address the effects of silencing TTK, we transiently silenced TTK using three independent siRNA duplexes. All three duplexes achieved over 50% knockdown in HCC1954 cells (Fig 2.2). In the CA assay, where we measured CA via pericentrin staining, knockdown of TTK reduced the percentages of CA in HCC1954 cells (Fig 2.2) from ~10% to less than 5%.

Pharmacologic inhibition of TTK also decreases centrosome amplification in Her2+ cells

To determine if the kinase activity of TTK is necessary to maintain CA in Her2+ breast cancer cells, we treated HCC1954 with TTK inhibitor NMS-P715 and completed CA assays (Fig. 2.3). We found no differences in CA at a low dose of NMS-P715 (1 uM), but moderate decreases in CA at higher doses (2.5 and 5 uM respectively). Overall, these data suggest that TTK protein and kinase activity is necessary to maintain CA in Her2+ breast cancer cells.

Silencing TTK does not alter DNA synthesis in Her2+ cells

To determine if alterations in DNA replication capacity is involved in the maintenance of CA by TTK overexpression, we utilized a BrdU incorporation assay to

measure DNA synthesis (Fig. 2.4). The BrdU incorporation was modestly affected by knockdown of TTK, but not significantly reduced. This result suggests the reduction in CA observed after transient knockdown of TTK is attributed to a mechanism independent of DNA changes in DNA replication.

Stable knockdown and pharmacologic inhibition of TTK attenuates cell proliferation in Her2+ breast cancer cells

Although we did not observe changes in DNA replication after silencing TTK, we wanted to determine if there were changes in cell proliferation upon silencing TTK or inhibiting TTK kinase activity. To address these questions, we first generated Her2+ cell lines stably downregulated for TTK and validated TTK knockdown. Then, we measured cell proliferation using the CCK-8 assay (Fig. 2.5). We observed modest decreases in cell proliferation in the stable HCC1954-shTTK cells, and more noticeable decreases in cell proliferation in JIMT-1/shTTK and SKBR3-shTTK cells. We also observed decreased cell proliferation in all three cell lines after treatment with the TTK inhibitor NMS-P715 (data not shown).

Association of TTK expression with the outcome of breast cancer patients.

To further explore the clinical relevance of genes examined in this study, we used the KM Plot resource that provides an online survival analysis of 22,277 genes to assess the effects on breast cancer prognosis. The database consists of microarray data from 1809 patients. For TTK expression, we found that high TTK RNA levels were correlated with decreased overall survival of luminal A breast cancer patient in this database. (Fig. 2.6)

2.4 Discussion

Centrosome amplification is a promoter of CIN and aneuploidy that supports the acquisition and maintenance of malignant phenotypes in breast tumors. TTK's most well characterized role is in the spindle assembly checkpoint (159) and has also shown a role in centriole duplication and assembly (160, 161). Tightly controlled levels and regulation of TTK is partially responsible for ensuring proper centriole duplication and assembly during the cell cycle. One mechanism for this regulation is control of TTK through the MPS1 degradation signal (160), which prevents TTK accumulation and centriole re-duplication. Although TTK is not required for normal centriole duplication, there is evidence that overexpressed TTK results in centriole re-duplication, which could lead to CA. In regards to breast cancer, increased TTK mRNA has been observed across many cell lines, specifically Her2+ and triple negative subtypes, and in tumor samples collected from patients with advanced disease (129, 133, 162). These correlations suggest there is a correlation between high TTK levels, increased proliferation and aggressive cell phenotypes in cancer. In summary, TTK presents a potential biomarker to predict patient prognosis. While previous studies revealed how modifying TTK affects cell viability, mitosis and tumor growth in vivo, none addressed the functional roles of TTK in breast cancer (129, 162). Through this study, we showed for the first time that attenuating TTK expression and kinase activity can decrease the percentages of cells displaying CA and cell proliferation in a subset of Her2+ breast cancer cells, without affecting DNA synthesis. The changes in cell proliferation observed after TTK inhibition could be due to apoptosis or changes in other parts of the cell cycle, but that was not in the scope of this study. Further understanding of how TTK drives CA and

genomic instability in breast cancer will be important for understanding the correlations between high TTK levels and aggressive and/or drug resistant breast tumors.

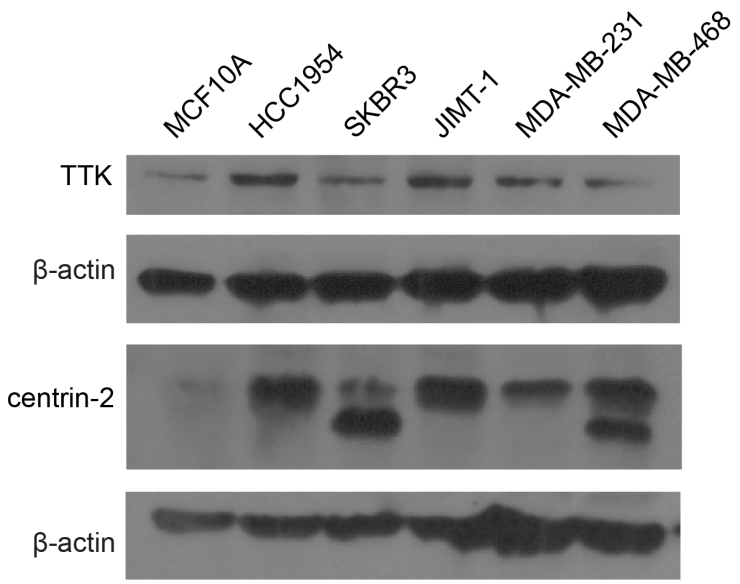


Figure 2.1: Basal levels of TTK are higher in breast cancer cells.

Basal levels of TTK and TTK substrate centrin-2 are higher in unsynchronized populations of Her2+ and triple negative breast cancer cell lines compared to MCF10A cells.

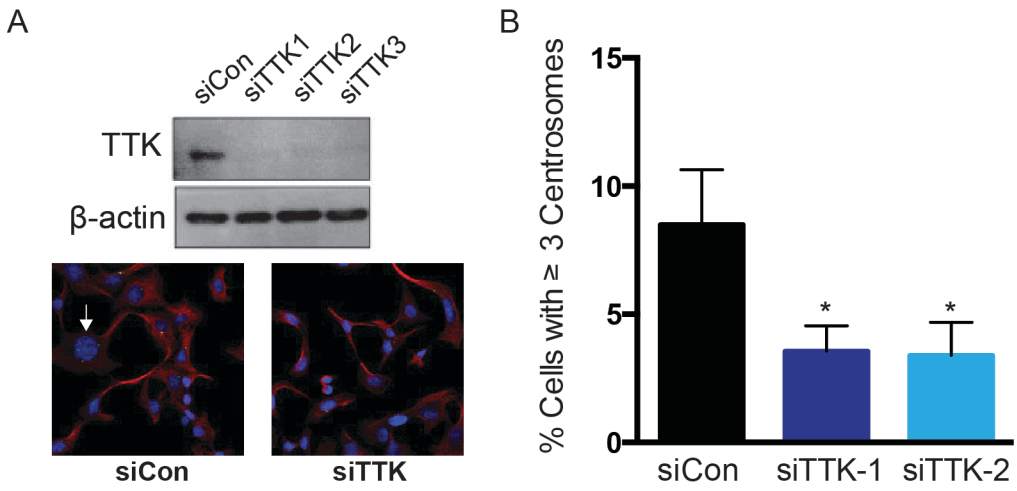


Figure 2.2: TTK maintains centrosome amplification in Her2+ breast cancer cells

(A) Western blots validating transient TTK knockdown in HCC1954 cells and representative Actin and pericentrin staining in HCC1954 cells. (B) Quantification of fluorescent microscopy for centrosome amplification by pericentrin staining, *p<0.05.

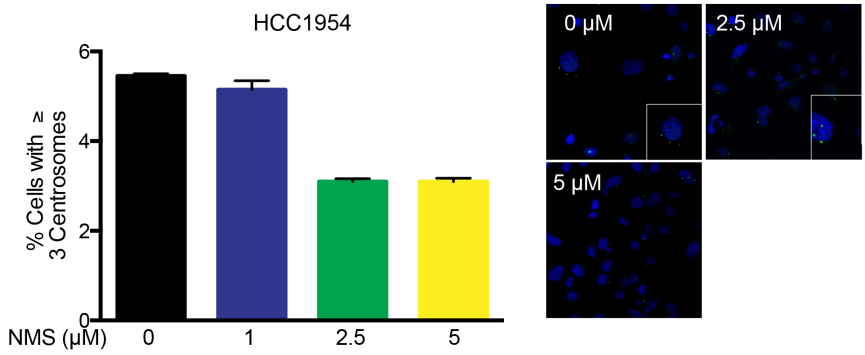


Figure 2.3: TTK kinase activity maintains centrosome amplification in Her2+ breast cancer cells

Quantification and representative fluorescent microscopy for pericentrin staining in HCC1954 cells treated with increasing doses of TTK inhibitor NMS-P715.

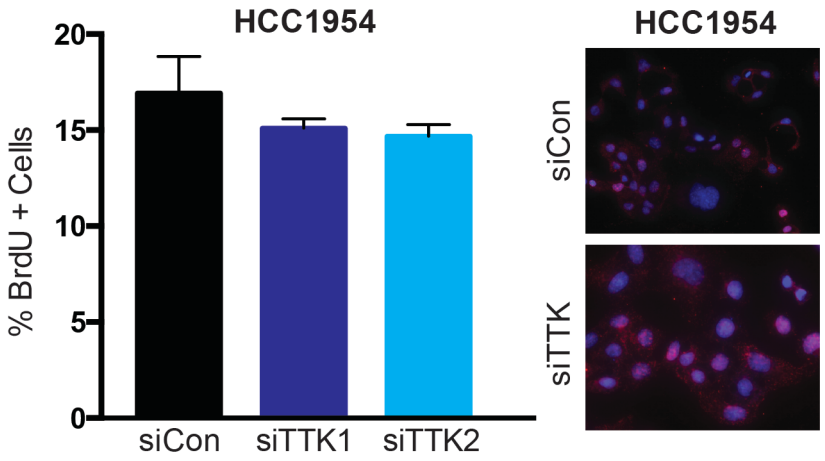


Figure 2.4: TTK silencing does not decrease BrdU incorporation

Quantification of BrdU+ cells and representative microscopy images in HCC1954 cells with transient TTK knockdown

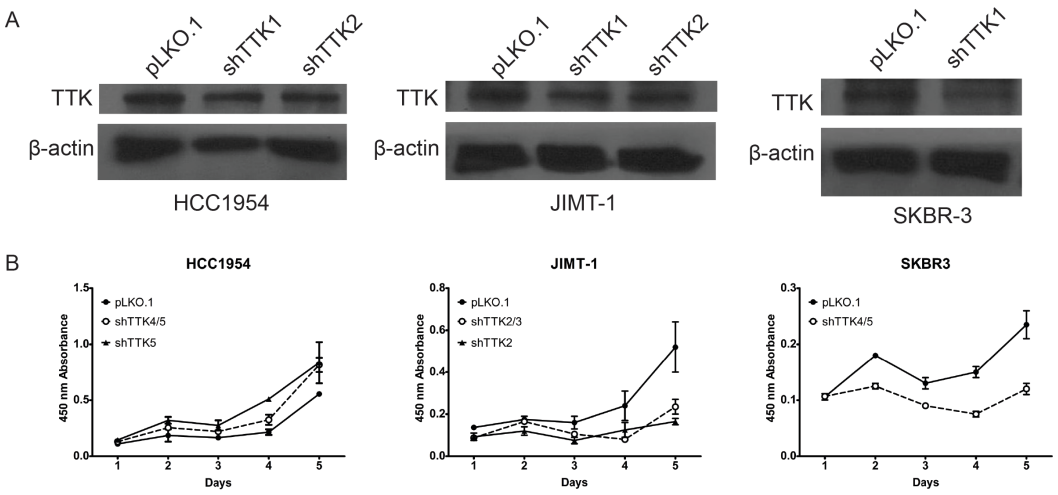


Figure 2.5: Stable TTK inhibition suppresses the proliferation of Her2+ breast cancer cells.

(a) Validation of stable TTK downregulation via viral delivery of shRNA in HCC1954, JIMT-1 and SKBR-3 cells. (b) Cell viability in HCC1954, JIMT-1, and SKBR-3 cells as determined by the CCK-8 assay.

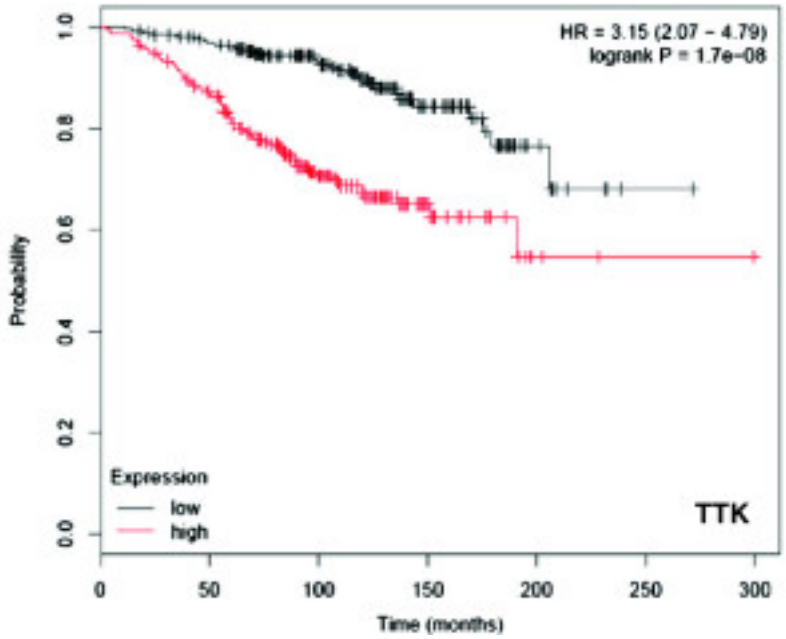


Figure 2.6: Correlation between TTK and overall survival in Luminal A breast cancer patients.

High TTK mRNA is correlated with decreased overall survival of luminal A breast cancer patients, as analyzed in the KM Plotter database.

Chapter 3

TTK promotes mesenchymal signaling via multiple mechanisms in triple negative breast cancer.

This chapter is adapted from a manuscript submitted by JL King, B Zhang, Y Li, KP Li, J Ni, H Saavedra, and JT Dong. ***TTK promotes mesenchymal signaling via multiple mechanisms in triple negative breast cancer.*** Under revision to Oncogenesis, 2018

3.1 ABSTRACT

Abnormal expression of TTK kinase has been associated with the initiation, progression and therapeutic resistance of breast and other cancers, but its roles remain to be clarified. In this study, we examined the role of TTK in triple negative breast cancer (TNBC), and found that higher TTK expression correlated with the mesenchymal and proliferative phenotypes in TNBC cells. Pharmacologic inhibition and genomic silencing of TTK not only reversed the epithelial to mesenchymal transition (EMT) in TNBC cells, they also increased the expression of KLF5, an effector of TGF-β signaling and inhibitor of EMT. In addition, TTK inhibition decreased the expression of EMT associated micro-RNA miR-21 but increased miR-200 family members expression and suppressed TGF-β signaling. To test if upregulation of KLF5 plays a role in TTK induced EMT, TTK and KLF5 were silenced simultaneously which decreased EMT caused by loss of TTK. Consistently, the decrease in miR-21 expression and increase in miR-200 expression caused by TTK silencing were rescued by loss of KLF5. Altogether, this study highlights a novel role and signaling pathway for TTK in regulating EMT of TN breast cancer cells through TGF-β and KLF5 signaling, highlighting targetable signaling pathways for TTK inhibitors in aggressive breast cancer.

3.2 INTRODUCTION

Triple negative breast cancer (TNBC) remains a critical public health issue. TNBC is characterized by lack of progesterone receptor, estrogen receptor and HER2 expression and is more aggressive than other breast cancer subtypes. As a result of lacking ER, PR, and HER2, well established pharmaceutical targets for breast cancer treatments, targeted treatments against TNBC have yet to be developed. Also, while basal (composed of 76% TNBC) and Her2+ breast cancers respond better to chemotherapy than luminal subtypes, they have a higher probability of relapse if residual disease remains (21). Thus, finding novel directed therapies against TNBC would greatly improve survival outcomes of these patients.

Further molecular profiling within TNBC has revealed genetic differences within the subtype that could affect cellular behavior and responses to chemotherapy. These subtypes of TNBC include four groups categorized as BL1, BL2, M, and LAR (163). The mesenchymal subtype is enriched in genes associated with the epithelial to mesenchymal transition (EMT), an important cellular process that is associated with increased cell migration, invasion, tumor metastasis, and resistance to chemotherapy and radiotherapy (71, 164). Mesenchymal TNBC cells exhibit a loss of epithelial morphology, as well as increased cell migration and invasion. EMT may contribute to TNBC progression because it is considered one of the earliest events during cancer metastasis.

TNBC, Her2+ and ER- breast cancers are more prone to have elevated frequencies of centrosome amplification (CA) and chromosome instability (CIN) relative to other subtypes (6, 60, 153, 154, 156, 165) In our previous studies, we noted

overexpression of the TTK kinase in Her2+ (ER-PR-) breast cancer cells displaying elevated frequencies of CA compared to normal mammary epithelial cells (166). CA causes mammary tumorigenesis in mice and correlates with high stage, grade, and poor relapse free and overall survival of breast cancer patients (54, 165), which suggests TTK could have a role in mammary tumor development and progression. CA induces CIN, de-differentiation, and invasion, which could be the underlying mechanisms for CA's role in tumorigenesis (167, 168).

TTK plays critical roles in aneuploidy and genomic integrity across cancer types (95, 127, 129, 131, 133, 169, 170), such as brain, pancreatic, hepatocellular and breast cancer. TTK has also been reported to have functions in promoting cell invasion(125). In breast cancer, high TTK expression is correlated with aggressive subtypes and therapeutic resistance (129, 133, 145, 152, 166). In addition, higher TTK expression has been noted amongst high expression of other spindle assembly regulators in a panel of breast cancer cell lines and patient samples (95). In our recent study, we found that TNBC cell lines exhibited the highest levels of TTK. Knockdown of TTK increased apoptosis and prevented tumor growth. Genomic silencing of TTK in Her2+ breast cancer cells attenuates centrosome amplification (CA) as well as pharmacological inhibition (unpublished data). Although TTK has a much higher expression in TN cells compared to other subtypes, distinct functions of TTK in TNBC cells are not clear.

In this study, we hypothesized that TTK could exert its functions in mesenchymal triple negative breast cancer cells by regulating KLF5 and associated miR-21 or miR-200's, since TTK has functions in regulating TGF-β signaling. Our key findings were that the mesenchymal status of TNBC cells could be decreased by silencing or inhibiting TTK and this effect is dependent KLF5 being upregulated. We

also found that silencing TTK reversed expression patterns of the onco-miR miR-21 and miR-200 family members, in parallel with the decreased mesenchymal phenotype. Our study highlights a distinct TTK induced signaling pathway in TNBC in which TTK maintains the proliferative and EMT phenotype by suppression of KLF5, which in turn facilitates upregulation of miR-21 expression and downregulation of miR-200's. Promising clinical therapeutic strategies could be developed for TNBC by targeting this novel signaling pathway.

3.3 Materials and Methods

Bioinformatic data and analyses

GOBO and BreastMark online databases were used to analyze the expression levels of TTK and KLF5 in different molecular and clinical subtypes of breast cancer. In the BreastMark database, we analyzed overall survival as the clinical endpoint. Publically available RNAseq data was obtained from the Cancer Genome Atlas (TCGA) to analyze TTK and KLF5 z-scores in breast cancer patients. Patients were categorized into groups by subtype and z-scores were analyzed using the RStudio software (*http://www.rstudio.com/*).

Cell culture, inhibitor treatment and transfection

MCF10A non-transformed mammary epithelial cells were obtained from ATCC and cultured in DMEM-F12 media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 4 ug/mL insulin, 1 ug/mL hydrocortisone and EGF. Human breast cancer cell lines MDA-MB-231, Hs578t and all other breast cancer cell lines were obtained from ATCC or collaborators and grown in DMEM media supplemented with

10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were cultured in a humidified chamber at 37 degrees Celsius and 5% CO2. NMS-P715 (TTK inhibitor) was purchased from Millipore. Scrambled control and custom TTK siRNA duplexes were purchased from Integrated DNA technologies and the sequences used were from our previous publication (166). KLF5 custom siRNA sequences were described in our previous publication (84). MiR-21 mimics and negative controls were a gift from the Vertino laboratory and originally purchased from Thermo Fisher. MiR-200 mimics and inhibitors were purchased from RiboBio. Transfections were performed using JetPrime Polyplus reagent per manufacturer's instructions.

Viral infection

HEK293T cells were cultured to 80% confluence and transfected with packaging plasmids along with control shRNA pLKO.1 or pLHCX or TTK shRNA or flag tagged KLF5 constructs. Lipofectamine 2000 was used for transfections per manufacturer's instructions. At 48 hours post-transfection, cell culture medium containing viruses was collected and aliquoted with a sterile filter. Target cells were plated and allowed to reach 60% confluence before infection with viruses and 8 ug/mL polybrene. Stable cells expressing shTTK or KLF5 constructs were selected with 2 ug/mL puromycin or 200 ug/mL hygromycin for one week post infection before the antibiotic dose was decreased for cell maintenance.

Colony formation assay

For the colony formation assay, 2000 cells were plated in each well of a 6-well culture plate. Cells were allowed to grow for 11 days then fixed with 75% ethanol, 1%

crystal violet solution. Colonies (clusters of >50 cells) were counted via ImageJ and colony intensity was also calculated in ImageJ.

SRB viability assay

Prior to treatment with NMS-P715, 1500 cells per well were plated into 96 well plates and cultured overnight to allow cells to attach. For NMS-P715 treatment, the inhibitor was added to the media at designated doses. To end the assay, cells were fixed with 10% TCA for one our at 4 degrees prior to adding 0.05% SRB dye for 30 minutes followed by dissolving in 10 mM Tris-HCL for 30 minutes. Absorbance was read at 562 nm to obtain quantification of cell viability.

Western blot analysis

For protein collection, cells were rinsed with PBS and lysed directly with Laemmli buffer (Bio-Rad) containing β-mercaptoethanol. Total protein lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes and subjected to Western blot analysis. Primary antibodies for EMT markers (#9782), TTK (#5469), and secondary rabbit antibody (#7074) were obtained from Cell Signaling. The primary antibody for β -actin was from Sigma (A2006) and the antibody for KLF5 was generated and described in a previous study (171).

Immunofluorescent microscopy

Cells were plated in 4 well chamber slides and allowed to adhere overnight prior to fixation. Cells were fixed with 4% paraformaldehyde prepared in PBS, permeabilized with 1% Triton X-100, blocked with 10% goat serum. Cells were stained with primary

antibodies against Vimentin (Cell Signaling #5741) and AlexaFluor fluorescent secondary antibodies from ThermoFisher, counterstained with DAPI (1mg/mL), then imaged on a Zeiss Confocal LSM 510.

Quantitative RT-PCR

For traditional quantitative real time PCR, total RNA was isolated from cultured cells using Trizol according to manufacturer's instructions. CDNA was synthesized using the MMTV reverse transcription kit from Promega and quantitative real time PCR was performed using Takara SYBR green on a 7500 Fast qPCR machine from Applied Biosystems. For micro-RNA PCR, RNA was isolated using the miRNeasy mini kit. CDNA was synthesized with primers for miR-21 or miR-200 family members and U6 using the TaqMan MicroRNA Reverse Transcription Kit from ThermoFisher. Real Time PCR was conducted with Taqman Universal Master Mix and custom Taqman probes for miR-21, miR-200's and U6 non-coding RNA. The $2^{-\Delta\Delta Ct}$ method was used to analyze and quantify mRNA and miRNA levels. Values were normalized against actin or U6 as internal controls then compared to experimental controls to obtain differences in fold changes. Primer sequences for PCR are: *ZEB1*: 5'-TGCACTGAGTGTGGAAAAGC-3' (forward) and 5'-TGGTGATGCTGAAAGAGACG-3' (reverse); *ZO1*: 5'-CCCCACTCTGAAAATGAGGA-3' (forward) and 5'-ACAGCAATGGAGGAAACAGC-3' (reverse; *CDH1*: 5'-TGAAGGTGACAGAGCCTCTGGAT-3' (forward) and 5'-TGGGTGAATTCGGGCTTGTT-3' (reverse); *FN1*: 5'-CCATAAAGGGCAACCAAGAG-3' (forward) and 5'-ACCTCGGTGTTGTAAGGTGG-3' (reverse); *VIM*: 5'-CAGGCGATATATTACCCAGGCAAG-3' (forward) and 5'-CTTGTAGGAGTGTCGGTTGTTAAG-3' (reverse); *MMP9*: 5'-

ACGTGAACATCTTCGACGCCATC-3' (forward) and 5'-TCAGAGAATCGCCAGTACTTCCC-3' (reverse); *KLF5*: 5'-AAGGAGTAACCCCGATTTGG-3' (forward) and 5'-CAGCCTTCCCAGGTACACTT-3' (reverse); *TTK*: 5'-CGCAGCTTTCTGTAGAAATGGA-3' (forward) and 5'-GAGCATCACTTAGCGGAACAC-3' (reverse); *GAPDH*: 5'-GGTGGTCTCCTCTGACTTCAACA-3' (forward) and 5'-GTTGCTGTAGCCAAATTCGTTGT-3' (reverse).

Transwell motility and invasion assays

To begin cell motility and invasion assays, 35,000 cells per well were plated on the top of a transwell chamber. For invasion assays, chambers were coated with Matrigel prior to plating. Cells were incubated for 24 hours and non-invading cells the upper chambers were removed with cotton swabs. Cells that migrated to the lower part of the chamber were fixed with 0.5% crystal violet prepared in methanol. Cells were counted in 12 random fields per treatment group. Average motile cells or percent of invaded cells were calculated for three independent experiments.

To begin the wound healing assay, 75,000 cells were plated per well in 12 well plates with serum free media and serum starved for 48 hours. At 48 hours, the cell monolayer was scratched with a p200 pipet tip in a straight line. Cell debris was washed away with 1x PBS and the media was replaced with media containing serum. Images were acquired at designated time points following the addition of serum containing media. Wound widths were measured using ImageJ.

Phosphoantibody array

The Cancer Signaling phosphoantibody array kit was purchased from Full Moon Biosystems. Briefly, cells were washed with ice cold 1X PBS 3-5 times then lysed in lysis buffer according to manufacturer's instructions. Cell lysates were then purified, biotinylated and coupled to the pre-blocked array slides. Cy-3 streptavidin was added to the array slides with detection buffer then slides were sent to Full Moon Biosystems for detection of phosphorylated proteins.

Statistical analysis

Graphpad Prism was used to analyze data. All values are represented as mean +/- SEM for n=3 experiments and are considered significant if $p < .05$. Student's t-test was used to compare differences between experimental groups. For TCGA data, data was acquired from online repositories. For statistical analysis, the Hmisc package (https://CRAN.R-project.org/package=Hmisc) was used to analyze correlations of gene expression in each population.

3.4 RESULTS

Increased TTK expression is correlated with higher tumor grade, triple negative status, and worse overall survival in breast cancer

To determine the significance of higher TTK expression in breast cancer, we analyzed TTK expression in three publically available platforms. First, TTK expression was analyzed in the GOBO online database (http://co.bmc.lu.se/gobo/gsa.pl) (172). Initial analysis of TTK expression across tumor grades indicated that high TTK expression was significantly correlated with grade III breast tumors compared to grades I or II tumors

(Fig. 3.1a). In the same database, TTK expression was compared between ER-negative and ER-positive tumors, and there was a significant correlation between higher TTK expression and ER negativity (Fig. 3.1b). Further analysis of tumors from different clinical subtypes of breast cancer in the GOBO database revealed that higher TTK expression was most significantly correlated with the triple negative (TN) subtype compared to all other subtypes (Fig. 3.1c). Analysis of breast cancer cell lines in the GOBO samples revealed that higher TTK expression was correlated with the Basal A and Basal B subtypes compared to the luminal subtype in these cell lines (Fig. 3.1d). When the cell lines were categorized by clinical subtypes, higher TTK expression was correlated with the TN subtype compared to HER2+ and ER+ and/or PR+ cell lines (Fig. 3.1d). Following analysis in the GOBO database, we analyzed TTK expression in RNAseq data generated from breast cancers from the Cancer Genome Atlas (TCGA) database. The level of TTK expression, as indicated by z-scores, was significantly higher in the TNBC cases (n=89) when compared to all non-TNBC cases (n=439) (Fig. 3.1e). In the third analysis, we tested whether TTK expression increase correlates with patient survival using the BreastMark database (http://glados.ucd.ie/BreastMark/) (173). Among 2091 breast cancer patients that had both TTK expression and survival data available, higher TTK expression was significantly correlated with decreased overall survival (Fig. 3.1f). These results indicate that higher TTK expression is correlated with aggressive features of breast cancer, including higher tumor grade, ER negativity, worse patient survival, and TN status.

TNBC cell lines are sensitive to anti-proliferative activity of TTK Inhibitor

Previous studies demonstrated siRNA-mediated silencing TTK inhibits the proliferation of TNBC cells (129). Cell proliferation in other cancer cell lines from other tissues is also inhibited by treatment with a targeted TTK inhibitor, NMS-P715 (139), although the response of TNBC cells to NMS-P715 has not been tested. We previously reported that silencing TTK could decrease centrosome amplification in HER2+ breast cancer cell lines. To investigate other functional effects of inhibiting TTK with NMS-P715 in breast cancer, we first treated a panel of cell lines with the inhibitor and measured cell proliferation using the SRB assay. The most profound changes in cell viability caused by NMS-P715 treatment occurred in MDA-MB-231 and Hs578t cell lines (Fig. 3.2a-b). This finding was interesting, because both of these cell lines belong to the mesenchymal stem cell like subgroup of TNBC cell lines, and they both have previously been reported to express higher levels of TTK (133). To further examine the effects of inhibiting TTK in the context of TNBC, we generated MDA-MB-231 cells stably expressing shRNA's against TTK. Significant decrease in colony formation and changes in cell morphology, including fewer cells with protrusions and more rounded cells were detected over an 11-day growth period in cells with stable knockdown of TTK (Fig. 3.2c-e). Upon further examination, we observed that the colonies formed in the shTTK group appeared to be more rounded with fewer protruding cells, suggesting an epithelial phenotype of these cells. These morphological changes were not observed in HCC1954 cells expressing TTK shRNA (data not shown).

TTK inhibition attenuates the mesenchymal phenotype of MDA-MB-231 and Hs578t TNBC cells

After observing decreased mesenchymal morphology in cells with stable downregulation of TTK, we performed additional experiments to confirm the effect of TTK on the epithelial to mesenchymal transition (EMT). In MDA-MB-231 cells expressing the pLKO.1 vector control or shRNA against TTK (shTTK), western blotting demonstrated an increased expression of the E-cadherin epithelial marker and decreased expression of the vimentin mesenchymal marker, along with fewer cells with spindle like morphology, in the shTTK group (Fig. 3.3a-b). Consistently, treatment with the NMS-P715 TTK inhibitor also decreased vimentin expression and caused changes in morphology in both MDA-MB-231 and Hs578t cell lines (Fig. 3.3c,f). Changes in EMT markers were also measured at the mRNA level by real-time PCR, and there were moderate decreases in mesenchymal markers vimentin, fibronectin, Zeb1 and MMP-9 and increases in epithelial markers Cdh1 and ZO1 in MDA-MB-231 cells treated with 2 μM NMS-P715 (Fig. 3.3e, h). Changes in mesenchymal markers were consistent
in Hs578t cells, while the changes in epithelial markers were slightly different in this cell line, which could be attributed to cell density at the time of RNA collection. These results suggest that higher TTK expression facilitates the mesenchymal phenotype of TNBC cells.

Lower KLF5 expression is correlated with TN status of breast cancer and can be increased by TTK inhibition

Our recent study indicates that the KLF5 transcription factor maintains the epithelial phenotype of cells (84). In addition, among TNBC cell lines, those with a mesenchymal

phenotype, including MDA-MB-231 and Hs578T, express a lower level of KLF5 than those of epithelial phenotype (83, 84). We therefore tested whether KLF5 plays a role in TTK promoted EMT in TNBC cells. We first determined whether TTK affects KLF5 expression. In MDA-MB-231 cells, knockdown of TTK by RNAi increased KLF5 expression at both the RNA and protein levels (Fig. 3.4a-b), and the increase in KLF5 expression induced by TTK knockdown could be counteracted by the knockdown of KLF5 (Fig. 3.4a-b). A panel of EMT markers was analyzed by real-time PCR for expression, and silencing KLF5 eliminated the effect of silencing TTK on the expression of EMT markers fibronectin (FN), vimentin (VIM), ZEB1 and E-cadherin (CDH1) in MDA-MB-231 cells (Fig. 3.4c). Morphologically, cells with TTK knockdown (siTTK) showed less filamentous vimentin, but knockdown of KLF5 eliminated the effect of siTTK (Fig. 3.4d). We also analyzed the functional effects of silencing TTK and KLF5 on the migration and invasion of MDA-MB-231 cells using the Boyden chamber assay. Silencing TTK alone did not significantly affect cell migration, but significantly decreased cell invasion through matrigel coated membranes (Fig. 3.4e-f); and expression of MMP-9, an enzyme involved in degradation of the extracellular matrix during cell invasion, was markedly decreased in the siTTK group (Fig. 3.4g). Similar to its effects on EMT markers, knocking down KLF5 eliminated the changes in cell invasion and MMP-9 expression caused by TTK silencing (Fig. 3.4f, g). These results indicate that downregulation of KLF5 is required for TTK to induce a mesenchymal phenotype in TNBC cells.

To evaluate the importance of the inverse relationship between TTK and KLF5 expression in TNBC, we first analyzed TTK and KLF5 protein expression in a panel of cell lines. In the MCF10A mammary epithelial and DU-145 prostate cancer cell lines,

TTK was expressed at low levels while KLF5 was expressed at moderate levels. Meanwhile, the TNBC cell lines used in the current study expressed higher TTK but lower KLF5 compared to MCF10A cells (Fig. 3.4h). In the GOBO database, lower KLF5 expression was significantly correlated with the Basal B subtype (which includes MDA-MB-231 and Hs578t) and the overall TN status but not with the Basal A status in breast cancer cell lines (Fig. 3.4i). In the TCGA database, higher KLF5 expression, as indicated by the z-score, was also correlated with the TN status in breast cancer (Fig. 3.4j). TNBCs in the TCGA database have not been classified into subgroups of Basal A, Basal B and Mesenchymal stem cell like, which prevented us from testing which subtype is correlated to the highest KLF5 expression. To address this shortcoming, we selected genes that are overexpressed in different subtypes of TNBC identified in a previous study (174), and analyzed if their expression correlates with KLF5 expression in samples from the TCGA dataset. In this analysis, the basal like-immunosuppressed group and luminal androgen receptor subtypes had the highest percentages of genes correlated with KLF5 expression (90.9 and 92.9% respectively), with the basal-like immunosuppressed group having the most genes with spearman's coefficients over 0.40 (36.4%), which is indicative of a moderate correlation (Fig. 4k). The basal-like immunoactivated group had 64.3% of genes associated with KLF5 expression, but only 7% had moderate spearman's correlation. The mesenchymal subgroup only had 40% of genes associated with KLF5 expression, and none of them had strong Spearman's coefficients. These results further suggest that there is minimal correlation between high KLF5 within mesenchymal TNBC's as compared to other subtypes of TNBC.

In the BreastMark cohort, lower KLF5 expression significantly correlated with decreased overall survival in TNBC patients (Fig. 3.4l). Taken together, these results

suggest that the inverse correlation between TTK and KLF5 plays a role in the development of TNBC, particularly in the Mesenchymal subtype of TNBC.

TTK inhibition attenuates TGF-β signaling and miR-21 expression

TGF-β signaling is the most potent known signaling pathway inducing EMT, and KLF5 was previously demonstrated to participate in TGF-β signaling (86, 175). We therefore tested if TGF-β signaling is involved in the inverse relationship between TTK and KLF5 and their role in EMT regulation in TNBC cells. TTK has been reported to regulate the TGF-β signaling pathway, as TTK modulates SMAD3 phosphorylation(123, 124, 150). In MCF10A cells, which clearly respond to TGF-β, we confirmed that TGF-β induced EMT, as indicated by morphological and EMT marker changes (Fig. 3.5b). This induction of EMT was mildly prevented when MCF10A cells were treated with NMS-P715 in conjunction with TGF-β (Fig. 3.5b). We further tested the effect of TTK inhibition on SMAD3 phosphorylation in MDA-MB-231 cells, and found that inhibition of TTK by NMS-P715 slightly decreased TGF-β-induced Smad3 phosphorylation (Fig. 3.5a). TGF-β treatment downregulates KLF5 expression (84), so we tested if KLF5 downregulation by TGF-β treatment in TNBC cells can be counteracted by TTK inhibition. NMS-P715 treatment did not prevent the downregulation of KLF5 induced by TGF-β, suggesting that TTK inhibition cannot significantly counteract the effect of TGF-β.

Micro-RNAs are important mediators of EMT, and micro-RNA 21 (miR-21), a downstream target of TGF-β involved EMT, appears to be regulated by TTK in Glioblastoma cells(150) . To test if miR-21 is regulated by TTK in TNBC cells, miR-21 expression was measured in MDA-MB-231 cells after treatment with siRNA against TTK

or the NMS-P715 inhibitor. Expression of miR-21 was decreased by siTTK and simultaneous knockdown of KLF5 rescued the downregulation of miR-21 by siTTK (Fig. 3.5c). Expression of miR-21 was also decreased in MDA-MB-231 cells by NMS-P715 (Fig. 3.5d). Meanwhile, ectopic expression of KLF5 decreased miR-21 expression in MDA-MB-231 cells, and this effect was augmented by TTK silencing (Fig. 3.5e). These results suggest that TTK modulates miR-21 expression in TNBC cells, and the effect of TTK is likely dependent on the downregulation of KLF5.

Functionally, ectopic expression of miR-21 partially attenuated the effects of TTK silencing on EMT and invasion in MDA-MB-231 cells (Fig. 3.5g,h), further suggesting a partial role of miR-21 in TTK mediated EMT in TNBC cells (Fig. 3.5f).

Inhibitory effects of TTK on KLF5 activated miR-200 expression

The miR-200 family of micro-RNAs regulates EMT as part of a feedback loop with ZEB1 and ZEB2, which bind promoter regions of miR-200s to prevent their transcription and repress the epithelial phenotype (80). In a previous study, we showed that KLF5 maintains the epithelial phenotype by directly binding to the GC boxes in the promoters of miR-200 genes to activate their expression(84) . In the same study, we also showed that TGF-β induced EMT requires the KLF5 downregulation and subsequent downregulation of miR-200s . Finally, miR-200 family members were upregulated in MDA-MB-231 cells following ectopic KLF5 expression. We therefore tested whether the inhibitory effect of TTK on KLF5 expression also leads to the repression of KLF5 activated miR-200s during TTK induced EMT in TNBC cells.

In MDA-MB-231 cells, we observed significantly increased miR-200a and miR-200c expression following treatment with the TTK inhibitor NMS-P715 (Fig. 3.6a).

Changes in other members of the miR-200 family were insignificant. Silencing TTK by RNAi showed similar effects on the expression of miR-200a and miR-200c in MDA-MB-231 cells (Fig. 3.6b). Expression of miR-200a was also decreased by silencing both TTK and KLF5 (Fig. 3.6c).

Functionally, we tested if combining TTK inhibition with miR-200 overexpression increases the effect of inhibiting TTK on EMT in TNBC cells. Cells were transfected with a pool of miRNA mimics containing miR-200a, miR-200b and miR-200c and treated with the NMS-P715 inhibitor. As expected, NMS-P715 treatment alone downregulated mesenchymal markers vimentin, Zeb1 and Slug, while upregulating epithelial marker CDH1 (Fig. 3.6d, 6e). Overexpression of miR-200 family members alone had similar effects as NMS-P715 treatment alone. Interestingly, combination of miR-200 overexpression with NMS-P715 treatment further downregulated vimentin, Slug, Zeb-1 and upregulated CDH1 compared to the other groups (Fig. 3.6d,e).

We also transfected cells with inhibitors against miR-200a and miR-200c to determine if inhibiting these microRNAs attenuates the effect of TTK inhibition on EMT changes. Morphological changes were observed in MDA-MB-231 cells treated with inhibitors of miR-200a and miR-200c, indicative of a mesenchymal phenotype, which was not prevented when these groups were treated with NMS-P715 (Fig. 3.6f). Downregulation of vimentin by TTK inhibition in MDA-MB-231 cells was not reversed by miR inhibition (Fig. 3.6g). Inhibiting the miR's also did not revert the upregulation of KLF5 and CDH1 and downregulation of Zeb1 induced by TTK inhibition (Fig. 3.6e), indicating that silencing miR-200a or miR-200c does not completely revert the effect of TTK inhibition on EMT.

Molecules with altered phosphorylation in MDA-MB-231 cells expressing shTTK.

To characterize molecules with altered phosphorylation in cells stably downregulated for TTK, we completed a Phosphoantibody array in MDA-MB-231 pLKO.1 vs. MDA-MB-231 shTTK cells. Through this pilot experiment, we did observe decreased Smad3 phosphorylation and AKT signaling in shTTK cells (Figure 3.8) and upregulated phosphorylation of caspase 9 and Bcl2-2. However, the conclusions about the MAPK signaling pathways from these data were inconclusive.

3.5 DISCUSSION

TTK overexpression leads to the mesenchymal phenotype of triple negative breast cancer

Previous studies have thoroughly characterized TTK's role in mitotic regulation (101, 106, 176, 177), and overexpression of mitotic kinases including TTK in general facilitates genomic instability in cancer cells. In addition to promoting genomic instability and aneuploidy in cancer, TTK overexpression is also involved in promoting cancer cell proliferation and invasion. Based on these tumor-promoting roles, TTK has recently become an attractive therapeutic target. However, cellular mechanisms for how TTK facilitates these processes are lacking. Some proposed pathways for how TTK promotes cell survival and invasion include the AKT signaling pathway and the regulation of miR-21 via TGF- β. In this study, we correlated TTK expression with clinical and pathological characteristics of breast cancer using multiple publically available databases, and confirmed the correlation between higher levels of TTK

expression with adverse features of breast cancer, including triple negative status, higher tumor grade, and worse overall survival (Fig. 3.1). Further supporting an oncogenic function of TTK, inhibition of TTK activity by either RNAi or a chemical inhibitor significantly suppressed cell proliferation or survival (Fig. 3.2).

EMT is associated with aggressive behaviors of cancer including increased motility, invasion and metastasis. In this study, we report for the first time that TTK promotes the mesenchymal status of TNBC cells, because silencing the expression of TTK or inhibiting its activity caused a reversion from the mesenchymal to epithelial phenotype, including decreased expression mesenchymal markers vimentin and ZEB1, decreased invasion, and increased expression of epithelial markers CDH1 and ZO1 (only in MDA-MB-231 but not in Hs578T cell line) (Fig. 3.3). Maintaining a mesenchymal morphology further supports a role of TTK in the progression of TNBC.

Maintenance of EMT by TTK involves the downregulation of KLF5 and its transcriptional targets miR-200s

KLF5 maintains the epithelial phenotype by activating the expression of miR-200s and likely other molecules, and downregulation of KLF5 is essential for TGF-β to induce EMT. In some TNBC cells, KLF5 has been shown to promote cell proliferation(87, 88) . The function of KLF5 could be context dependent, including a role in TTK mediated EMT due to specific cell signaling, tumor microenvironment, and the subtype of TNBCs. In this regard, higher *KLF5* expression was associated with the triple negative status of breast cancer, and more specifically, KLF5 expression is higher in the basal A subtype of TNBC than the basal B subtype (Fig. 3.4H), which inversely correlates with TTK expression (Fig. 3.1D). In the MDA-MB-231 mesenchymal stem cell

like TNBC cells, KLF5 expression was indeed at a lower level, which is partially attributed to increased TTK expression in the cell line, because inhibiting TTK by RNAi upregulated KLF5 expression (Fig. 3.4a). How TTK downregulates KLF5 is unknown, but could be related to the TGF-β signaling pathway, as TGF-β downregulates KLF5 , and TTK inhibition reduced the activation of SMAD3 by TGF-β (Fig. 3.5a).

Downregulation of KLF5 by TTK indeed contributes to TTK-mediated EMT, as knockdown of KLF5 by RNAi attenuated the effect of TTK inhibition on multiple parameters of EMT, including expression of EMT markers and cell invasion (Fig. 3.4A-4E). In addition, the expression of miR-200a and miR-200c was also upregulated by the inhibition of TTK (Fig. 3.6). These miRNA's are not only well established repressors of EMT that downregulate ZEB1 and Zeb2 expression but are also activated by KLF5 to suppress EMT and maintain the epithelial phenotype. In addition to supporting a role of TTK in the induction of EMT, these findings also provide a mechanism for how TTK induces EMT, i.e., higher TTK expression suppresses KLF5 expression in mesenchymal TNBC cells, and decreased KLF5 expression leads to reduced miR-200 expression and subsequent upregulation of mesenchymal genes and downregulation of epithelial genes (Fig. 3.7).

TTK likely interacts with the TGF-β signaling pathway to induce EMT

SMAD3 is a key downstream effector of TGF-β, and TGF-β is the most potent known inducer of EMT (82). TGF-β signaling has also been demonstrated to be more active and associated with worse patient survival in TNBC (178). TTK has been reported to preferentially phosphorylate Smad3 but not Smad2 in the SSXS motif, which is

similar to TGF-β (123). We found that TTK indeed modulates TGF-β signaling, as TTK inhibition decreased SMAD3 phosphorylation induced by TGF-β (Fig. 3.5A).

In addition to miR-200a and miR-200c, TTK has previously been shown to indirectly regulate the expression of micro-RNAs miR-132 and miR-21 (149, 150). MiR-21 is also a downstream target of TGF-β that is transactivated to promote the mesenchymal cell status. In addition, miR-21 is generally considered an onco-miR(179-181). TTK has been reported to upregulate miR-21, although this regulation has not been fully characterized . We demonstrated that in TNBC cells, TTK also upregulates miR-21 expression, as inhibition of TTK by siRNA or inhibitor decreased miR-21 expression, and restoration of miR-21 moderately prevented the effect of TTK on EMT marker expression (Fig. 3.5). Therefore, promotion of TGF-β-induced miR-21 expression is likely another mechanism for TTK to promote EMT.

Analysis using the TargetScan online resource (http://www.targetscan.org/vert_71/) , which predicts biological targets of miRNAs by detecting for the presence of evolutionarily conserved binding sites that match the seed region of each miRNA (182), demonstrated that KLF5 also has a target sequence for miR-21. Taken together with the observation that TTK inhibition decreased miR-21 expression while increasing KLF5 expression, TTK-upregulated miR-21 is likely a mechanism for TTK-mediated downregulation of KLF5 and induction of EMT (Fig. 3.7).

In addition to downregulating the expression of KLF5 and miR-200s during EMT induction, TGF-β has been shown to induce the acetylation of KLF5, and acetylated KLF5 binds with Smads to mediate TGF-β's inhibitory function in cell proliferation and tumor growth. It is currently unknown whether acetylated KLF5 plays a role in TTK-induced EMT(85).

Clinical implications of TTK induced EMT in TNBC

The present study highlights the role of TTK in EMT induction and mesenchymal TNBC involving TGF-β signaling, KLF5 and miRNAs. Targeting TTK with small molecule inhibitors has become a viable therapeutic approach in recent years, since aneuploid tumors are usually more sensitive to TTK inhibition(126). Targeting TTK could also improve existing therapeutic strategies such as taxane therapy or radiation treatment (183-185). For example, targeting TTK appears to radiosensitize Glioblastoma cells via miR-21 signaling, and enhances the efficacy of Docetaxel in the treatment of breast cancer (145).

However, signaling pathways associated with high TTK expression have not been comprehensively characterized, and whether and how TTK contributes to various disease statuses is still poorly understood. The current study provides evidence for the role of TTK in EMT and invasion, which predicts a role of TTK in tumor progression and metastasis. It is thus likely that targeting TTK could attenuate tumor progression.

The current study also provides novel information about the signaling pathways associated with TTK overexpression and tumor aggressiveness in TNBC, including its interaction with TGF-β signaling and its regulation of EMT regulators KLF5, miR-21 and miR-200s. Such mechanistic insight into how TTK mediates EMT could facilitate the study of TTK expression as a clinical biomarker for the prediction of tumor aggressiveness in the context of TGF-β signaling and treatment responses in breast cancer patients.

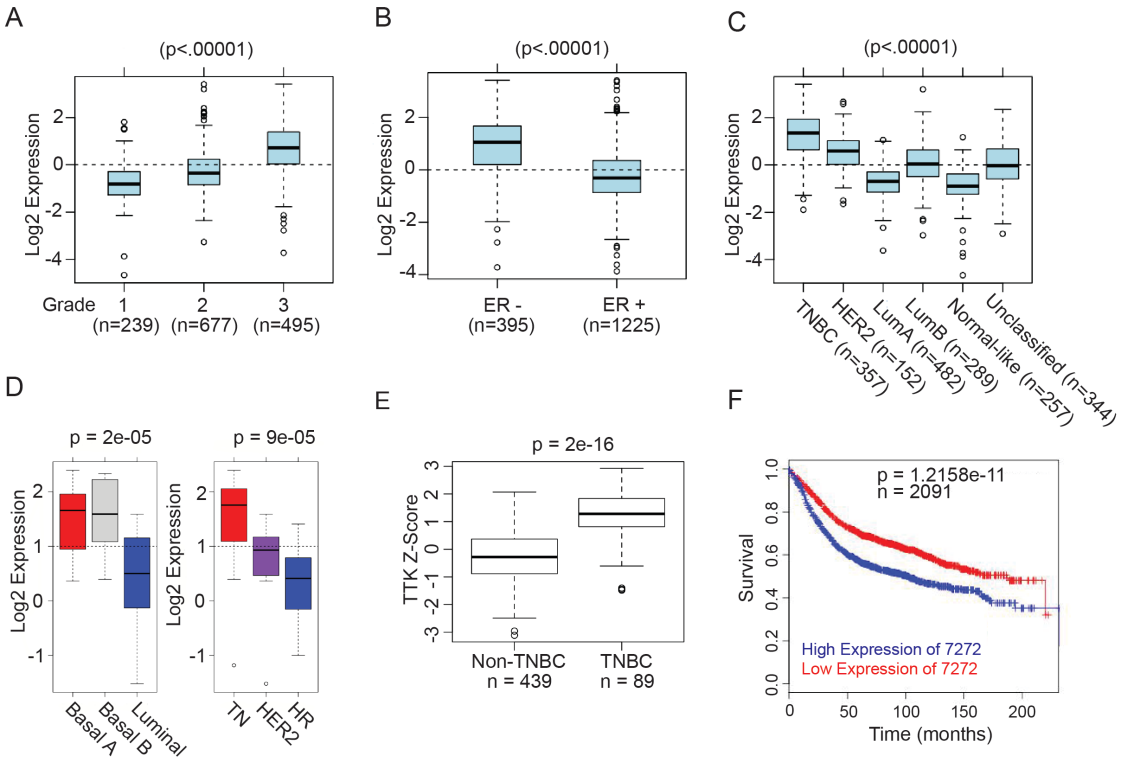


Figure 3.1: Increase in TTK expression and its correlation with higher tumor grade, triple negative status, and worse overall survival in breast cancer. (a-d) Box plots of TTK gene expression across tumor grades (a), between ER positive and ER negative breast cancers (b), across Pam50 subtypes of tumors (c), and across different subtypes in breast cancer cell lines from the GOBO database (d). **(e)** Box plots comparing TTK z-scores between triple negative breast cancers (TNBCs) and non-TNBCs from patients in the TCGA database. **(f)** Kaplan Meier survival analysis illustrating the correlation between higher TTK expression and decreased overall survival in breast cancer patients from the BreastMark database.

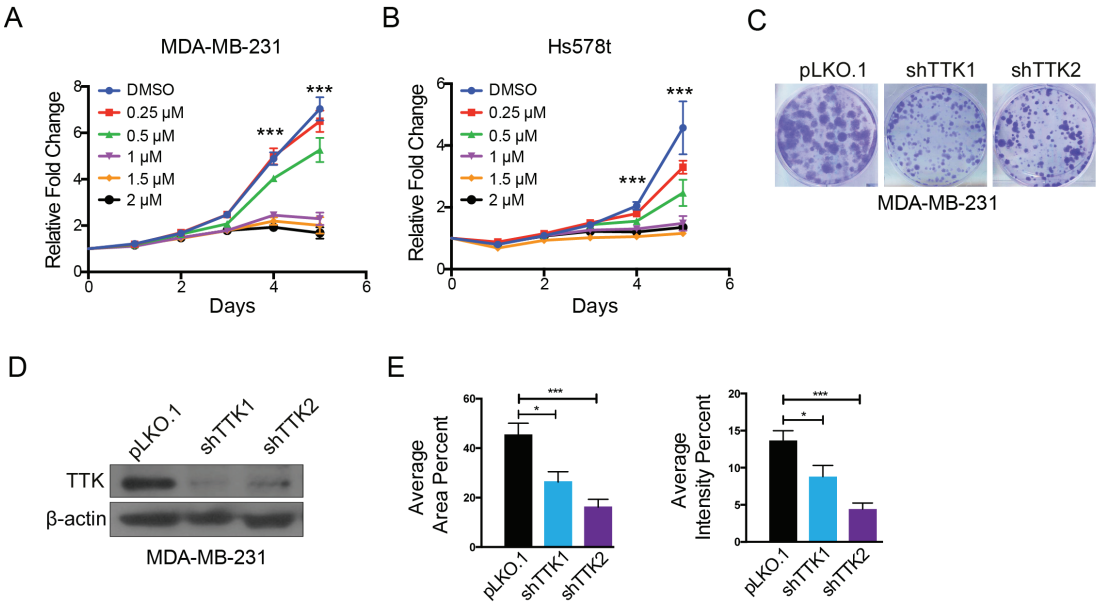


Figure 3.2: TTK inhibition suppresses the proliferation of TNBC cells. (a, b) Cell viability in MDA-MB-231 and Hs578t TNBC cells as determined by the SRB assay. *** p<.01, **(c-e)** Representative colony formations and validation of stable TTK downregulation via viral delivery of shRNA in MDA-MB-231 cells. *, p<. 05,***, p<.01.

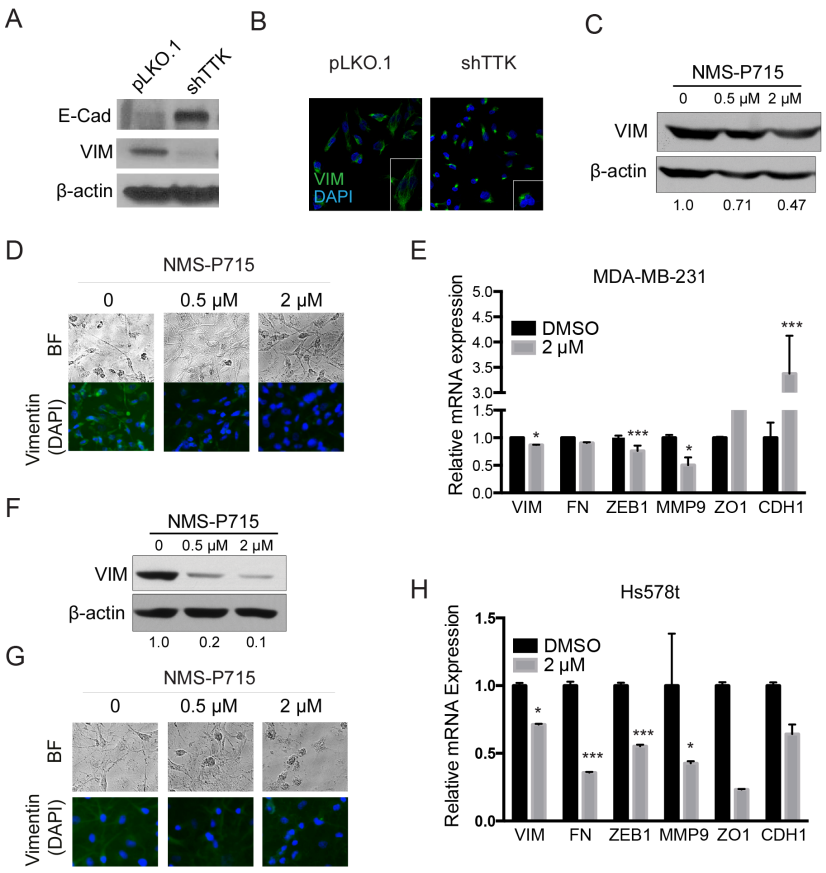


Figure 3.3: TTK Inhibition attenuates the mesenchymal status of MDA-MB-231 and Hs578t TNBC cells. (a) Vimentin and E-cadherin protein expression in MDA-MB-231 cells stably expressing the pLKO.1 vector or the shTTK shRNA, as detected by western blotting. **(b)** Detection of vimentin expression by immunofluorescent staining in MDA-MB-231 cells stably expressing pLKO.1 or shTTK. **(c-h)** Detection of EMT markers in MDA-MB-231 (c-e) and Hs578t (f-h) TNBC cell lines treated with the NMS-P715 TTK inhibitor by western blotting for protein (c and f, upper), immunofluorescent staining for vimentin (d and g lower), and by real time PCR for mRNA (e and h). * ,p<.05,***, p<.01. Bright field (BF) cell morphology is also shown (d and g upper).

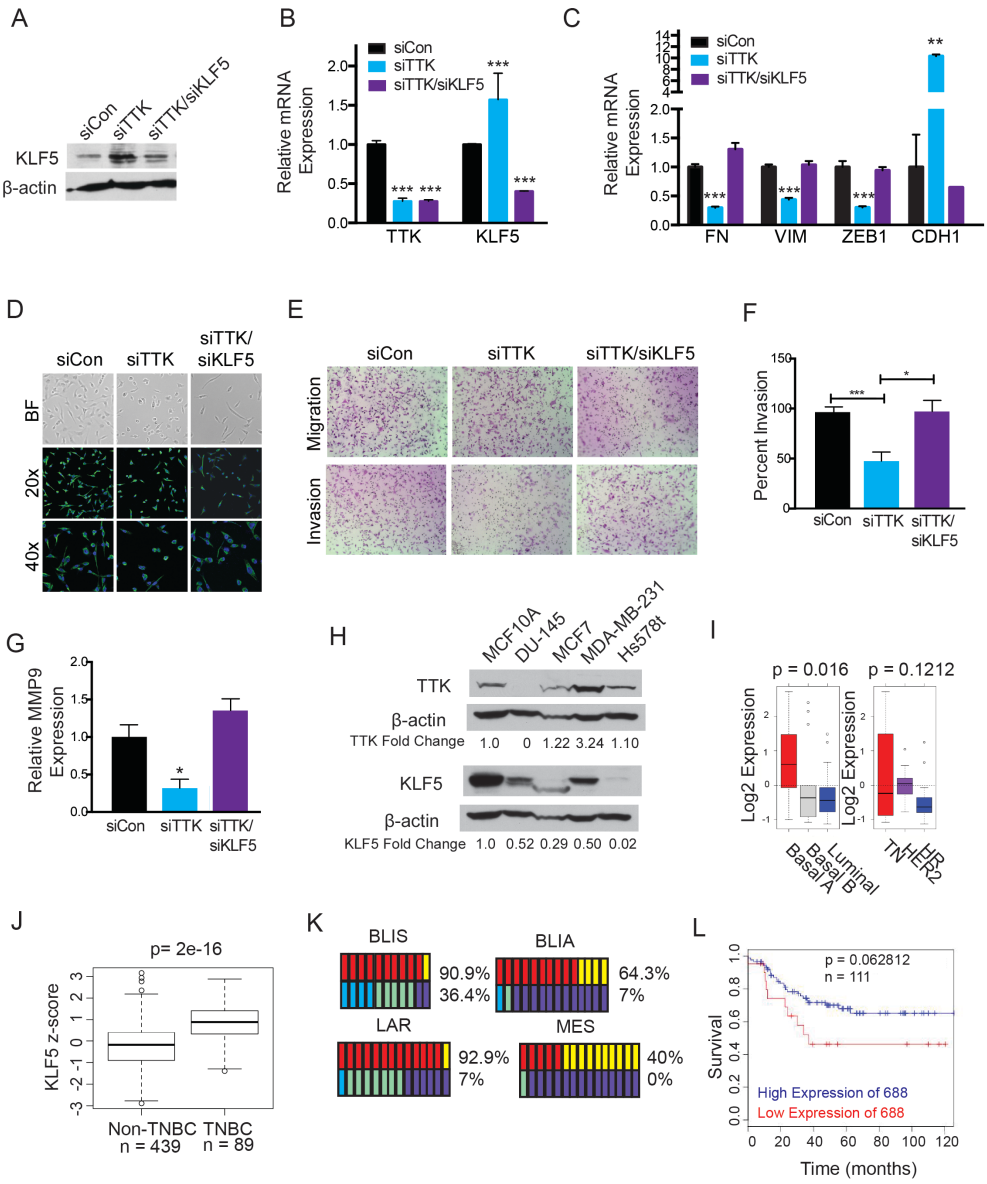

A
siCon
siTTK
siTTK/siKLF5
KLF5
β-actin
B
siCon
siTTK
siTTK/siKLF5
Relative mRNA Expression
TTK
KLF5
C
siCon
siTTK
siTTK/siKLF5
Relative mRNA Expression
FN
VIM
ZEB1
CDH1
D
siCon
siTTK
siTTK/ siKLF5
BF
20x
40x
E
siCon
siTTK
siTTK/siKLF5
Migration
Invasion
F
Percent Invasion
siCon
siTTK
siTTK/ siKLF5
G
Relative MMP9 Expression
siCon
siTTK
siTTK/ siKLF5
H
MCF10A
DU-145
MCF7
MDA-MB-231
Hs578t
TTK
β-actin
TTK Fold Change 1.0 0 1.22 3.24 1.10
KLF5
β-actin
KLF5 Fold Change 1.0 0.52 0.29 0.50 0.02
I
p = 0.016
p = 0.1212
Log2 Expression
Basal A
Basal B
Luminal
TN
HR
HER2
J
p= 2e-16
KLF5 z-score
Non-TNBC n = 439
TNBC n = 89
K
BLIS
90.9%
36.4%
BLIA
64.3%
7%
LAR
92.9%
7%
MES
40%
0%
L
p = 0.062812
n = 111
Survival
High Expression of 688
Low Expression of 688
Time (months)

Figure 3.4: Downregulation of KLF5 plays a role in TTK-induced EMT and worse patient survival in TNBC. (a-b) Validation of RNAi-mediated TTK and KLF5 knockdown in MDA-MB-231 TNBC cells by western blotting (a) and realtime PCR (b). **, p<.01 **(c)** Expression of EMT markers, as detected by realtime PCR, in MDA-MB-231 cells with the knockdown of TTK or TTK/KLF5. **(d)** Cellular morphology and detection of vimentin by immnunoflourescent staining in MDA-MB-231 cells after TTK or TTK/KLF5 silencing. **(e)** Representative images of invaded cells in the invasion assay. **(f)** Quantification of cell invasion. *, p<.05 ***, p<.01. **(g)** Expression of MMP-9 mRNA as measured by realtime PCR in cells with TTK or TTK/KLF5 knockdown. *, p<.05 **(h)** Detection of TTK and KLF5 protein expression by western blotting in different breast cancer cell lines. **(i)** Box plots of KLF5 expression in different subtypes of breast cancer cell lines from the GOBO database. **(j)** Box plots comparing KLF5 z-scores between non-TNBC subtypes and TNBC patients using data from the TCGA database. **(k)** Distribution of genes significantly correlated with KLF5 for each TNBC subtype. Top row indicates genes with significant p values (<.05) in red or non-significant p-values (yellow) for each subtype. Bottom row indicates moderate (blue), weak (green) or very weak (purple) Spearman's coefficient for each gene. **(l)** Kaplan Meier survival analysis for the correlation between KLF5 expression and overall survival in TNBC patients using data from the BreastMark database.

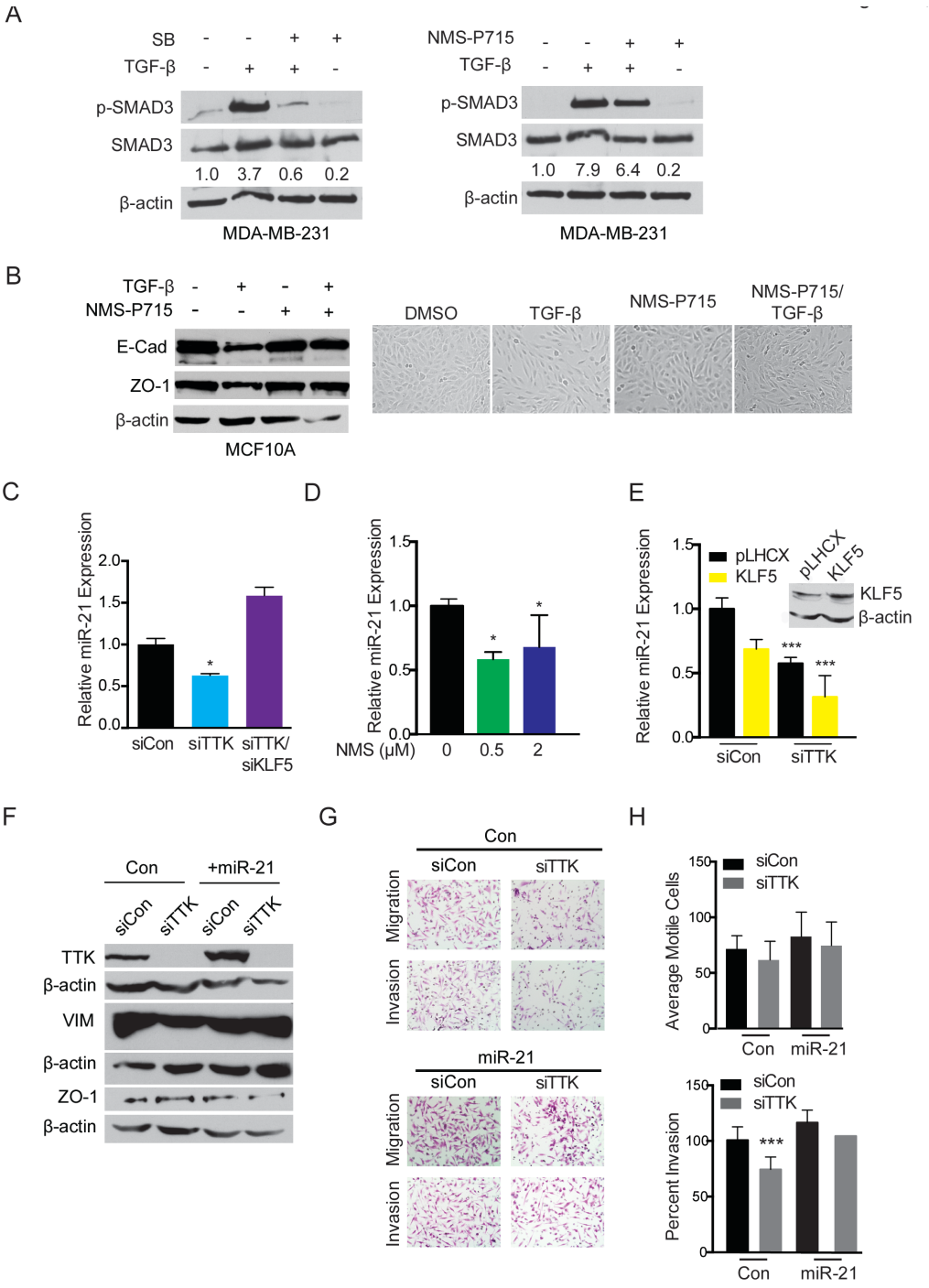

A
SB
TGF-β
p-SMAD3
SMAD3
1.0 3.7 0.6 0.2
β-actin
MDA-MB-231
NMS-P715
TGF-β
p-SMAD3
SMAD3
1.0 7.9 6.4 0.2
β-actin
MDA-MB-231
B
TGF-β
NMS-P715
E-Cad
ZO-1
β-actin
MCF10A
DMSO
TGF-β
NMS-P715
NMS-P715/ TGF-β
C
Relative miR-21 Expression
siCon
siTTK
siTTK/ siKLF5
D
Relative miR-21 Expression
NMS (μM) 0 0.5 2
E
Relative miR-21 Expression
pLHCX
KLF5
pLHCX
KLF5
KLF5
β-actin
siCon
siTTK
F
Con
+miR-21
siCon
siTTK
siCon
siTTK
TTK
β-actin
VIM
β-actin
ZO-1
β-actin
G
Con
siCon
siTTK
Migration
Invasion
miR-21
siCon
siTTK
Migration
Invasion
H
Average Motile Cells
siCon
siTTK
Con
miR-21
Percent Invasion
siCon
siTTK
Con
miR-21

Figure 3.5: Effects of TTK inhibition on TGF-β signaling and miR-21 expression in MDA-MB-231 breast cancer cells. (a) Comparison of the SB505124 TGF-β inhibitor and the NMS-P715 TTK inhibitor for their effects on Smad3 phosphorylation in MDA-MB-231 cells, as measured by western blotting. **(b)** Detection of EMT markers E-cadherin and ZO1 in and images of MCF10A mammary epithelial cells following treatments with TGF-B, the NMS-P715 TTK inhibitor, and their combination. **(c-e)** Detection of mir-21 expression by real-time PCR in MDA-MB-231 cells with the knockdown of TTK or TTK/KLF5 by siRNA (c), treatment of the NMS-P715 TTK inhibitor (d), and lentivirus mediated KLF5 overexpression (e). *, $p<.05$, ***, $p<.01$. **(f)** Protein expression of EMT markers in MDA-MB-231 cells with siRNA knockdown of TTK and miR-21 overexpression. **(g, h)** Representative images of invaded cells (g) and their quantification in motility and invasion assays. ***, $p<.01$.

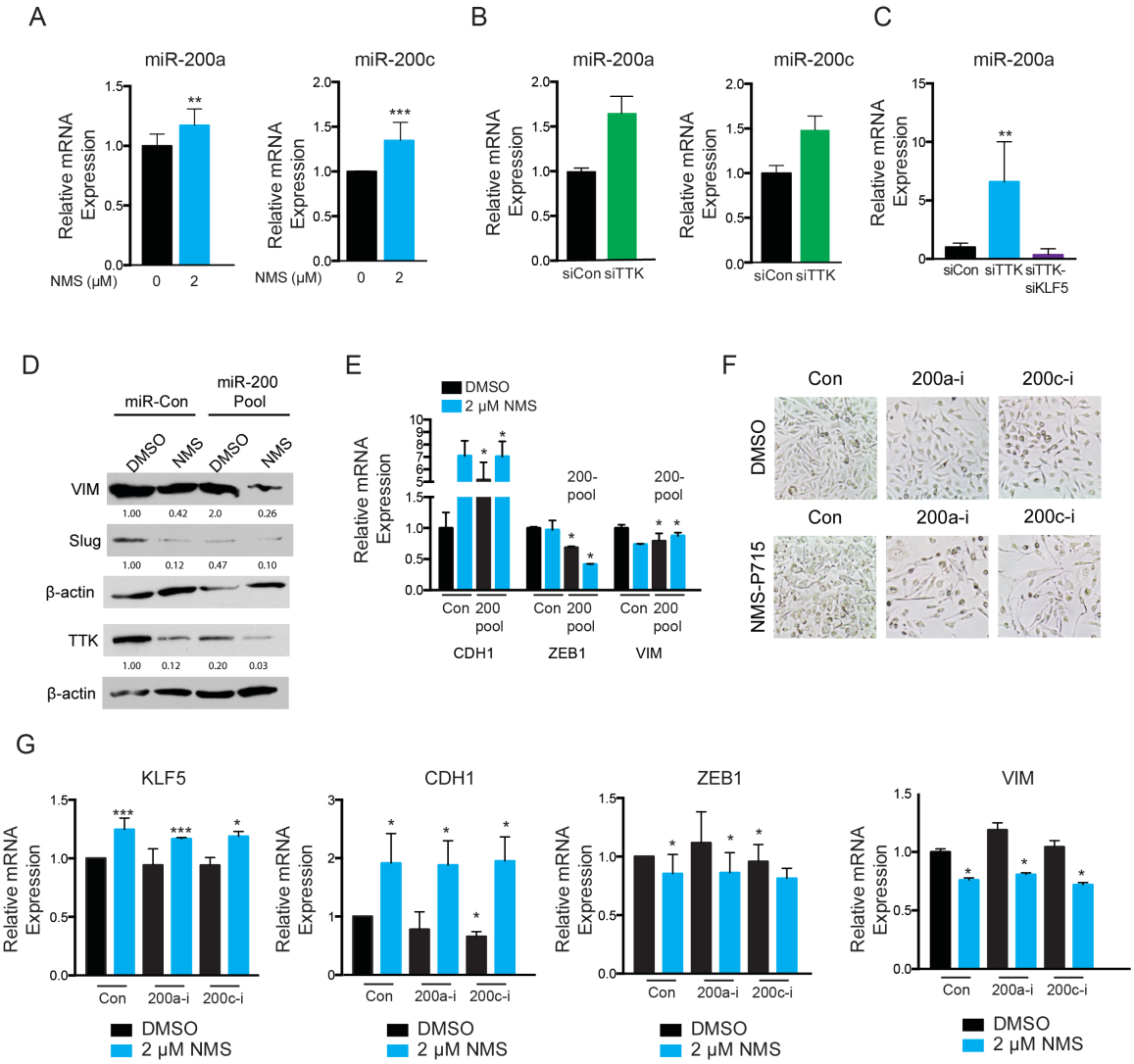

A
miR-200a
miR-200c
Relative mRNA Expression
NMS (μM) 0 2
B
miR-200a
miR-200c
siCon siTTK
C
miR-200a
siCon siTTK siTTK-siKLF5
D
miR-Con
miR-200 Pool
DMSO NMS DMSO NMS
VIM
Slug
β-actin
TTK
β-actin
E
DMSO
2 μM NMS
Con 200 pool
CDH1 ZEB1 VIM
F
Con 200a-i 200c-i
DMSO
NMS-P715
G
KLF5
CDH1
ZEB1
VIM
Con 200a-i 200c-i
DMSO
2 μM NMS

Figure 3.6: TTK inhibition upregulates the expression of miR-200a and miR-200c to attenuate EMT in MDA-MB-231 cells

(a, b) Detection of miR-200a and miR-200c expression by real-time PCR in MDA-MB-231 cells treated with the NMS-P715 TTK inhibitor (a) or TTK and KLF5 siRNA (b,c). **(d-e)** Expression of EMT markers in MDA-MB-231 cells treated with NMS-P715 in combination with pools of miR-200 mimics. (f**-g)** Representative images of cells (f) and detection of mRNA expression of KLF5, CDH1, ZEB1, and VIM by real-time PCR (g) in MDA-MB-231 cells treated with NMS-P715 in the presence or absence of inhibitors against miR-200a or miR-200c. ***, $p<.01$; *, $p<.05$.

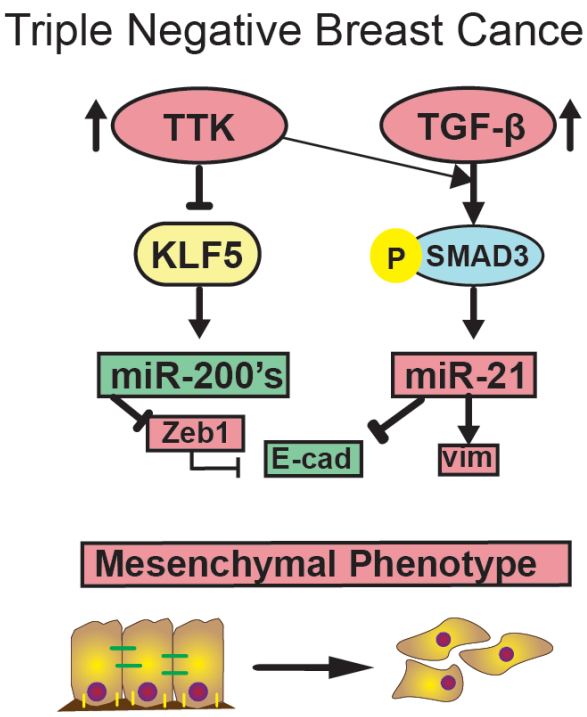


Figure 3.7: A potential model for how TTK promotes EMT in TNBC cells. In normal breast cells, the TTK expression level is low while that for KLF5 is higher, and, KLF5 induces the transcription of miR-200 family members and represses miR-21 expression, leading to the epithelial phenotype. However, in TNBC cells, higher TTK expression leads to decreased KLF5 expression, resulting in the downregulation of miR-200s and upregulation of miR-21 and the induction of EMT in TNBC cells.

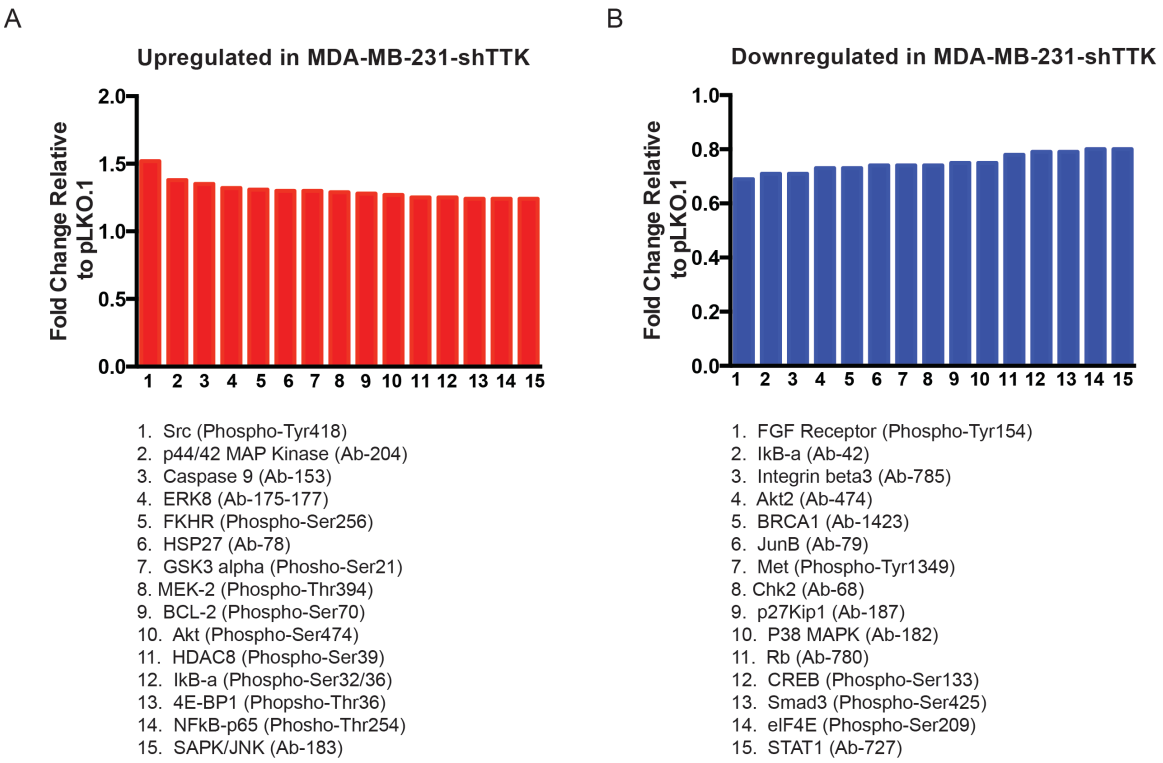


Figure 3.8 Genes with altered phosphorylation levels in shTTK TNBC cells.

Genes with (a) upregulated and (b) downregulated phosphorylation levels in MDA-MB-231 cells stably downregulated for TTK, as identified by FullMoon Biosystems antibody array.

Chapter 4

Effects of targeting TTK in radioresistant breast cancer cells

4.1 Introduction

Radiation therapy remains an additional treatment in breast cancer that can be combined with chemotherapy to reduce local tumor recurrence. Even though this standard therapeutic approach is effective, some caveats include cardiac toxicity, metastatic potential, as well as intrinsic and acquired resistance (186). For specific breast cancer subtypes, radiation treatment is used for triple negative breast cancer (TNBC) and Her2+ breast cancer. Some patients have an initial response to radiation therapy, while some don't respond to radiation treatment at all (187). Another clinical issue in breast cancer patients is that patients treated with radiotherapy who have recurrent tumors have poor outcomes because their tumors are radioresistant at the time of relapse (188). Both of these responses (intrinsic and acquired radioresistance) represent a critical clinical challenge. There are also inherent differences in breast tumor biology that could be incorporated into clinical decision making for radiation therapy (189). At the molecular level, one goal in radiation biology is to find molecules that mediate radioresistance (190) to develop treatments that could enhance the initial response to radiation treatment and counteract radioresistance. This approach could also contribute to the development of molecular signatures to predict radiation response (191).

In radioresistant breast tumors, several mechanisms have been illustrated to promote insensitivity to radiation treatment. One mechanism is the emergence of resistant stem cells, which can elude frontline treatment and expand in recurrent tumors (192, 193). As expected, alterations in cell cycle and apoptotic pathways are associated with diminished response to radiation in breast cancer, since these pathways control how the cell responds to cellular damage induced by radiation. There is some

crosstalk between cell cycle pathways such as the CDK4/cyclin D1 pathway, AKT survival and the DNA damage repair pathways (194, 195), which means these could be viable targets to improve radiosensitivity. For example, therapeutic intervention targeting CDK4/6 was shown to modify the DNA damage response in cancer cells (196). We also showed in previous studies that silencing CDK4 could radiosensitize breast cancer cells by promoting apoptosis (158). Targeting anti-apoptotic molecules such as BCL-2 and BCL-XL can also reverse acquired radioresistance in TNBC cells (197). Intervention targeting the G2/M checkpoint has also shown to be effective in improving the response to radiation (198).

In addition to crosstalk between cell cycle and DNA damage repair pathways, there is also crosstalk between cell cycle and DNA repair and EMT signaling pathways such as microRNAs and homing receptors, which mediate the response to radiation. RAD51 facilitates TNBC metastasis (199) and increased miR-144 expression decreases response to radiation by regulating cell migration (200). Altered expression of the ATM transducer of DNA damage can also stabilize Zeb1 to promote radioresistance through Chk1 (201). Conversely, overexpression of the EMT suppressor miR-200c can radiosensitize breast cancer cells (202). High expression of miR-21 in breast cancer cells is an example of another micro-RNA associated with EMT that also causes G2/M checkpoint arrest to attenuate response to radiation (183). Since our studies and others have shown that miR-21 is also associated with TTK expression, we explored how TTK might be involved in radiation resistance in breast cancer.

TTK is a serine threonine kinase with well characterized roles in centriole duplication and the spindle assembly checkpoint (SAC). In addition to these traditional roles, TTK is also involved in the DNA repair response, which is induced upon cellular

insults such as radiation. Upon detection of DNA strand breaks, TTK phosphorylates CHK2 (CHK2-Thr68) (121). This phosphorylation event controls the G2/M arrest caused by ultraviolet radiation or ionizing radiation (121). CHK2 is the same substrate used by ATM to detect DNA damage and is a transducer of the DNA repair response. More recently, TTK was shown to be required for DNA damage repair and cell survival by phosphorylating MDM2 (203). Targeting TTK has been shown to radiosensitize glioblastoma cells by modulating DNA repair proteins (184). Similar to aneuploidy, there is a delicate balance between the effects on DNA damage repair and TTK expression. In regards to breast cancer, TTK is overexpressed in TN and Her2+ breast cancers. Since TTK regulates the DNA damage response and is overexpressed in some radioresistant TN and Her2+ breast cancer cell lines, we hypothesized that overexpression of TTK may play a role in radioresistance and tested how blocking TTK expression would impact the responses of resistant breast cancer cells to radiation treatment.

4.2 Materials & methods

Cell culture, inhibitor and radiation treatment

Human breast cancer cell lines MDA-MB-231, HCC1954, and MDA-MB-468 were obtained from ATCC or provided as gifts from the Nahta and Vertino laboratories and grown in DMEM or RPMI media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were cultured in a humidified chamber at 37 degrees Celsius and 5% CO2. NMS-P715 (TTK inhibitor) was purchased from Millipore. Cells were irradiated at indicated doses in a X-RAD 320 irradiator (Precision X-Ray, CA).

Viral infection

HEK293T cells were cultured to 80% confluence and transfected with packaging plasmids along with control shRNA pLKO.1 or TTK shRNA. Lipofectamine 2000 was used for transfections per manufacturer's instructions. At 48 hours post-transfection, cell culture medium containing virus particles was collected and aliquoted with a sterile filter. Target cells were plated and allowed to reach 60% confluence before infection with viruses and 8 ug/mL polybrene. Stable cells expressing shTTK were selected with 2 ug/mL puromycin or 200 ug/mL hygromycin for one week post infection before the antibiotic dose was decreased for cell maintenance.

Western blot analysis

Western blotting was performed according to previously published protocols (41, 62, 158). The antibodies for cleaved caspase 3, γ-H2A.X, pCHK2 and B-actin were all purchased from Cell Signaling. Signals were detected with Lumigen TMA-6 reagent.

Immunofluorescent microscopy

Cells were plated in 4 well chamber slides and allowed to adhere overnight prior to NMS-P715 and radiation treatment. Cells were fixed with 4% paraformaldehyde prepared in PBS, permeabilized with 1% Triton X-100, blocked with 10% goat serum. Cells were stained with primary antibodies against γ-H2A.X and AlexaFluor fluorescent secondary antibody from ThermoFisher, counterstained with DAPI (1mg/mL), then imaged on a Zeiss Confocal LSM 510. Two-hundred cells were counted for each group and the percentage of γ-H2A.X positive cells was calculated.

Annexin V Assay

Following NMS-P715 or radiation treatment, cells were washed twice with cold 1X PBS, then resuspended in 1X binding buffer (10X recipe 0.1 M Hepes/NaOH pH 7.4), 1.4 M NaCl, 25 mM CaCl2) at a concentration of $1x10^6$ cells/mL (in triplicate). Then 100 μL of the solution was transferred to a 5 mL flow cytometry tube and 5 μL FITC Annexin V and 5 μL PI was added to each tube, vortexed briefly and incubated at room temperature in the dark for 15 minutes. Immediately before analysis by flow cytometry, 400 μL of 1X binding buffer was added to each tube. For control purposes, a tube containing unstained cells, cells stained with FITC/Annexin V only and a tube with PI stained cells only were first analyzed to set up compensation and gating on the flow cytometer. A BD LSR II Flow Cytometer was used for Annexin V analysis and data was analyzed using FlowJo software.

Tunel Assay

The APO-BrdU TUNEL assay kit was purchased from Invitrogen and cells were processed for the assay per manufacturer's instructions for microscopy. Slides were imaged on a Zeiss Axioplan 2 and the percentage of TUNEL positive cells (out of 200 cells) was calculated for each treatment group.

Phosphoantibody array

The Cancer Signaling phosphoantibody array kit was purchased from Full Moon Biosystems. Prior to processing, cells were irradiated at 2 Gy and processed for the array 48 hours later. Briefly, cells were washed with ice cold 1X PBS 3-5 times then lysed

in lysis buffer according to manufacturer's instructions. Cell lysates were then purified, biotinylated and coupled to the pre-blocked array slides. Cy-3 streptavidin was added to the array slides with detection buffer then slides were sent to Full Moon Biosystems for detection of phosphorylated proteins.

Statistical Analysis

Student's t-test was used to calculate statistical significance between groups for immunofluorescence and the Annexin V assays.

4.3 Results

We previously established that TTK is overexpressed in radioresistant breast cancer cell lines HCC1954 and MDA-MB-231, but the role of TTK in radioresistance of breast cancer is unclear. One hypothesis is that processes deregulated by TTK, including DNA damage signaling can mediate radioresistance and targeting TTK could radiosensitize cells.

Stable downregulation of TTK or TTK inhibition decreases rates of DNA break repair in radioresistant breast cancer cells.

To determine if targeting TTK could attenuate increased DNA repair in radioresistant cells that overexpress TTK, we measured DNA strand breaks by detecting pH2A.X foci. pH2Ax is a mark of DNA strand breaks since this histone is phosphorylated by DNA damage sensors like ATM or ATR to recruit DNA repair enzymes to sites of DNA damage (204). Clearance of pH2A.x after irradiation treatment can be used as a measure of DNA damage repair capacity (204). In pilot experiments, we irradiated cells

at increasing doses of radiation treatment (0, 1, 2, 4, and 8 Gy respectively) to assess levels of H2A.X phosphorylation in control vs. experimental groups. In HCC1954 cells expressing pLKO.1 vector controls, we observed some baseline pH2AX expression at all radiation doses that was increased in shTTK cells combined with radiation treatment (Fig. 4.1). In HCC1954 cells treated with DMSO only and IR, there was minimal pH2AX at low radiation doses, but pH2AX was detectable at high radiation doses. In comparison, HCC1954 cells pre-treated with NMS-P715 had high pH2AX phosphorylation in all groups treated with IR (Fig 4.2).

Similar trends were observed in MDA-MB-231 cells stably silenced for TTK (Fig. 4.3). To further measure repair capacity of HCC1954 or MDA-MB-231 cells expressing shTTK shRNA or pre-treated with NMS-P715, we irradiated cells with 2 Gy IR treatment and detected pH2A.X foci by immunofluorescence at different time points post-irradiation treatment. In the control group for MDA-MB-231 cells (pLKO.1) p-H2A.x foci peaked at 24 hours, but returned to levels comparable to basal levels by 48 hours (Fig. 4.4). However, in the groups expressing shTTK, the pH2A.X foci remained at 48 hours. These experiments indicate that decreasing TTK expression in combination with radiation treatment could attenuate the DNA repair capacity of radioresistant breast cancer cells.

Irradiated breast cancer cell lines silenced for TTK or treated with TTK Inhibitor display increased frequencies of apoptosis.

We previously showed that radiosensitization of breast cancer cells silenced for CDK4 could be caused by enhanced frequencies of apoptosis (158). Therefore, we hypothesized a similar mechanism could be responsible for altered responses of

resistant cells silenced for TTK. To assess the changes in apoptotic responses, we measured cleaved caspase 3 protein expression at increased doses of radiation treatment in control vs. experimental groups. Similar to the pH2A.X findings, we did not observe high cleaved caspase 3 levels in control groups at increased doses of radiation treatment (Fig. 4.1-4.3). In contrast, TTK inhibition combined with radiation treatment increased the cleaved caspase 3 levels in both HCC1954 and MDA-MB-231 cells (Fig. 4.1-4.3). As another means to measure apoptosis, we employed Annexin V assays to measure the percentage of cells undergoing apoptosis. Due to technical difficulty staining Annexin V in inhibitor treated cells, we transiently silenced TTK and irradiated these cells prior to Annexin V staining and flow cytometry analysis. We did not observe significant changes in the percentage of cells in late apoptosis in the scrambled control group combined with radiation treatment. In cells silenced with two independent siRNAs against TTK, we did observe moderate increases in apoptotic cells when combined with radiation treatment (Fig. 4.5). In another pilot experiment, we also assessed apoptosis through the TUNEL assay (Fig. 4.5). We utilized this assay as a secondary technique to measure DNA breaks induced by IR and TTK inhibition, since the TdT enzyme can incorporate into DNA breaks. In this experiment, we also observed significant changes in TUNEL positive cells in the siTTK/2 Gy group when compared to the others. Overall, these data suggest that targeting TTK in combination with radiation treatment can induce increased apoptotic responses in resistant cell lines.

TTK inhibition does not alter radiation responses through changes in pCHK2.

In similar experiments used to detect changes in p-H2A.X, we also measured levels of pCHK2. In both HCC1954 and MDA-MB-231 cells, we did not observe significant changes in pCHK2 when targeting TTK in combination with IR.

Molecules with altered phosphorylation in MDA-MB-231 cells expressing shTTK combined with irradiation.

In another pilot experiment, we utilized a phosphoantibody array to examine what cancer signaling molecules could have altered phosphorylation in shTTK cells when combined with 2 Gy radiation treatment. This pilot experiment validated some of our findings, in that Histone H2A.X and caspase 9 both had increased phosphorylation in the shTTK cells plus 2 Gy radiation (Fig. 4.6). Some other interesting findings were PTEN upregulation and BRCA1, while p44/42 MAPK had a slightly increased phosphorylation level in these cells. As further validation, CHK2 did emerge as a molecule with downregulated phosphorylation, as well as Myc and some members of the AKT signaling pathway. This antibody array data provides some starting points for investigation in addition to the previously presented experimental evidence on cellular signaling changes induced by combining TTK inhibition with radiation.

4.4 Discussion

Over the course of radiation treatment, breast cancer cells with acquired signaling alterations can comprise a population within tumors that emerge as resistant to further treatment, eventually forming a primary or recurrent tumor that is not responsive to radiation treatment(205). Through these pilot experiments to examine TTK's role in radioresistance of breast cancer, we found that TTK overexpression might

enhance radiation resistance by maintaining overactive DNA break repair and increased survival, even in the presence of radiation.

Similar to CDK4 and other mitotic kinases that have been shown to be involved in radioresistance, alterations in TTK associated signaling pathways could alter the sensing and response to DNA damage induced by radiation treatment. For example, aberrant spindle assembly checkpoint signaling facilitated by TTK overexpression could bypass necessary cell death that should occur upon radiation treatment, allowing for cancer cells to maintain genomic defects in the presence of radiation treatment. When we examined the apoptotic response of radioresistant breast cancer cells with overexpressed TTK, we did not observe completely functional apoptotic responses, as measured by caspase 3 cleavage and Annexin V staining. This is in contrast to cells with transient (siRNA-mediated) silencing of TTK, which shows an apoptotic response (129). Thus, cells can adjust to anti-apoptotic signaling. However, when we combined radiation treatment with various methods of inhibiting TTK, we observed enhanced cleaved caspase 3, annexin V + cells in late apoptosis and increased TUNEL positive cells, suggesting that targeting TTK can improve the cell death response in resistant cells. Further studies can elucidate the changes that may be present in the upstream intrinsic apoptotic signaling pathway, such as changes in Bcl-2 family members.

Relating to the DNA damage repair signaling pathways, we observed active DNA repair in resistant cell populations that was attenuated when TTK inhibition was combined with radiation treatment. We tested the activity of Chk2, a direct substrate of TTK that is involved in DNA repair and did not observe significant differences between experimental groups. Overall these results suggest that while TTK functions to mediate radioresistance through changing DNA damage response, this function is occurring

independent of the Chk2 pathway. Other molecules that may be altered could be Rad51 or Ku70.

While these studies are preliminary in regards to fully characterizing the role of TTK in radioresistance, they provide some interesting insights into how TTK overexpression can promote differential responses to treatment in breast cancer patients. Some future directions could involve examining TTK expression in radioresistant tumor tissue from breast cancer patients or cellular studies to examine how combining other cell cycle based treatments with TTK inhibitors alters the response to radiation treatment.

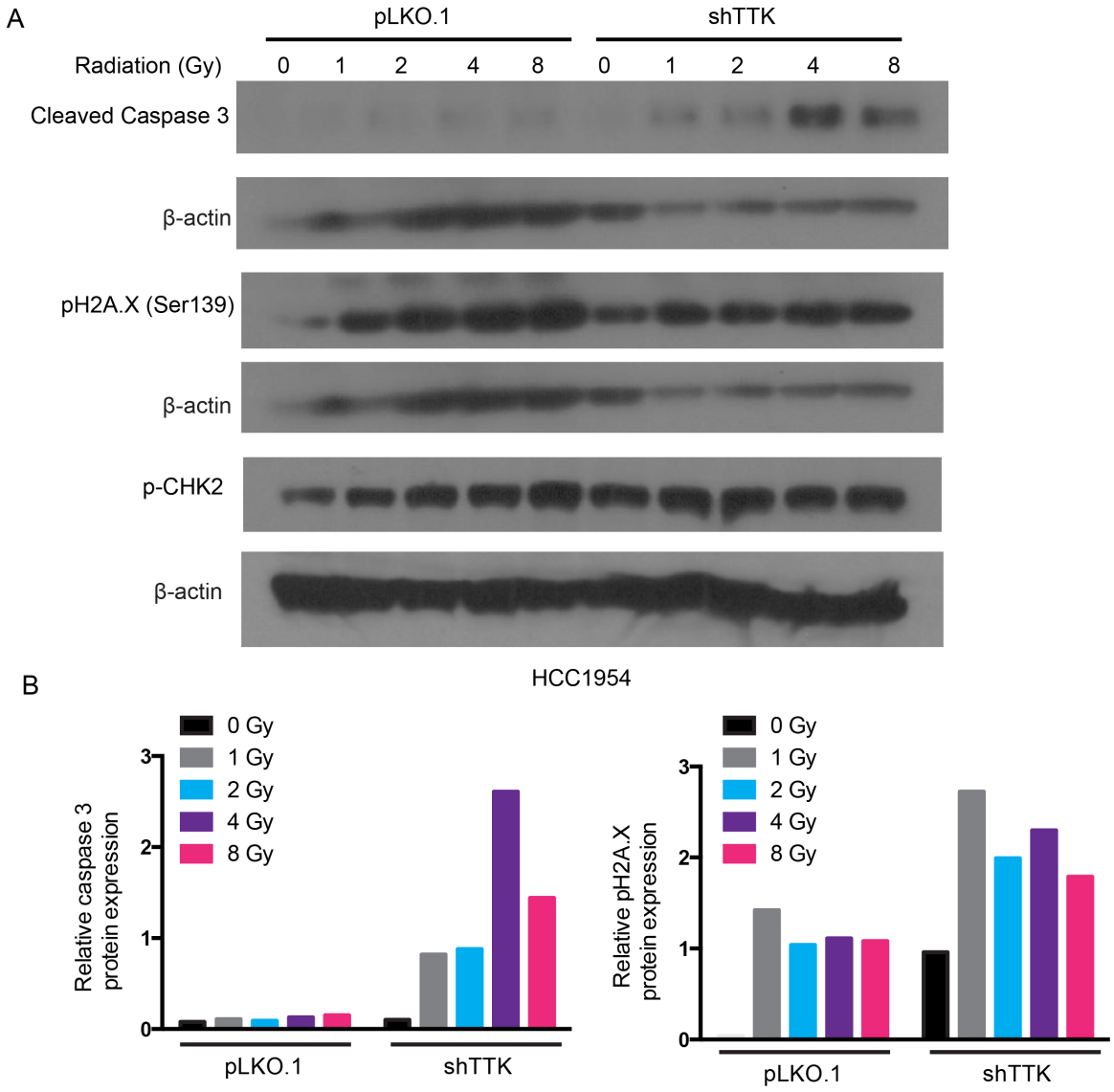


Figure 4.1: Irradiated Her2+ breast cancer cells with silenced TTK display increased apoptosis and lack DNA repair capacity.

HCC1954 cells stably expressing pLKO.1 or shTTK were unirradiated (basal) or irradiated at increasing doses. Protein lysates were collected 48 hours later and subjected to Western blotting to analyze cleaved caspase 3, pH2A.X and p-CHK2 protein expression.

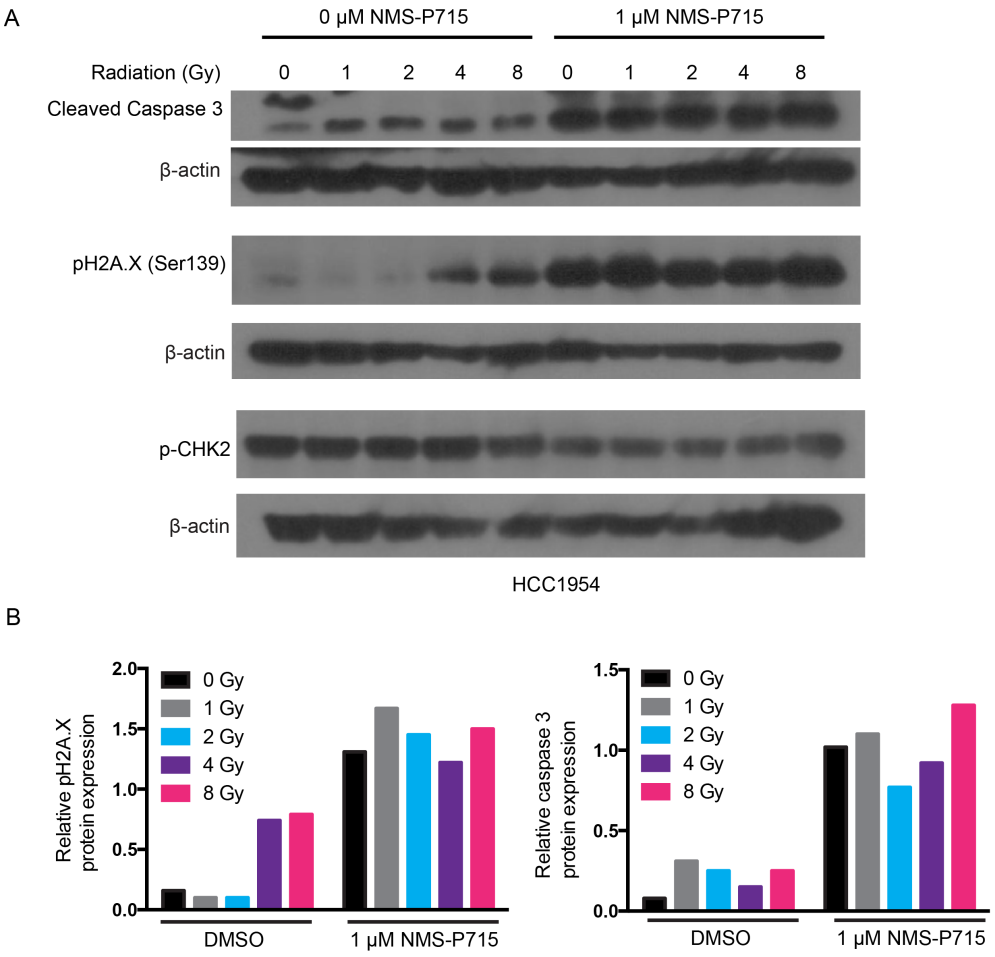


Figure 4.2: Irradiated Her2+ breast cancer cells treated with TTK inhibitor NMS-P715 display increased apoptosis and lack DNA repair capacity.

HCC1954 cells treated with vehicle or 1 μM NMS-P715 were unirradiated (basal) or irradiated at increasing doses. Protein lysates were collected 48 hours later and subjected to Western blotting to analyze cleaved caspase 3, pH2A.X and p-CHK2 protein expression.

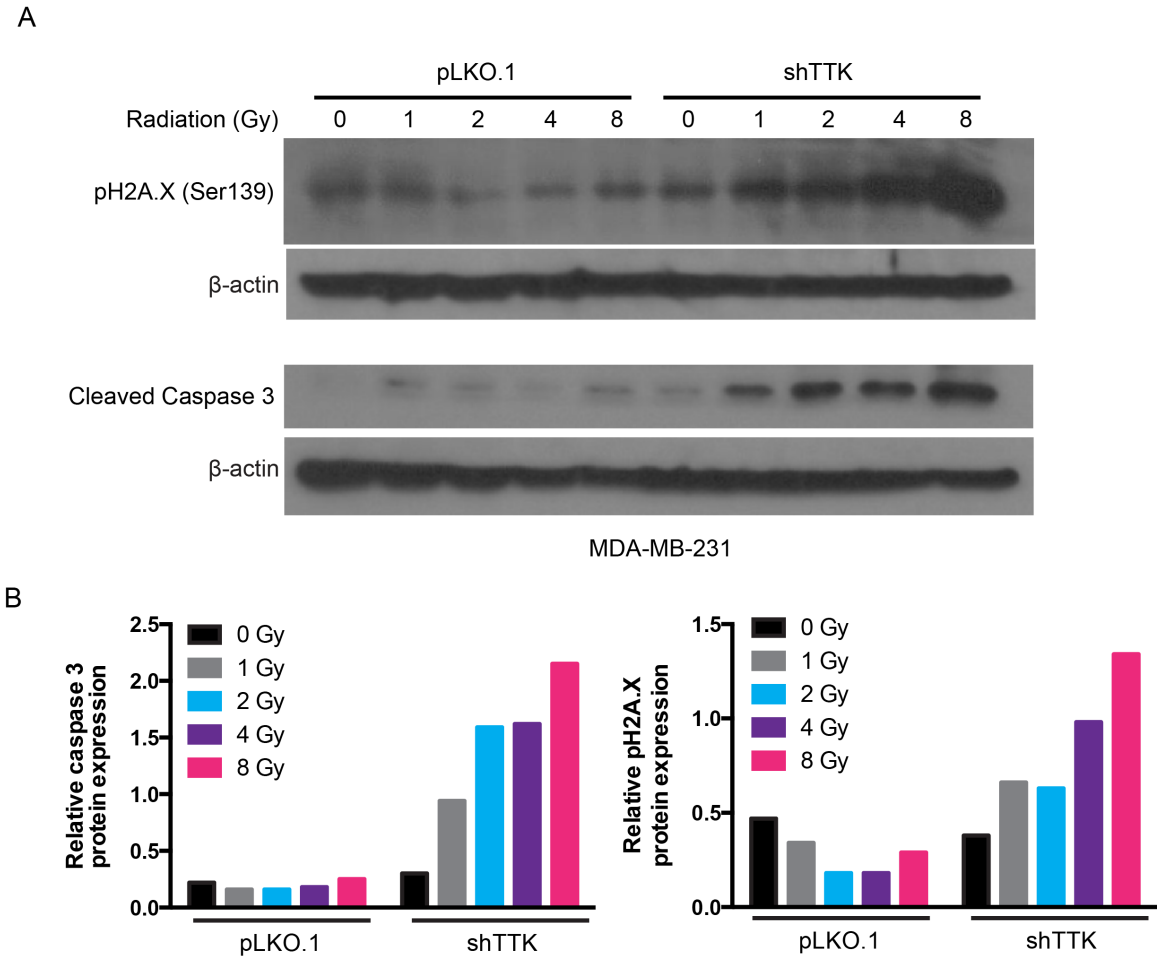


Figure 4.3: Irradiated TNBC cells with silenced TTK display increased apoptosis and lack DNA repair capacity.

MDA-MB-231 cells stably expressing pLKO.1 or shTTK were unirradiated (basal) or irradiated at increasing doses. Protein lysates were collected 48 hours later and subjected to Western blotting to analyze cleaved caspase 3 and pH2A.X protein expression.

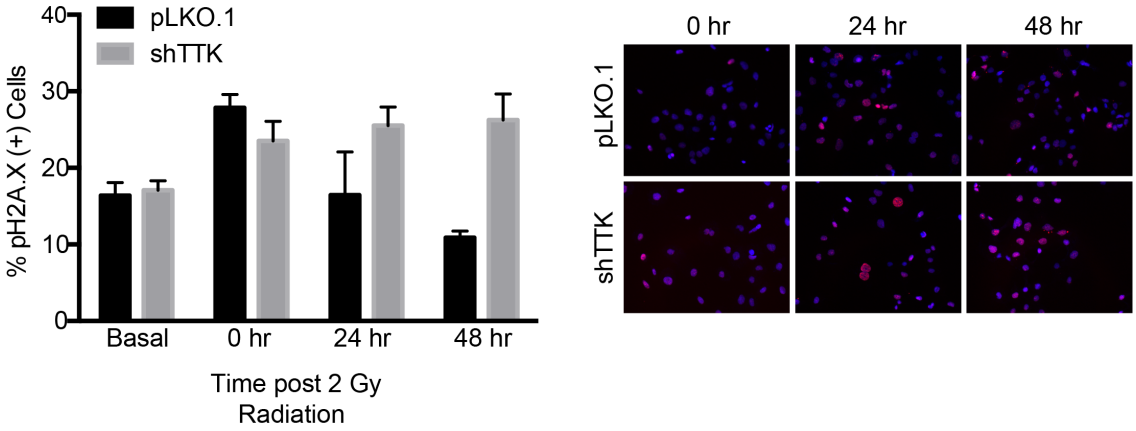
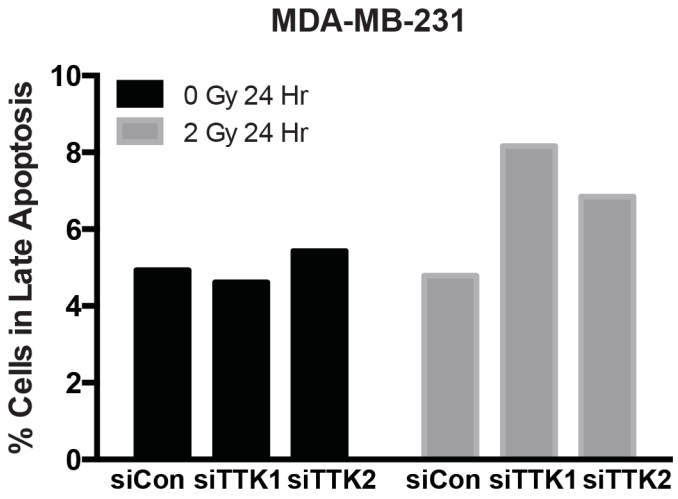


Figure 4.4: Irradiated triple negative breast cancer cells with silenced TTK display lack of DNA repair capacity.

MDA-MB-231 cells stably expressing pLKO.1 or shTTK were irradiated at 2 Gy. Cells were fixed at time points post-irradiation (0 hr, 24 hr or 48 hr) and subjected to immunostaining for pH2A.x. pH2A.X foci were counted to calculate percentage of pH2A.X positive cells.


MDA-MB-231
% Cells in Late Apoptosis
0 Gy 24 Hr
2 Gy 24 Hr
0
2
4
6
8
10
siCon
siTTK1
siTTK2
siCon
siTTK1
siTTK2

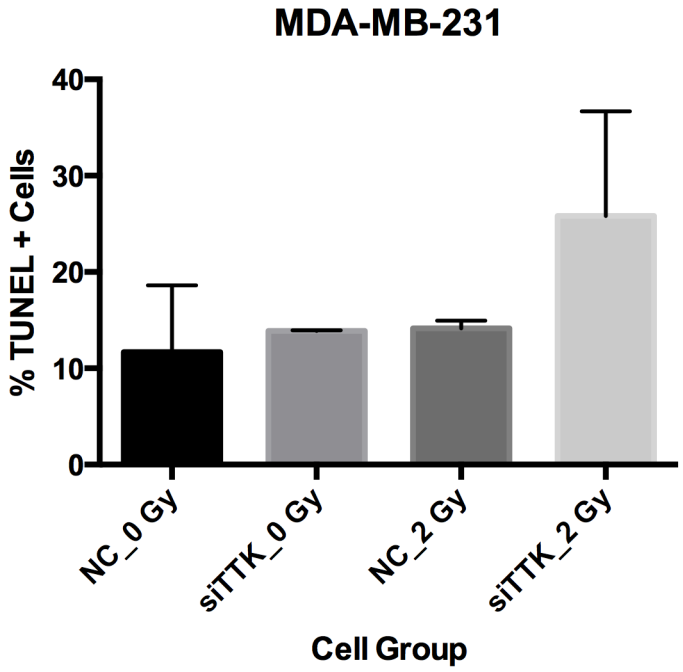

MDA-MB-231
% TUNEL + Cells
0
10
20
30
40
NC_0 Gy
siTTK_0 Gy
NC_2 Gy
siTTK_2 Gy
Cell Group

Figure 4.5: Silencing TTK in combination with irradiation enhances rates of apoptosis in TNBC cells.

Representative quantifications of apoptotic cells as determined by Annexin V staining and TUNEL staining in MDA-MB-231 cells with transiently silenced TTK and 2 Gy irradiation treatment. Cells were transfected with siRNA then irradiated 48 hours later. Cells were subjected to Annexin V and TUNEL staining, followed by flow cytometry or microscopy processing 48 hours later.

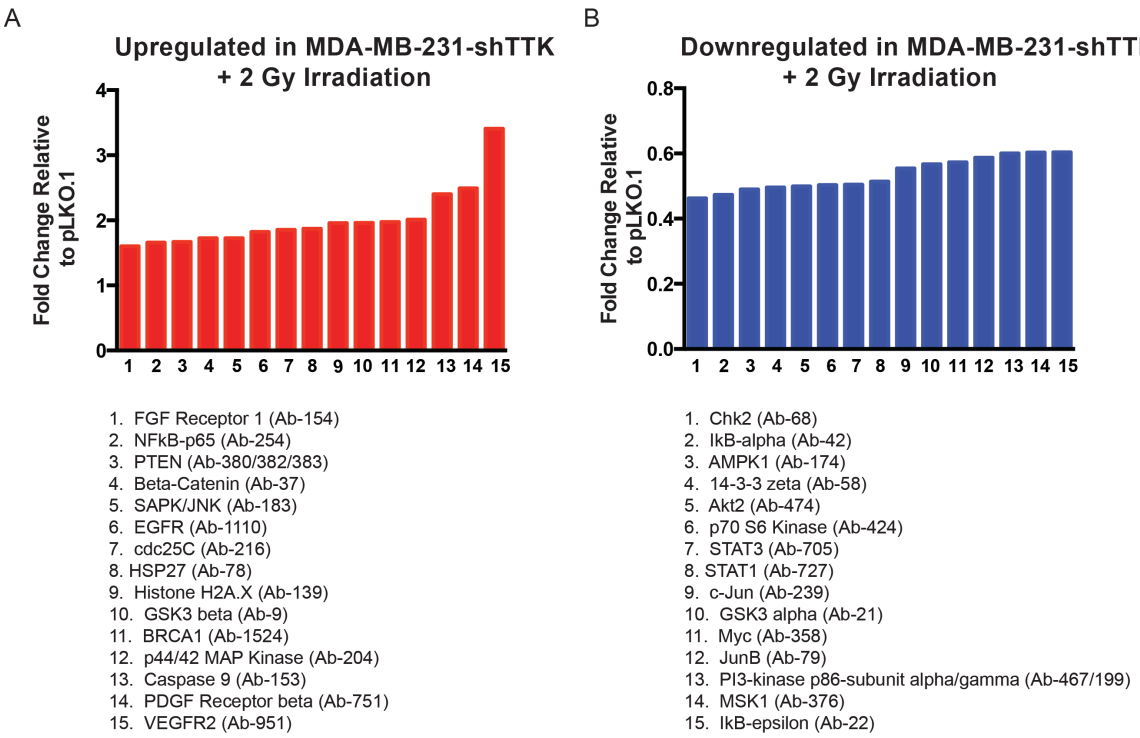


Figure 4.6: Genes with altered phosphorylation levels in shTTK TNBC cells combined with 2 Gy radiation dose.

Genes with (a) upregulated and (b) downregulated phosphorylation levels in MDA-MB-231 cells stably downregulated for TTK, combined with 2 Gy radiation dose, as identified by FullMoon Biosystems antibody array.

Chapter 5

Discussion

5.1 Summary and conclusions

This dissertation focused on molecular mechanisms and phenotypes associated with the TTK kinase and breast tumorigenesis. Taken together, our data indicate that TTK contributes to breast tumorigenesis through three interconnected aspects of tumorigenesis that are also involved in responses of breast tumor cells to therapeutic interventions. The three aspects that were the main focus of this research were centrosome amplification, EMT and radioresistance (Fig. 5.1). Our initial interest in investigating TTK in a breast cancer model grew out of our previous studies illustrating the powerful roles of Cdk4 mediated CA in breast cancer and the impacts of this cell cycle kinase in oncogenic signaling in breast cancer. At the beginning of this dissertation project, there was a gap in cancer research regarding the links between TTK overexpression in tumors and associated cell signaling changes. Through the approaches of genomic silencing TTK or inhibiting kinase activity in breast cancer cells, we show that targeting TTK can attenuate tumorigenic phenotypes and signaling in breast cancer.

5.2 Novel role of TTK in promoting CA in breast cancer

The roles of cell cycle regulators in promoting centrosome amplification and associated genomic instability have been extensively studied. Genomic instability results from alterations in a slew of molecules at various levels, including kinases like TTK that control necessary checkpoints within the cell cycle. Previously, the functions of TTK in centrosome regulation have been well established (132, 161). However, the links between high TTK expression and CA in breast cancer had not been previously studied. This is an important area of research, since understanding how kinases like

TTK contribute to genomic instability is important for determining what effects targeted therapies will have on pathways such as CA that facilitate aneuploidy in cancer cells. The studies in this dissertation elucidated that targeting TTK could attenuate a specific promoter of genomic instability in breast cancer cells, CA. We found that this attenuated CA was accompanied by decreased cell proliferation that was independent of changes in DNA replication. Therefore, we concluded that the changes in proliferation associated with TTK inhibition could be attributed to changes in a pathway independent of altered S phase, such as apoptotic response or arrest in mitosis. Additionally, targeting TTK could be functioning similar to targeting CDK4, as inhibiting TTK could specifically target cells that have CA, but still allow normal cells to proliferate, which may explain why we did not detect obvious cell cycle changes when inhibiting TTK.

5.3 Novel role of TTK in mediating EMT and KLF5 expression in aggressive breast cancer

Although several mitotic kinases have recently been shown to mediate invasive phenotypes in various cancer models, there was only one study showing that targeting TTK could change the invasive capabilities of cancer cells (125). Through our initial studies examining the effects of stably downregulating TTK in TNBC cells, our incidental findings of dramatic morphological changes induced after stable TTK downregulation led us to study changes in EMT signaling relating to TTK inhibition. The changes we observed were in a subset of breast cancer cells that are highly aggressive and well established as model for EMT. Our detailed analysis of TTK expression in breast cancer patients and cell lines across multiple publically available platforms revealed that the highest TTK expression was observed in TNBC patients,

which matched our observation of the morphological change only occurring in TNBC cells. In addition, a survey of related literature revealed that TTK could mediate TGF-β related signaling through micro-RNA regulation (123, 150) as well as a phospho-antibody array in MDA-MB-231-shTTK cells where SMAD3 emerged as an altered substrate of TTK (123, 124). From this compounded evidence, we hypothesized that the KLF5 transcription factor, which is also controlled by TGF-β, involved in EMT, and associated micro-RNA signaling (84-86) could also be involved in the EMT phenotype associated with TTK overexpression. Indeed, our studies showed that targeting TTK in mesenchymal TNBC cells could restore the epithelial phenotype through upregulating KLF5 and micro-RNAs associated with EMT repression, while suppressing mesenchymal markers and EMT promoting micro-RNAs. These studies also began to characterize changes in the TGF-β signaling pathway associated with TTK overexpression. These studies also opened up a novel area of research in regards to mitotic kinases in EMT, since the Saavedra lab has now found that other centrosome and mitotic regulators, such as Nek2, trigger EMT and invasion (168).

5.4 Exploration of TTK as a mediator of radiosensitivity

In addition to reports of TTK as a mediator of genomic instability and clinical correlations between high TTK and aggressive tumor phenotypes, there were some reports on TTK in mediating the response of cells to DNA damage (122). Also, TTK had been reported to modulate micro-RNA signaling that is associated with radioresistance (184) and we observed TTK overexpression in radioresistant breast cancer cells. Although radiation therapy is not a standalone treatment for breast cancer patients, radioresistance is a clinical barrier, as it contributes to tumor populations that emerge

and are resistant to further therapeutic intervention (191, 206). No previous studies had aimed to dissect changes in the response to radiation treatment that could be changed by targeting TTK in breast cancer. In our preliminary explorations of TTK's functions in radioresistance, we noted that the major cell responses changed by targeting TTK in breast cancer cells were the apoptotic response and DNA damage repair capacity. Our studies revealed that combining TTK inhibition with radiation treatment could enhance the rates of apoptosis in breast cancer cells and impair their ability to clear sites of DNA damage. In regards to therapeutic development, these findings provide a molecular mechanism for how TTK inhibition could enhance the effects of existing radiation therapy.

5.5 Proposed mechanisms for how TTK overexpression facilitates CA, EMT and radioresistance

The studies presented here illustrate how targeting TTK can attenuate three processes associated with tumorigenesis independently, but in fact, attenuating CA could be interconnected with reversion of EMT and radiosensitization in cancer cells. There is compelling evidence illustrating correlations between enhanced EMT in radioresistant cell populations that are also resistant to apoptosis (200, 207, 208). Many of these studies implicate alterations in pro-survival signaling pathways such as the PI3K-AKT signaling pathway (207, 209). In regards to how TTK overexpression could be facilitating CA that is linked to EMT and radioresistance, there are several possibilities. One possibility is that CA facilitated by TTK overexpression results in mechanical changes in cell division which could lead to centrosome clustering and altered contractility (210), ultimately leading to decreased expression of cellular

junction proteins like E-Cadherin (68, 211). In addition, altered phosphorylation of centrosome target genes could lead to incorrect phosphorylation of intermediate filaments (212, 213) or binding of downstream molecules that impacts binding of downstream molecules to promoter regions of EMT genes. Also, the cooperation between the TGF-B pathway and TTK overexpression, leading to increased Smad3 phosphorylation could stabilize signaling at invasive fronts, possibly through transcription factors such as Snail (214). Coordination of these events would support the ability of cancer cells to become invasive and migrate from the primary site of origin.

Another possibility is that oncogenic signaling occurring through TTK overexpression results in altered expression of micro-RNAs and contributes to feedback loops leading to EMT and radioresistance. One example is that miR-21 overexpression and/or miR-200 downregulation, as highlighted by the studies presented in chapter three, could promote ZEB1 expression. ZEB1 is involved in homologous recombination repair and stabilizes CHK1 (201). This relationship could ultimately lead to resistance to radiation treatment. Restoration of the miR-200 family in breast cancer cells has been shown to prevent proliferation, increase apoptosis, and inhibit pro-survival signaling in radioresistant cells (209, 215). The suppression of miR-200's that occurs as a result of TTK overexpression and correlated KLF5 downregulation, could result in the failure of miR-200's to prevent proliferation and increase apoptosis in response to DNA damage.

5.6 Future Directions

The overarching goal of the research in this dissertation was to characterize phenotypes and pathways associated with TTK and breast tumorigenesis. This goal was

built on the foundation that TTK accumulation in cells contributes to centrosome overduplication and associated genomic instability in cancer cells. Some unexpected but interesting directions also revealed that TTK has roles in mediating the aggressive signaling in breast cancer and response to treatment (Fig. 5.2).

Regarding the roles of TTK in promoting CA in breast cancer, one future direction would be to determine how specific components of the TTK structure contribute to CA in breast cancer cells. This could contribute to understanding exactly which substrates and associated processes lead to CA in cells that overexpress TTK. This line of study could also provide information for how TTK interacts with transcription factors like KLF5. For example, it would be advantageous to learn if modifications to the MPS1 degradation signal or kinase domain affect localization of TTK to centrosomes in breast cells or utilize an induced model of CA in breast cells to study this. We completed some preliminary experiments to examine TTK overexpression in MCF10A cells, but did not pursue this direction for the scope of these dissertation studies. We also completed some preliminary experiments to determine if targeting TTK alters downstream targets of TTK associated with its roles in centrosome duplication. We did not observe consistent changes in TACC2, but some future directions would be to measure live cell localization of TTK targets throughout the cell cycle in various conditions to see how silencing TTK impacts their roles.

We observed that the overexpression of TTK contributes to EMT phenotypes of TNBC cells and unraveled a novel signaling relationship between TTK, KLF5 and micro-RNAs. Regarding the regulation of KLF5, the full relationship between TTK and KLF5 is unclear. TTK and KLF5 could directly interact in the nucleus or cytoplasm at specific times, especially if KLF5 is temporally regulated in the cell cycle. An approach to

answer this question would be to flag both proteins and visually track them throughout different cell cycle phases. Another possibility is that KLF5 is phosphorylated directly by TTK to impact its transcription or degradation by E3 ubiquitin ligases. By analyzing phosphorylation sites in GPS 3.0, there were several predicted TTK phosphorylation sites on KLF5, which could be used in future studies to determine if any of them are involved in TTK suppressing KLF5 expression in specific breast cancer models. The effects of TTK inhibition on additional transcriptional targets of KLF5, such as C-MYC and P21 are also avenues for future studies on the relationships between TTK and KLF5.

Similar to further testing for TTK's role in CA, a future direction in this role of TTK would be to study how modifications to different regions in the kinase impact EMT in a breast cell model. One approach would be to generate a system similar to the classical TGF-β induced model of EMT in MCF10A cells. Our studies showed that inhibiting TTK in this model could attenuate EMT, so further tests to determine what components of TTK are involved in this process would be informative. In addition to microRNA's like the miR-200 family controlling classical markers of EMT such as E-Cadherin and vimentin, they are also involved in controlling signaling associated with cell motility and migration, such as the Rho kinases and FAK signaling. Another future direction would be to examine how these pathways are altered in breast cancer or other cancer models upon targeting TTK.

The studies relating to the role of TTK in radioresistance did not fully characterize how TTK controls the DNA repair sensing and apoptotic response to promote radioresistance in breast cancer; therefore many future directions could be explored for this role of TTK. In depth screening could be conducted in cancer cells with or without TTK to determine of downstream molecules associated with DNA damage

repair are altered by TTK inhibition. This approach could help answer the question of what type of DNA damage is mediated by EMT (i.e. non-homologous end joining or homologous repair). Another approach would be conduct similar screens with the TTK inhibitor to determine which DNA damage repair molecules are altered when the kinase activity of TTK is impaired.

5.7 Contributions to the field and larger implications for clinical investigations of TTK

Large advances have been made in developing mitotic kinases as therapeutic targets in breast and other cancers. During the course of this dissertation project, CDK4 inhibitors were FDA approved for clinical use in breast cancer patients (216). There is currently a clinical trial underway for a TTK inhibitor as well. The fundamental signaling pathways associated with TTK overexpression and inhibition have not been well studied. Therefore, the studies presented here highlight avenues to understand the complex pathways that are controlled by TTK. This information will be useful for understanding mechanisms of action for TTK inhibitors and how resistance to these inhibitors may arise. Furthermore, these studies provide experimental evidence to develop TTK as a biomarker in cancer, which could help inform clinical decisions, based on predicted tumor behavior and treatment response.

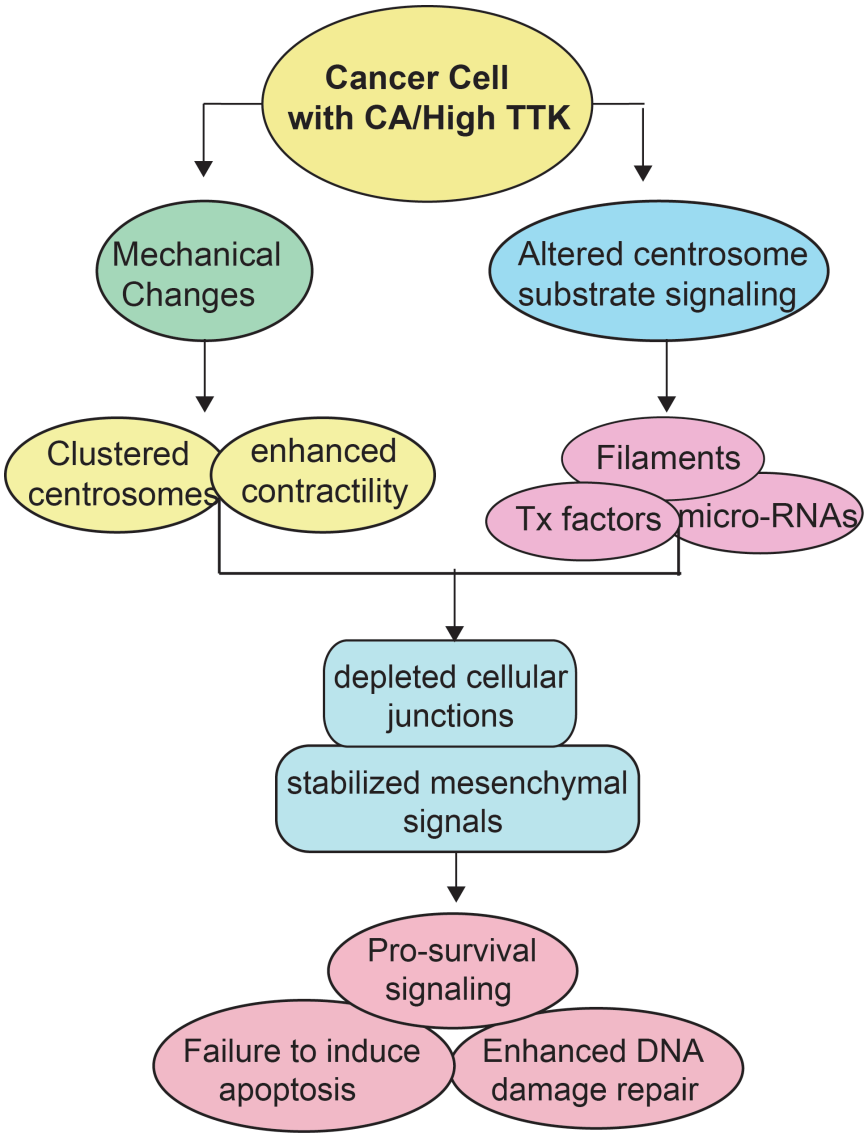


Figure 5.1 Potential model illustrating how CA & high TTK expression contribute to EMT and radiosensitivity.

TTK levels peak during centriole initiation and spindle assembly during the cell cycle, and high TTK expression facilitates CA. In these studies, we found that TTK also promotes oncogenic processes by facilitating CA, EMT and radioresistance in breast cancer cells.

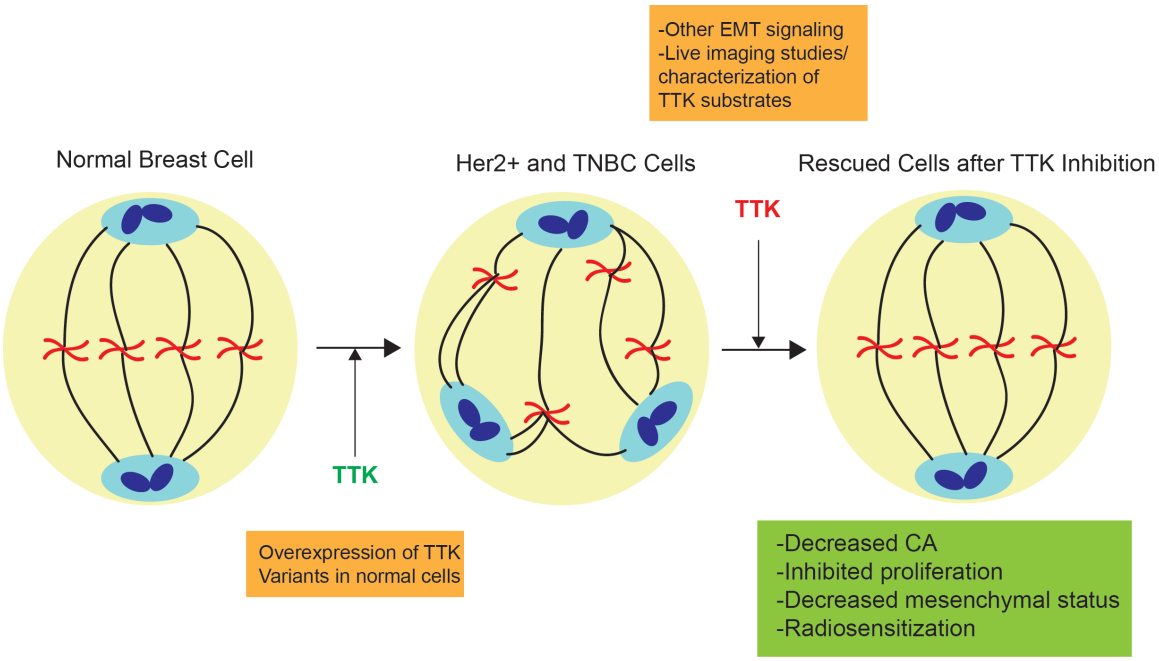


Figure 5.2 Summary of dissertation findings and future directions

Through the studies in this dissertation, we observed that overexpressed TTK in breast cancer cells contributes to amplified centrosomes and CIN accompanied by tumorigenic phenotypes (EMT and radioresistance) in Her2+ and TNBC cells. Through our experimental approaches, we observed that silencing TTK expression or preventing TTK kinase activity can attenuate these phenotypes.

(Summarized findings are indicated in the green box and areas for future studies are indicated in orange boxes).

References

1. Cancer Facts & Figures [press release]. 2018.
2. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.
3. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
4. Fernandez-Medarde A, Santos E. Ras in cancer and developmental diseases. Genes & cancer. 2011;2(3):344-58.
5. Dyson NJ. RB1: a prototype tumor suppressor and an enigma. Genes & development. 2016;30(13):1492-502.
6. Fukasawa K. Centrosome amplification, chromosome instability and cancer development. Cancer letters. 2005;230(1):6-19.
7. Saavedra HI, Fukasawa K, Conn CW, Stambrook PJ. MAPK mediates RAS-induced chromosome instability. The Journal of biological chemistry. 1999;274(53):38083-90.
8. Breast Cancer Facts & Figures [press release]. 2017.
9. Peppercorn J, Perou CM, Carey LA. Molecular subtypes in breast cancer evaluation and management: divide and conquer. Cancer investigation. 2008;26(1):1-10.
10. Prat A, Perou CM. Deconstructing the molecular portraits of breast cancer. Molecular oncology. 2011;5(1):5-23.
11. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(19):10869-74.
12. Brufsky AM, Dickler MN. Estrogen Receptor-Positive Breast Cancer: Exploiting Signaling Pathways Implicated in Endocrine Resistance. The oncologist. 2018.
13. Reinert T, Barrios CH. Overall survival and progression-free survival with endocrine therapy for hormone receptor-positive, HER2-negative advanced breast cancer: review. Therapeutic advances in medical oncology. 2017;9(11):693-709.
14. Tremont A, Lu J, Cole JT. Endocrine Therapy for Early Breast Cancer: Updated Review. The Ochsner journal. 2017;17(4):405-11.
15. Baselga J, Coleman RE, Cortes J, Janni W. Advances in the management of HER2-positive early breast cancer. Critical reviews in oncology/hematology. 2017;119:113-22.
16. Corraliza-Gorjon I, Somovilla-Crespo B, Santamaria S, Garcia-Sanz JA, Kremer L. New Strategies Using Antibody Combinations to Increase Cancer Treatment Effectiveness. Frontiers in immunology. 2017;8:1804.
17. Gligorov J, Richard S, Todorovic V. New anti-HER2 agents: from second-generation tyrosine kinases inhibitors to bifunctional antibodies. Current opinion in oncology. 2017;29(6):405-10.
18. Apuri S. Neoadjuvant and Adjuvant Therapies for Breast Cancer. Southern medical journal. 2017;110(10):638-42.
19. Lee CI, Chen LE, Elmore JG. Risk-based Breast Cancer Screening: Implications of Breast Density. The Medical clinics of North America. 2017;101(4):725-41.
20. Newman LA, Kaljee LM. Health Disparities and Triple-Negative Breast Cancer in African American Women: A Review. JAMA surgery. 2017;152(5):485-93.

21. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, et al. The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(8):2329-34.
22. DeSantis CE, Ma J, Goding Sauer A, Newman LA, Jemal A. Breast cancer statistics, 2017, racial disparity in mortality by state. CA: a cancer journal for clinicians. 2017;67(6):439-48.
23. Lund MJ, Butler EN, Hair BY, Ward KC, Andrews JH, Oprea-Ilies G, et al. Age/race differences in HER2 testing and in incidence rates for breast cancer triple subtypes: a population-based study and first report. Cancer. 2010;116(11):2549-59.
24. Huo D, Hu H, Rhie SK, Gamazon ER, Cherniack AD, Liu J, et al. Comparison of Breast Cancer Molecular Features and Survival by African and European Ancestry in The Cancer Genome Atlas. JAMA oncology. 2017;3(12):1654-62.
25. Cooper G. The Eukaryotic Cell Cycle. In: Associates S, editor. The Cell: A Molecular Approach2000.
26. Barnum KJ, O'Connell MJ. Cell cycle regulation by checkpoints. Methods in molecular biology. 2014;1170:29-40.
27. Aarts M, Linardopoulos S, Turner NC. Tumour selective targeting of cell cycle kinases for cancer treatment. Current opinion in pharmacology. 2013;13(4):529-35.
28. Otto T, Sicinski P. Cell cycle proteins as promising targets in cancer therapy. Nature reviews Cancer. 2017;17(2):93-115.
29. Holland AJ, Cleveland DW. Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. Nature reviews Molecular cell biology. 2009;10(7):478-87.
30. Holland AJ, Cleveland DW. Losing balance: the origin and impact of aneuploidy in cancer. EMBO reports. 2012;13(6):501-14.
31. Scheer U. Historical roots of centrosome research: discovery of Boveri's microscope slides in Wurzburg. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2014;369(1650).
32. Azimzadeh J, Bornens M. Structure and duplication of the centrosome. Journal of cell science. 2007;120(Pt 13):2139-42.
33. Rivera-Rivera Y, Saavedra HI. Centrosome - a promising anti-cancer target. Biologics : targets & therapy. 2016;10:167-76.
34. Hinchcliffe EH, Sluder G. "It takes two to tango": understanding how centrosome duplication is regulated throughout the cell cycle. Genes & development. 2001;15(10):1167-81.
35. Vorobjev IA, Chentsov Yu S. Centrioles in the cell cycle. I. Epithelial cells. The Journal of cell biology. 1982;93(3):938-49.
36. Hollander MC, Fornace AJ, Jr. Genomic instability, centrosome amplification, cell cycle checkpoints and Gadd45a. Oncogene. 2002;21(40):6228-33.
37. Kaneko N, Okuda M, Toyama N, Oikawa T, Watanabe M, Kanaya N, et al. Detection of centrosome amplification as a surrogate marker of dysfunction in the p53 pathway -p53 gene mutation or MDM2 overexpression. Veterinary and comparative oncology. 2005;3(4):203-10.
38. Kryukov F, Dementyeva E, Smetana J, Sevcikova S, Kuglik P, Hajek R. Association of aneuploidy category with centrosome amplification in multiple myeloma. Leukemia & lymphoma. 2011;52(10):2020-2.

39. Pihan GA. Centrosome dysfunction contributes to chromosome instability, chromoanagenesis, and genome reprograming in cancer. Frontiers in oncology. 2013;3:277.
40. Salisbury JL, D'Assoro AB, Lingle WL. Centrosome amplification and the origin of chromosomal instability in breast cancer. Journal of mammary gland biology and neoplasia. 2004;9(3):275-83.
41. Adon AM, Zeng X, Harrison MK, Sannem S, Kiyokawa H, Kaldis P, et al. Cdk2 and Cdk4 Regulate the Centrosome Cycle and Are Critical Mediators of Centrosome Amplification in p53-Null Cells. Molecular and Cellular Biology. 2010;30(3):694-710.
42. Cai Y, Liu YF, Li SL, Pan YX, Zhu Y, Yu YN. [Cyclin E overexpression and centrosome amplification in squamous cell carcinoma of oral cavity]. Zhonghua bing li xue za zhi Chinese journal of pathology. 2007;36(6):375-8.
43. Duensing A, Liu Y, Tseng M, Malumbres M, Barbacid M, Duensing S. Cyclin-dependent kinase 2 is dispensable for normal centrosome duplication but required for oncogene-induced centrosome overduplication. Oncogene. 2006;25(20):2943-9.
44. Hanashiro K, Kanai M, Geng Y, Sicinski P, Fukasawa K. Roles of cyclins A and E in induction of centrosome amplification in p53-compromised cells. Oncogene. 2008;27(40):5288-302.
45. Hinchcliffe EH, Li C, Thompson EA, Maller JL, Sluder G. Requirement of Cdk2-cyclin E activity for repeated centrosome reproduction in Xenopus egg extracts. Science. 1999;283(5403):851-4.
46. Fukasawa K, Choi T, Kuriyama R, Rulong S, Vande Woude GF. Abnormal centrosome amplification in the absence of p53. Science. 1996;271(5256):1744-7.
47. Saavedra HI, Maiti B, Timmers C, Altura R, Tokuyama Y, Fukasawa K, et al. Inactivation of E2F3 results in centrosome amplification. Cancer cell. 2003;3(4):333-46.
48. Bergmann S, Royer-Pokora B, Fietze E, Jurchott K, Hildebrandt B, Trost D, et al. YB-1 provokes breast cancer through the induction of chromosomal instability that emerges from mitotic failure and centrosome amplification. Cancer research. 2005;65(10):4078-87.
49. Pihan GA, Purohit A, Wallace J, Knecht H, Woda B, Quesenberry P, et al. Centrosome defects and genetic instability in malignant tumors. Cancer research. 1998;58(17):3974-85.
50. Basto R, Brunk K, Vinadogrova T, Peel N, Franz A, Khodjakov A, et al. Centrosome amplification can initiate tumorigenesis in flies. Cell. 2008;133(6):1032-42.
51. Castellanos E, Dominguez P, Gonzalez C. Centrosome dysfunction in Drosophila neural stem cells causes tumors that are not due to genome instability. Current biology : CB. 2008;18(16):1209-14.
52. Chan JY. A clinical overview of centrosome amplification in human cancers. International journal of biological sciences. 2011;7(8):1122-44.
53. Pihan GA, Wallace J, Zhou Y, Doxsey SJ. Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas. Cancer research. 2003;63(6):1398-404.
54. Levine MS, Bakker B, Boeckx B, Moyett J, Lu J, Vitre B, et al. Centrosome Amplification Is Sufficient to Promote Spontaneous Tumorigenesis in Mammals. Developmental cell. 2017;40(3):313-22 e5.

55. Ogden A, Rida PC, Aneja R. Prognostic value of CA20, a score based on centrosome amplification-associated genes, in breast tumors. Scientific reports. 2017;7(1):262.
56. D'Assoro AB, Lingle WL, Salisbury JL. Centrosome amplification and the development of cancer. Oncogene. 2002;21(40):6146-53.
57. Ogden A, Rida PCG, Aneja R. Centrosome amplification: a suspect in breast cancer and racial disparities. Endocrine-related cancer. 2017;24(9):T47-T64.
58. Pannu V, Mittal K, Cantuaria G, Reid MD, Li X, Donthamsetty S, et al. Rampant centrosome amplification underlies more aggressive disease course of triple negative breast cancers. Oncotarget. 2015;6(12):10487-97.
59. Lingle WL, Lutz WH, Ingle JN, Maihle NJ, Salisbury JL. Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(6):2950-5.
60. Lingle WL, Barrett SL, Negron VC, D'Assoro AB, Boeneman K, Liu W, et al. Centrosome amplification drives chromosomal instability in breast tumor development. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(4):1978-83.
61. Harrison MK, Adon AM, Saavedra HI. The G1 phase Cdks regulate the centrosome cycle and mediate oncogene-dependent centrosome amplification. Cell division. 2011;6:2.
62. Harrison Pitner MK, Saavedra HI. Cdk4 and nek2 signal binucleation and centrosome amplification in a her2+ breast cancer model. PloS one. 2013;8(6):e65971.
63. Krzywicka-Racka A, Sluder G. Repeated cleavage failure does not establish centrosome amplification in untransformed human cells. The Journal of cell biology. 2011;194(2):199-207.
64. Weaver BA, Silk AD, Cleveland DW. Low rates of aneuploidy promote tumorigenesis while high rates of aneuploidy cause cell death and tumor suppression. Cellular oncology : the official journal of the International Society for Cellular Oncology. 2008;30(5):453.
65. Milunovic-Jevtic A, Mooney P, Sulerud T, Bisht J, Gatlin JC. Centrosomal clustering contributes to chromosomal instability and cancer. Current opinion in biotechnology. 2016;40:113-8.
66. Ogden A, Rida PC, Aneja R. Heading off with the herd: how cancer cells might maneuver supernumerary centrosomes for directional migration. Cancer metastasis reviews. 2013;32(1-2):269-87.
67. Godinho SA, Picone R, Burute M, Dagher R, Su Y, Leung CT, et al. Oncogene-like induction of cellular invasion from centrosome amplification. Nature. 2014;510(7503):167-71.
68. Rhys AD, Monteiro P, Smith C, Vaghela M, Arnandis T, Kato T, et al. Loss of E-cadherin provides tolerance to centrosome amplification in epithelial cancer cells. The Journal of cell biology. 2018;217(1):195-209.
69. Mehlen P, Puisieux A. Metastasis: a question of life or death. Nature reviews Cancer. 2006;6(6):449-58.
70. van Zijl F, Krupitza G, Mikulits W. Initial steps of metastasis: cell invasion and endothelial transmigration. Mutation research. 2011;728(1-2):23-34.

71. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. The Journal of clinical investigation. 2009;119(6):1420-8.
72. Spano D, Heck C, De Antonellis P, Christofori G, Zollo M. Molecular networks that regulate cancer metastasis. Seminars in cancer biology. 2012;22(3):234-49.
73. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nature reviews Molecular cell biology. 2014;15(3):178-96.
74. Ma L, Weinberg RA. Micromanagers of malignancy: role of microRNAs in regulating metastasis. Trends in genetics : TIG. 2008;24(9):448-56.
75. Sui X, Wang X, Han W, Li D, Xu Y, Lou F, et al. MicroRNAs-mediated cell fate in triple negative breast cancers. Cancer letters. 2015;361(1):8-12.
76. Liu CY, Lin HH, Tang MJ, Wang YK. Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation. Oncotarget. 2015.
77. Yan L, Cao R, Liu Y, Wang L, Pan B, Lv X, et al. MiR-21-5p Links Epithelial-Mesenchymal Transition Phenotype with Stem-Like Cell Signatures via AKT Signaling in Keloid Keratinocytes. Scientific reports. 2016;6:28281.
78. Ferrari-Amorotti G, Chiodoni C, Shen F, Cattelani S, Soliera AR, Manzotti G, et al. Suppression of invasion and metastasis of triple-negative breast cancer lines by pharmacological or genetic inhibition of slug activity. Neoplasia (New York, NY). 2014;16(12):1047-58.
79. Grzegrzolka J, Biala M, Wojtyra P, Kobierzycki C, Olbromski M, Gomulkiewicz A, et al. Expression of EMT Markers SLUG and TWIST in Breast Cancer. Anticancer research. 2015;35(7):3961-8.
80. Hill L, Browne G, Tulchinsky E. ZEB/miR-200 feedback loop: at the crossroads of signal transduction in cancer. International journal of cancer Journal international du cancer. 2013;132(4):745-54.
81. Humphries B, Wang Z, Li Y, Jhan JR, Jiang Y, Yang C. ARHGAP18 Downregulation by miR-200b Suppresses Metastasis of Triple-Negative Breast Cancer by Enhancing Activation of RhoA. Cancer research. 2017;77(15):4051-64.
82. Massague J. TGFbeta in Cancer. Cell. 2008;134(2):215-30.
83. Chen C, Bhalala HV, Qiao H, Dong JT. A possible tumor suppressor role of the KLF5 transcription factor in human breast cancer. Oncogene. 2002;21(43):6567-72.
84. Zhang B, Zhang Z, Xia S, Xing C, Ci X, Li X, et al. KLF5 activates microRNA 200 transcription to maintain epithelial characteristics and prevent induced epithelial-mesenchymal transition in epithelial cells. Molecular and cellular biology. 2013;33(24):4919-35.
85. Li X, Zhang B, Wu Q, Ci X, Zhao R, Zhang Z, et al. Interruption of KLF5 acetylation converts its function from tumor suppressor to tumor promoter in prostate cancer cells. International journal of cancer Journal international du cancer. 2015;136(3):536-46.
86. Guo P, Dong XY, Zhang X, Zhao KW, Sun X, Li Q, et al. Pro-proliferative factor KLF5 becomes anti-proliferative in epithelial homeostasis upon signaling-mediated modification. The Journal of biological chemistry. 2009;284(10):6071-8.
87. Li Z, Dong J, Zou T, Du C, Li S, Chen C, et al. Dexamethasone induces docetaxel and cisplatin resistance partially through up-regulating Kruppel-like factor 5 in triple-negative breast cancer. Oncotarget. 2017;8(7):11555-65.

88. Liu R, Shi P, Nie Z, Liang H, Zhou Z, Chen W, et al. Mifepristone Suppresses Basal Triple-Negative Breast Cancer Stem Cells by Down-regulating KLF5 Expression. Theranostics. 2016;6(4):533-44.
89. Hagos EG, Ghaleb AM, Dalton WB, Bialkowska AB, Yang VW. Mouse embryonic fibroblasts null for the Kruppel-like factor 4 gene are genetically unstable. Oncogene. 2009;28(9):1197-205.
90. Fan G, Sun L, Shan P, Zhang X, Huan J, Zhang X, et al. Loss of KLF14 triggers centrosome amplification and tumorigenesis. Nature communications. 2015;6:8450.
91. Chen Y, Yeh PC, Huang JC, Yeh CC, Juang YL. The spindle checkpoint protein MAD1 regulates the expression of E-cadherin and prevents cell migration. Oncology reports. 2012;27(2):487-91.
92. Wu J, Yang L, Shan Y, Cai C, Wang S, Zhang H. AURKA promotes cell migration and invasion of head and neck squamous cell carcinoma through regulation of the AURKA/Akt/FAK signaling pathway. Oncology letters. 2016;11(3):1889-94.
93. Ikawa K, Satou A, Fukuhara M, Matsumura S, Sugiyama N, Goto H, et al. Inhibition of endocytic vesicle fusion by Plk1-mediated phosphorylation of vimentin during mitosis. Cell cycle. 2014;13(1):126-37.
94. Kazazian K, Go C, Wu H, Brashavitskaya O, Xu R, Dennis JW, et al. Plk4 Promotes Cancer Invasion and Metastasis through Arp2/3 Complex Regulation of the Actin Cytoskeleton. Cancer research. 2017;77(2):434-47.
95. Finetti P, Cervera N, Charafe-Jauffret E, Chabannon C, Charpin C, Chaffanet M, et al. Sixteen-kinase gene expression identifies luminal breast cancers with poor prognosis. Cancer research. 2008;68(3):767-76.
96. Al-Ejeh F, Simpson PT, Saunus JM, Klein K, Kalimutho M, Shi W, et al. Meta-analysis of the global gene expression profile of triple-negative breast cancer identifies genes for the prognostication and treatment of aggressive breast cancer. Oncogenesis. 2014;3:e124.
97. Marina M, Saavedra HI. Nek2 and Plk4: prognostic markers, drivers of breast tumorigenesis and drug resistance. Frontiers in bioscience. 2014;19:352-65.
98. Winey M, Goetsch L, Baum P, Byers B. MPS1 and MPS2: novel yeast genes defining distinct steps of spindle pole body duplication. The Journal of cell biology. 1991;114(4):745-54.
99. Mills GB, Schmandt R, McGill M, Amendola A, Hill M, Jacobs K, et al. Expression of TTK, a novel human protein kinase, is associated with cell proliferation. The Journal of biological chemistry. 1992;267(22):16000-6.
100. Schmandt R, Hill M, Amendola A, Mills GB, Hogg D. IL-2-induced expression of TTK, a serine, threonine, tyrosine kinase, correlates with cell cycle progression. Journal of immunology. 1994;152(1):96-105.
101. Fisk HA, Mattison CP, Winey M. A field guide to the Mps1 family of protein kinases. Cell cycle. 2004;3(4):439-42.
102. Marquardt JR, Perkins JL, Beuoy KJ, Fisk HA. Modular elements of the TPR domain in the Mps1 N terminus differentially target Mps1 to the centrosome and kinetochore. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(28):7828-33.
103. Nijenhuis W, von Castelmur E, Littler D, De Marco V, Tromer E, Vleugel M, et al. A TPR domain-containing N-terminal module of MPS1 is required for its kinetochore localization by Aurora B. The Journal of cell biology. 2013;201(2):217-31.

104. Nigg EA, Holland AJ. Once and only once: mechanisms of centriole duplication and their deregulation in disease. Nature reviews Molecular cell biology. 2018.
105. Morin V, Prieto S, Melines S, Hem S, Rossignol M, Lorca T, et al. CDK-dependent potentiation of MPS1 kinase activity is essential to the mitotic checkpoint. Current biology : CB. 2012;22(4):289-95.
106. Yang CH, Kasbek C, Majumder S, Yusof AM, Fisk HA. Mps1 phosphorylation sites regulate the function of centrin 2 in centriole assembly. Molecular biology of the cell. 2010;21(24):4361-72.
107. Dou Z, Liu X, Wang W, Zhu T, Wang X, Xu L, et al. Dynamic localization of Mps1 kinase to kinetochores is essential for accurate spindle microtubule attachment. Proceedings of the National Academy of Sciences of the United States of America. 2015;112(33):E4546-55.
108. Boutros R, Mondesert O, Lorenzo C, Astuti P, McArthur G, Chircop M, et al. CDC25B overexpression stabilises centrin 2 and promotes the formation of excess centriolar foci. PloS one. 2013;8(7):e67822.
109. Lee M, Rivera-Rivera Y, Moreno CS, Saavedra HI. The E2F activators control multiple mitotic regulators and maintain genomic integrity through Sgo1 and BubR1. Oncotarget. 2017;8(44):77649-72.
110. Weiss E, Winey M. The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint. The Journal of cell biology. 1996;132(1-2):111-23.
111. Yeh CW, Yu ZC, Chen PH, Cheng YC, Shieh SY. Phosphorylation at threonine 288 by cell cycle checkpoint kinase 2 (CHK2) controls human monopolar spindle 1 (Mps1) kinetochore localization. The Journal of biological chemistry. 2014;289(22):15319-27.
112. Conde C, Osswald M, Barbosa J, Moutinho-Santos T, Pinheiro D, Guimaraes S, et al. Drosophila Polo regulates the spindle assembly checkpoint through Mps1-dependent BubR1 phosphorylation. The EMBO journal. 2013;32(12):1761-77.
113. von Schubert C, Cubizolles F, Bracher JM, Sliedrecht T, Kops GJ, Nigg EA. Plk1 and Mps1 Cooperatively Regulate the Spindle Assembly Checkpoint in Human Cells. Cell reports. 2015;12(1):66-78.
114. Zhao Y, Chen RH. Mps1 phosphorylation by MAP kinase is required for kinetochore localization of spindle-checkpoint proteins. Current biology : CB. 2006;16(17):1764-9.
115. Zhu T, Dou Z, Qin B, Jin C, Wang X, Xu L, et al. Phosphorylation of Microtubule-binding Protein Hec1 by Mitotic Kinase Aurora B Specifies Spindle Checkpoint Kinase Mps1 Signaling at the Kinetochore. The Journal of biological chemistry. 2013;288(50):36149-59.
116. Hardwick KG, Weiss E, Luca FC, Winey M, Murray AW. Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. Science. 1996;273(5277):953-6.
117. London N, Biggins S. Mad1 kinetochore recruitment by Mps1-mediated phosphorylation of Bub1 signals the spindle checkpoint. Genes & development. 2014.
118. Zich J, May K, Paraskevopoulos K, Sen O, Syred HM, van der Sar S, et al. Mps1Mph1 Kinase Phosphorylates Mad3 to Inhibit Cdc20Slp1-APC/C and Maintain Spindle Checkpoint Arrests. PLoS genetics. 2016;12(2):e1005834.

119. Shimogawa MM, Graczyk B, Gardner MK, Francis SE, White EA, Ess M, et al. Mps1 phosphorylation of Dam1 couples kinetochores to microtubule plus ends at metaphase. Current biology : CB. 2006;16(15):1489-501.
120. Dou Z, Ding X, Zereshki A, Zhang Y, Zhang J, Wang F, et al. TTK kinase is essential for the centrosomal localization of TACC2. FEBS letters. 2004;572(1-3):51-6.
121. Wei JH, Chou YF, Ou YH, Yeh YH, Tyan SW, Sun TP, et al. TTK/hMps1 participates in the regulation of DNA damage checkpoint response by phosphorylating CHK2 on threonine 68. The Journal of biological chemistry. 2005;280(9):7748-57.
122. Yeh YH, Huang YF, Lin TY, Shieh SY. The cell cycle checkpoint kinase CHK2 mediates DNA damage-induced stabilization of TTK/hMps1. Oncogene. 2009;28(10):1366-78.
123. Hirschhorn T, Barizilay L, Smorodinsky NI, Ehrlich M. Differential regulation of Smad3 and of the type II transforming growth factor-beta receptor in mitosis: implications for signaling. PloS one. 2012;7(8):e43459.
124. Zhu S, Wang W, Clarke DC, Liu X. Activation of Mps1 promotes transforming growth factor-beta-independent Smad signaling. The Journal of biological chemistry. 2007;282(25):18327-38.
125. Liu X, Liao W, Yuan Q, Ou Y, Huang J. TTK activates Akt and promotes proliferation and migration of hepatocellular carcinoma cells. Oncotarget. 2015;6(33):34309-20.
126. Libouban MAA, de Roos J, Uitdehaag JCM, Willemsen-Seegers N, Mainardi S, Dylus J, et al. Stable aneuploid tumors cells are more sensitive to TTK inhibition than chromosomally unstable cell lines. Oncotarget. 2017;8(24):38309-25.
127. Kaistha BP, Honstein T, Muller V, Bielak S, Sauer M, Kreider R, et al. Key role of dual specificity kinase TTK in proliferation and survival of pancreatic cancer cells. British journal of cancer. 2014;111(9):1780-7.
128. Liang XD, Dai YC, Li ZY, Gan MF, Zhang SR, Yin P, et al. Expression and function analysis of mitotic checkpoint genes identifies TTK as a potential therapeutic target for human hepatocellular carcinoma. PloS one. 2014;9(6):e97739.
129. Maire V, Baldeyron C, Richardson M, Tesson B, Vincent-Salomon A, Gravier E, et al. TTK/hMPS1 is an attractive therapeutic target for triple-negative breast cancer. PloS one. 2013;8(5):e63712.
130. Tannous BA, Kerami M, Van der Stoop PM, Kwiatkowski N, Wang J, Zhou W, et al. Effects of the selective MPS1 inhibitor MPS1-IN-3 on glioblastoma sensitivity to antimitotic drugs. Journal of the National Cancer Institute. 2013;105(17):1322-31.
131. Xie Y, Wang A, Lin J, Wu L, Zhang H, Yang X, et al. Mps1/TTK: a novel target and biomarker for cancer. Journal of drug targeting. 2017;25(2):112-8.
132. Kasbek C, Yang CH, Fisk HA. Mps1 as a link between centrosomes and genomic instability. Environmental and molecular mutagenesis. 2009;50(8):654-65.
133. Daniel J, Coulter J, Woo JH, Wilsbach K, Gabrielson E. High levels of the Mps1 checkpoint protein are protective of aneuploidy in breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(13):5384-9.
134. Ling Y, Zhang X, Bai Y, Li P, Wei C, Song T, et al. Overexpression of Mps1 in colon cancer cells attenuates the spindle assembly checkpoint and increases aneuploidy. Biochemical and biophysical research communications. 2014;450(4):1690-5.
135. Gradishar WJ. Taxanes for the treatment of metastatic breast cancer. Breast cancer : basic and clinical research. 2012;6:159-71.

136. de Duenas EM, Gavila-Gregori J, Olmos-Anton S, Santaballa-Bertran A, Lluch-Hernandez A, Espinal-Dominguez EJ, et al. Preclinical and clinical development of palbociclib and future perspectives. Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico. 2018.
137. de Groot AF, Kuijpers CJ, Kroep JR. CDK4/6 inhibition in early and metastatic breast cancer: A review. Cancer treatment reviews. 2017;60:130-8.
138. Liewer S, Huddleston A. Alisertib: a review of pharmacokinetics, efficacy and toxicity in patients with hematologic malignancies and solid tumors. Expert opinion on investigational drugs. 2018;27(1):105-12.
139. Colombo R, Caldarelli M, Mennecozzi M, Giorgini ML, Sola F, Cappella P, et al. Targeting the mitotic checkpoint for cancer therapy with NMS-P715, an inhibitor of MPS1 kinase. Cancer research. 2010;70(24):10255-64.
140. Faisal A, Mak GWY, Gurden MD, Xavier CPR, Anderhub SJ, Innocenti P, et al. Characterisation of CCT271850, a selective, oral and potent MPS1 inhibitor, used to directly measure in vivo MPS1 inhibition vs therapeutic efficacy. British journal of cancer. 2017;116(9):1166-76.
141. Kusakabe K, Ide N, Daigo Y, Itoh T, Yamamoto T, Hashizume H, et al. Discovery of imidazo[1,2-b]pyridazine derivatives: selective and orally available Mps1 (TTK) kinase inhibitors exhibiting remarkable antiproliferative activity. Journal of medicinal chemistry. 2015;58(4):1760-75.
142. Wengner AM, Siemeister G, Koppitz M, Schulze V, Kosemund D, Klar U, et al. Novel Mps1 Kinase Inhibitors with Potent Antitumor Activity. Molecular cancer therapeutics. 2016;15(4):583-92.
143. Phase I Dose Escalation of Oral BAY1161909 in Combination With Intravenous Paclitaxel 2018 [updated January 3, 2018March 27, 2018]. Available from: https://clinicaltrials.gov/ct2/show/NCT02138812.
144. Cescon DW, Hansen AR, Razak ARA, Stayner L-A, Hilton JF, Renouf DJ, et al. Phase I study of CFI-402257, an oral TTK inhibitor, in patients with advanced solid tumors. Journal of Clinical Oncology. 2017;35(15_suppl):TPS2619-TPS.
145. Maia AR, de Man J, Boon U, Janssen A, Song JY, Omerzu M, et al. Inhibition of the spindle assembly checkpoint kinase TTK enhances the efficacy of docetaxel in a triple-negative breast cancer model. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2015;26(10):2180-92.
146. Tardif KD, Rogers A, Cassiano J, Roth BL, Cimbora DM, McKinnon R, et al. Characterization of the cellular and antitumor effects of MPI-0479605, a small-molecule inhibitor of the mitotic kinase Mps1. Molecular cancer therapeutics. 2011;10(12):2267-75.
147. Stratford JK, Yan F, Hill RA, Major MB, Graves LM, Der CJ, et al. Genetic and pharmacological inhibition of TTK impairs pancreatic cancer cell line growth by inducing lethal chromosomal instability. PloS one. 2017;12(4):e0174863.
148. Jemaa M, Manic G, Lledo G, Lissa D, Reynes C, Morin N, et al. Whole-genome duplication increases tumor cell sensitivity to MPS1 inhibition. Oncotarget. 2016;7(1):885-901.
149. Chen S, Wang Y, Ni C, Meng G, Sheng X. HLF/miR-132/TTK axis regulates cell proliferation, metastasis and radiosensitivity of glioma cells. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2016;83:898-904.

150. Maachani UB, Tandle A, Shankavaram U, Kramp T, Camphausen K. Modulation of miR-21 signaling by MPS1 in human glioblastoma. Oncotarget. 2015.
151. Riggs JR, Nagy M, Elsner J, Erdman P, Cashion D, Robinson D, et al. The Discovery of a Dual TTK Protein Kinase/CDC2-Like Kinase (CLK2) Inhibitor for the Treatment of Triple Negative Breast Cancer Initiated from a Phenotypic Screen. Journal of medicinal chemistry. 2017;60(21):8989-9002.
152. Sugimoto Y, Sawant DB, Fisk HA, Mao L, Li C, Chettiar S, et al. Novel pyrrolopyrimidines as Mps1/TTK kinase inhibitors for breast cancer. Bioorganic & medicinal chemistry. 2017;25(7):2156-66.
153. Roylance R, Endesfelder D, Gorman P, Burrell RA, Sander J, Tomlinson I, et al. Relationship of extreme chromosomal instability with long-term survival in a retrospective analysis of primary breast cancer. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2011;20(10):2183-94.
154. Guo HQ, Gao M, Ma J, Xiao T, Zhao LL, Gao Y, et al. Analysis of the cellular centrosome in fine-needle aspirations of the breast. Breast cancer research : BCR. 2007;9(4):R48.
155. Lingle WL, Salisbury JL. Altered centrosome structure is associated with abnormal mitoses in human breast tumors. The American journal of pathology. 1999;155(6):1941-51.
156. Schneeweiss A, Sinn HP, Ehemann V, Khbeis T, Neben K, Krause U, et al. Centrosomal aberrations in primary invasive breast cancer are associated with nodal status and hormone receptor expression. International journal of cancer Journal international du cancer. 2003;107(3):346-52.
157. Lee MY, Moreno CS, Saavedra HI. E2F activators signal and maintain centrosome amplification in breast cancer cells. Molecular and cellular biology. 2014;34(14):2581-99.
158. Hagen KR, Zeng X, Lee MY, Tucker Kahn S, Harrison Pitner MK, Zaky SS, et al. Silencing CDK4 radiosensitizes breast cancer cells by promoting apoptosis. Cell division. 2013;8(1):10.
159. Lan W, Cleveland DW. A chemical tool box defines mitotic and interphase roles for Mps1 kinase. The Journal of cell biology. 2010;190(1):21-4.
160. Kasbek C, Yang CH, Fisk HA. Antizyme restrains centrosome amplification by regulating the accumulation of Mps1 at centrosomes. Molecular biology of the cell. 2010;21(22):3878-89.
161. Pike AN, Fisk HA. Centriole assembly and the role of Mps1: defensible or dispensable? Cell division. 2011;6:9.
162. Yuan B, Xu Y, Woo JH, Wang Y, Bae YK, Yoon DS, et al. Increased expression of mitotic checkpoint genes in breast cancer cells with chromosomal instability. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006;12(2):405-10.
163. Lehmann BD, Jovanovic B, Chen X, Estrada MV, Johnson KN, Shyr Y, et al. Refinement of Triple-Negative Breast Cancer Molecular Subtypes: Implications for Neoadjuvant Chemotherapy Selection. PloS one. 2016;11(6):e0157368.
164. Savagner P. Epithelial-mesenchymal transitions: from cell plasticity to concept elasticity. Current topics in developmental biology. 2015;112:273-300.

165. Denu RA, Zasadil LM, Kanugh C, Laffin J, Weaver BA, Burkard ME. Centrosome amplification induces high grade features and is prognostic of worse outcomes in breast cancer. BMC cancer. 2016;16:47.
166. Lee MY, Marina M, King JL, Saavedra HI. Differential expression of centrosome regulators in Her2+ breast cancer cells versus non-tumorigenic MCF10A cells. Cell division. 2014;9:3.
167. Godinho SA, Pellman D. Causes and consequences of centrosome abnormalities in cancer. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2014;369(1650).
168. Lee M, Oprea-Ilies G, Saavedra HI. Silencing of E2F3 suppresses tumor growth of Her2+ breast cancer cells by restricting mitosis. Oncotarget. 2015;6(35):37316-34.
169. Ahn CH, Kim YR, Kim SS, Yoo NJ, Lee SH. Mutational analysis of TTK gene in gastric and colorectal cancers with microsatellite instability. Cancer research and treatment : official journal of Korean Cancer Association. 2009;41(4):224-8.
170. Niittymaki I, Gylfe A, Laine L, Laakso M, Lehtonen HJ, Kondelin J, et al. High frequency of TTK mutations in microsatellite-unstable colorectal cancer and evaluation of their effect on spindle assembly checkpoint. Carcinogenesis. 2011;32(3):305-11.
171. Chen C, Sun X, Ran Q, Wilkinson KD, Murphy TJ, Simons JW, et al. Ubiquitin-proteasome degradation of KLF5 transcription factor in cancer and untransformed epithelial cells. Oncogene. 2005;24(20):3319-27.
172. Ringner M, Fredlund E, Hakkinen J, Borg A, Staaf J. GOBO: gene expression-based outcome for breast cancer online. PloS one. 2011;6(3):e17911.
173. Madden SF, Clarke C, Gaule P, Aherne ST, O'Donovan N, Clynes M, et al. BreastMark: an integrated approach to mining publicly available transcriptomic datasets relating to breast cancer outcome. Breast cancer research : BCR. 2013;15(4):R52.
174. Burstein MD, Tsimelzon A, Poage GM, Covington KR, Contreras A, Fuqua SA, et al. Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(7):1688-98.
175. Guo P, Zhao KW, Dong XY, Sun X, Dong JT. Acetylation of KLF5 alters the assembly of p15 transcription factors in transforming growth factor-beta-mediated induction in epithelial cells. The Journal of biological chemistry. 2009;284(27):18184-93.
176. Kasbek C, Yang CH, Yusof AM, Chapman HM, Winey M, Fisk HA. Preventing the degradation of mps1 at centrosomes is sufficient to cause centrosome reduplication in human cells. Molecular biology of the cell. 2007;18(11):4457-69.
177. Mattison CP, Old WM, Steiner E, Huneycutt BJ, Resing KA, Ahn NG, et al. Mps1 activation loop autophosphorylation enhances kinase activity. The Journal of biological chemistry. 2007;282(42):30553-61.
178. Wahdan-Alaswad R, Harrell JC, Fan Z, Edgerton SM, Liu B, Thor AD. Metformin attenuates transforming growth factor beta (TGF-beta) mediated oncogenesis in mesenchymal stem-like/claudin-low triple negative breast cancer. Cell cycle. 2016;15(8):1046-59.
179. Han M, Wang F, Gu Y, Pei X, Guo G, Yu C, et al. MicroRNA-21 induces breast cancer cell invasion and migration by suppressing smad7 via EGF and TGF-beta pathways. Oncology reports. 2016;35(1):73-80.

180. Wang JY, Gao YB, Zhang N, Zou DW, Wang P, Zhu ZY, et al. miR-21 overexpression enhances TGF-beta1-induced epithelial-to-mesenchymal transition by target smad7 and aggravates renal damage in diabetic nephropathy. Molecular and cellular endocrinology. 2014;392(1-2):163-72.
181. Yan LX, Wu QN, Zhang Y, Li YY, Liao DZ, Hou JH, et al. Knockdown of miR-21 in human breast cancer cell lines inhibits proliferation, in vitro migration and in vivo tumor growth. Breast cancer research : BCR. 2011;13(1):R2.
182. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005;120(1):15-20.
183. Anastasov N, Hofig I, Vasconcellos IG, Rappl K, Braselmann H, Ludyga N, et al. Radiation resistance due to high expression of miR-21 and G2/M checkpoint arrest in breast cancer cells. Radiation oncology (London, England). 2012;7:206.
184. Maachani UB, Kramp T, Hanson R, Zhao S, Celiku O, Shankavaram U, et al. Targeting MPS1 Enhances Radiosensitization of Human Glioblastoma by Modulating DNA Repair Proteins. Molecular cancer research : MCR. 2015;13(5):852-62.
185. Miao R, Wu Y, Zhang H, Zhou H, Sun X, Csizmadia E, et al. Utility of the dual-specificity protein kinase TTK as a therapeutic target for intrahepatic spread of liver cancer. Scientific reports. 2016;6:33121.
186. Zhou KX, Xie LH, Peng X, Guo QM, Wu QY, Wang WH, et al. CXCR4 antagonist AMD3100 enhances the response of MDA-MB-231 triple-negative breast cancer cells to ionizing radiation. Cancer letters. 2018;418:196-203.
187. Jameel JK, Rao VS, Cawkwell L, Drew PJ. Radioresistance in carcinoma of the breast. Breast. 2004;13(6):452-60.
188. Gabos Z, Thoms J, Ghosh S, Hanson J, Deschenes J, Sabri S, et al. The association between biological subtype and locoregional recurrence in newly diagnosed breast cancer. Breast cancer research and treatment. 2010;124(1):187-94.
189. Horton JK, Jagsi R, Woodward WA, Ho A. Breast Cancer Biology: Clinical Implications for Breast Radiation Therapy. International journal of radiation oncology, biology, physics. 2018;100(1):23-37.
190. Begg AC, Stewart FA, Vens C. Strategies to improve radiotherapy with targeted drugs. Nature reviews Cancer. 2011;11(4):239-53.
191. Speers C, Pierce LJ. Molecular Signatures of Radiation Response in Breast Cancer: Towards Personalized Decision-Making in Radiation Treatment. International journal of breast cancer. 2017;2017:4279724.
192. Rycaj K, Tang DG. Cancer stem cells and radioresistance. International journal of radiation biology. 2014;90(8):615-21.
193. Wang Y, Li W, Patel SS, Cong J, Zhang N, Sabbatino F, et al. Blocking the formation of radiation-induced breast cancer stem cells. Oncotarget. 2014;5(11):3743-55.
194. Shimura T, Kobayashi J, Komatsu K, Kunugita N. DNA damage signaling guards against perturbation of cyclin D1 expression triggered by low-dose long-term fractionated radiation. Oncogenesis. 2014;3:e132.
195. Andrade D, Mehta M, Griffith J, Panneerselvam J, Srivastava A, Kim TD, et al. YAP1 inhibition radiosensitizes triple negative breast cancer cells by targeting the DNA damage response and cell survival pathways. Oncotarget. 2017;8(58):98495-508.

196. Dean JL, McClendon AK, Knudsen ES. Modification of the DNA damage response by therapeutic CDK4/6 inhibition. The Journal of biological chemistry. 2012;287(34):29075-87.
197. Li JY, Li YY, Jin W, Yang Q, Shao ZM, Tian XS. ABT-737 reverses the acquired radioresistance of breast cancer cells by targeting Bcl-2 and Bcl-xL. Journal of experimental & clinical cancer research : CR. 2012;31:102.
198. Dillon MT, Good JS, Harrington KJ. Selective targeting of the G2/M cell cycle checkpoint to improve the therapeutic index of radiotherapy. Clinical oncology. 2014;26(5):257-65.
199. Wiegmans AP, Al-Ejeh F, Chee N, Yap PY, Gorski JJ, Da Silva L, et al. Rad51 supports triple negative breast cancer metastasis. Oncotarget. 2014;5(10):3261-72.
200. Yu M, Lin Y, Zhou Y, Jin H, Hou B, Wu Z, et al. MiR-144 suppresses cell proliferation, migration, and invasion in hepatocellular carcinoma by targeting SMAD4. OncoTargets and therapy. 2016;9:4705-14.
201. Zhang P, Wei Y, Wang L, Debeb BG, Yuan Y, Zhang J, et al. ATM-mediated stabilization of ZEB1 promotes DNA damage response and radioresistance through CHK1. Nature cell biology. 2014;16(9):864-75.
202. Sun Q, Liu T, Yuan Y, Guo Z, Xie G, Du S, et al. MiR-200c inhibits autophagy and enhances radiosensitivity in breast cancer cells by targeting UBQLN1. International journal of cancer Journal international du cancer. 2015;136(5):1003-12.
203. Yu ZC, Huang YF, Shieh SY. Requirement for human Mps1/TTK in oxidative DNA damage repair and cell survival through MDM2 phosphorylation. Nucleic acids research. 2016;44(3):1133-50.
204. Mah LJ, El-Osta A, Karagiannis TC. gammaH2AX: a sensitive molecular marker of DNA damage and repair. Leukemia. 2010;24(4):679-86.
205. Jiang Y, Liu Y, Hu H. Studies on DNA Damage Repair and Precision Radiotherapy for Breast Cancer. Advances in experimental medicine and biology. 2017;1026:105-23.
206. Boyages J. Radiation therapy and early breast cancer: current controversies. The Medical journal of Australia. 2017;207(5):216-22.
207. Jin Y, Xu K, Chen Q, Wang B, Pan J, Huang S, et al. Simvastatin inhibits the development of radioresistant esophageal cancer cells by increasing the radiosensitivity and reversing EMT process via the PTEN-PI3K/AKT pathway. Experimental cell research. 2018;362(2):362-9.
208. Song N, Jing W, Li C, Bai M, Cheng Y, Li H, et al. ZEB1 inhibition sensitizes cells to the ATR inhibitor VE-821 by abrogating epithelial-mesenchymal transition and enhancing DNA damage. Cell cycle. 2017:1-32.
209. Koo T, Cho BJ, Kim DH, Park JM, Choi EJ, Kim HH, et al. MicroRNA-200c increases radiosensitivity of human cancer cells with activated EGFR-associated signaling. Oncotarget. 2017;8(39):65457-68.
210. Bijnsdorp IV, Hodzic J, Lagerweij T, Westerman B, Krijgsman O, Broeke J, et al. miR-129-3p controls centrosome number in metastatic prostate cancer cells by repressing CP110. Oncotarget. 2016;7(13):16676-87.
211. Vargas-Hurtado D, Basto R. When E-cadherin is away, centrosomes can play. The Journal of cell biology. 2018;217(1):11-3.

212. Ando J, Sugimoto K, Tamayose K, Sasaki M, Ando M, Oshimi K. Changes in cell morphology and cytoskeletal organization are induced by human mitotic checkpoint gene, Bub1. Biochemical and biophysical research communications. 2008;365(4):691-7.
213. Bollong MJ, Pietila M, Pearson AD, Sarkar TR, Ahmad I, Soundararajan R, et al. A vimentin binding small molecule leads to mitotic disruption in mesenchymal cancers. Proceedings of the National Academy of Sciences of the United States of America. 2017;114(46):E9903-E12.
214. Xu W, Liu H, Liu ZG, Wang HS, Zhang F, Wang H, et al. Histone deacetylase inhibitors upregulate Snail via Smad2/3 phosphorylation and stabilization of Snail to promote metastasis of hepatoma cells. Cancer letters. 2018;420:1-13.
215. Lin J, Liu C, Gao F, Mitchel RE, Zhao L, Yang Y, et al. miR-200c enhances radiosensitivity of human breast cancer cells. Journal of cellular biochemistry. 2013;114(3):606-15.
216. Shah A, Bloomquist E, Tang S, Fu W, Bi Y, Liu Q, et al. FDA Approval: Ribociclib for the Treatment of Postmenopausal Women with Hormone Receptor-Positive, HER2-Negative Advanced or Metastatic Breast Cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2018.