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EDUCATION

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Projects:

Tristetraprolin induces an antitumorigenic phenotype in triple negative breast cancer (TNBC) via a novel non-canonical mechanism

- Performed functional and cell biology assays to assess the phenotypic impact of tristetraprolin in triple-negative breast cancer (TNBC)
- Identified tissue-specific expression patterns of TTP across 25 cancer types (TCGA) and matched healthy tissues (GTEx) using R scripts
- Investigated key pathways and gene networks altered by tristetraprolin re-expression through RNA-sequencing and Gene Set Enrichment Analysis (GSEA)
- Supervised two Nathan Schnaper Intern Program students in molecular biology and tissue culture experiments

Excitation/contraction-coupling gene expression is decreased in the muscle of mice with Huntington's Disease

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- Vickroy Scholarship recipient, Lebanon Valley College – 2015-2018
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PUBLICATIONS

1. **Rutherford JL**, Stemberger MB, Mahmud R, Ross CR, Lee NC, Low AS, Winter A, Patrón Fidalgo GM, White EJJ, Wilson GM. Tristetraprolin induces antitumorigenic phenotypes in TNBC cell models via a novel non-canonical mechanism. *Submitted for review.*
2. Miranda DR, Burke SRA, Kamalabadi PHM, **Rutherford JL**, Kamau J, Bibollet H, Talmadge RJ, Wilson GM, Singh A, Bahn V, Ren H, Hernandez-Ochoa EO, Voss AA. Integrated Effects of Mutant Huntingtin Expression on Action Potentials and Calcium Release in Skeletal Muscle. *Submitted for review.*
3. Roy BG, **Rutherford JL**, Weaver AE, Beaver K, Rasmussen M. A Self-Powered Biosensor for the Detection of Glutathione. *Biosensors*. 2020;10(9). PubMed PMID: 32899114

4. McLaughlin LJ, Stojanovic L, Kogan AA, **Rutherford JL**, Choi EY, Yen RWC, Xia L, Zou Y, Lapidus RG, Baylin SB, Topper MJ, Rassool FV. Pharmacologic induction of innate immune signaling directly drives homologous recombination deficiency. *Proc Natl Acad Sci U S A*. 2020;117(30). PubMed PMID: 32651270
5. VandeZande GR, Olvany JM, **Rutherford JL**, Rasmussen M. Enzyme Immobilization and Mediation with Osmium Redox Polymers. In: Minteer SD, editor. *Enzyme Stabilization and Immobilization*. New York, USA: Springer; 2017. p. 165–79. PubMed PMID: 27770421

PRESENTATIONS

1. **Rutherford JL**, Stemberger MB, Mahmud R, Ross CR, White EJF, Wilson GM. “Tristetraprolin induces an antitumorigenic phenotype in TNBC via a novel non-canonical mechanism.” Biochemistry Department Annual Retreat. University of Maryland Baltimore, Baltimore, MD. January 10, 2025.
2. **Rutherford JL**, Stemberger MB, Mahmud R, Ross CR, White EJF, Wilson GM. “Tristetraprolin induces an antitumorigenic phenotype in TNBC via a novel non-canonical mechanism.” Cancer Biology Research Retreat. University of Maryland Baltimore, Baltimore, MD. June 6, 2024.
3. **Rutherford JL**, Stemberger MB, Mahmud R, Ross CR, White EJF, Wilson GM. “Tristetraprolin induces an antitumorigenic phenotype in TNBC via a novel non-canonical mechanism.” American Association of Cancer Research Annual Meeting. San Diego, CA. April 9, 2024.
4. **Rutherford JL**, Stemberger MB, Mahmud R, Ross CR, White EJF, Wilson GM. “Tristetraprolin induces an antitumorigenic phenotype in TNBC via a novel non-canonical mechanism.” Biochemistry and Molecular Biology Departmental Retreat. University of Maryland Baltimore, Baltimore, MD. January 12, 2024.
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5. **Rutherford JL**, Stemberger MB, Mahmud R, Wilson GM. “Tristetraprolin induces an antiproliferative phenotype in TNBC via a novel non-canonical mechanism.” Graduate Research Conference. University of Maryland Baltimore, Baltimore, MD. March 31, 2023.
Selected for an oral presentation
6. **Rutherford JL**, Stemberger MB, Mahmud R, Wilson GM. “Non-canonical role of tristetraprolin on triple negative breast cancer cell proliferation.” Biochemistry and Molecular Biology Annual Departmental Retreat. University of Maryland Baltimore, Baltimore, MD. January 13, 2023.
7. **Rutherford JL**, McLaughlin LJ, Topper MJ, Carter-Cooper B, Lapidus RG, Baylin SB, Rassool FV. “Pharmacologic induction of innate immune signaling drives metastasis suppression in triple-negative breast cancer.” Cancer Biology Research Retreat. University of Maryland Baltimore, Baltimore, MD. June 8, 2021.
8. **Rutherford J**, Roy B, Rasmussen M. “Self-Powered Enzymatic Biosensor for Detection of Glutathione in Serum.” Inquiry Symposium, Lebanon Valley College, April 26, 2018.
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10. **Rutherford J**, VandeZande G, Rasmussen M. “Self-Powered Enzymatic Biosensor for Simultaneous Detection of Two Biomarkers of Parkinson’s Disease.” Inquiry Symposium, Lebanon Valley College, April 27, 2017
11. VandeZande G, **Rutherford J**, Rasmussen M. “Self-Powered Enzymatic Biosensor for Simultaneous Detection of Two Biomarkers of Parkinson’s Disease.” ACS National Meeting, Philadelphia, PA, August 22, 2016.

ABSTRACT

Title of Dissertation: Beyond mRNA Decay: Novel Antitumor Mechanisms of Tristetraprolin in Triple Negative Breast Cancer

Julia Rutherford, Doctor of Philosophy, 2025

Dissertation directed by: Dr. Gerald Wilson, Professor and Chair, Department of Biochemistry and Molecular Biology

The leading cause of mortality in patients with triple negative breast cancer (TNBC) is metastatic disease, due in large part to the absence of effective targeted therapies, reflecting substantial disease heterogeneity. Consequently, there is a critical need to identify novel molecular mechanisms that can be leveraged to suppress TNBC progression and improve patient survival. Tristetraprolin (TTP) is an RNA-binding protein that recognizes AU-rich elements (AREs) in the 3' untranslated regions (UTRs) of select mRNAs and promotes their rapid degradation, including many encoding proteins that contribute to cancer-related processes. Loss of TTP expression has been correlated with more aggressive disease and reduced overall survival in patients, implicating TTP as a tumor suppressor across multiple cancer types, including breast cancer. To discover how TTP modulates tumorigenic phenotypes in advanced breast cancer, we generated stable cell lines expressing FLAG-tagged TTP in three highly aggressive and metastatic TNBC models. Transcriptomic profiling by RNA sequencing revealed that TTP broadly altered gene expression patterns across all three cell lines. Gene Set Enrichment Analysis (GSEA) further demonstrated that TTP significantly suppressed pathways involved in cell growth, metastasis, and stemness. Functional assays revealed that TTP robustly suppressed cell proliferation, stem cell

frequency, migration, and invasiveness *in vitro*. Consistent with these findings, *in vivo* experiments showed that TTP expression significantly reduced tumor growth. We next sought to identify the mechanism underlying the anti-proliferative effect of TTP. Surprisingly, actinomycin D experiments demonstrated that TTP did not alter the decay kinetics of several well-characterized TTP target mRNAs, indicating that its tumor-suppressive activity in TNBC cells may occur independently of RNA destabilization. To directly test this, we engineered TNBC cell lines expressing an RNA-binding-deficient TTP mutant (C147R). Remarkably, this mutant recapitulated the suppression of all *in vitro* and *in vivo* tumor phenotypes observed with wild-type TTP. Together, these findings demonstrate that TTP exerts potent tumor-suppressive effects in TNBC cell models, inhibiting multiple oncogenic phenotypes and significantly attenuating tumor growth *in vivo*. Importantly, these effects occur through a mechanism that is independent of TTP's canonical RNA-binding and RNA-destabilizing functions, revealing a previously unrecognized mode of TTP-mediated tumor suppression.

Beyond mRNA Decay: Novel Antitumor Mechanisms of Tristetraprolin in Triple
Negative Breast Cancer

by
Julia L. Rutherford

Dissertation submitted to the Faculty of the School of Graduate Studies of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2025

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Dedication

In the words of Leslie Knope, main character of the NBC sitcom Parks and Recreation, “It’s a lesson I have learned over and over again, but it bears repeating: No one achieves anything alone.” This thesis is dedicated to my family, friends, and community, as it was only with their support and strength that I completed this dissertation and earned my degree.

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Mahmud, contributed to this project before my time, and I am grateful for the work they did. Thank you also to my summer interns, Anne-Fleur Winter and Noa Deutsch, who taught me a lot about teaching and added levity to my days in the lab.

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A huge thank you goes out to all of the friends who have been with me through the past 7 years of this journey! Thank you Hyla Howe, Rebecca Pierpont, and Dr. Ebeth Drda, for all the amazing memories; I cherish our friendships so much. Thank you to my former lab mates, Dr. Rachel Abbotts, Dr. Anna Dellomo (and honorary lab mate Matt Castanga!) who were always down for foodie adventures and science troubleshooting. Special thanks to all my amazing Baltimore friends, especially Ella Brunsting, Amara Ejikemeuwa, Julie Leon, and Colleen McMullen (and Minnie!). I am so grateful to all of my friends for having so much fun in the city (and beyond) with me, and for their support when I needed it most.

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List of Abbreviations

231td	Tumor-derived MDA-MB-231	G418	G418 sulfate
ABP	ARE-binding protein	GSEA	Gene set enrichment analysis
actD	Actinomycin D	GTE _x	Gene-Tissue Expression
ADC	Antibody-drug conjugate	H	Histidine
AGO	Argonaute	HER2	Human epidermal growth factor
APA	Alternative polyadenylation	hygB	Hygromycin B
ARE	AU-rich element	ICI	Immune checkpoint inhibition
AUF1	ARE/poly(U)- binding/degradation factor 1	IDC	Invasive ductal carcinoma
BCA	Bicinchoninic assay	ILC	Invasive lobular carcinoma
C	Cysteine	iNOS	Inducible nitric oxide synthase
DCIS	Ductal carcinoma in situ	Inr	Initiator element
DOX	Doxycycline	KH	K homology
DTT	Dithiothreitol	KSRP	KH-type splicing regulatory protein
ECAR	Extracellular acidification rate	lncRNA	long non-coding RNA
eCLIP	Enhanced crosslinking immunoprecipitation	MDSC	Myeloid-derived suppressor cells
ECM	Extracellular matrix	miRNA	microRNA
eIF	Eukaryotic initiation factor	MK2	p38/MAPKAP kinase 2
ELDA	Extreme limiting dilution analysis	MSK1/2	Mitogen- and stress- activated kinase 1/2
ER	Estrogen receptor	N7	Nitrogen-7
EV	Empty vector	NES	Normalized enrichment score

OCR	Oxygen consumption rate	SERM	Selective estrogen receptor modulator
OS	Overall survival	SMD	Staufen-mediated decay
PABP	Poly(A) binding protein	STAU-1	Staufen-1
PARP	Poly-ADP ribose polymerase	t1/2	Half-life
PAS	Poly(A) signal	TAM	Tumor-associated macrophage
PBS-T	PBS containing 0.05% Tween-20	TCGA	The Cancer Genome Atlas
PDAC	Pancreatic ductal carcinoma	TME	Tumor microenvironment
PFS	Progression free survival	TNBC	Triple negative breast cancer
PR	Progesterone receptor	TPA	12-O-tetradecanoylphorbol-13-acetate
PYK2	Protein tyrosine kinase 2 β	TTP	Tristetraprolin
RBP	RNA-binding protein	UMGCCC	University of Maryland Greenebaum Comprehensive Cancer Center
RFS	Relapse-free survival	UTR	Untranslated region
RIPA	Radioimmunoprecipitation assay	ZnF	Zinc finger
RISC	RNA-induced silencing complex		
RNase	Ribonuclease		
RRM	RNA recognition motif		
RTCA	Real-time cell analysis		
RT-qPCR	Reverse transcription quantitative polymerase chain reaction		

Chapter 1: Introduction

1.1 Molecular features, subtypes, and treatments of breast cancer

Though breast cancer is a disease that predominantly affects women, it is the most diagnosed cancer in the United States. Through decades of research, novel targeted therapies and increased early screening methods have improved the overall survival rate to around 90%. However, breast cancer is still the fourth highest cancer-related cause of mortality, with approximately 42,250 deaths predicted in the United States for 2024 (1). Carcinomas of the breast arise from the two epithelial tissues present in the breast, lobules and ducts. Lobular tissue is a glandular tissue responsible for milk production, while ductal tissue forms the thin tubes of the milk ducts, responsible for the transportation of milk through the breast (Figure 1.1) (2). Cells that face the inside of the ducts and lobules, or lumen, are called luminal cells, which can be identified by the expression of keratins 8/18. Cells that attach to the extracellular matrix (ECM) are known as basal cells, which in the

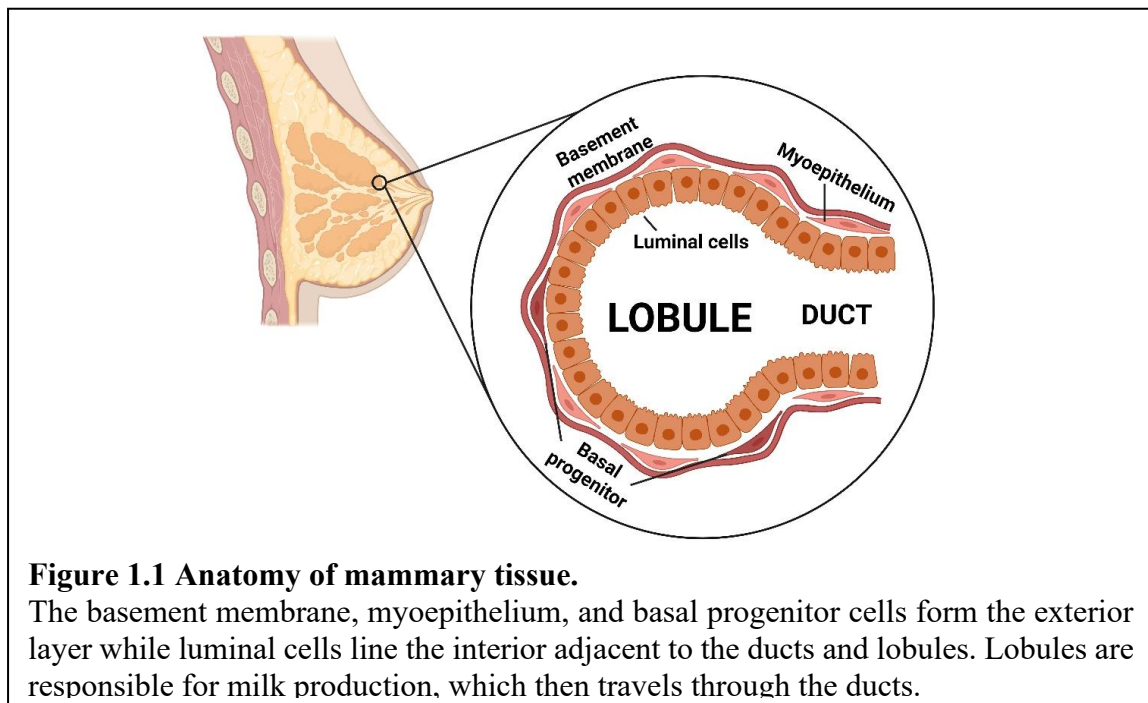


Figure 1.1 Anatomy of mammary tissue.

The basement membrane, myoepithelium, and basal progenitor cells form the exterior layer while luminal cells line the interior adjacent to the ducts and lobules. Lobules are responsible for milk production, which then travels through the ducts.

breast are made up of myoepithelial cells and stain positive for keratins 5/6 (3). This differential staining allows for the identification of the respective cell types. Interspersed among these two layers is a second small subpopulation of basal cells, made up of stem cells and immature progenitor cells, which also stain positive for keratin 5/6 (4). Basal myoepithelial cells do not form tumors; therefore, the current hypothesis is that basal breast cancers arise from the small subset of stem cells and potentially immature progenitors (5). In support of this, several groups have profiled both mammary stem cells and basal breast cancers and observed similar gene expression patterns between the two (6,7). Research has described luminal breast cancers as being derived from luminal progenitor cells, and basal cancers as being derived from stem cells (5). However, recent research has presented a competing hypothesis that all breast cancers originate from luminal progenitors, which can sometimes de-differentiate to acquire the basal phenotype and become more stem cell-like in nature (8).

Ductal carcinoma in situ (DCIS) is the earliest form of breast neoplasia, and, as it is localized to the inside of the ducts without invading through the wall of the duct, has the highest survival rate. Due to the development of advanced screening for breast cancer, DCIS now accounts for 25% of newly diagnosed breast cancers. Current treatment guidelines recommend that DCIS tumors be removed surgically, through either breast-conserving surgery or mastectomy, depending on the size of the lesion. Adjuvant therapy is prescribed to only a small subset of patients and usually consists of hormone therapy or radiation (9). Once tumor cells have invaded into nearby tissues through the walls of the ducts, this is referred to as invasive ductal carcinoma (IDC), which is the most common type of invasive breast cancer. Invasive cancers also arise in the lobular tissue, termed

invasive lobular carcinoma (ILC), however this cancer is less common. Together these cancers make up the majority of breast cancer cases, though rare subtypes exist, which have been thoroughly reviewed by Weigelt and Reis-Filho (10). The standard of care treatment regimen for these early-stage breast cancers is surgical resection of the tumor and any involved lymph nodes. Depending on the size of the tumor, neoadjuvant therapy may be recommended, which consists of a sequence of anthracycline-taxane chemotherapy or radiation therapy, both of which serve to shrink the tumor volume, leading to improved surgical outcomes. Based on the risk of recurrence, patients are prescribed adjuvant chemotherapy and applicable targeted treatments (11). To help clinicians evaluate patient risk of recurrence and guide clinical treatment practice, several biomarker-based tools have been developed, such as *Oncotype DX* and *TAILORx* (12).

While the goal of early-stage breast cancer is to eliminate the tumor entirely, the treatment goal changes when a patient is diagnosed with metastatic breast cancer, which is virtually incurable. Current treatment goals for metastatic disease include prolongation of life and symptom palliation (13). Many approaches have been developed over the past decades to target the primary tumor, but the deeper issue has continued to be the cells that remain or escape to form metastases. Though originally thought to be a late event in cancer development, more recent studies have revealed that metastasis-initiating cells break off from the primary tumor early in cancer development (14,15), emphasizing the need for novel therapeutic strategies that reach and attack not only the primary tumor, but also circulating tumor cells and growing tumor colonies in secondary locations. In the primary tumor microenvironment (TME), increased expression and secretion of ECM proteins leads to the formation of a stiffer matrix, which allows tumor cells to migrate more easily.

Tumor-associated macrophages play a key role in the early stages of metastasis, by enhancing tumor cell motility and promoting angiogenesis (16). Additionally, tumors can preemptively induce the formation of favorable microenvironments in distant locations, or premetastatic niches, to supplement metastasis (17). The first study to provide direct evidence of a premetastatic niche showed that tumor cells influence bone marrow-derived cells to migrate to distant locations to attract and support circulating tumor cells (18), and many supporting studies have followed (19–21). The discovery of the premetastatic niche presents new opportunities for therapeutic intervention to combat its development in ways that decrease metastasis formation.

1.1.1 Targeted treatments for breast cancer subtypes

Where applicable, targeted therapies for breast cancer are often more effective, and better tolerated, than chemotherapy and radiation. These therapies are based on the molecular and clinical profile of the tumor, which can be subdivided into four categories (Figure 1.2). Based on these molecular subtypes, recent research now classifies breast cancer as not one overarching disease, but rather several heterogeneous cancers.

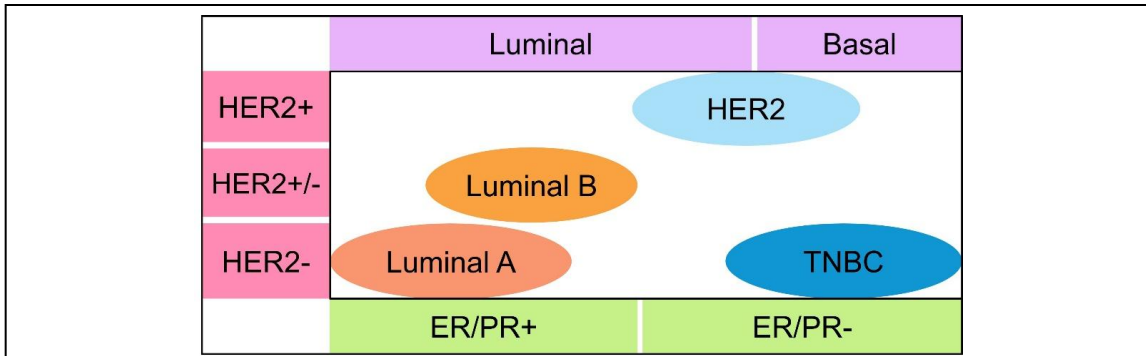


Figure 1.2 Clinical subtypes of breast cancer characterized by their molecular features.

Luminal A breast cancers arise from luminal tissue, are ER/PR+, and HER2-. Luminal B breast cancers arise from luminal tissue, are ER/PR+, are either HER2+/-, and are often marked by a high proliferation rate. HER2+ cancers can be either luminal or basal in origin, are ER/PR-, and must have HER2 expression. Approximately 25% and 75% of TNBC cancers are luminal and basal, respectively. TNBCs are ER/PR/HER2-.

The luminal breast cancers, comprised of the luminal A and B subtypes, make up around two-thirds of all breast cancers (22) and are often combined into one subtype. Luminal tumors are characterized by expression of both the estrogen receptor (ER) and progesterone receptor (PR) and have a 4-year survival rate of around 90% (23). Luminal A tumors lack the human epidermal growth factor receptor 2 (HER2). Additionally, Luminal A breast cancers are characterized by low expression of Ki67, a marker of proliferation. To be characterized as Luminal B however, the tumor must either express high levels of HER2, or have high expression of Ki67, indicating a high proliferation rate. Luminal B breast cancers with HER2 positivity are sometimes called triple positive breast cancer (ER/PR/HER2+). These triple-positive breast cancers are more aggressive but still have a 4-year survival rate of 90% (23).

Treatment for hormone positive breast cancers involves targeted endocrine therapy, including selective estrogen receptor modulators (SERM, *e.g.*, tamoxifen) and aromatase inhibitors. SERMs are most prescribed to pre-menopausal women, as they block the

binding of estrogen to the ER. Aromatase inhibitors, which block the production of estrogen and effectively induce menopause, are more commonly prescribed to women who are post-menopausal. Virtually all patients are prescribed adjuvant endocrine therapy; tamoxifen taken for five years after diagnosis decreases a patient's risk of cancer relapse by 50% (13). In January 2025, the FDA approved a new antibody-drug conjugate (ADC), datopotamab deruxtecan, which is indicated for HR+/HER2- breast cancers. The antibody targets Trop-2, a transmembrane calcium signal transducer that is highly expressed in more than 90% of breast cancers, and is combined with deruxtecan, a potent topoisomerase I inhibitor (24,25).

As the name suggests, HER2 breast cancers have high expression of the HER2/ERBB2 protein, and/or amplification of the *ERBB2* gene, which occurs in 15-25% of breast cancers (26). However, HER2 cancers can be either basal or luminal in origin and do not express the estrogen or progesterone receptor, differentiating them from the Luminal B/triple-positive subtype (27). These tumors comprise one of the most high-risk subtypes of breast cancer, but the advent of the HER2 monoclonal antibody therapy, trastuzumab, and subsequent derivatives, has led to huge increases in both overall and disease-free survival. The first clinical study of trastuzumab reported that one year of trastuzumab treatment after primary therapy reduced the rate of tumor recurrence by 50% (26), leading to FDA approval of this drug as an adjuvant treatment for HER2 early breast cancer, as well as a first-line treatment for metastatic HER2 breast cancer (28). Trastuzumab can also be used as the antibody component for an ADC, which delivers deruxtecan to cells expressing high levels of HER2 (29).

Unlike the other three subtypes, triple negative breast cancer (TNBC) is unique because it does not express the estrogen and progesterone receptors and has low expression of the HER2 protein. TNBC is actually the second most common breast cancer, after Luminal A breast cancer (1). The cells that give rise to TNBC tumors are most often basal or stem-like, with 80% of cases presenting as such, but in rare cases can arise from luminal tissues (30). Thus, the name TNBC is often interchanged with basal breast cancer, though they are two distinct conditions. Patients diagnosed with TNBC have a lower age at diagnosis than other breast cancer patients and are more likely to experience a short duration of response to therapy and high likelihood of distant recurrence. Of these patients, those with metastatic disease have an extremely low survival rate, with a median overall survival (OS) of 9-13 months (31,32). Race plays an important, yet understudied, role in TNBC, as black women are more likely than white women to be diagnosed with TNBC (33,34). This combination of an aggressive stem-like subtype with a lack of targetable receptors creates a major challenge to both the development and utilization of effective therapies, resulting in the lowest survival rates compared to the other subtypes (Table 1.1).

5-Year Relative Survival Rate, SEER Combined Summary Stage			
Subtype	Localized	Regional	Distant
ER/PR+ and HER2-	100%	90.8%	36.5%
ER/PR+ and HER2+	99.5%	91.0%	46.7%
HER2+	97.7%	85.4%	40.8%
TNBC	92.4%	67.5%	14.9%

Table 1.1 5-year survival rates of breast cancer across different stages. Localized refers to tumors that are contained to mammary tissue, regional indicates that tumors have invaded locally through the basement membrane into surrounding tissues, distant refers to metastatic spread to a secondary organ.

1.2 Advances in TNBC treatment options and subsequent challenges

While patients diagnosed with the other three breast cancer subtypes have the benefit of targeted therapies that have been developed for identified receptors, patients diagnosed with TNBC often rely on the standard treatment regimen of chemotherapy, radiation, and surgery. Development of these original therapies occurred from the late 1880's through the mid-1900's. Though subsequent iterations of these drugs and treatment modalities have been developed, they are still remarkably like the original therapies, thus are still very invasive, often ineffective, and poorly tolerated by patients (13). Patients who are eligible for hormone or HER2+ therapies often use these three basic treatment options in combination with targeted therapies (35), and targeted therapies that prove effective in one subtype of breast cancer are often expanded to other subtypes, suggesting that further development of novel TNBC therapies could also have increased benefit to the overall breast cancer patient population.

In an effort to identify novel molecular targets and inform new treatment options, Lehmann et al. used gene expression profiles and cluster analysis to further divide TNBC into six subclasses: Basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen receptor (36). These six subclasses were later revised, after identification of contaminating immune and stromal cells in the tumor samples, into four subclasses: basal-like 1 and 2, mesenchymal, and luminal androgen receptor (37). Overall, this research emphasizes the heterogeneity of TNBC tumors, which has been and continues to be the biggest challenge to targeted therapy development and underscores the need for personalized medicine, or perhaps the identification of a regulatory node common in many breast cancers and TNBCs.

One notable exception to this is the development of poly-ADP ribose polymerase (PARP) inhibitors, which are indicated for TNBC, HER2- BC, and several other cancers that have homologous recombination deficiencies, usually from the loss of one or both BRCA proteins (BRCA1/2) that are involved in the repair of double strand DNA breaks that develop naturally as cells grow and divide. When cells lose these proteins, their DNA has a higher chance of mutating, which often results in the formation of tumors (38). People with BRCA1 mutations have a 55-70% chance of developing breast cancer and around 40% chance of ovarian cancer by age 70, while BRCA2 mutations confer a 45-70% and 11-17% lifetime risk of developing breast and ovarian cancers, respectively (39–41). The loss of these proteins allows cells to bypass the checks on DNA integrity, allowing the cells to grow faster, a characteristic that is preferred by tumor cells. However, the loss of these proteins means that the cells must rely solely on other DNA repair proteins, such as PARP. Using PARP inhibitors to target PARP often results in the formation of catastrophic DNA damage lesions that result in cell death, a concept defined as synthetic lethality (38). To date, two PARP inhibitors, Olaparib and Talazoparib (42), have been FDA-approved for metastatic TNBC with BRCA1/2 mutations.

Development of the ADC, sacituzumab govitecan, combines a Trop-2 antibody directing the conjugate to neoplastic cells with the active metabolite of the topoisomerase I inhibitor, irinotecan, which is a chemotherapeutic drug. Topoisomerase inhibitors have historically proven to be too toxic for patients, but the addition of the Trop-2 antibody has led to its success in clinical trials (43,44). Progression free survival (PFS) for patients receiving sacituzumab govitecan increased from 1.7 to 5.6 months, while OS increased from approximately 7 to 12 months, compared to standard chemotherapy regimens.

Treatment with sacituzumab govitecan is FDA-approved for patients with advanced TNBC that have received two or more systemic therapies, with at least one for metastatic disease (45). Particularly for patients whose tumors have been heavily pre-treated, this therapy provides clinicians with an additional therapeutic option, though the survival benefits are limited to months.

One of the breakthrough cancer therapies of past decade has been immune checkpoint inhibition (ICI). Initially developed and tested in melanoma (46), pembrolizumab, a selective, monoclonal anti-PD-1 antibody, has shown efficacy and is FDA-approved for advanced TNBC, encompassing locally advanced, unresectable or metastatic tumors, as well as early-stage high-risk TNBC (47–50). Pembrolizumab acts by blocking the binding of the PD-1 receptor, which is present in activated CD8+ and CD4+ T cells, as well as B cells, to its ligand, PD-L1, which is often expressed by tumor cells as a mechanism of immune evasion. The inhibition of this immunosuppressive interaction reactivates these cytotoxic immune cells to attack the tumors, inducing programmed cell death (51). When used in combination with chemotherapy, which induces cell death in the tumors, various tumor-associated antigens are released, and newly re-activated immune cells are drawn to the tumor microenvironment to further induce tumor cell killing (52). In a clinical trial of advanced TNBC, pembrolizumab in combination with chemotherapy increased OS from 16 to 23 months (47). The efficacy of this combination therapy was also tested in early-stage, high-risk TNBC, where patients, especially those with high PD-L1 expression, receiving combination therapy had a higher pathologic complete response compared to the placebo group (48).

Another monoclonal antibody, atezolizumab, was developed to target the PD-L1 ligand, instead of the PD-1 receptor. Atezolizumab in combination with paclitaxel in unresectable locally advanced or metastatic TNBC was recently investigated in the IMpassion130 clinical trial. The combination of atezolizumab with nab-paclitaxel, or paclitaxel bound to albumin protein, showed promising results with significant improvements in PFS (53), leading to accelerated FDA approval. However, a phase III study combining atezolizumab with conventional paclitaxel revealed that there was no appreciable difference in OS and PFS compared to standard chemotherapy regimens (54), culminating in the rescission of FDA approval. New clinical trials are trying to resurrect the use of atezolizumab, this time in combination with carboplatin, which has already shown early improvements in PFS and OS in patients with metastatic TNBC (55).

Though these treatment options constitute significant advances to the field, there are still major challenges to resolve. While patients utilizing these therapies have significantly higher survival rates, the length of survival improves only by months, not years. Though targeted therapy and ICIs can be extremely effective, one of the major challenges is that this efficacy is limited to a small subset of patients. For example, BRCA1/2 mutations are only found in up to 31% of TNBC patients (56), and patients who respond to pembrolizumab typically have a PD-L1 combined positive score higher than 10, which is less than half of TNBC patients (48). One way to combat this is the utilization of combination therapies. However, while various combinations of ICIs and chemotherapies have been tested in clinical trials, many studies exhibited a lack of benefit compared to standard regimens (52). For example, CTLA-4 is another essential immune receptor located on T cells, for which a monoclonal antibody has been developed. There have been

several clinical trials testing the efficacy of this inhibitor in TNBC as well as BC, but so far, the lack of response and the development of severe immune-related adverse effects, a common issue across ICI therapy, have prevented its translation to the clinic (57). Another key challenge is the development of resistance. Resistance to PARPi therapy often develops when BRCA1/2 become reactivated, most commonly by secondary activating mutations (58). In the tumor microenvironment, the induction of adaptive immunity is one proposed mechanism for resistance to ICI. Cytokines that are secreted by tumor infiltrating lymphocytes drive expression of PD-L1, leading to overexpression on the cell surface, which overwhelms the newly reactivated immune cells, allowing for the re-induction of immunosuppression (59).

Though the cancer field has evolved dramatically since its inception, there is still great need for new therapies and perhaps even novel approaches to target both the primary tumor and secondary metastases.

1.3 Post-transcriptional regulation of gene expression

In 2000, Hanahan and Weinberg proposed a set of six phenotypic guidelines that regulate the development of neoplasia known as the hallmarks of cancer (60). Over the past 20 years, these hallmarks have been refined twice (61,62). These phenotypic hallmarks are often mechanistically controlled at the level of gene expression. Control of gene expression can happen at both the transcriptional and post-transcriptional levels. At the transcriptional level, transcription factors are a key modulator of gene expression. Because these proteins are capable of altering transcription, and thereby gene expression, they are often drivers of various disease states, especially cancer.

To advance the field of cancer therapeutics and clinical practice, it is important to identify and subsequently target regulatory nodes of these hallmarks that have so far been overlooked. One such node is mechanisms of post-transcriptional regulation. When analyzing gene expression, many studies solely ascribe steady-state mRNA levels to alterations in transcription, but do not consider transcript stability, when this is a co-equal factor in the regulation of mRNA levels (63). Furthermore, mRNA levels confer no information on possible regulation at translational and post-translational levels.

1.3.1 *Cis*-acting elements and *trans*-acting factors

Gene regulation at the RNA level is directed by regulatory *cis*-acting sequences and structural elements encoded in the mRNA. The most ubiquitous examples are the 5'-cap and 3' polyA tails of almost all mature mRNAs (Figure 1.3), which protect transcript termini from exonucleolytic degradation and enhance ribosome recruitment to initiate protein synthesis (64). While these are examples of global *cis*-acting RNA regulators, there are also many gene-specific regulatory sequences, which can be located throughout the



Figure 1.3 Structure of mRNA.

transcript. An example is the internal ribosome entry sites (IRES). These were first discovered in viral transcripts and are sequences that mediate non-canonical translation initiation. Viral mRNAs often lack the 5' methyl-guanosine cap structure, the primary mechanism by which translation is initiated, and require cap-independent translation for protein synthesis. Cap-independent translation through IRESes has also been shown for a

subset of eukaryotic mRNAs, mainly under conditions of stress, such as hypoxia or nutrient deprivation, and for specific mRNAs encoding proteins involved in the stress response (65,66). *Cis*-acting sequences can be regulated by a variety of nucleotidic and proteinaceous factors that influence gene expression. These *trans*-acting factors control all aspects of mRNA metabolism including stability, translation, localization, and even transcript identity (64). Examples of major RNA-targeted *cis-trans* regulatory mechanisms are described below.

1.3.2: RNA-based regulatory factors

Endogenous RNA-based regulatory factors were first recognized in the early 2000's (67), and include the non-coding microRNA (miRNA), and long non-coding RNA (lncRNA). Most miRNAs are generated by pre-RNA processing of primary transcripts by the ribonucleases (RNase) Drosha and Dicer (68). After endonucleolytic cleavage, one strand of the miRNA is loaded into the RNA-induced silencing complex (RISC), where it functions as a targeting system that directs the RISC complex to respective mRNA targets. The Argonaute (AGO) family of proteins are key components of the RISC complex that directly bind miRNAs. AGO2, the only AGO family member with an RNase domain, is the principal catalytic agent of RNA interference and gene silencing as part of the RISC complex. AGO proteins are also engaged by a second class of proteins, the GW182 proteins, which drive repression of protein synthesis (69). miRNAs are approximately 20 nucleotides long and form imperfect hybrids with sequences that are frequently located in the 3' UTR (70). Thus, when loaded into the RISC complex, base-pairing of miRNAs to mRNA can trigger mRNA destabilization initiated by endonucleolytic cleavage or

translational repression (71). Though most regulation by miRNA takes place in the cytoplasm, there are now examples of miRNA acting inside the nucleus to regulate pre-mRNA processing (72).

lncRNAs are defined as non-coding transcripts greater than 200 nucleotides in length. In the nucleus, lncRNAs are involved in transcriptional control by participating in chromatin remodeling and associating with enhancer regions (67). Post-transcriptionally, they can interact with various RNA-binding proteins (RBPs) to affect mRNA splicing and stabilization or interact with miRNAs to induce their sequestration and subsequent inhibition. For example, Staufen-1 (STAU-1) is recruited to Alu sequences in the 3' UTRs of some mRNA and initiates their degradation via a pathway called Staufen-mediated decay (SMD). As STAU-1 is a double-stranded RNA (dsRNA)-binding protein, Alu targeting in mRNAs often requires recruitment and base-pairing of complementary Alu-containing lncRNAs, which thus generate the dsRNA substrates for STAU-1 in SMD target transcripts, and direct their degradation (73).

1.3.3 RNA binding proteins

RBPs are the proteinaceous components of post-transcriptional regulation, and compared to other classes of proteins that regulate gene expression, like transcription factors, they are arguably more understudied and therefore are likely to contain untapped therapeutic potential. Current estimates put the number of RBPs present in human cells at over 4000, which is significantly higher than the amount of transcription factors in human cells. In the cell, RBPs impact almost all cellular processes. RBPs bind a variety of RNA targets, including non-coding RNA and mRNA.

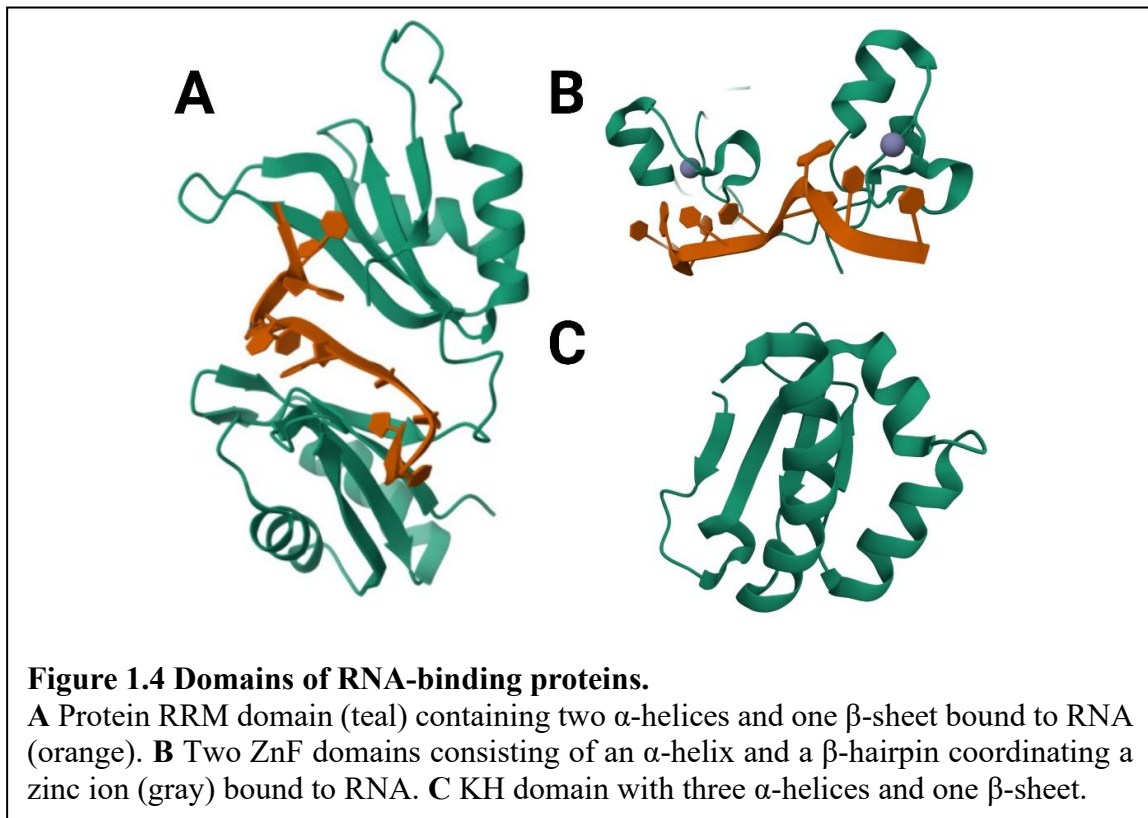
While all nucleic acid-binding proteins use common features such as positively charged and polar amino acid residues to bind substrates, specificity of a protein for RNA over DNA binding is based on structural differences between these two molecules. Since DNA and RNA form distinct double-stranded and single-stranded structures, respectively, the proteins that bind them often have structural differences leading to the preferential binding of one molecule over the other. While most DNA binding proteins are structured to bind the major or minor grooves of the DNA helix, RNA is synthesized as a single-stranded molecule, and as such can exist in a wide spectrum of unpaired and locally folded structures, such as stem-loops or bulges, to which RBPs must be able to accommodate or adapt (74). Another feature unique to RNA is the 2' hydroxyl group, responsible for the stabilization of around 20% of RNA-protein interactions (74).

1.3.3.a RNA-binding domains of RBPs

A wide variety of RNA-binding domains have been identified (75), though recent proteomic studies have identified hundreds of proteins that bind RNA but do not contain canonical RNA-binding domains (76). The most common of these domains is the RNA recognition motif (RRM). This motif forms a four-stranded anti-parallel β -sheet set against two α -helices, with RNA binding occurring in the two central β -strand domains, using π -stacking interactions between aromatic residues in the protein and the nitrogenous bases of the RNA (Figure 1.4). Relatively few RBPs use single RRMs, and for those with multiple RRMs, not all are required for binding. The use of more than one RRM increases binding affinity and target specificity. In contrast, K homology (KH) domains lack RNA-protein stacking interactions, and bind instead using hydrophobic interactions, leading to RNA

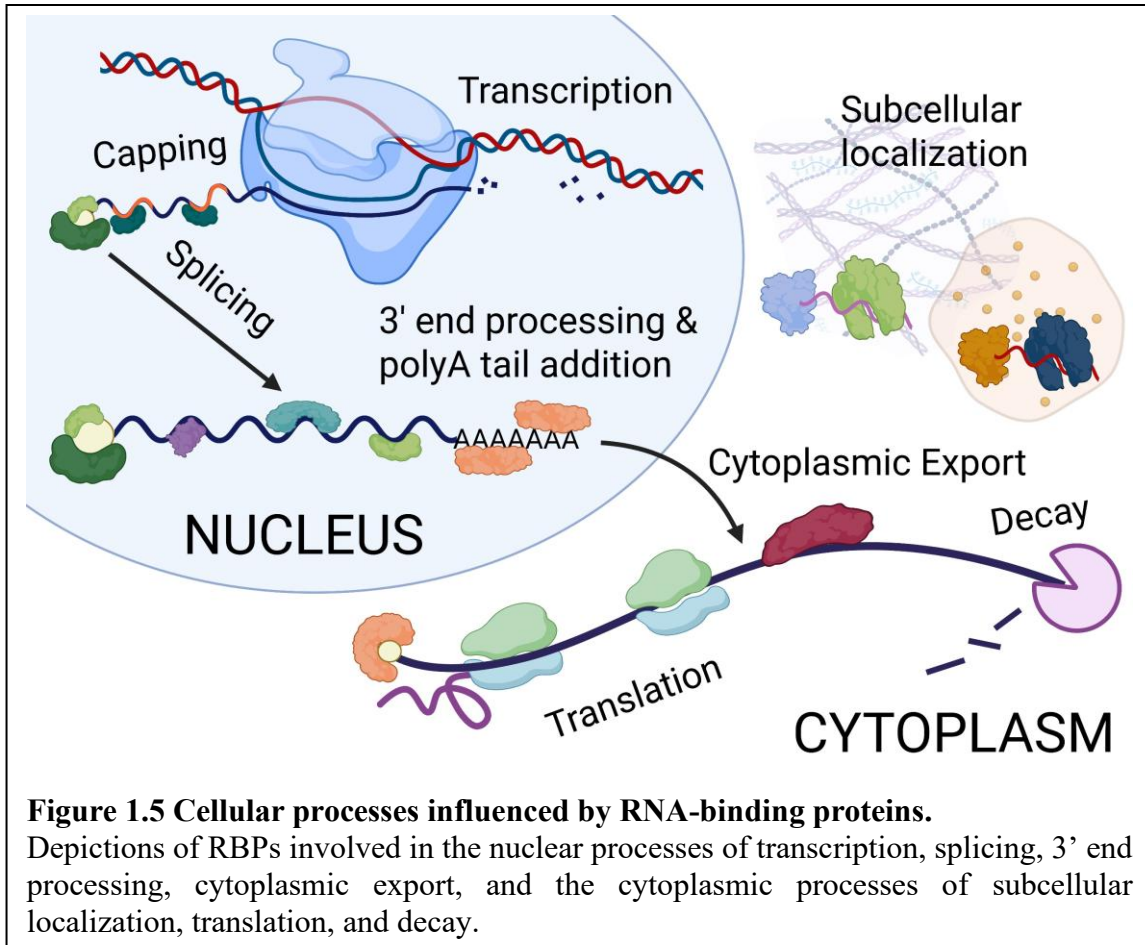
affinities in the micromolar range (Figure 1.4). To counter this, KH proteins utilize more than one KH domain or extend the domain by combination with another RNA binding domain when binding to target mRNAs (77).

A rare but important RNA-binding domain, the zinc finger (ZnF) domain is a key feature of the ZnF class of proteins, a diverse group containing members with distinct RNA- or DNA-binding abilities, as well as non-nucleotide factors such as proteins and small molecules (78). ZnF domains consist of a β -hairpin and an α -helix, which are responsible for the coordination of a zinc ion. ZnF domains bind zinc ions through coordinated interactions with four spaced cysteine (C) and/or histidine (H) residues, the order of which distinguishes each ZnF family: CCHH, CCHC, CCCC, or CCCH (75,77) (Figure 1.4).



1.3.4 Dysregulation of post-transcriptional mechanisms in cancer

There is clear evidence that a multitude of these post-transcriptional regulatory mechanisms are dysregulated in cancer. A single RBP can bind and post-transcriptionally regulate subsets of mRNAs that are involved in similar processes. This coordinated control of multiple mRNA targets by activation or inhibition of a common RBP is known as an RNA regulon (79). Furthermore, one *cis*-acting regulatory sequence can bind to multiple RBPs, often with disparate effects. Finally, transcript regulation can occur at multiple levels of post-transcriptional control, allowing cells to respond efficiently to changes in their environment (63). RNA-centric mechanisms of post-transcriptional gene regulation involve many functions, including assisting mRNAs in nuclear export to the cytoplasm, subcellular localization, and direct effects on translation. However, the most important functions involve effects on synthesis processes such as transcription, 3' end processing, and pre-mRNA splicing, as well as cytoplasmic turnover processes, or mRNA decay (Figure 1.5) (80).



1.3.4.a Splicing

Three essential sequences regulate the splicing of pre-mRNAs: the 5' splice site (GU), the 3' splice site (AG), and the branch point sequence, all of which exist within longer, partially conserved sequences. Since these sequences direct splicing of the vast majority of introns, they are known as the core splicing elements. Auxiliary sequences, such as splicing enhancers and silencers, play important roles in regulating these processes, but vary widely between different transcripts (81,82). Under normal conditions, nascent pre-mRNA transcripts undergo intronic splicing to form mature mRNAs. During the development of neoplasia, however, genetic mutations can disrupt normal splice sites or

introduce novel alternative splice junctions, leading to alternative splicing. Analysis of patient tumors across multiple cancers reveals that *TP53*, a key tumor suppressor gene, has an increased number of splice-site creating-mutations in tumor compared to normal tissue (83). Aberrantly spliced mRNAs can either be degraded by nonsense mediated decay, an RNA surveillance pathway that can silence tumor suppressor genes or generate novel transcripts that encode proteins with altered functions (84). Further, mutations in *trans*-acting factors, such as the proteins that mediate splicing, can affect the expression or sequence of a multitude of mRNAs. In glioblastoma, the activity of the splicing regulator MBNL1 is inhibited, which results in alternative splicing switching in multiple target genes. Restoration of MBNL1 activity inhibits stem cell renewal and prolongs survival in an *in vivo* mouse model (85).

1.3.4.b Cleavage and polyadenylation

One of the key steps of 3' pre-mRNA end processing is cleavage and polyadenylation, which involves recognition of the poly(A) signal (PAS), which directs cleavage of the 3' end and subsequent addition of approximately 200 adenosine residues to the 3' end (86). Alternative polyadenylation (APA) is the creation of multiple isoforms via cleavage at different PAS sites located within a transcript. Proximal sites are common in both the 3'UTR, as well as introns. In proliferating cells, early development, and cancer, the enhanced use of proximal sites and APA contributes to the expression of some unique protein isoforms, which in cancer are often oncogenic, and transcripts with variable 3' UTR lengths, which can alter expression of an encoded protein owing to the inclusion or absence of specific binding sites for *trans*-acting regulatory factors (87).

1.3.4.c Export to the cytoplasm

Many RBPs are involved in packaging RNAs and their subsequent nucleocytoplasmic transport. Export almost always involves movement of these RNA-protein complexes through the nuclear pore complex, so that they can be translated in the cytoplasm. Many types of RNA, such as tRNA, miRNA, and mRNA undergo export, which has been thoroughly reviewed by Sloan *et al* (88). There is some evidence that dysregulation of nucleocytoplasmic export plays a role in the development of neoplasia. Increased expression of eukaryotic initiation factor (eIF) 4E is seen in approximately 30% of cancers, and this contributes to decreased expression of RanBP2 and Nup214, components of the pore complex, which are thought to enhance flux through this portal. One consequence of this is an increased abundance of eIF4E targets, including oncogenic mRNAs like *MYC*, *CCND1*, *NBS1*, and *HDM2* in the cytoplasm (89).

1.3.4.d Translation

Cis-acting mRNA elements that affect global mRNA translational control include the 5' cap and the poly(A) tail, both of which confer stability to transcripts containing these elements and strongly promote the initiation of translation. The 5' cap, which is made of a guanosine residue with an added methyl group on nitrogen-7 (N7), is attached at the 5' end of mRNA using a 5'-5' triphosphate linkage. In contrast, the poly(A) tail is a long string of adenine residues located at the 3' end of mRNA. It was initially thought that ~100% of mRNAs are capped at steady state, but recent reports put this number at around 30-50%

(90). The modified nucleotidic mRNA cap is directly bound by eIF4E, which then initiates recruitment and binding of the rest of the translation initiation complex.

One of the additional members of the translation initiation and cap-binding complex, eIF4G, acts as a scaffolding protein. It enhances initiation frequency by binding both the 40S ribosomal subunit and poly(A) binding protein (PABP), thereby circularizing the mRNA and bringing the 5' and 3' ends within relative proximity (70,91). The interaction of the 5' cap complex and eIF4G is stabilized by the subsequent interaction with PABP. Further, the interaction between eIF4G and PABP is stabilized when PABP is bound to the poly(A) tail (92).

In addition to pre-mRNA processing, widespread dysregulation of translational processes is a key feature contributing to the development of many cancers. To affect global RNA translation, elevated expression of eIF4E leads to increased capping efficiency by driving expression of proteins involved in the capping process. For a subset of transcripts, most of which are oncogenic, this results in increased mRNA expression of these targets, which include *MYC*, *MDM2*, and *CTNNB1* (90). Several therapeutic options have been proposed to target these mechanisms, such as inhibiting phosphorylation of eIF4E by proteasomal degradation of the kinases MNK1/2, which results in attenuated translation and suppressed growth of TNBC tumors *in vivo* (93).

In contrast to global translational control, gene-specific translational control affects a small subset of mRNAs, often ones containing a particular binding sequence. As discussed previously, IRESes are one such example of this subset-specific translational control. Additionally, the RNA binding protein hnRNP A18 has been shown to stabilize a subset of mRNAs containing an A18-binding site in their 3'UTR. These mRNAs are

involved in cancer progression and interact with and recruit eIF4G to promote their translation. One of those mRNAs is CTLA-4, which encodes a key target of ICI therapy. Treatment of a variety of cancer cell lines with chemical inhibitors that block mRNA binding by the RRM of hnRNP A18 leads to preferential tumor cell death (94).

1.3.4.e Subcellular localization

RNAs often contain *cis*-acting “zipcodes,” which direct their cellular localization. Zipcodes are bound by specific RBPs, often in protein complexes. Localization of mRNAs and subsequent translation allow for efficient spatial and temporal control, but conditions of cell stress can often alter mRNA localization (95). Interestingly, during cell migration, ribosomal protein-coding mRNAs localize to actin filaments, which are abundant in cell protrusions that direct migration. This causes localized increases in translation, which supports cellular movement. Epithelial-to-mesenchymal transition (EMT) increases cellular mobility, and interestingly, upregulates *LARP6*, the RBP responsible for the localization of ribosomal mRNAs (96).

1.3.4.f Decay

Beyond stabilization of the translation initiation complex, a key function of the 3'-polyA tail is to protect mRNAs from degradation by exonucleases, which extends the amount of time that an individual mRNA can be translated. Added by the enzyme poly(A) polymerase during 3' processing of nascent pre-mRNAs, poly(A) tails are present on almost all mRNAs, with the exception of those encoding the mammalian core histone proteins. Interactions between the tail and PABP physically prevent access to the mRNA

by exonucleases (97,98), slowing the process of deadenylation-dependent decay, the major mechanism of mRNA decay.

Removal of the tail occurs via two major deadenylation complexes: the PAN2-PAN3 and CCR4-NOT complexes. PAN2-PAN3 is responsible for the initial phase of deadenylation, which takes place at the distal end of the poly(A). In the second phase, CCR4-NOT, which contains the exonucleases CCR4 and CAF1, completes deadenylation by removing the residues close to the 3' UTR; this is the rate-limiting step of deadenylation-dependent mRNA decay (97,98). The inclusion of two exonucleases in this complex seems redundant, but distinct roles have been discovered for each exonuclease. CCR4 mediates the release of PABP bound to the poly(A) tail, which then undergoes degradation, whereas CAF1 is limited to the deadenylation of mRNA that is already free of PABP (99,100).

Because of its role in the deadenylation of residues close to the 3'UTR, CCR4-NOT is actively recruited to the mRNA through the action of proteins that bind in the 3' UTR of an mRNA and regulate its decay. This highlights the significance of the spatial relationship between the 5' and 3' ends of the mRNA. While translation begins at the 5' end, the majority of gene-specific regulatory sequences are located within the 3' UTR. These sequences, such as the AU-rich element (ARE), act as docking sites for various RBPs that affect a variety of processes including translation and mRNA turnover.

1.4 *Cis*-acting AU-rich elements and their role in disease

The best characterized class of *cis*-acting regulatory sequences in mammalian mRNA 3'UTRs are the AREs, an evolutionarily conserved sequence motif present in

approximately 5-8% of transcripts that targets mRNAs for rapid degradation (101). The canonical sequence motif of AREs is AUUUA. This motif often occurs as dispersed copies in a U-rich sequence, or in multiple clustered copies. Many studies have tried to determine the minimum sequence needed to destabilize mRNA, with some reports indicating that UAUUUAU is sufficient, while others indicate that UUAUUUAUU is the optimal functional site. Interestingly, neither of these sequences is present in some archetypal ARE-containing mRNAs, such as *MYC* and *FOS* (102). Transcripts containing these sequences are usually highly labile, and interactions with *trans*-acting factors often affect their stability. Extracellular stimuli can result in the rapid stabilization or destabilization of these mRNAs, giving cells the ability to rapidly respond to their environment. To alter stability of these transcripts, these sites are hybridized with miRNA or bound by RBPs. (101–103).

1.4.1 Interactions between *cis*-acting AREs and *trans*-acting ARE-binding proteins

RBPs that bind AREs, collectively termed ARE-binding proteins (ABPs), are involved in multiple aspects of post-transcriptional regulation. As it became clear that AREs are present in many mRNAs involved in disease, many studies interrogated the dysregulation of mechanisms that either stabilize or destabilize these transcripts in disease, especially cancer. Since ABPs are key players regulating the turnover of ARE-containing transcripts, they can reprogram gene expression to support either pro-tumorigenic or anti-tumorigenic programs. Additionally, binding of some ABPs to their mRNA targets can either promote or inhibit translation. Both mRNA stability and translational efficiency can alter the amount of protein available to effect cellular functions.

The mRNAs that encode several well-known oncogenes and tumor suppressors contain AREs. For example, *MYC* contains an ARE that can be bound by multiple proteins. (104,105). The fate of *MYC* mRNA depends on the RNA-protein interaction, with examples in the literature of both stabilizing and destabilizing roles. c-Myc, the protein encoded by *MYC*, has many oncogenic functions, such as promoting cell proliferation through accelerating the cell cycle, pro-tumorigenic metabolism, and evading the immune response (106). The mRNA encoding the oncogenic protein CXCL1 (107) includes an ARE sequence that can be regulated by the ABP tristetraprolin. In cholangiocarcinoma, gut dysbiosis results in increased bacteria in the liver, which induces CXCL1 expression to attract granulocytic myeloid-derived suppressor cells (MDSCs), to evade immune surveillance (108). CXCL1 is also associated with premetastatic niche formation and metastasis in colorectal cancer. Secreted VEGFA from colorectal tumors stimulates CXCL1 production in tumor-associated macrophages (TAMs), resulting in CXCL1 accumulation in the premetastatic liver, thus attracting MDSCs to create a favorable tumor environment (107). As discussed earlier, BRCA1 is a protein that is often mutated in breast and other cancers (38). Interestingly, *BRCA1* contains an ARE site that can be bound by the HuR protein, which results in decreased BRCA1 protein expression, though the mechanism by which this occurs is still unclear (109). Another study reported that expression of BCR-ABL in leukemia induces endoplasmic reticulum cell stress, causing inhibition of BRCA1 translation, mediated by the ABP TIAR (110). This research could hold interesting translational potential, as PARP inhibitors already take advantage of a loss of BRCA1/2 to selectively kill tumor cells (111) PARP inhibitors can be used in the clinic for tumors displaying a “BRCAness,” or homologous recombination-deficient phenotype

(112). *MCL1* mRNA, encoding a member of the anti-apoptotic BCL2 family, contains multiple regulatory sequences in its 3'UTR, one of which is an ARE. In glioma, this sequence can be bound by HuR, causing mRNA stabilization and increased expression. Indeed, HuR can bind to mRNAs encoding all BCL2 family members via ARE sites in their 3' UTRs. Accordingly, silencing HuR resulted in functional decreases in cell proliferation, tumor growth and cell survival (113). Other mRNAs contributing to the Hallmarks of Cancer and containing ARE sites are listed in Table 1.2.

ARE-containing mRNAs contributing to the Hallmarks of Cancer	
Enabling replicative immortality	<i>UBE3A</i>
Activating invasion and metastasis	<i>MMP1</i> <i>CXCL1</i> <i>uPA</i> <i>uPAR</i> <i>MUC4</i> <i>IL1B</i> <i>FOS</i> <i>CCL2/3</i> <i>CLDN7</i> <i>NOS2</i>
Inducing or accessing vasculature	<i>VEGFA</i> <i>IL6</i> <i>PTGS2</i> <i>FGF2</i> <i>HIF1A</i> <i>GM-CSF</i>
Resisting cell death	<i>BIRC3/4/5</i> <i>TP53</i> <i>BCL2L1</i> <i>CDKN1A</i> <i>AKT1</i> <i>BCL2</i> <i>BNIP2</i> <i>MCL1</i> <i>PTGS2</i> <i>SIRT1</i>
Deregulating cellular metabolism	<i>OPA1</i> <i>HIF1A</i> <i>SLC2A1</i> <i>PFKFB3</i>
Sustained proliferative signaling	<i>CCNA1/B1/D1/E1</i> <i>CDKN1A</i> <i>CXCL2</i> <i>PIM1</i> <i>PLK3</i> <i>CDK2</i> <i>JUN</i> <i>FOS</i> <i>MYC</i>
Evading growth suppressors	<i>DUSP1</i> <i>LATS2</i> <i>TP53</i>
Avoiding immune destruction	<i>PDL1</i> <i>MYC</i> <i>TGFB1</i> <i>IL10</i>
Tumor promoting inflammation*	<i>TNFA</i> <i>PTGS2</i> <i>CXCL8</i> <i>CCL2/3</i> <i>CD86</i> <i>GM-CSF</i>
Genome instability and mutation*	<i>TP53</i> <i>BRCA1</i> <i>CLSPN</i> <i>GADD45A</i> <i>LATS2</i>
Unlocking phenotypic plasticity [^]	<i>SNAI1</i> <i>TWIST1</i>
Non-mutational epigenetic reprogramming [^]	<i>SIRT1</i>
Senescent cells [^]	<i>UBE3A</i> <i>CDKN1A</i>
Polymorphic microbiomes [^]	<i>CXCL1</i>

Table 1.2 mRNAs containing AREs that contribute to the development of cancer.
List of mRNAs containing AREs grouped by their associated Hallmark of Cancer (60).
Listed mRNA substrates were compiled from Refs (105,109,114–126).
*Hallmarks of Cancer, Next Generation (62).
[^]Hallmarks of Cancer, New Dimensions (61).

1.4.2 Regulation of translation by AREs

TIA-1 and TIAR are ABPs that have defined translational regulatory roles. Under conditions of cellular stress, TIA-1 and its close homologue, TIAR, recruit mRNAs to stress granules, which inhibits their translational initiation. TIA-1 is known to affect the translation of *TNFA* and *PTGS2* mRNAs (127,128).

While TIA-1 and TIAR are the prototypical examples of translational regulation by ABPs, HuR, a member of the ELAVL/Hu family of proteins, also has been reported to have this function. Structurally, the Hu protein family all use RRM domains to bind mRNAs. HuR is one of the best-characterized ABPs, though mainly with regard to mRNA stability. However, HuR can also interact with the 3'UTR of the mRNA encoding the vitamin D receptor (VDR) in order to promote its translation. In a polysome profiling experiment, association of HuR with *VDR* increased the amount of *VDR* mRNA in actively translating fractions. Silencing of HuR resulted in lower translational efficiency, as measured by a luciferase reporter assay (129).

1.4.3 Regulation of mRNA turnover via coordinated action of AREs and ABPs

As it became clear that ABPs regulate the stability of mRNAs involved in disease, it became important to determine if these mechanisms were dysregulated in disease, especially cancer. This led to the hypothesis that in some cancers, reprogramming of gene expression might involve ABPs.

The first ABP to be identified and cloned was ARE/poly(U)-binding/degradation factor 1 (AUF1). Binding of AUF1 to ARE-containing mRNAs involves RRM domains and can either increase or decrease mRNA stability. In some contexts, differences in

stability depend on the transcript, however, it has also been shown that AUF1 affects transcripts in a cell-type specific manner (101). In a spontaneous tumor model, sarcoma tumors from AUF1-expressing mice had increased levels of *MYC*, *FOS*, *JUN*, and *CCND1* mRNAs, all of which contain AREs in their 3' UTRs. Interestingly, expression of *GM-CSF* and *TNFA* decreased in AUF1-expressing tumors. The authors state that these alterations in mRNA accumulation cannot be directly attributed to alterations in mRNA stability, as they relied on the previous reports of AUF1 altering mRNA turnover, and did not measure mRNA stability themselves (115).

The binding of HuR to ARE sites plays a well-established role in the stabilization of mRNA (116,122,130). Cellular processes modulated by HuR include inhibition of apoptosis, progression of cell division, and the response to immune stimuli (131). During tumor development, HuR acts as an oncogene, stabilizing mRNAs encoding proteins that contribute to the development of malignant phenotypes (130). Suppressing HuR expression in colorectal carcinoma leads to marked decreases in cell proliferation, which is mechanistically linked to an accumulation of cells in the S and G2/M phases of the cell cycle. HuR expression increases the steady state expression of the mRNAs encoding cyclin A and cyclin B1, resulting from increases in transcript turnover rates (116). Oncogenic targets of HuR also include *SIRT1* and *PTGS2* (116,121,122). Significant drug discovery work is underway to identify HuR inhibitor compounds, as targeted inhibition of HuR could prove to be a valuable strategy for the clinic (132).

In contrast to HuR's mRNA-stabilizing effect, KH-type splicing regulatory protein (KSRP) causes decreased mRNA stability when bound to AREs. KSRP uses KH-domains to bind single-stranded nucleic acids. In addition to altering mRNA turnover, KSRP also

mediates transcription, miRNA biogenesis, mRNA stability, and translational control. KSRP acts by recruiting the RNA exosome complex to target mRNAs, as well as other ribonucleases and deadenylases (133). KSRP has also been shown to bind and destabilize several ARE-containing mRNAs, such as *CXCL1*, *TNFA*, *IL8*, and *NOS2* (114,134).

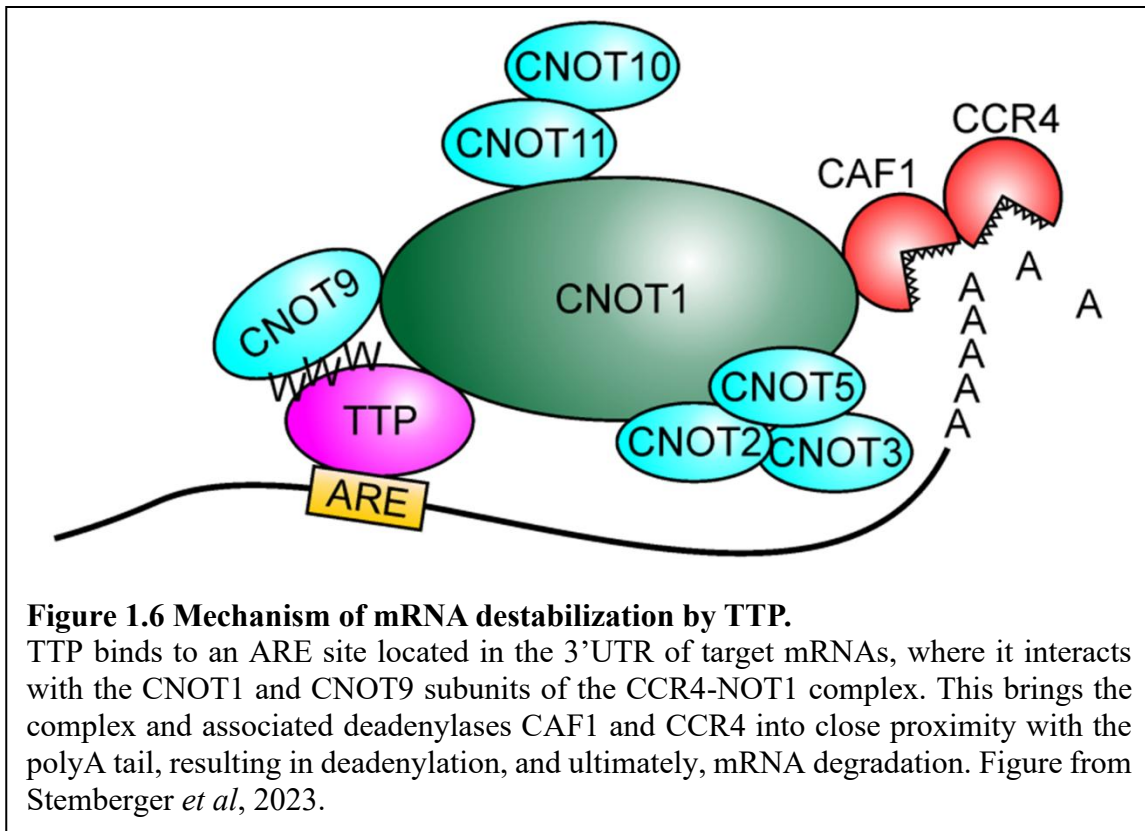
1.5 Tristetraprolin

One well-described ABP is tristetraprolin (TTP, aka ZFP36, Nup475, TIS11), which was first discovered and characterized in the 1990s. TTP is a proline-rich protein, which influenced its name; it contains three sets of four proline residues, ergo, tris-tetra-prolin. Based on its rapid mRNA and protein accumulation after insulin- and serum-stimulation in fibroblasts, TTP is classified as an immediate early response gene. TTP was initially thought to be a DNA-binding transcriptional activator due to its two tandem zinc finger domains and proline-rich region, features similar to other proteins involved in transcriptional activation (135–137). Additionally, initial localization studies suggested that TTP localized to nuclei in mouse fibroblasts, and research discovered that TTP can coordinate a zinc ion, but its lack of similarity to other ZnFs suggested that it was a novel type of ZnF protein (138). TTP is now characterized as the prototype CCCH motif ZnF protein (78).

Investigation into the physiological role of TTP in a mouse model revealed that loss of TTP results in chronic inflammation, predominated by symptoms of systemic inflammatory syndromes. As this phenotype mimicked the effect of chronic TNF α administration, and subsequent neutralization of TNF α via monoclonal antibody was sufficient to reverse the phenotype, the role of TTP in the regulation of TNF α expression

was revealed (139). Though prior research had suggested that TTP could be a transcriptional activator, subsequent research revealed that TTP does not affect the transcription of TNF α yet is able to post-transcriptionally affect gene expression by inducing decay of the TNF α transcript, redefining TTP as an RBP that bound the 3'UTR of TNF α mRNA. Further investigation identified ARE sequences as the specific binding sites within the TNF α 3'UTR, classifying TTP as an ABP (140).

Much research has focused on elucidating TTP's mechanism of action, which canonically leads to the destabilization and decreased translation of its target mRNAs. Unlike other ABPs, TTP binds specifically to mRNAs containing multiple clustered copies of the AUUUA pentamer. Like other ABPs, it can undergo nucleocytoplasmic shuttling (141), even though most research now defines its primary location as the cytoplasm (101,142). The interaction of TTP with AREs causes subsequent interaction with the NOT1 subunit of the CCR4-NOT complex (143,144). As discussed earlier, this complex is responsible for deadenylation of poly(A) tail residues proximal to the 3' UTR (Figure 1.6), which is the rate-limiting step of mRNA degradation (99,100). TTP can then recruit the RNA exosome to the mRNA, to further stimulate degradation of the mRNA body (145).



1.5.1 Tumor suppressive functions of tristetraprolin

With its RNA-destabilizing activity, TTP has been shown to decrease transcript abundance for many oncogenic proteins, which has led to the identification of a multitude of TTP targets, reinforcing the potential of TTP in cancer therapy. For example, in melanoma, the most common mutation in the BRAF kinase, V600E, results in constitutive BRAF activity and increased expression of the oncogene *CXCL8* (IL-8). Overexpression of TTP in melanoma cell lines leads to *CXCL8* destabilization and decay, resulting in suppression of neoplastic phenotypes, including cell proliferation and autophagy (146). In glioma cells stimulated with rapamycin, TTP binds to and destabilizes both *CCND1* and *MYC* mRNAs. Interestingly, in cells where Akt is active, TTP is capable of binding and destabilizing target mRNAs, however, in cells where Akt is inactive, TTP is sequestered

and cannot destabilize these mRNAs, illustrating a potential role for TTP in modulating drug resistance (147). In the context of cancer immunotherapy, TTP is responsible for the degradation of *PDL1* mRNA, which contains four ARE sites in its 3'UTR. In non-small cell lung cancer and colorectal cancer, oncogenic RAS signaling leads to the inhibition of TTP, which results in an abundance of PD-L1 on the tumor surface and immunoresistance. Restoration of TTP expression reverses this immunoresistance by returning PD-L1 expression to its normal levels (117).

Limited evidence suggests that TTP can also influence gene expression by its interactions with other proteins. In a colorectal adenocarcinoma cell line, TTP was shown to destabilize inducible nitric oxide synthase (iNOS), yet it did not bind to the ARE site. Co-immunoprecipitation studies revealed that TTP instead binds to KSRP, removing it, and the bound exosome, from iNOS mRNA. This is a unique example of TTP stabilizing, instead of destabilizing mRNA (134,148).

TTP target mRNAs encode an extensive list of proteins, many of which are either oncogenic or inflammatory, designating TTP as a candidate tumor suppressor gene (120). Because of its tumor-suppressive activities, TTP mRNA and protein expression are often suppressed during the development of neoplasia, resulting in significantly lower levels in tumors compared to the corresponding healthy tissue (118,149,150). There are several proposed mechanisms that may direct attenuation of TTP expression. At the transcriptional level, methylation of a single CpG site in a TGF β response element in the promoter of TTP results in TTP silencing in a hepatocellular cancer cell model (151). By binding to the initiator element (Inr) in the TTP promoter, c-MYC is also responsible for suppressing TTP expression in lymphoma (152). Though there are few mechanisms currently proposed at

the transcriptional level, it is likely that alternative suppressive mechanisms exist and are yet to be described.

Independent of inhibition at the transcriptional level, TTP expression and/or function can also be inhibited post-transcriptionally. TTP mRNA also contains an ARE in its 3'UTR, and as a consequence, increased TTP expression accelerates turnover of its own transcript (153,154). Currently, two mechanisms involving miRNA regulation have been identified that affect TTP mRNA turnover. In pancreatic and breast cancer, upregulation of miR-29a results in downregulation of TTP expression, likely due to the presence of two regions complementary to the miR-29a sequence located in the 3' UTR of TTP mRNA (155,156). In bone-marrow derived macrophages, a 3'UTR binding site for both HuR and miR-29 was identified, resulting in competitive binding, with increased miR-29 binding leading to decreased TTP expression (157). At the protein level, phosphorylation of TTP is known to inhibit its mRNA-binding abilities (158,159). TTP can be phosphorylated at multiple sites, and activation of the p38/MAPKAP kinase 2 (MK2) pathway induces the specific phosphorylation of TTP by MK2 at serines 52 and 178. Phosphorylation of Ser178 creates a binding site for the chaperone protein 14-3-3 to generate a TTP-14-3-3 protein complex (159), which sequesters TTP from binding target mRNAs (160). MK2 can be activated by other oncogenic mechanisms, such as RAS signaling, which subsequently inhibits TTP function (117).

Since loss of TTP is a common feature in a multitude of cancers, over-expression or restoration of TTP levels in tumors could hold therapeutic benefit. As this research is still in its infancy, strategies to do so are currently limited. Sorafenib, which targets RAF kinases, modestly increases TTP expression, while also decreasing tumorigenic

phenotypes in melanoma cell and animal models (146). While this therapy significantly increases PFS and OS in advanced renal cell carcinoma and advanced hepatocellular carcinoma, respectively, a clinical trial in metastatic melanoma using this drug in combination with chemotherapy unfortunately showed no therapeutic benefit (161). In HeLa, HepG2, and MDA-MB-231 cells, mitogenic stimulation followed by treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) strongly induced TTP expression (119), indicating the existence of other TTP-inducing transcriptional circuits that could be therapeutically manipulated in future studies.

Since pharmacological stimulation of TTP expression is yet to be fruitful, most studies that describe the effects of TTP in tumors utilize various ectopic overexpression strategies. The first study to characterize TTP as a tumor suppressor demonstrated that expression of TTP in a mast cell model delayed tumor progression *in vivo*. Mechanistically, this was the result of *IL3* mRNA destabilization, leading to decreased synthesis and secretion of IL-3 protein, which drives mast cell proliferation through an oncogenic autocrine loop (162). In lymphoma, re-expression of TTP can counteract c-Myc-induced proliferative signaling, slowing proliferation rates to levels similar to those of wild-type B cells (152).

In breast cancer, an inverse correlation exists between TTP mRNA level and breast tumor grade, which is also reflected at the protein level, where TTP expression is higher in normal tissue but extremely low in patient-matched tumor samples. Additionally, a gene-array dataset of 251 breast cancer patients separated into cohorts based on TTP mRNA level at tumorectomy reveals that the highest TTP expressors have a 15% mortality rate over 12 years, however, that rate increases to 45% for the lowest-expressing cohort (118).

Because of its importance in suppressing the development of neoplasia, re-expression of TTP and characterization of the downstream mechanisms it induces has the potential to yield valuable discoveries to field of cancer research.

1.6 Scope of work

The current goals of cancer research include the identification of novel regulatory nodes that can be exploited in the development of targeted therapy options to suppress malignancy. The heterogeneity of TNBC tumors continues to be a major challenge to this goal. Furthermore, the aggressive nature of TNBC necessitates the development of therapies that inhibit neoplastic growth. Significant advances in the last decade have led to FDA approval of several new therapies, expanding the reservoir of treatment options and increasing survival for a subset of TNBC patients. However, for the majority, patient response rates are low, and both resistance and relapse are common, emphasizing the need for the discovery and characterization of novel targets. Given the important roles that RBPs play in all aspects of the mRNA life cycle, these mechanisms of RNA regulation could prove to be important targets for translational medicine. Tsherniak *et al* developed a Cancer Dependency Map, which identifies cancer growth-related vulnerabilities, defined as a gene or a set of genes that a cancer cell relies on for growth and survival (163). A total of 759 vulnerabilities were identified across 501 cell lines and interestingly, the largest class of dependencies is nucleic acid binding proteins. Transcription factors, which are involved in key roles in the induction of oncogenes, are a separate category. The largest subsection of the nucleic acid binding proteins is RBPs, which still accounts for a greater number of

proteins than the transcription factor category (163), suggesting that this protein class could have much more clinical potential than previously thought.

We and others have previously reported that loss of TTP in tumors correlates with increased disease severity and decreased survival, suggesting it acts as a tumor suppressor across a wide spectrum of cancer types (118,152,164). This led us to hypothesize that re-expression of TTP in TNBC would attenuate multiple tumor phenotypes.

While previous research has shown that TTP can suppress proliferation in breast cancer, the mechanism remains weakly defined. It has been well established that TTP's antitumorigenic functions are in part mediated by its ability to bind and destabilize mRNAs that often encode oncogenic proteins. During the course of our research, we discovered an unexpected mechanism by which TTP acts to attenuate aggressive tumor phenotypes. We originally expected that the potentiation of TTP's anti-tumorigenic effects was through the destabilization of oncogenic transcripts, but quantitative measurements of mRNA decay kinetics revealed that TTP was not destabilizing its canonical mRNA targets in our TNBC cell models, leading us to hypothesize that there is a novel, non-canonical mechanism through which TTP can induce anti-tumorigenic phenotypes.

A greater understanding of the anti-tumorigenic mechanisms affected by TTP, and especially characterization of this non-canonical mechanism is needed. Based on its suppression in tumors and well-defined tumor-suppressive functions, pharmacological induction of TTP could be an attractive option for the development of novel targeted therapies. Another potential approach is intratumoral delivery of TTP protein using liposome packaging or by direct protein entry. A precedent for the latter comes from studies showing that short cationic tags enabled efficient uptake of the TTP family member

ZFP36L1 (BRF1) into COS7 cells (165). Collectively, our findings present clear evidence that TTP can attenuate aggressive tumor phenotypes in TNBC models. Further, this research defines a novel non-canonical mechanism, again displaying potent anti-tumorigenic activity, which is independent of TTP's mRNA-binding and -destabilizing capabilities. This research highlights the vital therapeutic potential of restoring TTP expression in tumors, regardless of its functional ability to bind and destabilize mRNA.

Chapter 2: Tristetraprolin induces antitumorigenic phenotypes in TNBC cell models via a novel non-canonical mechanism¹

2.1 Introduction

While roles for transcription in the development and severity of neoplasia are well-established, post-transcriptional control remains comparatively underexplored and could hold untapped therapeutic potential. Identifying new molecular targets is an ongoing challenge in oncology, and modulating post-transcriptional regulatory mechanisms—particularly through RNA-binding proteins (RBPs)—may present novel strategies for cancer therapy. RBPs govern nearly all stages of the mRNA life cycle, including nuclear pre-mRNA processing, export, and cytoplasmic regulation of mRNA turnover, translation, and localization (80).

RBP specificity for mRNA targets is mediated by interactions with cognate sequence motifs within each transcript. A key class of regulatory motifs are AU-rich elements (AREs), U-rich tracts often punctuated by AUUUA repeats that reside in the 3'-untranslated regions (3'UTRs) of unstable mRNAs encoding inflammatory and oncogenic proteins (101,166). ARE-mediated regulation depends on ARE-binding proteins (ABPs), whose functions vary based on ABP identity and RNA context. Some oncogenic ABPs, such as HuR, stabilize transcripts that encode pro-tumorigenic proteins including COX-2, cyclin A, and cyclin B1, thus increasing their steady-state levels and subsequent translational output (116,122). Anti-tumorigenic ABPs, like KSRP, destabilize select transcripts (167). ABPs such as AUF1 present more complex situations, positively or

¹ Rutherford JL, Stemberger MB, Mahmud R, Ross CR, Lee NC, Low AS, Winter A, Patrón Fidalgo GM, White EJF, Wilson GM. “Tristetraprolin induces antitumorigenic phenotypes in TNBC cell models via a novel non-canonical mechanism.” *Submitted for review.*

negatively regulating the decay kinetics and/or translational efficiency of different mRNA substrates (168,169). Since AREs are found in the 3'UTRs of up to 20% of human mRNAs (170), dysfunction or dysregulation of a single ABP can coordinately alter expression of hundreds of transcripts (63), with dramatic consequences for oncogenesis (105,171).

Tristetraprolin (TTP, also known as ZFP36) is an ABP that has garnered attention for its tumor-suppressive potential. The prototypical member of the CCCH class of tandem zinc finger binding proteins (135,138), TTP was initially described as an anti-inflammatory factor that binds and destabilizes *TNFA* mRNA (140). TTP mediates this function by recruiting the CCR4-NOT1 deadenylase complex to initiate poly(A) tail removal, the first and rate-limiting catalytic step of deadenylation-dependent mRNA decay (143,144,172,173). Since the discovery of TTP's first mRNA target, many TTP substrates have been identified that encode other inflammatory factors (174,175), or oncogenic proteins such as VEGFA, MYC, and cyclin D1 (118,147,151,176). Owing to the close links established between inflammation and neoplasia, there is significant overlap in the functions of genes associated with both pathologies (177).

Early evidence supporting a tumor-suppressive role for TTP came from mast cells, where TTP destabilized *IL-3* mRNA, inhibiting IL-3 production and disrupting an autocrine loop responsible for rapid cell proliferation and *in vivo* tumor growth (162). Multiple studies have since reported TTP repression in various cancers, often coinciding with enhanced oncogenic phenotypes or tumor growth in xenograft models (150,152,164). Our prior work showed that TTP expression was inversely correlated with tumor grade and associated with poor clinical outcomes in a modest cohort of breast cancer patients (118). Another study reported that expressing TTP in an aggressive breast cancer cell line slowed

cell proliferation and xenograft tumor growth (178); similar observations have been reported in prostate cancer and B cell lymphoma (152,164). TTP expression has been linked to attenuation of migration and invasiveness in glioma and head and neck cancer cell models (179,180), suggesting context-dependent anti-oncogenic roles.

Current models propose that the major mechanism by which TTP inhibits tumorigenic phenotypes is via targeted degradation of its substrate mRNAs (181,182). The wide range of oncogenic mRNA targets identified for TTP (120) coupled with the pathological consequences of TTP suppression in neoplasia suggest that modulating TTP expression may alter multiple tumorigenic properties in individual cell types, however, this area remains understudied. Using an aggressive TNBC cell line as a model, we used RNA-Seq to identify pathways altered by TTP expression and functionally validated multiple anti-tumorigenic effects across several TNBC cell models. Unexpectedly, we found that TTP's mRNA-binding or -destabilizing activity was not required for inhibition of these phenotypes, since a TTP mutant lacking RNA-binding capacity retained the ability to suppress tumorigenic phenotypes *in vitro* and *in vivo*. Collectively, these data show that expression of TTP can potently suppress several oncogenic phenotypes in TNBC cells via a mechanism independent of its canonical functions.

2.2: Materials and Methods

Plasmid constructs and cell lines

MDA-MB-231, MDA-MB-436, and BT-549 cells were obtained from ATCC (Manassas, VA). Tumor-derived MDA-MB-231 (231td) cells were a generous gift from

Dr. Stuart Martin and the University of Maryland Greenebaum Comprehensive Cancer Center's (UMGCCC) Translational Laboratory Shared Service. Transgene expression in parental MDA-MB-231 lines was performed in a regulatable Tet-Off background. First, MDA-231/Tet-Off cells were created by transfecting MDA-MB-231 with plasmid pTet-Off (Clontech, Mountain View, CA) and selecting in 800 µg/ml G418. Clones were screened by luciferase assay using plasmid pTRE2-luc (Clontech); only those that suppressed luciferase $\geq 99\%$ in 2 µg/ml doxycycline (Dox, Sigma, St. Louis, MO) were retained. Subsequently, MDA-231/EV, MDA-231/FLAG-TTP, and MDA-231/FLAG-TTP C147R lines were generated by transfecting MDA-231/Tet-Off cells with plasmids pTRE2hyg (Clontech), pT2hyg-FLAG-TTPwt, and pT2hyg-FLAG-TTP C147R (118), respectively, and selecting in 100 µg/ml hygromycin B. Dox was included (2 µg/ml) during selection and clonal expansion to minimize transgene effects on cell viability. Clonal lines were then maintained in DMEM (Corning, Corning, NY), 10% fetal bovine serum (FBS, Sigma and Biowest, Bradenton, FL), 1% penicillin/streptomycin (PS, Corning; final concentrations are 100 IU/ml penicillin, 100 µg/mL streptomycin), 400 µg/mL G418 sulfate (G418, GeminiBio, West Sacramento, CA), and 100 µg/mL hygromycin B (hygB, Invitrogen, Waltham, MA) supplemented with 2 µg/mL Dox. Cells were cultured in the absence of Dox for at least 3 days before experiments to permit transgene expression.

Constitutive TTP-expressing and empty vector (EV) control clonal lines in MDA-MB-436 and BT-549 were generated by stable expression of pcDNA3.1(-)FLAG-TTP or pcDNA3.1(-), respectively, while lines constructed in 231td cells used pcDNA3.1(-)zeo vectors. MDA-MB-436 EV and TTP lines were maintained in 50/50 DMEM/F12 (Corning), 10% FBS, 1% PS, and 400 µg/mL G418. BT-549 EV and TTP lines were

maintained in DMEM, 10% FBS, 1% PS, 1% L-glutamine (Corning), and 400 ug/mL G418, while tumor-derived 231td EV, TTP, and C147R lines were maintained in DMEM, 10% FBS, 1% PS, 400 µg/mL G418, 100 µg/mL hygB, and 400 µg/mL zeocin (Invitrogen). All cell lines were maintained in a humidified incubator at 37°C and 5% CO₂, regularly passaged, and regularly tested for mycoplasma contamination.

TCGA analyses

Gene expression data in normal and tumor tissues were generated by The Cancer Genome Atlas (TCGA) and Gene-Tissue Expression (GTEx, Broad Institute). Raw data were re-analyzed by the UCSC Toil Recompute Project to standardize the data analysis method and ensure uniformity of data. $\log_{10}(\text{tpm}+0.001)$ values used in this study were downloaded from the UCSC Xena (xena.ucsc.edu) database, filtered by cancer/tissue type, and plotted using R/ggplot2. Significance of differences in gene expression in unpaired normal versus tumor tissue was assessed with the Wilcoxon Rank Sum/Mann-Whitney test.

Kaplan-Meier survival analyses

Kaplan-Meier analysis was conducted to determine the clinical relevance of our genes of interest. The online software KM plotter (<https://kmplot.com/analysis/>) was used to determine relationships between gene expression and relapse-free survival (RFS) among breast cancer patients analyzed across multiple integrated microarray data sets (total $n = 4929$). Cutoffs for high vs low TTP-expressing tumors were autoselected by the software and relevant n values for each cohort are reported in the figure legend. Hazard ratios and logrank P values are shown on the individual KM plots.

RNA-Seq and analysis

Total RNA was extracted from two independent MDA-MB-231 EV and TTP clones (4 biological replicates of each) using the NucleoSpin RNA purification kit (Macherey-Nagel, Allentown, PA). RNA quality assessments, library preparation, sequencing, and initial analysis were performed by Azenta Life Sciences (Indianapolis, IN), according to their standardized protocols. Log₂ fold change data were used as input for the subsequent bioinformatic analyses. Low expression genes (normalized read count < 100 in both EV and TTP) were filtered out. Gene targets with an adjusted p-value of less than 0.05 and a log₂-fold change of <-1 or >1 were considered significantly differentially expressed.

Gene Set Enrichment Analysis (GSEA)

Target genes were ranked by Log₂ fold change between EV- vs TTP- expressing cells. These ranked lists were used as inputs for GSEA analysis conducted using the R/Bioconductor package clusterProfiler (183). Datasets were pulled from the Molecular Signatures Database from the Broad Institute and UC San Diego and queried against our ranked gene list. Pathways with false discovery rates of less than 0.25 were considered significantly enriched.

Cell cycle analyses

TTP-expressing or EV control lines were harvested with trypsin, resuspended in DMEM, and counted. 1.5×10^5 cells were resuspended in 0.5 mL 1× PBS and fixed by adding 3.5 mL of cold 70% ethanol while slowly vortexing. Cells were stored at -20°C for

at least 24 hours before staining. 3 mL of 1× PBS was added to the fixed cells, which were spun for 5 minutes at 500 × g. The supernatant was discarded, and cells were resuspended in 500 µL 1× PBS and moved to a 5 mL tube. RNase A was added to a final concentration of 10 µg/mL and incubated at room temperature for 20 minutes. Propidium iodide was then added at a concentration of 50 µg/mL and incubated for 30 minutes in the dark at room temperature. Tubes were then transferred to ice, covered with foil, and analyzed by flow cytometry using a BD Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ) at the UMGCCC Flow Cytometry Core facility. Data analysis was performed using BD FACS Diva software (BD Biosciences).

Protein extraction and western blotting

Cells were lysed with radioimmunoprecipitation assay (RIPA, Sigma) buffer supplemented with 1% protease inhibitor cocktail (Sigma) and 1% phosphatase inhibitor cocktails 2 and 3 (Sigma). Samples were incubated for 1 hour on ice, then centrifuged at 16,000 × g for 15 minutes at 4°C to pellet debris. Soluble protein concentrations were determined by bicinchoninic assay (BCA, ThermoFisher, Waltham, MA). 10 µg of extracts were mixed with 4× SDS buffer containing 10 mM dithiothreitol (DTT) and run on a 12% SDS-PAGE gel, then transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA). Membranes were blocked for 1 hour in 5% milk in PBS containing 0.05% Tween 20 (PBS-T) and incubated overnight with antibodies against rabbit monoclonal HRP-conjugated anti-GAPDH (HRP-60004, Proteintech, Rosemont, IL), rabbit monoclonal HRP-conjugated anti-FLAG (HRP-66008, Proteintech), rabbit monoclonal anti-TTP (71732, Cell Signaling, Danvers, MA), rabbit monoclonal anti-p21

(2947, Cell Signaling), rabbit polyclonal anti-cyclin D1 (sc-718, Santa Cruz Biotechnology, Dallas, TX), rabbit polyclonal anti-CDK4 (sc-601, Santa Cruz Biotechnology), rabbit polyclonal anti-cyclin E1 (sc-481, Santa Cruz Biotechnology), rabbit polyclonal anti-CDK2 (sc-163, Santa Cruz Biotechnology), mouse monoclonal anti-cyclin A2 (4656, Cell Signaling), mouse monoclonal anti-CDK1 (sc-54, Santa Cruz Biotechnology), rabbit monoclonal anti-cyclin B1 (12231, Cell Signaling), rabbit monoclonal anti-cyclin F (81045, Cell Signaling), rabbit monoclonal anti-MYC (5605, Cell Signaling), rabbit polyclonal anti-ERK (9102, Cell Signaling), rabbit polyclonal anti-phospho-ERK (9101, Cell Signaling), rabbit monoclonal anti-phospho-p38^{MAPK} (4511, Cell Signaling), rabbit polyclonal anti-p38^{MAPK} (9218, Cell Signaling) or rabbit monoclonal anti-phospho-MAPKAP K2 (3007, Cell Signaling). After washing, blots were incubated with species-appropriate horseradish peroxidase-conjugated secondary antibodies in PBS-T and incubated for 1 h at room temperature, then washed again. Bound antibodies were detected using Radiance ECL or Radiance Q reagents (Azure Biosystems, Dublin, CA) and a chemiluminescence imager (Azure Biosystems). Band intensity was quantified using ImageJ's densitometry analysis (National Institutes of Health, Bethesda, MD).

RNA extraction

Except for samples analyzed by RNA-Seq (above), total RNA was extracted from cultured cells using TRIzol (Invitrogen) and purified according to the manufacturer's protocol.

Reverse transcription quantitative PCR (RT-qPCR)

RNA was quantified using Nanodrop (ThermoFisher) and 50 ng of RNA was used per reaction. cDNA amplification and qRT-PCR were performed with the GoTaq Probe 1-Step RT-qPCR System (Promega, Madison, WI) using SYBR detection in a CFX Opus 96 Real-Time PCR System (Bio-Rad, Hercules, CA). Primer sets used were (all listed 5'→3'):

GAPDH forward GACAGTCAGCCGCATCTTC, reverse
ACTCCGACCTTCACCTTCC; *CCND1* forward CGCCTCACACGCTTCCTCTC,
reverse GTCCACCTCCTCCTCCTCCTC; *MYC* forward
CCCGCTTCTCTGAAAGGCTCTC, reverse GTTCTCCTCCTCGTCGCAGTAG;
VEGFA forward GCACCCATGGCAGAAGG, reverse
CTCGATTGGATGGCAGTAGCT; *PIMI* forward GACCTGCACGCCACCAAG,
reverse CGGCAAGTTGTCGGAGACG; *JUN* forward CCTTTCCCGCGCAACCC,
reverse GGC ACTGTCTGAGGCTCCT; *18S* rRNA forward
ACAGTGAAACTGCGAATGGC, reverse GATAAATGCACGCATCCCC.

Proliferation assays

MDA-MB-231, MDA-MB-436, and BT-549 lines were plated at a density of 1.0×10^4 per well in 6-well plates. 231td lines were plated at a density of 5.0×10^3 per well in 6-well plates. At indicated time points, cells were harvested with 0.25% Trypsin (Corning), resuspended in $1 \times$ PBS, and mixed with $2 \times$ trypan blue dye, which was diluted from $4 \times$ trypan blue dye (Invitrogen). Viable cell numbers were counted using a Countess II FL cell counter (Invitrogen).

Apoptosis Assay

MDA-MB-231, MDA-MB-436 and BT-549 lines expressing TTP or EV were analyzed for apoptotic cell populations using the eBioscience Annexin V Apoptosis Detection Kit PE Kit (Invitrogen) essentially as described by the manufacturer. Annexin V-PE staining data were collected using a Cytex Aurora cytometer (Cytex Biosciences, Fremont, CA), and data were analyzed with FCS Express (De Novo Software, Pasadena, CA).

Migration and invasion assays

Migration and invasion were assessed using xCELLigence Real-Time Cell Analysis (Agilent, Santa Clara, CA) at the UMGCCC Translational Laboratory Shared Service facility. Cells were resuspended in serum-free DMEM and counted. Cell suspensions were prepared to yield 1.0×10^4 cells/100 μ L DMEM and loaded on real-time cell analysis (RTCA) C_{im} plates (Agilent) in serum-free medium. Medium containing 10% FCS was used as a chemoattractant in the lower chamber. Cell migration was measured by electrical impedance across the membrane as a function of time following established protocols (184,185) relative to parallel reactions lacking serum. For invasion assays, RTCA plates were coated with Matrigel (BD Biosciences) diluted in serum-free medium (1:20 v/v) prior to cell loading.

Biotin RNA pulldown

Interactions between candidate FLAG-tagged proteins expressed in MDA-MB-231 cells and RNA substrates were evaluated in vitro using a modification of the biotin-RNA pull-down assay, essentially as described previously (119,186). Crude cytoplasmic extracts were prepared from MDA-MB-231 lines stably expressing wild type and C147R-mutated TTP by scraping into lysis/wash buffer (10 mM Tris-HCl (pH 7.5) containing 100 mM KCl, 2.5 mM MgCl₂, 2 mM DTT, and 1% IGEPAL-CA630) supplemented with 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride. Protein concentrations were measured by Bradford assay using the Bio-Rad Protein Assay reagent (Bio-Rad). Biotin-RNA pull-down reactions were assembled with 200 µg protein extract and 100 pmol 5'-biotin-tagged ARE (5'-GUGAUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAG-3') or Rβ (5'-UGGCCAAUGCCCUGGCUCACAAAUACCACUG-3') RNA ligands (Sigma). Reactions were incubated for 30 min at room temperature, then combined with streptavidin-agarose beads (Millipore) and incubated for a further 30 min. Complexes were washed twice in lysis/wash buffer and dissociated by re-suspension in 2x SDS-PAGE buffer supplemented with 20 mM DTT at 50°C for 5 min. Analysis of co-purifying TTP proteins was determined by Western blotting.

RNA decay assays

Cells were plated at a density of 1×10^5 cells/well in 6-well plates and incubated for 3 days, with media changed 1 day before measuring mRNA decay rates. Nascent transcription was inhibited by adding actinomycin D (actD, Sigma) to cells at a final concentration of 5 µg/mL. Thereafter, RNA was isolated at select time points using TRIzol

purification and analyzed by qRT-PCR. Time courses were limited to 4 h to avoid complications of actD-enhanced apoptosis (187). Relative *MYC*, *VEGFA*, and *PIMI* mRNA levels remaining at each time point were quantified by qRT-PCR (described above), normalized to *GAPDH* mRNA, and plotted as a function of time following actD treatment. From these plots, first-order mRNA decay constants (k) were resolved by nonlinear regression (PRISM v3.03, GraphPad, Boston, MA), from which mRNA half-lives were calculated using $t_{1/2} = \ln 2/k$ as described previously (188). Tabulated mRNA half-life values are based on the mean \pm standard deviation of n independent time-course experiments to permit pair-wise statistical comparisons.

Xenograft models

Six- to eight-week old female athymic nude Foxn1^{nu} mice were obtained from Inotiv (Indianapolis, IN) and housed under pathogen-free conditions in the American Association for Accreditation of Laboratory Animal Care-accredited animal facility at University of Maryland, Baltimore. All experiments conducted with these mice complied with Public Health Service guidelines for animal research and were approved by the University of Maryland Baltimore Institutional Animal Care and Use Committee. 3×10^6 231td/EV, TTP, or TTP-C147R cells were mixed with 50% Matrigel in a 50 μ L suspension and injected near the mammary fat pad. Tumor volumes were measured at least twice per week using electronic calipers and volume was calculated using the formula volume = length \times 2(width). Mice were weighed at least three times per week and observed daily. All mice were euthanized on Day 24 of the study.

Promoter Activity Assay

Transcription activation by NF- κ B was measured in select cell lines by co-transfection of plasmids pELAM-Luc and pRL-GAPDH using Attractene transfection reagent (Qiagen, Germantown, MD). pELAM-luc contains a fragment of the E-selectin promoter (positions -730 to +52) with three copies of the NF- κ B binding site upstream of the firefly luciferase gene(189) and was generously donated by Dr. Bret Hassel. pRL-GAPDH was generated by amplifying the core and proximal promoter sequences (-269 to +20) from the human GAPDH gene by PCR and subcloning it in place of the SV40 promoter upstream of the *Renilla* luciferase gene in plasmid pRL-SV40 (Promega). Two days post-transfection, luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) and analyzed by normalizing firefly luciferase to the GAPDH promoter-driven *Renilla* luciferase signal.

Statistical Analysis

Unless otherwise noted, quantitative data are presented as mean \pm SD of at least three independent replicate experiments. For simple binary comparisons, differences in means were assessed using the two-tailed unpaired Student's *t* test with $p < 0.05$ considered significant. Methods used to analyze larger datasets are defined in relevant figure legends. Graphical presentations are denoted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ unless noted otherwise.

2.3 Results

2.3.1 TTP expression is significantly suppressed in many tumors and correlates with patient survival in breast cancer

While several studies examined altered TTP expression in a single tissue, few have surveyed this across solid cancers. To identify any correlations between TTP expression in healthy human tissues compared to their respective tumor tissue, we performed a large scale bioinformatic analysis on 25 solid tissue types using Gene-Tissue Expression project (GTEx) and The Cancer Genome Atlas (TCGA) datasets. While the TCGA contains RNA-Seq data for many tumor samples, there is significantly less for normal tissues. To mitigate this, we supplemented normal tissue data from the TCGA with corresponding data from the GTEx in cases where TCGA listed fewer than 10 normal samples to expand the rigor of our analysis across more tumor types. Additionally, in some situations tissue type in the GTEx was further stratified, such as “Brain – Cerebellum” or “Skin – sun exposed”, allowing for closer matches of normal tissue to tumor tissue of origin. In 18 out of 25 solid cancer types analyzed, TTP expression was significantly decreased in tumor tissue compared to healthy tissue (Figure 2.1 and Table 2.1), with particularly potent repression observed in tumors of the bladder, lung, and uterus. TTP expression was also potently suppressed in breast tumors relative to normal controls (Figure 2.2A and Table 2.1), an observation further supported by analyses of patient-matched normal/tumor tissues in breast cancer (Figure 2.2B).

Additionally, relapse-free survival (RFS) in breast cancer was linked to TTP expression level; patients whose tumors express higher levels of TTP had significantly

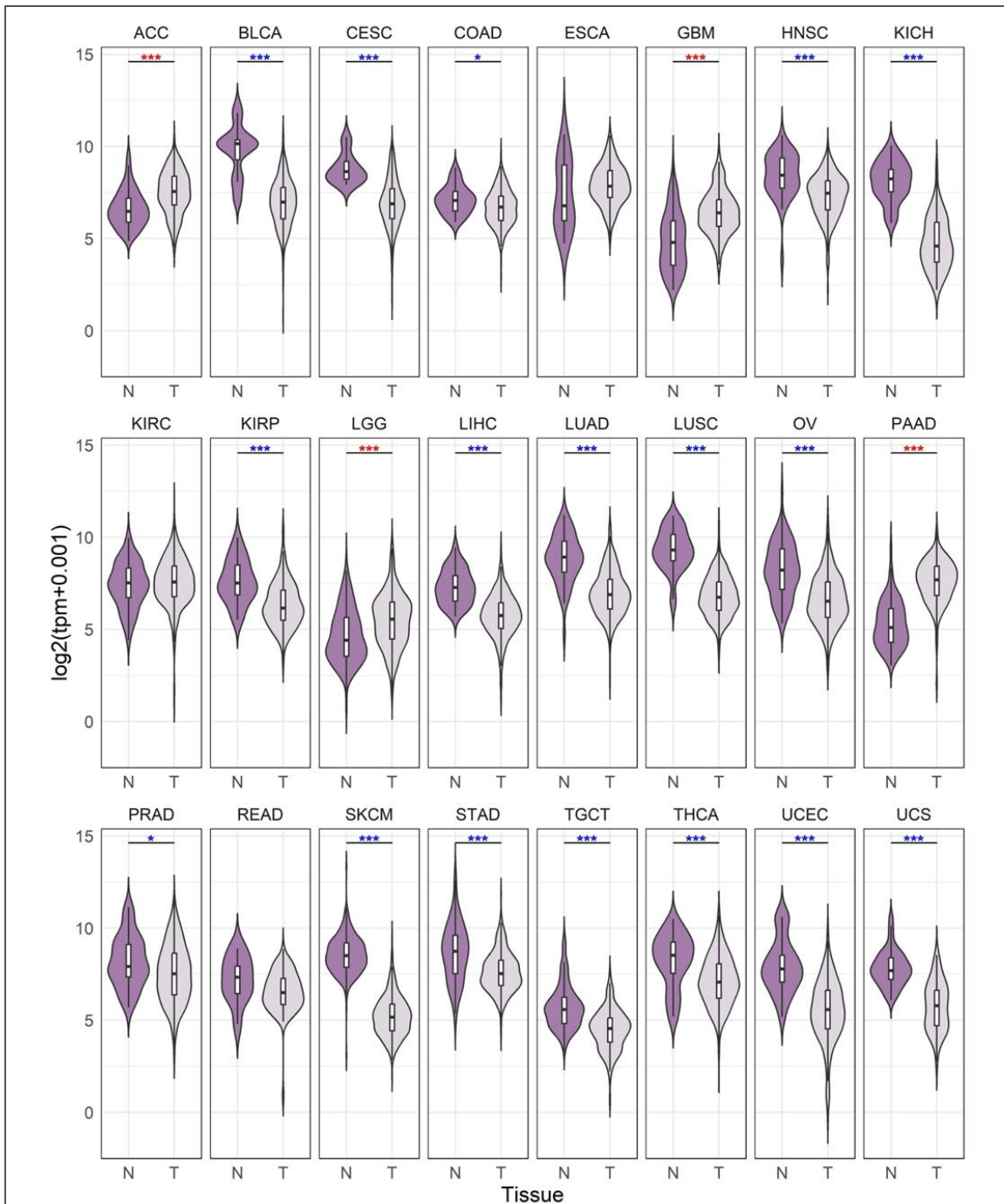


Figure 2.1 Expression of TTP in 25 solid cancers.

Expression of TTP in 25 solid tumor cancers, for which sufficient normal tissue and tumor tissue data was publicly available. For full names corresponding to abbreviations and statistical data, see Table 2.1. Lower hinge of box plot represents the 25% percentile (1st quartile), middle bar represents median, and upper hinge represents the 75% percentile (3rd quartile). Upper and lower whiskers display data that falls within 1.5 * inner quartile range. Width of violin plot represents data density. Blue asterisks: significantly downregulated, red asterisks: significantly upregulated. Raw data are presented in **Table 2.1**

Acronym	Cancer	Normal		Tumor		P-Value	Source of Normal Data
		Median	Mean	Median	Mean		
ACC	Adrenocortical Cancer	6.481	6.627	7.557	7.509	2.43E-11	all GTEX
BLAD	Bladder Urothelial Carcinoma	10.16	9.81	6.983	6.951	2.50E-10	all TCGA
BRCA	Breast Invasive Carcinoma	8.663	8.494	6.32	6.453	2.92E-36	all TCGA
	Triple Negative Breast Cancer			5.886	5.925	1.49E-25 *	
						4.60E-03 **	
CESC	Cervical and Endocervical Cancer	8.638	8.779	6.89	6.916	2.02E-06	4 TCGA and 7 GTEX - Cervix
COAD	Colon Adenocarcinoma	7.071	7.108	6.716	6.681	0.005406	all TCGA
ESCA	Esophageal Carcinoma	6.78	7.395	7.846	7.908	0.261572	all TCGA
GBM	Glioblastoma Multiforme	4.794	4.824	6.394	6.43	3.58E-15	all GTEX - Brain (Cortex)
HNSC	Head and Neck Squam Cell Carcinoma	8.436	8.45	7.472	7.291	2.03E-09	all TCGA
KICH	Kidney Chromophobe	8.245	8.118	4.597	4.749	7.82E-12	all TCGA
KIRC	Kidney Clear Cell Carcinoma	7.523	7.393	7.575	7.581	0.268026	all TCGA
KIRP	Kidney Papillary Cell Carcinoma	7.519	7.595	6.162	6.325	3.65E-07	all TCGA
LGG	Lower Grade Glioma (brain)	4.412	4.643	5.553	5.489	6.24E-13	all GTEX - Brain (Cortex and Cerebellum)
LIHC	Liver Hepatocellular Carcinoma	7.278	7.314	5.749	5.744	1.52E-16	all TCGA
LUAD	Lung Adenocarcinoma	8.919	8.861	6.889	6.98	3.51E-19	all TCGA
LUSC	Lung Squamous Cell Carcinoma	9.305	9.312	6.743	6.806	1.79E-24	all TCGA
OV	Ovarian Serous Cystadenocarcinoma	8.222	8.214	6.534	6.615	2.31E-17	all GTEX - Ovary
PAAD	Pancreatic Adenocarcinoma	5.109	5.283	7.695	7.596	2.56E-40	4 TCGA + 165 GTEX Pancreas
PRAD	Prostate Adenocarcinoma	7.92	8.19	7.519	7.54	0.005939	all TCGA
READ	Rectum Adenocarcinoma	7.34	7.223	6.504	6.568	0.085265	all TCGA
SKCM	Skin Cutaneous Melanoma	8.5	8.537	5.162	5.205	4.69E-119	1 TCGA + 325 GTEX - Skin (Sun exposed)
STAD	Stomach Adenocarcinoma	8.741	8.683	7.538	7.624	1.91E-05	all TCGA
TGCT	Testicular Germ Cell Tumor	5.582	5.73	4.557	4.482	2.54E-18	all GTEX - Testis
THCA	Thyroid Carcinoma	8.521	8.235	7.07	7.126	3.85E-08	all TCGA
UCEC	Uterine Corpus Endomet Carcinoma	7.784	7.914	5.586	5.492	7.71E-09	all TCGA
UCS	Uterine Carcinosarcoma	7.694	7.819	5.794	5.765	5.64E-15	all GTEX - Uterus

Table 2.1: Statistics for pan-cancer analyses of TTP expression from TCGA and GTEx datasets displayed in **Figures 2.1** and **2.2A**.

better outcomes than those whose tumors express low levels of TTP (Figure 2.2C). This is consistent with data from our previous study (118), which used a much smaller set of patients and was limited to breast carcinomas collected from a single county (190). Interestingly, when stratified by invasion into nearby lymph nodes, high TTP expression was strongly indicative of RFS in the lymph node positive cohort, while in the lymph node negative cohort, high TTP was still associated with significant improvement in RFS although the magnitude of this effect was minor (Figure 2.2D). Together, these data suggest that suppression of TTP expression may be more deleterious in advanced, as opposed to early, breast cancer.

We then examined TTP expression solely in triple negative breast cancer (TNBC). Of the subtypes of breast cancer, TNBC is the most aggressive, with survival rates ranging from 91% for localized cancer to 11% for metastatic cancer (191). When gene expression data in TNBC patients is compared to the overall breast cancer population, we observe a subsequent decrease in TTP expression (Figure 2.2A). As low survival rates in TNBC stem from a lack of targetable receptors, targeted treatment options are currently very limited, especially in comparison to breast cancer as a whole (5,13,192), which indicates that investigation into the tumor-suppressive role of TTP in this cancer subtype could be particularly influential.

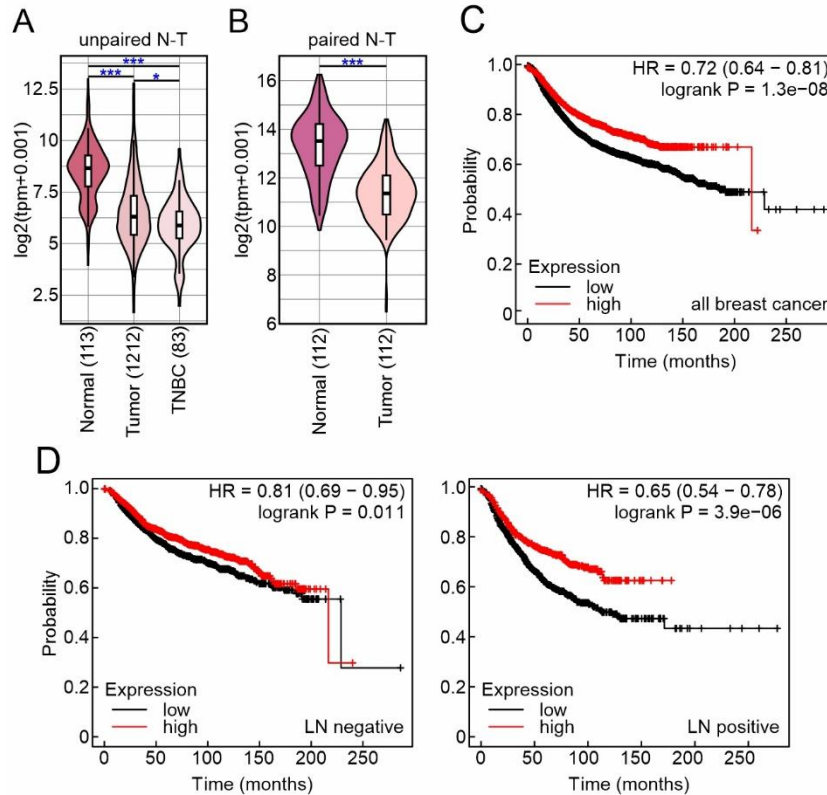


Figure 2.2 Analysis of TTP expression and survival in breast cancer patients. **A** Violin and box plots of TCGA data showing relative TTP expression in normal breast, breast tumor, and TNBC samples, as designated in Table 2.1. Significance of differences determined by Wilcoxon Rank Sum/Mann-Whitney test for unpaired data; * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$. **B** Violin and box plots of data from TNMplot.com showing relative TTP expression in 112 normal breast samples compared to patient-matched breast tumors. Significance determined by Wilcoxon Rank Sum test for paired data; * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$. **C** Kaplan-Meier survival curve of breast cancer patient relapse-free survival (RFS) stratified by TTP expression level across all patients sampled; low TTP, $n = 3331$; high TTP, $n = 1598$. **D** Kaplan-Meier survival curve of breast cancer patient RFS segregated by tumor lymph node (LN) status. LN negative: low TTP, $n = 1035$; high TTP, $n = 1333$, LN positive: low TTP, $n = 1069$; high TTP $n = 587$. For both C and D, TTP expression cutoffs were autoselected by kmplot.com software.

2.3.2 Expression of TTP coordinately downregulates several oncogenic pathways in MDA-MB-231 cells

The observation that TTP is repressed across a wide range of cancer types suggests that this may be a characteristic feature of aggressive neoplasia. This hypothesis is also

consistent with data from previous studies where ectopic expression of TTP attenuated select tumorigenic phenotypes (118,152,164,179). However, the population of regulated genes contributing to the diversity of TTP's anti-cancer functions is largely unknown. Given preliminary observations showing an antiproliferative role for TTP in a TNBC cell model (178), coupled with the unmet need for novel therapeutic targets in TNBC and extreme suppression of endogenous TTP expression in TNBC tumors (Figure 2.2A), we generated clonal MDA-MB-231 cell lines that stably express FLAG-tagged TTP along with EV controls (Figure 2.3A). Global consequences of TTP on gene expression in this cell line were evaluated by RNA-Seq using two independent clones of each cell model. This analysis revealed profound transcriptomic alterations including 279 upregulated and 170 downregulated genes in FLAG-TTP-expressing MDA-MB-231 cells relative to EV controls based on a log₂ fold-change cutoff of 1 (Figure 2.3B, C). Raw fastq files and processed log₂-fold change values have been compiled and deposited in the GEO database, under the accession number GSE292372. Subsequent interrogation of differentially-expressed genes by GSEA identified many cancer-related pathways that were potently suppressed in TTP-expressing cells, including several that regulate energy management, metabolism, and particularly, proliferation (Figure 2.3D, E). Interestingly, when using the same normalized enrichment score (NES) cutoff for pathways upregulated by TTP, we observed only nine, few of which are relevant to cancer. Among the oncogenic networks downregulated by TTP expression, we noted negative enrichment of cell cycle pathways (Fig. 2.3F); extraction of the leading edge genes driving this pathway reveals that a large subset is significantly downregulated. The second most downregulated pathway in our dataset was mTORC1 signaling (Fig. 2.3F), which has also been shown to be strongly

oncogenic (193). For example, in prostate cancer, mTOR signaling was functionally linked to proliferation, metabolism, protein synthesis, and invasion (194). Additionally, we saw significant downregulation of hypoxia genes and gene sets (Figure 2.3F). Together, these data

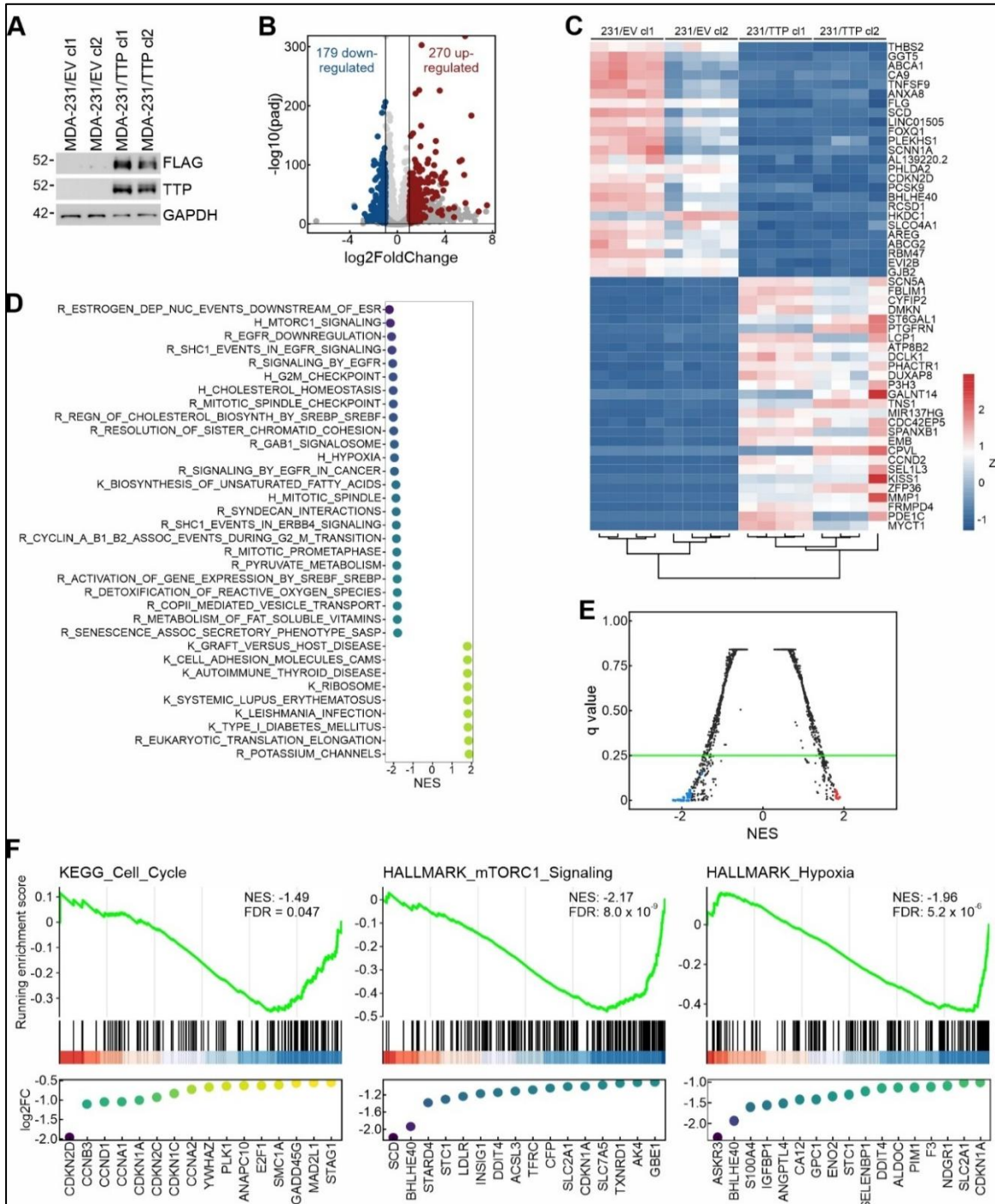


Figure 2.3 Transcriptomic analyses of global gene expression changes in TTP-expressing MDA-MB-231 cells.

A Western blot analysis of FLAG-TTP protein expression in MDA-MB-231 clonal cell lines, with GAPDH used as a loading control. **B** Volcano plot of total transcriptome RNA-seq DESeq2 analysis comparing MDA-MB-231/TTP versus EV control lines. Each dot is a single gene, red dots: genes with a log₂-fold change > 1 and a p-adjusted < 0.05; blue dots: genes with a log₂-fold change < -1 and a p-adjusted < 0.05; gray dots: genes that do not meet these criteria, or have a low read count, as defined in Materials and Methods. **C** Hierarchical clustering heatmap of the top 25 up- and down-regulated DEGs from z-score transformed MDA-MB-231/EV and TTP total transcriptome RNA-seq normalized read counts: blue, downregulated; red, upregulated. **D** Top HALLMARK (H), KEGG (K) and REACTOME (R) gene sets up- and down-regulated by TTP in MDA-MB-231. Color transitions reflect changes in normalized enrichment scores (NES) from GSEA analysis. **E** HALLMARK, KEGG, and REACTOME gene set NES volcano plot for all gene sets derived from GSEA analysis. Green line indicates q value significance cutoff of 0.25. Blue dots: NES less than -1.8 (25 sets); red dots: NES greater than 1.8 (9 sets); these are the sets listed in D. **F** KEGG Cell Cycle, HALLMARK mTORC1 Signaling, and HALLMARK Hypoxia NES plots from total transcriptome RNA-seq data. For each plot: y-axis, enrichment score; x-axis, compiled ranked genes. Below each plot is a scatter plot showing log₂ fold change values for the top 15 leading edge genes.

demonstrate that in MDA-MB-231 cells, expression of TTP can potentially suppress multiple oncogenic pathways.

2.3.3 Transcriptomic changes after TTP expression are accompanied by robust anti-tumorigenic phenotypes

To interrogate whether transcriptomic alterations observed in TTP-expressing MDA-MB-231 cells correspond to functional changes at the cellular level, we next analyzed select phenotypes characteristic of aggressive tumors. First, cell proliferation was dramatically inhibited in MDA-MB-231 cells that stably express FLAG-TTP relative to EV controls (Figure 2.4A), consistent with a previous study (178). To ensure that this observation was not unique to the MDA-MB-231 cell model, clonal lines stably expressing FLAG-TTP were also generated in MDA-MB-436 and BT-549 cell backgrounds along

with EV controls. In these independent TNBC models, TTP expression also potently suppressed proliferation (Figure 2.4B, C). Subsequent flow cytometry analyses of Annexin V-stained cells verified that the TTP-induced decreases in cell accumulation are due to slowed proliferation rather than increased cell death (Figure 2.4D).

Aggressive tumor cells also frequently display an increased propensity for migration and invasion, characteristics involved in the early local invasion and intravasation stages of the metastatic cascade. GSEA analyses of our MDA-MB-231 RNA-Seq data identified the HALLMARK_MTORC1_SIGNALING pathway as potently downregulated in response to TTP expression. Genes in this pathway have a number of pro-tumorigenic roles, including promoting cell growth, motility and the epithelial-to-mesenchymal transition (195). mTOR is also known to influence metabolic signaling, which can itself promote cell growth and metastatic processes (196,197). Interestingly, our analyses also indicated profound downregulation of multiple pathways relating to metabolism (Fig. 2.3D) in TTP-expressing cells as well as the pro-metastatic HALLMARK_ANGIOGENESIS gene set (NES = -1.24, FDR = 0.12, data not shown). Cumulatively, these data suggest that metastasis-associated functions might also be inhibited by TTP.

To test whether TTP expression altered cell migratory functions in TNBC models, we performed experiments using an xCELLigence RTCA system. In MDA-MB-231 and BT-549 cells, expression of TTP dramatically inhibited migration across a membrane towards a chemoattractant (Figure 2.4E, H). Migration of MDA-MB-436 cells was very slow even in the absence of TTP but was nonetheless modestly but significantly inhibited when TTP was expressed (Figure 2.4G). Finally, TTP expression also inhibited invasion

of MDA-MB-231 cells through Matrigel-coated membranes (Fig. 2.4F). Cumulatively these data indicate that TTP slows the rate of cell migration and decreases invasive potential in these TNBC models.

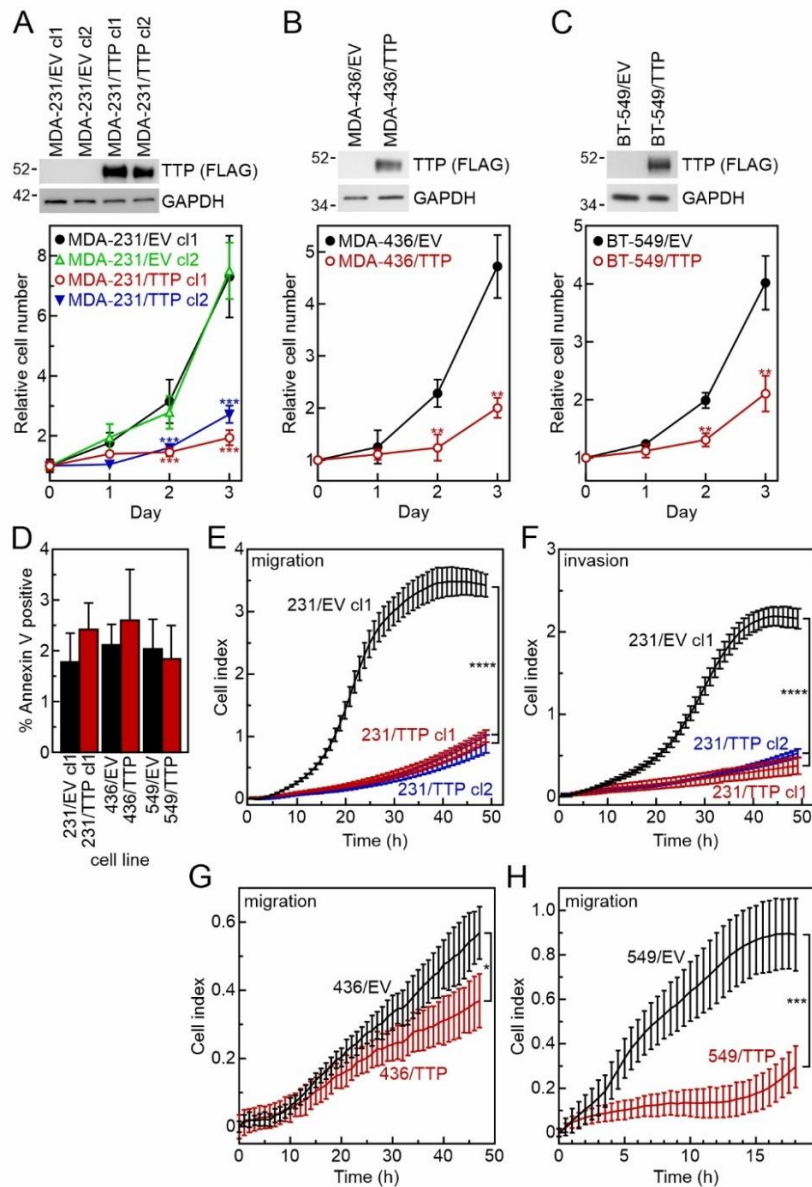


Figure 2.4 Effect of TTP on select tumorigenic phenotypes in TNBC models.

A-C (top) Western blots showing FLAG-TTP expression in indicated MDA-MB-231, MDA-MB-436, and BT-549 clonal cell lines with GAPDH used as loading control. (bottom) Cell proliferation analyses of each cell line. Symbols represent the mean \pm SD of $n \geq 4$ biological replicates. **D** Cell death analysis following Annexin V-PE staining analyzed by flow cytometry in indicated TNBC cell lines. Bars show the mean \pm SD of 3 independent replicates. **E** Cell migration and **F** invasion analyzed by xCELLigence RTCA in indicated MDA-MB-231 cell lines. **G, H** Migration analyzed by xCELLigence RTCA in indicated MDA-MB-436 and BT-549 cells. For migration and invasion assays, all points represent the mean \pm SD of 4 biological replicates except for the 231/EV cl1 invasion data in panel F and 436/EV migration data in panel G, which are based on $n = 3$. All statistical analyses were performed and annotated as described in Materials and Methods.

2.3.4 TTP expression does not alter cell cycle distributions or expression of major cell cycle regulatory proteins

After confirming that TTP inhibits proliferation and migratory potential in these cell models, we sought to define mechanism(s) by which these phenotypic changes were occurring. Based on our bioinformatic analyses, we hypothesized that inhibition of proliferation could be due to specific perturbations of the cell cycle. However, cell cycle analyses by propidium iodide staining and flow cytometry revealed no significant changes in cell distributions across the cell cycle in MDA-MB-231 cells apart from a slight decrease in the G1 subpopulation in a single MDA-MB-231/TTP clone (Figure 2.5A). Parallel experiments in MDA-MB-436 and BT-549 models also showed no effect of TTP expression on cell cycle distributions (Figures 2.5B, C). Western blots were then used to determine whether TTP expression altered levels of major cell cycle regulatory proteins in MDA-MB-231 cells. This panel included MYC, based on its roles in accelerating the cell cycle at multiple stages (198). However, apart from a modest decrease in cyclin D1 protein levels, we identified no TTP-dependent changes in expression of these regulatory proteins consistent with inhibition of the cell cycle (Figure 2.5D). If the observed decrease in cyclin D1 significantly contributed to impaired proliferation, we would expect to see a corresponding increase in the percentage of cells in the G1 stage, since cyclin D1 facilitates the G1/S transition. However, this was not observed in our cell cycle analyses.

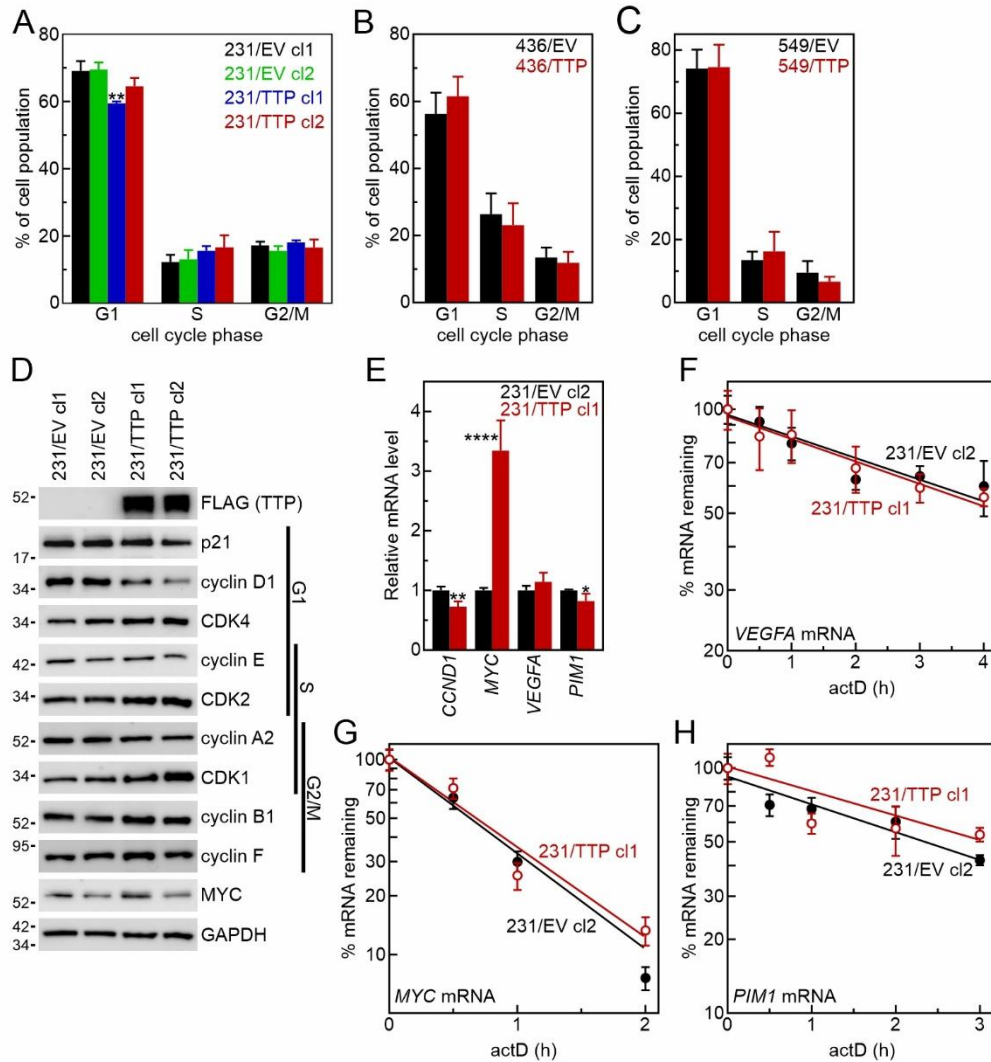


Figure 2.5 TTP does not alter cell cycle phases or destabilize canonical mRNA targets in TNBC models.

A-C Cell cycle distributions following propidium iodide (PI) staining analyzed by flow cytometry in indicated cell models. Bars represent the mean \pm SD of 3 biological replicates. **D** Western blot analysis of indicated cell cycle regulatory proteins in MDA-MB-231 clonal lines expressing TTP versus EV controls. Bars at right denote major cell cycle phase(s) regulated by each measured protein. MYC has no such designation as it can influence the cell cycle at multiple points. GAPDH was used as a loading control. **E** Relative levels of select TTP target mRNAs measured by RT-qPCR in indicated MDA-MB-231 cell lines normalized to 18S rRNA. Bars represent the mean \pm SD of 3 biological replicates. **F-H** Representative decay plots of indicated mRNAs in MDA-MB-231/EV (black) versus MDA-MB-231/TTP (red) cell models. Points represent mean \pm SD values of 4 technical replicates of each sample and lines are regression solutions to a single exponential decay function. mRNA half-lives resolved from multiple independent experiments are summarized in **Table 2.2**. All statistical analyses were performed and annotated as described in Materials and Methods.

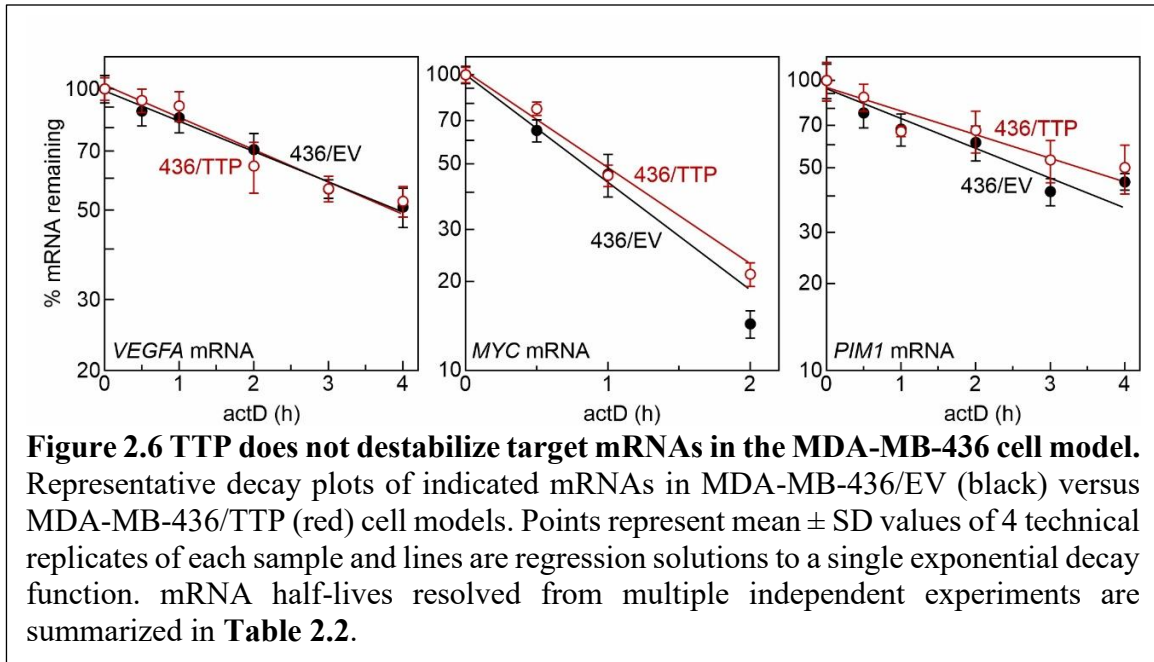
The limited effects of TTP on expression of major cell cycle regulatory factors in MDA-MB-231 cells were unexpected given that: (i) *CCND1* (encoding cyclin D1), *CCNE1* (encoding cyclin E1), and *MYC* mRNAs all contain ARE sequences in their 3'UTRs, and (ii) *CCND1* and *MYC* mRNAs are both targeted and destabilized by TTP in glioblastoma cells (147). To determine whether TTP might nonetheless regulate these genes at the mRNA level in MDA-MB-231 cells, we used RT-qPCR to compare transcript levels. *CCND1* mRNA levels were only modestly decreased in TTP-expressing MDA-MB-231 cells versus EV controls, while TTP induced *MYC* mRNA expression by over 3-fold (Fig. 2.5E). Parallel measurements with two other well-established TTP substrate mRNAs, *VEGFA* and *PIMI* (119,199), showed no or only slight suppression by TTP, respectively. These observations prompted two mechanistic hypotheses: (i) that TTP-dependent destabilization of these mRNAs was compensated by increased transcription, or (ii) that TTP does not destabilize these mRNAs in these cell models. To distinguish between these possibilities, the half-lives ($t_{1/2}$) of select TTP substrate mRNAs were measured using actinomycin D (actD) time course assays. In the MDA-MB-231 lines, TTP expression had no significant effect on the decay kinetics of *VEGF*, *MYC*, or *PIMI* mRNAs (Figure 2.5F-H and Table 2.2).

Table 2.2 Decay rates of select TTP substrate mRNAs are independent of TTP in MDA-MB-231 and MDA-MB-436 cell models

mRNA	Cell line	$t_{1/2}$ (h) ^a	<i>n</i>
VEGF	231/EV cl2	4.62 ± 0.54	3
	231/TTP cl1	4.73 ± 0.23	3
	436/EV	3.33 ± 0.60	3
	436/TTP	3.53 ± 0.39	3
<i>c-myc</i>	231/EV cl2	0.66 ± 0.12	3
	231/TTP cl1	0.65 ± 0.06	4
	436/EV	0.95 ± 0.18	4
	436/TTP	1.05 ± 0.30	3
PIM1	231/EV cl2	2.91 ± 0.23	3
	231/TTP cl1	3.38 ± 0.72	4
	436/EV	3.58 ± 0.86	4
	436/TTP	4.24 ± 0.47	3

^aDecay kinetics of indicated mRNAs measured by actD time course assays described in Figs. 4F-H and 2.6. Quoted mRNA half-lives ($t_{1/2}$) are the mean ± SD values from *n* independent time course experiments.

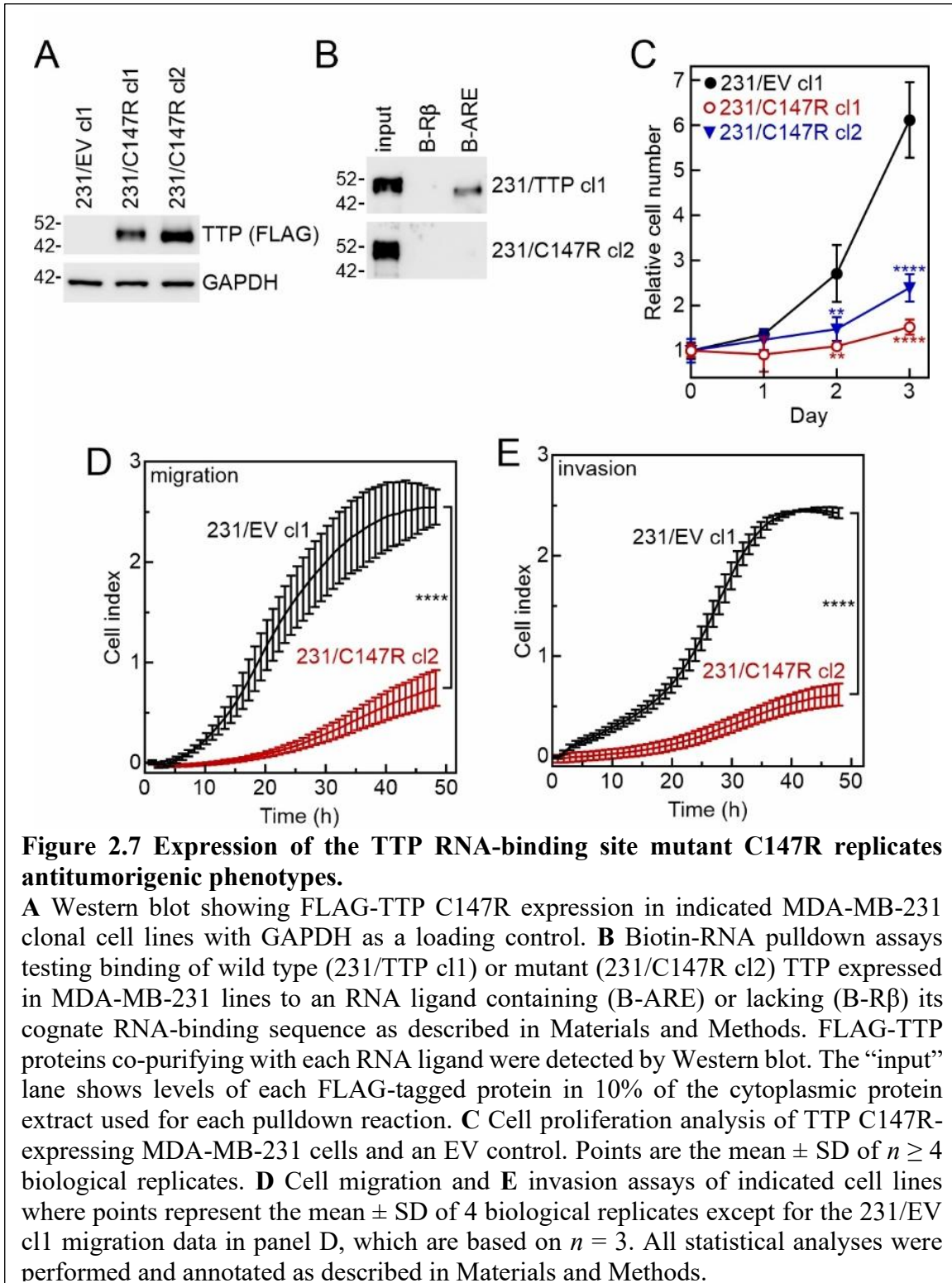
Similar results were observed in MDA-MB-436 cell models (Figure 2.6 and Table 2.2), indicating that inhibition of TTP’s mRNA-destabilizing activity was not limited to MDA-MB-231 cells. Together, these data indicate that the inhibition of cancer cell phenotypes mediated by TTP in these TNBC models is not the result of accelerated mRNA substrate decay but rather suggests that novel non-canonical mechanisms may be involved in the tumor suppressive roles of this protein.



2.3.5 TTP can suppress neoplastic phenotypes independent of its RNA-binding activity

The ability of TTP to attenuate oncogenic phenotypes independent of its mRNA-destabilizing activity prompts the question of whether these tumor suppressive roles even require its RNA-binding function. To test this possibility, we utilized a TTP mutant (C147R), where a zinc-coordinating cysteine located in the C-terminal zinc finger domain was mutated to an arginine, abrogating its RNA-binding activity (78). After constructing clonal MDA-MB-231 cell lines that stably express FLAG-tagged TTP C147R (Figure 2.7A), we used biotin-RNA pulldown assays to confirm its inability to bind a canonical ARE-containing RNA substrate (Figure 2.7B). We then tested whether expression of TTP C147R modulated any of the tumorigenic phenotypes suppressed by the wild type protein. Cell proliferation was markedly slowed in cells expressing TTP C147R (Figure 2.7C), consistent with the effects of wild type TTP (Figure 2.4A). Also, xCELLigence assays

revealed that cell migration and invasiveness were significantly inhibited in MDA-MB-231 cells expressing this mutant TTP protein (Figure 2.7D, E).



To extend our analyses to an *in vivo* model, we stably expressed TTP and its C147R mutant in a highly aggressive tumor-derived MDA-MB-231 cell line (231td, Figure 2.8A). *In vitro* growth assays confirmed that both wild type and mutant forms of TTP significantly inhibited cell proliferation relative to an EV control line (Figure 2.8B) analogous to findings in the standard MDA-MB-231 cell background (Figures 2.4A and 2.7C). Cells from each 231td cell line were then injected adjacent to the mammary fat pad in an athymic nude xenograft mouse model, where we observed significant inhibition of tumor growth from TTP- and TTP C147R-expressing cells relative to an EV control line in mice over a three-week period (Figure 2.8C). Upon study termination, tumors were excised and weighed, and tumors expressing TTP or TTP C147R were significantly smaller than EV controls (Figure 2.8D). These data demonstrate that TTP can function independently of its RNA-binding and -destabilizing roles and attenuates tumorigenic phenotypes by a novel, non-canonical mechanism. Further, to our knowledge, this is the first demonstration of anti-tumorigenic activity by an RNA-binding mutant form of TTP.

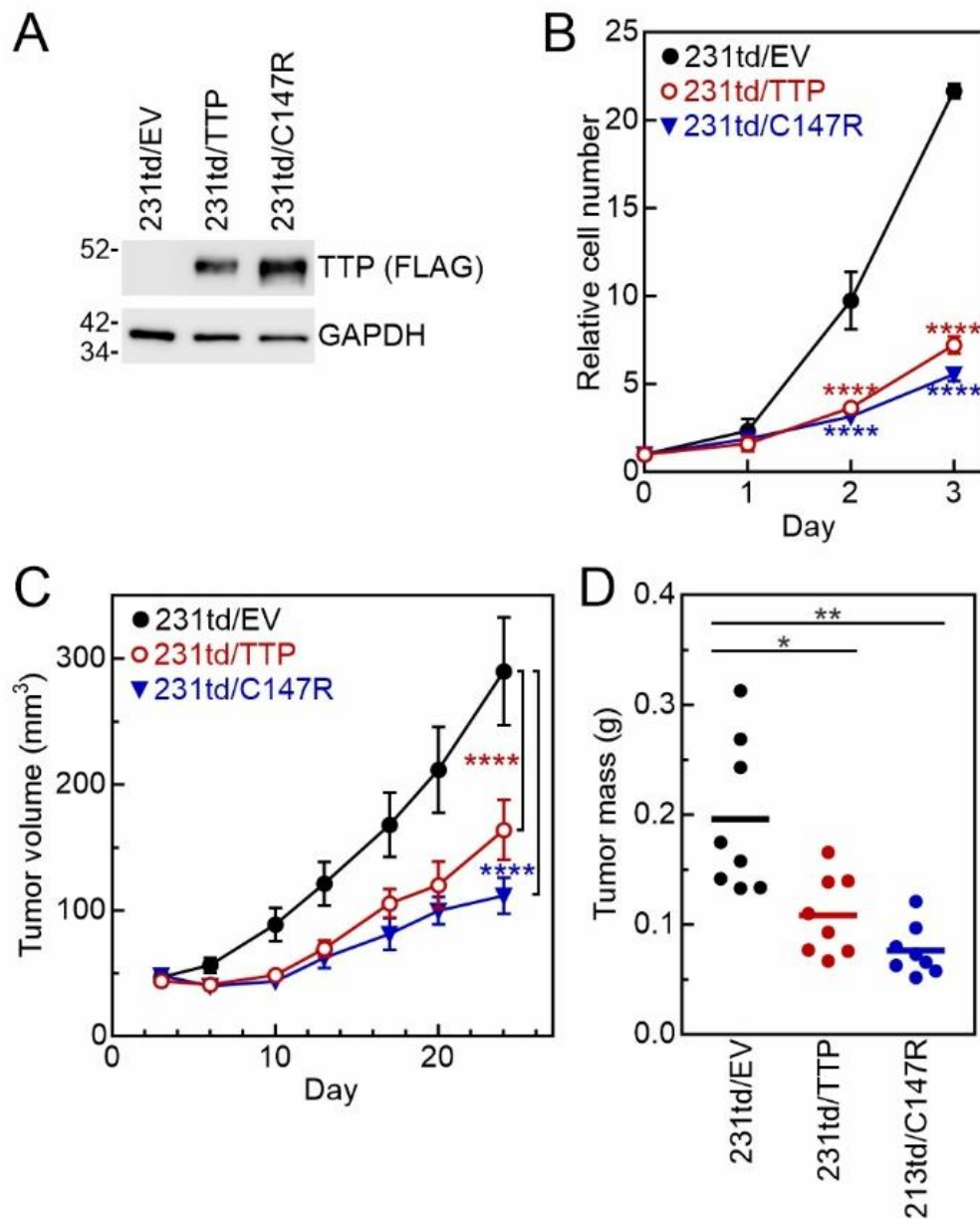


Figure 2.8 TTP and its C147R mutant inhibit proliferation and xenograft growth in a tumor-derived TNBC cell model.

A Western blot showing expression of FLAG-tagged TTPwt and C147R in tumor-derived MDA-MB-231 (231td) clonal lines with GAPDH used as a loading control. **B** Proliferation analyses of TTP- or TTP C147R-expressing 231td cells relative to an EV control. Points are the mean \pm SD of $n \geq 4$ biological replicates. **C** *In vivo* xenograft model measuring tumor volume (mm^3) of 231td/EV, TTP, or TTP-C147R cells over a 24-day period. Points represent the mean \pm SEM from $n = 8$ biological replicates. Differences were analyzed using two-way ANOVA. **D** Scatter plots showing masses of tumors excised from indicated xenografts on day 24. Lines indicate mean tumor mass for each group. Differences were compared using the Mann-Whitney test. For all panels, p values were designated as described in Materials and Methods.

2.4 Discussion

While numerous studies have investigated alterations in TTP expression in individual tumor types, few have evaluated their prevalence across the spectrum of solid cancers. Here, we show that suppression of TTP is a widespread phenomenon in many malignancies, reinforcing the importance of pre-clinical efforts to elucidate mechanisms by which TTP constrains tumor phenotypes. However, our data also indicate that TTP expression is significantly increased in four cancer types: adrenocorticoid carcinoma, glioblastoma, lower grade glioma, and pancreatic adenocarcinoma (Figure 2.1). While these data may reflect genuine TTP induction in some tumors, limitations in sample procurement could influence this interpretation. First, while the TCGA includes many tumor samples, the selection of normal tissues is much more limited, prompting us to supplement these with GTEx data. Second, “normal” samples are organ-specific, but not cell-type specific. In tissues with diverse cellular composition, it is possible that control samples may overrepresent cell types unrelated to the tumor’s origin. For instance, healthy pancreatic tissue primarily consists of acinar cells, with small fractions of ductal and endocrine cells. However, pancreatic ductal carcinoma (PDAC), the most common pancreatic cancer, arises from the ductal subpopulation (200). In fact, a previous study reported that TTP mRNA and protein levels were decreased in a cohort of 35 pancreatic tumor samples when compared to patient-matched adjacent normal tissue (150). Similarly, although our TCGA and GTEx analyses suggest increased TTP expression in glioblastoma and lower-grade glioma compared to normal tissue, prior studies have reported that TTP has tumor suppressor functions in glioma cell lines (180). Future studies incorporating

single-cell approaches will be essential to resolve alterations in TTP expression between tumors and their corresponding cells of origin.

Our study shows that TTP robustly inhibits multiple oncogenic phenotypes in TNBC models, including proliferation, migration, invasion, and *in vivo* tumor growth. Unexpectedly, we found that these inhibitory effects are independent of TTP's canonical activity of destabilizing ARE-containing mRNAs. A previous study reported that TTP suppressed metastasis-associated transcripts such as *PLAU* (*uPA*), *PLAUR* (*uPAR*), and *MMP1* in MDA-MB-231 cells by accelerating their degradation (201). While our RNA-Seq data showed modest (<50%) downregulation of *PLAU* and *PLAUR* in TTP-expressing MDA-MB-231 cells (GEO dataset GSE292372), *MMP1* was among the genes most potently upregulated by TTP (Figure 2.3C). Furthermore, TTP expression did not alter decay kinetics of several well-characterized TTP target mRNAs in either MDA-MB-231 or MDA-MB-436 cells (Table 2.2). Several factors could account for differences observed between these studies. First, the previous report used transient transfection to express TTP, while we used stably-transfected clonal lines. Conceivably, the consequences of TTP on gene regulation and cellular physiology may differ between short-term and sustained expression. Second, mRNA decay kinetics in the prior study was assessed using TTP^{+/+} and TTP^{-/-} mouse embryonic fibroblasts and analyzed using a bimodal model assuming that a large proportion (as much as 70%) of each mRNA population is decay-resistant. In contrast, our mRNA decay studies were performed in TNBC cell models and were well-resolved by a first-order decay function that did not assume differentially decaying subpopulations (Figures 2.5F–H and 2.6). Finally, while we cannot fully exclude that a subset of mRNAs may still be degraded by TTP in MDA-MB-231 cells, the potent

antineoplastic effects observed for the TTP C147R mutant (Figures 2.7 and 2.8) confirm that targeted mRNA destabilization is not required for TTP's tumor suppressor function in these models.

Another intriguing question arising from this study is how TTP's mRNA-destabilizing function is disabled in MDA-MB-231 and MDA-MB-436 cells, since TTP does destabilize mRNA targets in some other cancer cell models (118,179,201). Well-established regulators of TTP include the ERK and p38^{MAPK} pathways, which can modulate its function, abundance, and location via post-translational modification (146,202,203). To test whether these pathways inhibited TTP-mediated mRNA decay in our TNBC models, we treated MDA-MB-231/TTP cells with the MEK inhibitor U0126 or the p38^{MAPK} inhibitor SB202190. Western blot analyses verified that phosphorylation of downstream substrates ERK and MAPKAP K2, respectively, were inhibited by each compound (Figure 2.9A, B) while decay kinetics of three known TTP substrate mRNAs were unaffected by these inhibitors (Figure 2.9C and Table 2.3). These results suggest that neither pathway independently regulates TTP activity in this context, although functional redundancy or involvement of independent kinase pathways cannot be ruled out.

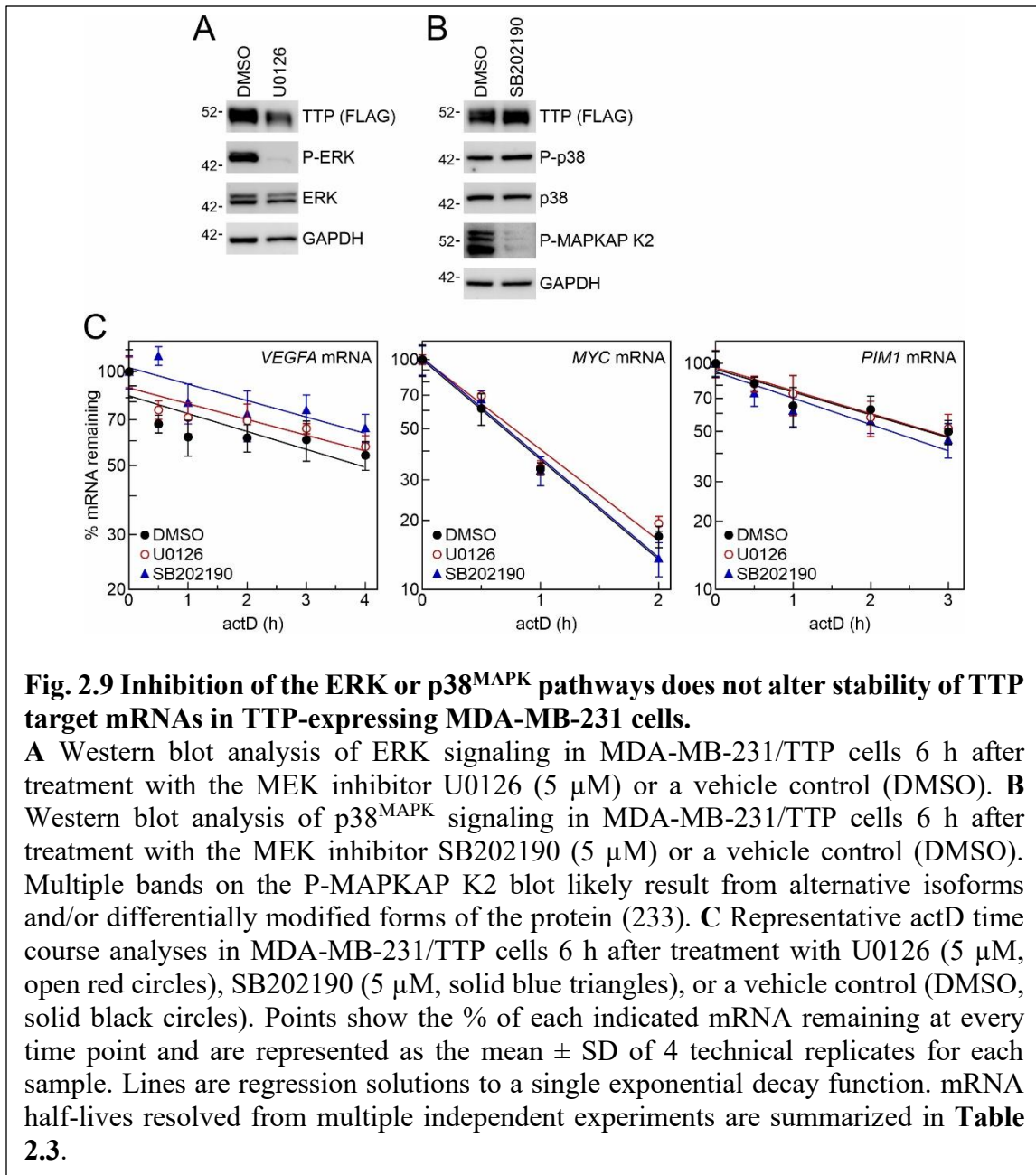


Table 2.3: Decay rates of select TTP substrate mRNAs are unaffected by MAP kinase inhibitors in TTP-expressing MDA-MB-231 cells

mRNA	treatment ^a	$t_{1/2}$ (h) ^b	n
<i>VEGFA</i>	DMSO	4.47 ± 1.35	4
	U0128	6.05 ± 0.28	3
	SB202190	6.35 ± 0.74	3
<i>MYC</i>	DMSO	0.72 ± 0.03	3
	U0128	0.74 ± 0.04	3
	SB202190	0.66 ± 0.04	3
<i>PIMI</i>	DMSO	2.66 ± 0.57	3
	U0128	2.43 ± 0.47	3
	SB202190	2.62 ± 0.53	3

^aMDA-MB-231/TTP clone 1 cells were treated with MEK inhibitor U0128 (5 μ M), p38^{MAPK} inhibitor SB202190 (5 μ M), or an equivalent volume of DMSO for 6 h prior to induction of actD time course assays.

^bDecay kinetics of indicated mRNAs measured by actD time course assays described in **Fig. 2.9**. Quoted mRNA half-lives ($t_{1/2}$) are the mean \pm SD values from n independent time course experiments.

Since TTP's tumor-suppressive effects are independent of both its RNA-binding and RNA-destabilizing activities, a major question prompted by this work is how TTP attenuates tumorigenic phenotypes in this model. A previous study reported that TTP induces S-phase arrest in MDA-MB-231 cells by inhibiting activation and nuclear translocation of the p65 subunit of NF- κ B, leading to reduced JUN expression and increased levels of the G2 checkpoint kinase Wee1 (178). However, several observations argue against this mechanism in our models. First, TTP did not significantly alter cell cycle distributions in any of the three TNBC models tested (Figure 2.5A–C).

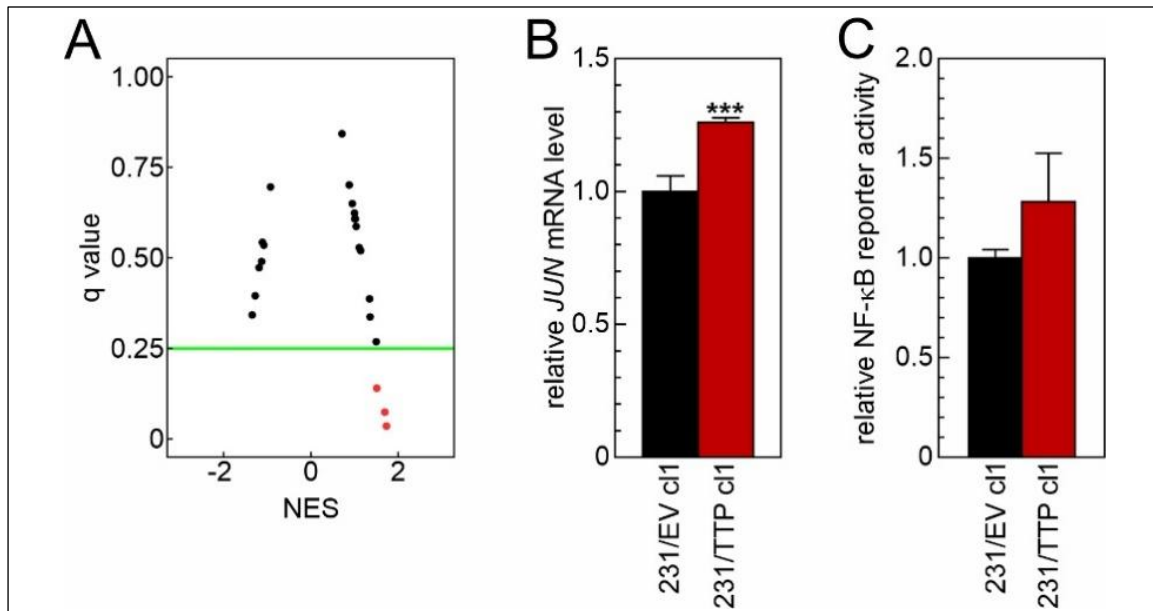


Fig. 2.10 Re-expression of TTP does not alter NF-κB transcription factor activity in MDA-MB-231 cells.

A NES volcano plot created from GSEA analysis of NF-κB-related pathways from the C2 subset of the Molecular Signatures Database (MSigDB). The green line indicates the q value significance cutoff of 0.25. Red dots: Significantly enriched pathways, black dots: pathways that do not meet significance criteria. **B** Relative expression of *JUN* mRNA measured by qRT-PCR in MDA-MB-231 cells expressing TTP versus EV. Bars represent the mean \pm SD of three biological replicates. *** $p < 0.001$. **C** Relative NF-κB reporter activity from plasmid pELAM-luc measured by luciferase assay after normalization to *Renilla* luciferase expressed from plasmid pRL-GAPDH in co-transfected MDA-MB-231/EV or TTP cells as described under Methods and Materials. Bars represent the mean \pm SD of three biological replicates.

Second, we expanded the GSEA analysis of our RNA-Seq data to include the entire C2 subset of the Molecular Signatures database. Among the 22 gene sets designated as NF-κB-related, only three were significantly modulated in TTP-expressing cells, and notably, all three were positively enriched (Figure 2.10A). Third, RNA-Seq showed no significant difference in *JUN* mRNA expression, while RT-qPCR showed a modest but significant increase in *JUN* expression in TTP-expressing cells (Figure 2.10B), rather than the decrease reported previously. Finally, luciferase reporter assays revealed no change in

activity of an NF- κ B-responsive promoter between EV and TTP-expressing MDA-MB-231 cells (Figure 2.10C). A major difference between these two studies is that the former largely used adenoviral vectors to deliver TTP, while our approach uses integrated plasmid vectors. It is conceivable that the previously observed effects on the NF- κ B-Jun-Wee1 axis leading to cell cycle arrest at S phase could be coupled to amplitude and/or duration of TTP expression. Alternatively, adenoviral transduction can activate stress response pathways (204), potentially confounding results, particularly because TTP plays regulatory roles during stress and inflammatory responses, including viral infection (205,206).

If RNA destabilization and NF- κ B signaling are not required for TTP's anti-tumor effects in TNBC, how does TTP mediate these phenotypes? Evidence for other TTP-interacting partners is emerging but remains incomplete. For example, TTP can function as a transcriptional co-repressor for estrogen receptor α (207), although this is unlikely to be relevant in TNBC models. Other known TTP partners include RNaseL, hnRNP F, and the translational repressor eIF4E2 (208–210), although all of these appear to be involved in RNA-targeted functions of TTP. Yet other examples are associated with regulation of TTP protein turnover, including PKM2 and PIM2 (211,212). Elucidation of potential mechanisms by which TTP suppresses tumorigenic phenotypes would significantly benefit from a comprehensive interactome analysis for TTP-binding proteins. In particular, a screen for factors binding TTP-C147R might prove useful, since it should exclude those recruited only to RNA-bound proteins.

In conclusion, our study shows that TTP extensively reprograms gene regulation leading to robust attenuation of several tumorigenic phenotypes in several TNBC cell models. However, these antioncogenic functions do not require RNA binding by TTP,

indicating a novel mechanism of action involving heretofore unknown binding partners. Elucidating these mechanisms in TNBC and other cancers will further support the potential of restoring TTP expression or activity as a viable therapeutic strategy.

Chapter 3: Summary and Perspectives

Post-transcriptional regulation is likely to be a key modulator of tumorigenic processes, but it is not well understood. Identification of novel molecular targets is an ongoing challenge in cancer research, and modulation of these post-transcriptional regulatory nodes may present new opportunities for therapeutic development. In the present study, we describe a novel role for TTP, previously characterized only as an RNA-binding and -destabilizing protein that accelerates degradation of its target ARE-containing mRNAs (78,140,152,203,209,213,214). Here, we first describe the anti-tumorigenic activity of TTP on multiple aggressive tumor phenotypes in TNBC, including proliferation, migration, invasion, and *in vivo* tumor growth. Next, we determine that the attenuation of the growth phenotypes is not mediated by cell cycle perturbations, as has been published previously (178). Our rigorous analysis of cell cycle regulators reveals that TTP does not alter the expression of these proteins in a manner consistent with global restriction through the cell cycle. Interestingly though, our tested candidates included two proteins encoded by mRNAs previously identified as targets for destabilization by TTP. This led us to the question, does TTP destabilize mRNA substrates in TNBC cell lines? To answer this, we performed actD time course assays using known TTP target mRNAs and discovered that TTP does not destabilize canonical targets in our cell models, contradicting an earlier report (201). We then asked, does TTP even need to bind mRNA in order to effect its anti-tumorigenic functions? To interrogate this, we used an RNA-binding mutant form of TTP, C147R, which we and others have shown incapable of binding to ARE-containing RNA

ligands. We found that expression of TTP C147R phenocopies the suppression of proliferation, migration, invasion, and *in vivo* tumor growth observed with the wild type protein. Collectively, our new insight indicates the presence of a novel mechanism by which TTP can attenuate aggressive tumor phenotypes, which is independent of its canonical RNA-binding and mRNA-destabilizing functions.

3.1 Non-canonical TTP mechanism

One of the key questions that arises from this work is, by what mechanism does non-canonical TTP activity suppress tumorigenic phenotypes? Given that we detected no alterations to mRNA stability in this study, but many gene alterations, it is likely that the mechanism by which TTP acts in these cell models involves influences on transcription. We propose that these changes in gene expression may arise from altered protein interactions and/or disruptions in cellular signaling pathways, ultimately leading to differential activation of one or more transcription factors. Downstream transcription factor activation is capable of changing multiple cellular programs, leading to the functional suppression of multiple tumor phenotypes demonstrated in Chapter 2. Future studies will interrogate the steps of this global pathway manipulation by TTP.

Using the BioID assay (215), we could investigate altered protein interactions using our TTP C147R protein. Here, a fusion protein of TTP C147R linked to a promiscuous biotin ligase would be expressed in cells. In the presence of biotin, other proteins in close proximity become biotinylated, and can then be isolated using streptavidin beads, allowing us to identify TTP binding partners by mass spectrometry. The advantage of using the mutant protein instead of the wild type is that the mutant protein is unable to bind RNA, so

any pulldown would not include other proteins associating with common mRNA substrates, such as translational machinery.

This is also the first time that anti-tumorigenic activity has been described for any RNA-binding mutant of TTP. One study had demonstrated that TTP suppressed invasiveness in HEK293 cells, but expression of the C124R TTP mutant, which is also incapable of binding RNA, was unable to recapitulate this phenotype (201). A survey of the literature revealed this work to be the only study besides ours to interrogate the effect of an RNA-binding mutant form of TTP on anti-tumorigenic phenotypes. This suggests that the TTP C147R variant may also possess anti-tumorigenic potential in cancers beyond breast cancer.

Since TTP is often phosphorylated to inhibit its activity, it is possible that differential phosphorylation in TNBC causes the switch to the non-canonical mechanism. In our present work, we showed that independent inhibition of ERK or p38^{MAPK} signaling did not restore the mRNA-destabilizing activity of TTP (Figure 2.9C and Table 2.4). Additionally, we generated preliminary data indicating that a combination of both ERK and p38^{MAPK} inhibitors does not rescue TTP's mRNA-destabilizing function (Figure 3.1). A proteomics approach would be particularly valuable here, as mass spectrometry could reveal the specific sites and identities of TTP post-translational modifications in our TNBC cell models. Comparing these data to a cell model where TTP has been identified to destabilize mRNA, such as HeLa cells (118), would allow us to dissect the differences in post-translational modifications between the two backgrounds.

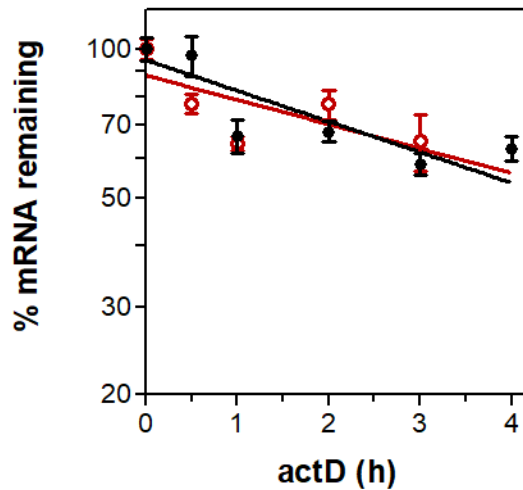


Figure 3.1. Decay of VEGF mRNA after combination ERK and p38^{MAPK} inhibition in MDA-231/TTP c11 cells

Decay plot of VEGF mRNA after 4 h treatment with either DMSO (solid black circles) or the combination of the p38^{MAPK} inhibitor SB202190 and the MEK inhibitor U0126 (5 μ M each, open red circles). Half-lives of VEGF mRNA were 4.8 h and 6.1 h, respectively.

Another key question that arises from this body of research is whether the RNA-destabilizing independent mechanism is unique to triple negative breast cancer. To our knowledge, this study is the first to identify this non-canonical mechanism. We hypothesize that this mechanism is dependent on the tumor of origin, and most likely results from genetic and epigenetic changes that are unique to those tumors, analogous to how the consequences of losing BRCA1/2 are largely limited to breast, ovarian, prostate and pancreatic cancer (216). Further complicating our understanding, a survey of the literature revealed that many papers that look at the role of TTP in suppressing neoplastic phenotypes fail to investigate whether this mechanism is RNA-decay dependent, which makes it difficult to know how often this phenomenon occurs.

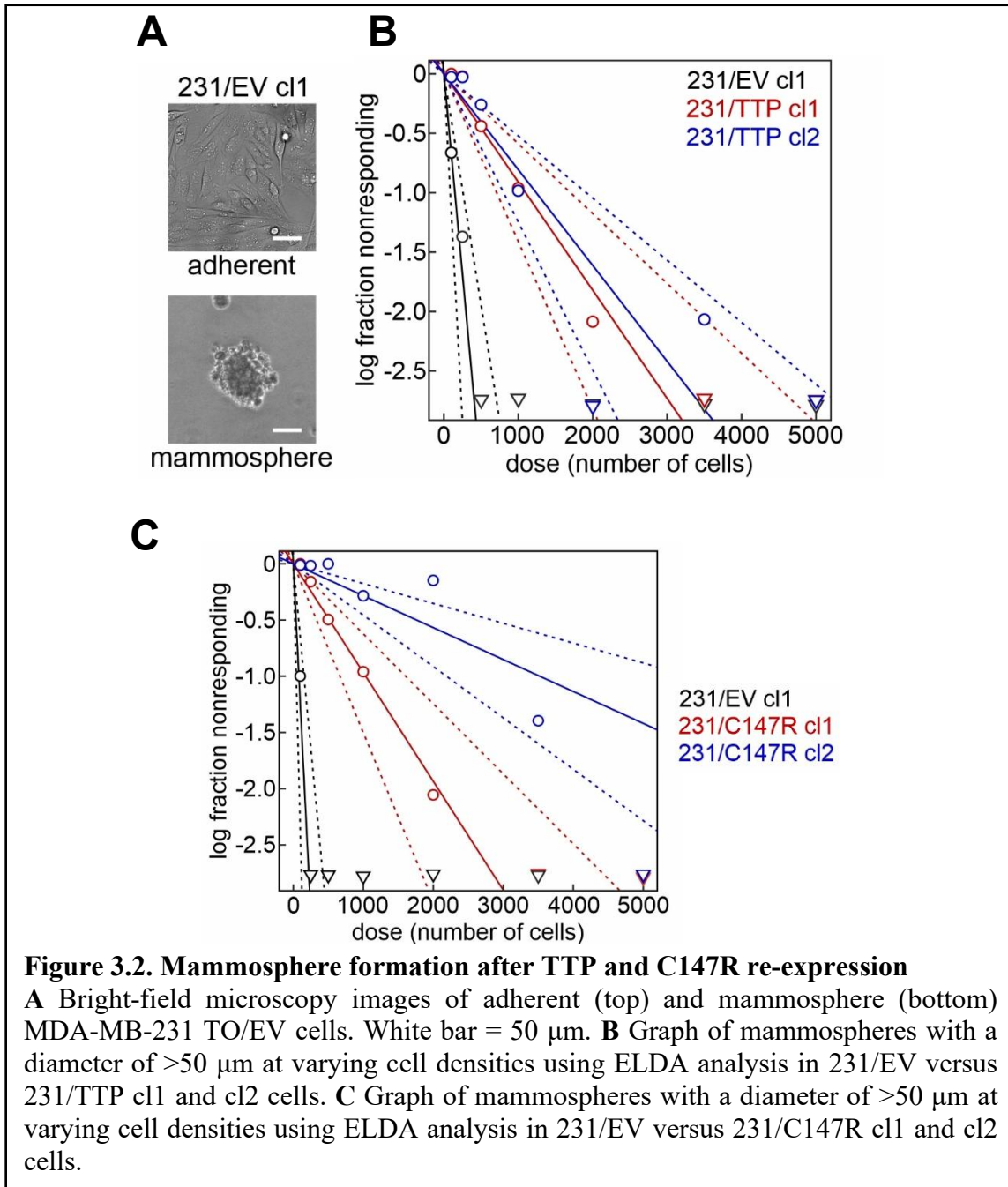
To test this, a high-throughput screen of multiple cell lines from multiple cancers could be helpful. Cell lines would be plated into a 96- or 384-well plate, where they would be transduced with a lentivirus encoding either the TTP C147R mutant, to selectively test

for models where TTP can act without binding RNA, or an empty vector. Both constructs would contain GFP to allow for fluorescence-based tracking and a puromycin resistance marker for selection. To avoid issues from stress responses that accompany viral transduction, we would select for the integrated subpopulation using puromycin, then perform growth assays a few days later to minimize these potential complications. In our present study, in all three TNBC cell lines examined, TTP significantly reduced proliferation by day two, indicating that a short time course, such as 3 days, is sufficient to evaluate alterations in cell growth (204). Monitoring GFP fluorescence over time would approximately measure cell growth. Cell lines displaying decreased proliferation after TTP C147R expression would be selected for further study. Additionally, existing mutations in these cell lines would be interrogated for the purpose of identifying whether TTP's non-canonical function is driven by a particular genomic driver event.

3.2: TTP-driven mechanisms that suppress neoplastic phenotypes

In addition to expanding the diversity of cell models that permit this non-canonical mechanism, defining additional anti-tumorigenic phenotypes induced by TTP expression could also be beneficial. To that end, we generated preliminary data that indicates that TTP can also suppress the development of stem cells. Cancer stem cells are a small subset of the tumor cell population which have undergone the epithelial-to-mesenchymal transition (EMT) and contribute to metastatic initiation due to their increased resilience in unfavorable environments, a characteristic that also contributes to chemoresistance (217). To quantify the stem cell fraction in our EV compared to TTP-expressing cells, we performed Extreme Limiting Dilution Analysis (ELDA), which scores stemlike cells based

on their ability to form mammospheres. In the MDA-MB-231 cell background, re-expression of TTP and C147R drastically lowers the fraction of stem cells in the bulk tumor cell population (Figure 3.2). The fraction of stem cells in the MDA-MB-436 cell background is similar between EV and TTP cell lines (data not shown), and we were unable to grow BT-549 cells as mammospheres in either our EV- or TTP- expressing cell line,

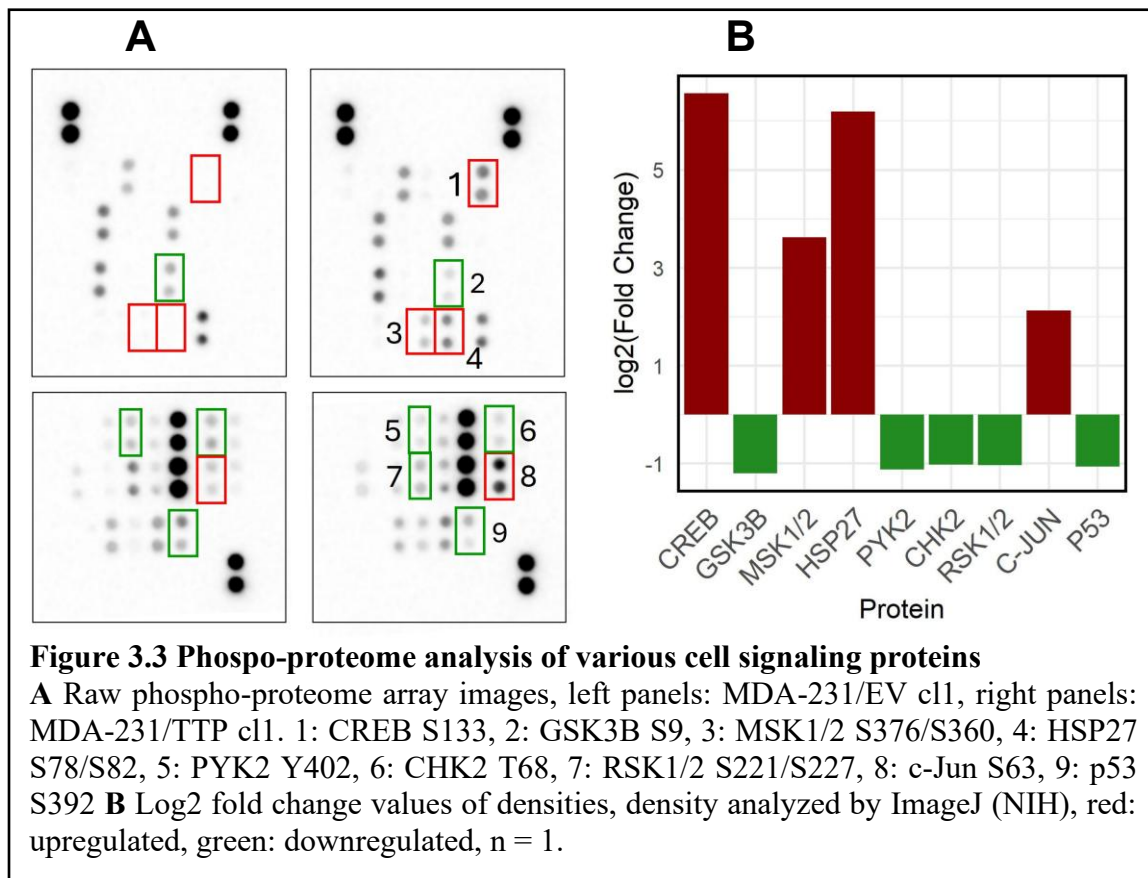


inhibiting our ability to analyze stemness in this model. However, these data indicates that in limited cell contexts, stem cell formation, an independent phenotype, is similarly suppressed by both the wild-type and RNA-binding-defective mutant form of TTP.

In a mouse xenograft model, we saw a significant decrease in primary tumor growth between our EV- and TTP-expressing MDA-MB-231 tumor-derived lines (Figure 2.8). However, a limitation of our study was the inability to evaluate metastasis given the experimental timeframe. One of the contributors to metastasis is the priming of the pre-metastatic niche, which is often driven by the actions of immune cells like MDSCs (17). Since our xenograft model utilized immune-deficient mice, we were unable to assess the effects of TTP on these processes. As discussed in Chapter 1, TTP is known to impact the tumor immune microenvironment in positive ways. In cancer cells resistant to pembrolizumab, TTP re-expression results in *PDL1* mRNA degradation, returning its expression to normal levels and restoring therapeutic efficacy (117). TTP can also destabilize *CXCL1* mRNA, which is associated with premetastatic niche formation, metastasis, and immune evasion (218). Loss of TTP in T47D breast cancer cells exacerbated tumorigenic phenotypes, including cell proliferation and survival, as well as *in vivo* tumor growth. TTP suppression in T47D organoids enhanced macrophage infiltration, with increased levels of M0, the precursor to tumor-associated macrophages (TAM), in cells lacking TTP expression (219). This was accompanied by increased cytokine production, suggesting that the impact of TTP extends beyond the tumor. While this study indicates overall increased intratumoral M0 infiltration, it does not address TaMs, which are known to differentiate into discrete pro- and anti-tumorigenic populations, commonly named M1 and M2, respectively. An interesting follow-up study

based on our current results would involve modifications to our original xenograft study by: (i) using an immunocompetent mouse model, such as the 4T1 TNBC model to analyze tumor growth (220), (ii) allowing the tumors to grow longer to study the effects of metastasis to the lungs, and (iii) analyzing immune cell infiltration into the primary and metastatic tumor microenvironments.

As discussed in Chapter 2, metastasis can be influenced by a variety of mechanisms, such as mTOR signaling, which was significantly negatively enriched in our GSEA analysis. Using a phospho-kinase array that measured phosphorylation of 33 signaling proteins, we generated preliminary data to analyze the effect of TTP expression on signaling pathway activation. One of the proteins showing the greatest increases in phosphorylation was mitogen- and stress-activated kinase (MSK) 1/2. Interestingly, in



glioma cells, inhibition of mTOR signaling leads to a compensatory upregulation of MSK1/2 activity, which has been linked to resistance against mTOR inhibitors (221). Conversely, protein tyrosine kinase 2 β (PYK2) showed decreases in phosphorylation, and has been positively correlated with mTOR pathway activation in cholangiocarcinoma (222). Decreased PYK2 expression has been linked to inhibition of metastasis in a breast cancer model (223). While some of these consequences of TTP expression are counterintuitive (e.g., increased phospho-CREB is pro-tumorigenic in several cancer models (224)), future gain- and loss-of-function experiments will need to be performed to assess whether they contribute to or possibly restrict the antitumorigenic consequences of TTP expression.

mTOR signaling is also closely tied to alterations in metabolism, another set of pathways that was heavily downregulated in our GSEA analysis. As increases in metabolic pathway flux have recently been linked to an increased propensity for proliferation and metastasis (197), we hypothesize that these alterations could be linked to the functional decreases we see in proliferation, migration and invasion. There is some evidence for regulation of metabolism by TTP, albeit involving a mechanism dependent on RNA-destabilization. The mRNA *SLC2A1*, encoding Glut1 and responsible for bringing glucose into cells, is a TTP target mRNA (105). The mRNAs encoding HK2 and PFKFB3, key enzymatic regulators of rate-limiting steps of glycolysis, can also be destabilized by TTP, leading to suppression of glycolysis in some lung and breast cancer cells (225,226). Interestingly, TTP destabilization of *HK2* mRNA leads to decreases in the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR)(225). In an *in vivo* retinal angiogenesis model, TTP can bind and destabilize *ENO2* mRNA, which encodes a

glycolytic enzyme, during neonatal development. This study utilized enhanced crosslinking immunoprecipitation (eCLIP)-seq to identify mRNAs to which TTP can bind, which included many that encode protein involved in various metabolic pathways, including glycolysis, oxidative phosphorylation, and fatty acid synthesis (227). Regulation of these mRNAs by TTP still needs to be interrogated in a future study.

To determine whether TTP might influence cellular energy management in MDA-MB-231 cells, we performed a preliminary glucose starvation assay and noted that while TTP-expressing MDA-MB-231 cells maintained their slow growth rates under low glucose conditions, our EV-expressing MDA-MB-231 cells grew at a much slower rate in low versus high glucose conditions, approaching the growth rate of TTP-expressing cells (Figure 3.4). In healthy aerobic conditions, glycolysis converts glucose into pyruvate, which then feeds the citric acid cycle coupled to oxidative phosphorylation through the electron transport chain. Under anerobic conditions, glucose is converted into lactate,

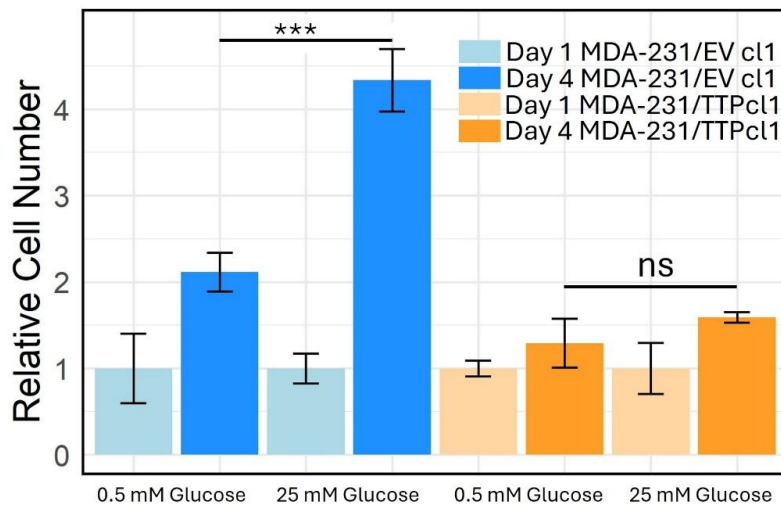


Figure 3.4 Growth of EV- and TTP-expressing cells under different glucose concentrations

Relative cell number at baseline (Day 1) or 3 days later (Day 4). Blue: MDA-MB-231/EV cl1, orange: MDA-MB-231/TTP cl1, in either low (0.5 mM) or high (25 mM) glucose, for all conditions, n = 3. Statistical significance determined by Student's *t* test.

which does not enter the citric acid cycle. Cancer cells often use anaerobic glycolysis, even under aerobic conditions, to quickly generate ATP and cellular intermediates that can be used to generate proteins, nucleic acids, and lipids, in a process that has been named the Warburg effect (228). We hypothesize that EV-expressing cells might preferentially use anaerobic glycolysis, to support their rapid proliferation rate, while TTP-expressing cells have converted back to aerobic glycolysis and normal metabolic processes, which we could test by performing a Seahorse assay (229), which measures OCR and ECAR. To further support this hypothesis, we could use metabolomics to analyze differential metabolite abundance between EV- and TTP-expressing cells.

3.3 Translating this study to the clinic

Even though the RNA-destabilizing effect of TTP is neutralized in our study, our results clearly demonstrate the therapeutic benefit of re-expression of TTP, regardless of its canonical activity. To begin to translate this research into the clinic, we must first identify a way to therapeutically increase TTP expression. Based on our research, TTP is significantly decreased at both the mRNA and protein levels in TNBC models. As we hypothesize that this inhibition starts at the gene level, modulating transcription could be an effective way to restore TTP expression. To identify potential ways to increase TTP expression, we could stably transfect a cell line that does not express TTP, such as MDA-MB-231, with a construct containing the TTP promoter upstream of the luciferase gene. This transgenic cell line would be used for a high-throughput drug screen against the Spectrum Collection drug library (MicroSource), which contains 2,560 compounds, composed of ~50% FDA-approved drugs, 30% natural products, and 20% other bioactive

compounds. Re-purposing FDA-approved drugs is an attractive approach that speeds up the drug development and clinical trial process, as well as reducing costs (230). Compounds that increase luciferase activity, would by proxy increase TTP expression, which would be followed by rigorous validation for effects on the endogenous gene locus, toxicity, and specificity across the genome. A successful trial of this screening platform on a pilot library could also justify a much broader drug discovery plan to identify new compounds that might re-activate transcription of TTP.

In a similar vein, we could also assess the ability of TTP to sensitize cancer cells to chemotherapy in TNBC. Combination therapies have shown efficacy in the clinic, as chemosensitization by one agent often allows for administration of lower doses, resulting in less toxicity and adverse events for patients (231). There is already evidence that TTP can sensitize head and neck squamous cell carcinoma cells to cisplatin. The anti-apoptotic protein Bcl2 is commonly overexpressed in cancer and contributes to cell survival and chemoresistance. Expression of TTP results in decreased Bcl2 expression, increased apoptosis, and enhanced cisplatin sensitivity (232). We have already generated three TNBC cell lines that express robust levels of TTP. These lines and their empty vector controls could be used in paired high-throughput drug screens against an anti-cancer drug library (Selleck Chemicals) to test if TTP expression results in increased cell death after compound administration compared to EV controls, as monitored by a luminescence-based cell viability assay.

Given the consistent and significant TTP suppression observed across a spectrum of solid tumors, along with the inhibition of tumorigenic phenotypes described in our present study, future studies investigating ways to increase endogenous TTP expression or

sensitize tumors to chemotherapy could prove beneficial in the clinical setting. Furthermore, continued research into TTP and its anti-tumorigenic mechanisms will likely benefit not only TNBC patients, but cancer patients as a whole.

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