Mutant P53-specific Differential Regulation of Yap1/Taz Proteins in Promoting Invasion of Mammary Epithelial Cells

by

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ABSTRACT

The *TP53* tumor suppressor gene is the most frequently mutated gene in human cancers. In the highly aggressive triple negative breast cancer (TNBC), *TP53* is mutated in 80% of cases. TNBC lacks viable drug targets, resulting in a low prognosis (12.2% 5 year survivability rate). As such, the discovery of druggable targets in TNBC would be beneficial. Mutated p53 protein typically occurs as a missense mutation and often endows cancer cells with gain of function (GOF) properties by dysregulating metabolic pathways. One of these frequently dysregulated pathways is the Hippo/Yes-associated protein-1 (YAP1)/WW Domain Containing Transcription Regulator 1 (TAZ) tumor suppressor pathway. This study therefore analyzed the involvement of the Hippo/YAP1/TAZ pathway in p53-mediated breast cancer cell invasion. From an RNA-seq screen in MCF10A cell lines harboring different *TP53* missense mutations, each with a differing invasive phenotype, components of the Hippo pathway were found to correlate with cell invasion. To this end, the active and inactive forms of YAP1 and TAZ were studied. Phosphorylated (inactive) YAP1 and TAZ are retained in the cytoplasm and eventually degraded. Unphosphorylated (active) YAP1 and TAZ translocate to the nucleus to activate TEAD-family transcription factors, inducing cell survival and proliferation genes leading to increased cell invasion. Using quantitative western blot analysis, it was found that inactive TAZ expression was lower in the most invasive cell lines and higher in the least invasive cell lines ($p = 0.003$). Moreover, the ratio of inactive TAZ protein to total TAZ protein was also shown to be predominantly lower in the invasive cell lines compared to the non-invasive lines ($p = 0.04$). Finally, active TAZ expression was primarily higher in p53-mutant invasive cell lines and lower in non-invasive p53 mutant cells. Additionally, although YAP1 and TAZ are thought to be functionally redundant, the pattern seen in TAZ was not seen in the YAP1 protein. Taken together, the results demonstrated here suggest that TAZ holds a more dominant role in governing TNBC cell invasion compared to YAP1 and further highlights TAZ as a potential therapeutic target in TNBC.

DEDICATION

This thesis is dedicated to my late aunt Terri Sanders. Aunt Terri will always be a joy in our hearts and will always remind us that there is so much beauty in this world. In my eyes, she will always be the enlightened and incredibly artistically driven person I love.

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CHAPTER 1

ANALYZING THE HIPPO-PATHWAY AS IT RELATES TO BREAST CANCER INVASION AND METASTASIS: BACKGROUND, HYPOTHESIS, AND APPROACHES

INTRODUCTION

An introduction into human breast cancer

The development of breast cancer occurs when a normal breast cell is transformed to one that is capable of forming a non-invasive or pre-invasive tumor (Sun, 2017). A tumor typically initially starts as an in situ carcinoma and is the result of several heterogeneous genetic mutations that give the abnormal cancer cells a survival and proliferative advantage over the non-transformed normal cells. The breast pre-invasive carcinoma in situ, typically a ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS), is hypothesized to begin with transformed luminal epithelial cells undergoing uncontrolled proliferation but are not yet capable of invading the surrounding tissue. The tumor cells can then become invasive by proliferating into the surrounding myoepithelial cell layer, basement membrane, and other surrounding tissues such as blood vessels and lymph nodes. The breast tumor cells then become metastatic when the breast cancer cells spread to other organs by traveling via the lymphatic or circulatory system (Akram, 2017). For breast cancer, the most common metastatic sites are the brain, bones, lungs, and liver.

Breast cancer is divided into several types depending on gene expression analysis where each type has a different genetic make-up, behavior, and risk factor. Luminal A breast cancers are estrogen receptor (ER) and progesterone receptor (PR) positive, human epidermal growth factor receptor 2 (HER2) negative, and possess a low amount of Ki67. Luminal B breast cancers are ER/PR+, either HER2+ or -, and have a higher amount of Ki67. HER2 amplified breast cancers are ER- and PR-, but have the HER2 gene over expressed. Because these breast cancers are hormone receptor positive, they can accurately be targeted with hormonal drugs, with

HER2 amplified breast cancers responding particularly well to trastuzumab (Yin, 2020). The basal-like triple negative breast cancer (TNBC), however, does not contain PR, ER, or the overexpression/amplification of HER2. Because of its molecular make-up, TNBC is not sensitive to endocrine or molecularly targeted therapy, leaving chemotherapy often as the most viable option but with little success. TNBC is also further splits into different subtypes where each subtype likewise contains different genetic expression profiles. The basal-like 1 subtype has dysregulated cell-cycle, DNA-damage repair, and proliferative gene expressions where the basal-like 2 subtype has dysregulated growth factor, glycolysis, and myoepithelial pathways. The immunomodulatory subtype often has the IL-7 pathway dysregulated along with other immune cell processes where the mesenchymal-like subtype has abnormal cell motility and differentiated gene expression. Finally, the luminal androgen receptor subtype has a dysregulated androgen signaling pathway (Abramson, 2015).

Because of the complex heterogeneity that breast cancer presents, the prognosis for the disease is often not encouraging. The cancers that are hormone receptor positive (ER/PR), but HER2-, account for almost 75% of all breast cancers, whereas TNBC accounts for around 10% of diagnosed breast cancers. Despite this smaller occurrence that TNBC occupies, its 5-year survival rate is much worse compared to the other types where the 5-year survival rate for metastatic TNBC cases is 12.2% compared to 30-40% for hormone receptor and/or HER2 positive metastatic cancers according to the National Cancer Institute for the years 2014-2018 (American Cancer Society, 2022). Because of this complex heterogeneity and lethality that predominantly TNBC, yet all other human breast cancers possess, especially at metastasis, it is no surprise that this disease proves difficult to find more successful therapeutics. This matter is even further complicated as breast cancer has been documented to acquire resistance to targeted therapies. Several approved breast cancer therapies include receptor targeted treatments such as anthracyclines and doxorubicin (chemotherapies), platinum agents, epidermal growth factor receptor (EFGR) inhibitors, PARP inhibitors, androgen receptor inhibitors, and immunotherapies (Yin, 2020). These therapies, however, have had little success in treating TNBC where observations show that TNBC is very aggressive in resisting against therapeutics (Bai,

2021). Intriguingly, TNBC patients having undergone neoadjuvant therapy subsequently develop a higher rate of pathological complete response (pCR) in comparison to non-TNBC patients and actually have a worse prognosis than the non-TNBC patients who underwent neoadjuvant chemotherapy, a phenomenon known as the *Paradox of Triple Negative Breast Cancer* (Fornier, 2011). This not only implies that TNBC has more instances of resisting targeted therapies because of the complex heterogeneity, but also highlights the importance of finding new successful therapeutics for TNBC (Nedeljković, 2019). As a result, new avenues are being pursued to target TNBC and breast cancer as a whole with a common theme that gravitates towards discovering underlying genetic mutations as the cause of cancer.

One of these genetic mutations is the *TP53* gene, which encodes for the p53 protein and is frequently mutated within breast cancer. p53 is mutated in just 30-35% of all breast cancers but interestingly is mutated in 80% of all TNBC cases, highlighting the importance that p53 has for TNBC development (Duffy, 2021). Deemed the 'guardian of the genome' the p53 protein is a homo-tetrameric protein responsible for the inducement of apoptosis and/or cell-cycle arrest genes upon cell genetic damage from stresses (Figure 1.1). When a cell's DNA is damaged (such as from certain stresses), p53 which is typically expressed in low levels, canonically undergoes post-translational modifications to convert into its active form. In its active form, p53 then transcribes several cell-cycle arrest genes such as *CDKN1A* (p21) while also encouraging DNA-damage repair genes such as *DDB2*. Should the DNA damage be too great to repair, p53 will also transactivate pro-apoptosis genes like *PUMA* to prevent the exchange and retention of damaged DNA to daughter cells (Ozaki, 2011). Tumors often arise when DNA-damaged cells achieve a gain of function (GOF) mutation giving them both a survival and proliferative advantage over non-mutated cells and are subsequently able to avoid cell-cycle arrest and apoptosis. As a tumor suppressor, p53 works to prevent these tumors from arising. Yet, it is no surprise that p53 is frequently mutated in human cancers, thus compromising p53's tumor suppressive capability and highlighting its role in cancer development.

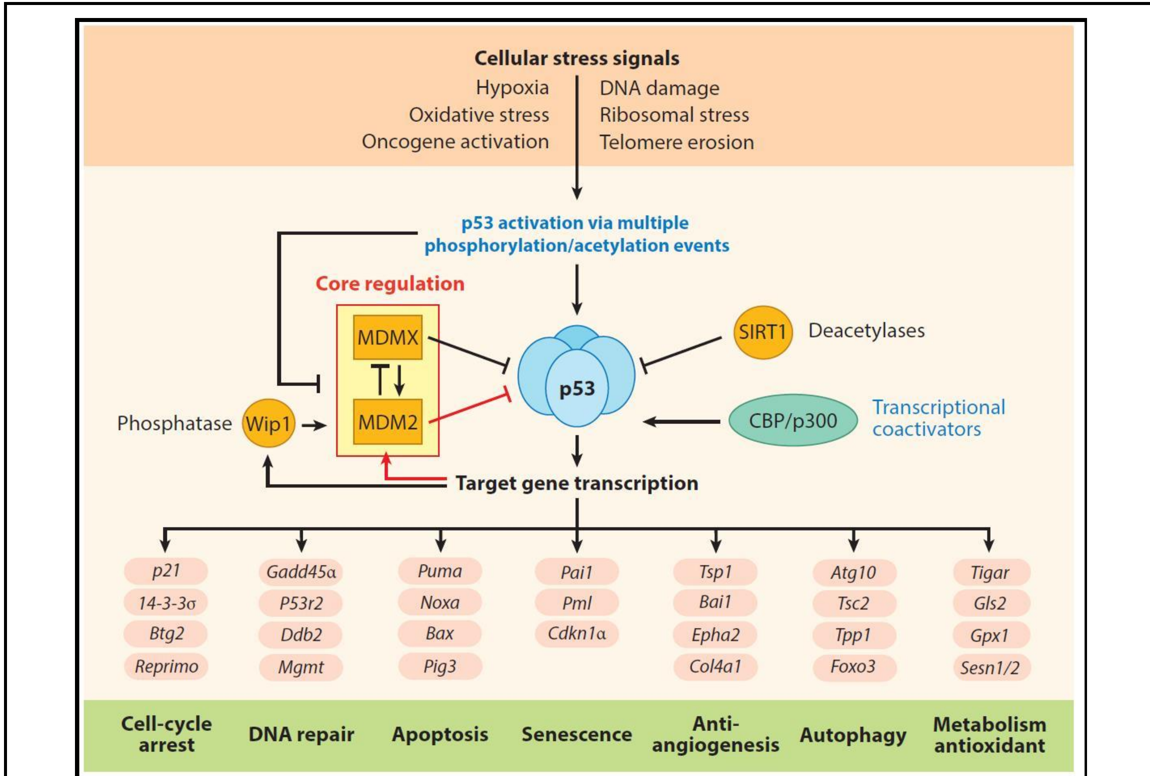

Figure 1.1. **Pathways of p53 protein activation and response.** DNA damage and other cellular stresses that activate the tumor suppressor capability in the p53 protein. In the event that the cell cannot repair itself correctly, p53 will also initiate cell-cycle arrest, apoptosis, and other tumor suppressor genes (Joerger, 2016).

The mutations that occur in the *TP53* gene are biased in both location of the gene and type of mutation. The majority of p53 mutations in cancer cells occur within the p53 DNA-binding domain (DBD), but only 10% of these mutations result in either no p53 protein or a truncated p53 protein. Instead, the remaining majority of the cancerous *TP53* mutations are missense mutations resulting in a full-length, but often faulty p53 protein. This basis towards missense mutations is not typically seen within tumor suppressor genes. It is noteworthy that several individual p53 missense mutations are often repeated in several cancer types and cancer cases, implying that there are several mutation hot spots within the p53 protein. In fact, it has been reported that the top 10 mutations of p53 in cancer account for 30% of all p53 missense mutations. Several p53 mutations are loss of function (LOF) mutations in which p53 loses its functions that are normally

accomplished by the wild-type (WT) p53, such as losing its capability to properly bind to DNA and promote gene transcription (Baugh, 2018).

This mutant-p53 LOF effect is especially pronounced if the mutant p53 can no longer promote the expression of cell-cycle arrest and apoptosis genes, which has been reported in several studies. Even more intriguing, however, are the reported cases in which mutant p53 in cancer cells actually have a gain of function (GOF) oncogenic effect, endowing the cancer cells with selective survival advantage over the non-cancer p53-WT cells. These reported neomorphic phenotypes in the cancer cells have been shown to contribute to some of the hallmarks of cancer. WT-p53 normally inhibits cell invasion, uncontrolled proliferation, migration, angiogenesis, and chemoresistance. Mutp53, however, tends to evade canonical p53 pathways and instead creates novel p53 functions, often involving itself in non-canonical pathways (Mantovani, 2019). In their report, Tan et al., 2015, found that the p53 mutant *TP53-R273H* was responsible for endowing cells with more resistance to anoikis by driving *AKT* signaling and suppressing BMF protein expression, showing that mutant p53 can have oncogenic effects for some cells. Yet this effect is not the case for every p53 mutant in which some p53 mutants may not give a survival advantage in one hallmark of cancer but may in another. In the same report, the authors demonstrated that only the silenced p53-R273H mutant increased mitochondrial-dependent apoptosis, but the same results were not seen with the p53-R175H mutant (Tan, 2015). In a different report, the R175H p53 mutant was shown to promote the expansion of initial breast tumor cells by suppressing ATM which typically initiates the DNA-damage checkpoint (Lu, 2013). This once again shows that not all p53 mutants are equivalent in their neomorphic and oncogenic properties. Taken together, it is fascinating to see what oncogenic/neomorphic properties each p53 mutant may possess in both phenotype and disrupted pathways. Doing so may reveal novel therapeutic targets for TNBC and all breast cancers.

Overarching goal

The aim of this research is to identify and analyze disrupted pathways in triple negative breast cancer. Previously, our group performed an in-depth analysis on the phenotypic effects that 10 top hotspot p53 mutants instilled on various hallmarks of cancer, including apoptosis resistance, anoikis resistance, growth factor independent survival, cell migration, cell invasion, and disrupted mammosphere polarity. Using MCF-10A cells as a breast epithelial model, each harboring a different p53 mutant, we demonstrated an overall trend in which p53 mutants such as R248W and R273C were most aggressive compared to p53 mutants G245S and Y234C as the least aggressive (Figure 1.2). Following, RNA-seq pathway analysis was also performed on each of the p53 mutants to uncover if there was a trend in disrupted pathways. One intriguing result was the Hippo-pathway in which transcriptome expression of the Hippo-pathway correlated with p53 mutant invasiveness. A thorough analysis of the Hippo-pathway as it relates to breast cancer invasion and mutant p53 could uncover potential key-drivers in cancer invasion and subsequently lead to new and more successful breast cancer therapeutics.

The remainder of this chapter will give a thorough overview of the Hippo-pathway, its involvement in breast cancer development, invasiveness, and other hallmarks of cancer. Although only discovered 2 decades ago in *Drosophila melanogaster,* the Hippo-pathway has since blossomed in the field of cancer research and is consistently gaining more attention, especially in the recent discoveries suggesting that the pathway may have both oncogenic and tumor suppressive roles. The remaining portions of this chapter will first go into the mechanisms governing the Hippo-pathway, followed by how this pathway has been shown to contribute towards cancer initiation, metastasis, and invasion. The last section will also look into the supposed two-faces that the Hippo-pathway shows, be it an oncogenic pathway or tumor suppressive pathway. With its discovery and relation to cancer quite recent, the Hippo-pathway remains difficult to comprehend.

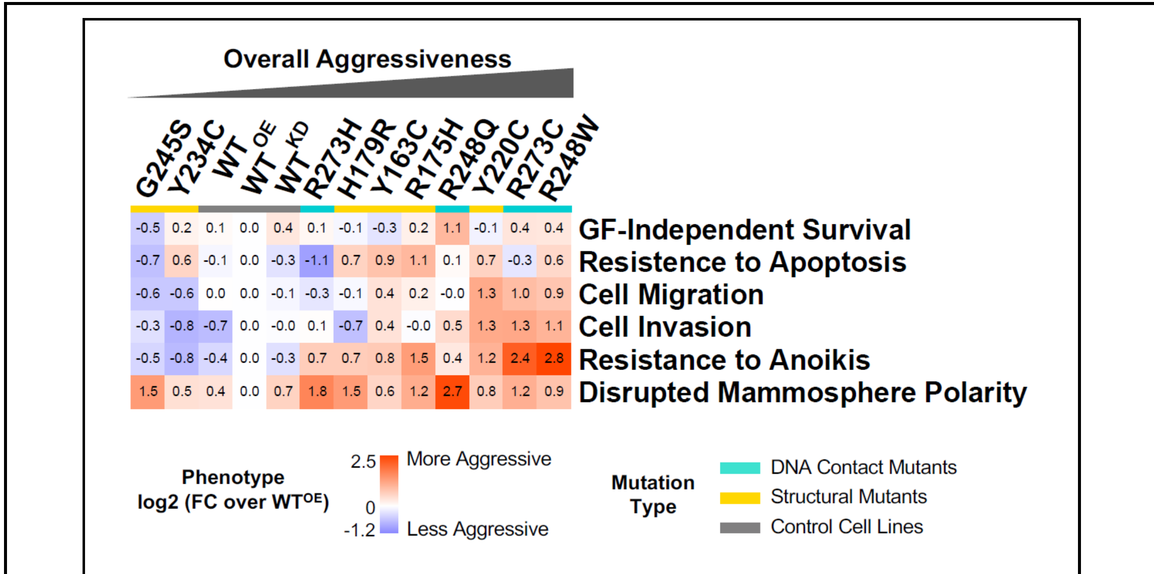

Figure 1.2. **Mutant p53 proteins and their phenotypic heterogeneity across hallmarks of cancer.** Different p53 mutants display heterogeneity when tested for various hallmarks of cancer phenotypes. Overall, mutants on the right side of the figure display higher aggression and more-so contain increased cancer hallmarks. Mutants on the far left side of the figure (such as G245S and Y234C) instead display overall less aggression than WT-p53.

The Hippo tumor suppressor pathway

An overview of the Hippo-pathway cascade, activation, and its involvement in cancer

The Hippo-pathway was first discovered in *D. melanogaster* and has since been documented to have human orthologues that regulate organ size and growth, stem cell maintenance, tissue regeneration, and wound healing (Figure 1.3). The Hippo pathway serves as a regulatory pathway by inhibiting the transcription co-activators *Yes-associated protein* (YAP1) and *WW-containing Transcription Regulator 1* (WWTR1 or TAZ) (Kyriazoglou, 2021). The Hippo-pathway is made up of the core proteins MST1/2, LATS1/2, TEAD1-4, and YAP1/TAZ. Initially, cell stress signals such as increased cell-cell contact, stiff extracellular matrix, nutrient starvation, DNA-damage, etc result in the phosphorylation and activation of Mammalian-STE20-like kinase 1 and 2 (MST1/2). Upon MST1/2 activation, MST1/2 also phosphorylates and activates the adaptor protein SAV1 which stabilizes the MST1/2 protein. The mechanism of MST1/2 activation is not

entirely known, though several reports have suggested that the KIBRA protein contributes to this process in addition to the RASSF pathway having a large role (Xiao, 2011). Following MST1/2 activation, the kinase then phosphorylates and activates the Large Tumor Suppressors 1 and 2 (LATS1/2) along with the LATS1/2 adaptor protein MOB1 which along with SAV1 helps facilitate the interaction of MST1/2 with LATS1/2. MST1/2 phosphorylates and activates LATS1 at threonine-1079 and LATS2 at threonine-1041. Once activated, LATS1/2 will also then phosphorylate YAP1 at the serine residues 61, 109, 127, 164, 381, and will also phosphorylate TAZ at the serine residues 66, 89, 117, and 311. The phosphorylation of YAP1 at S127 and TAZ at S89 enables its interaction with 14-3-3 proteins for cytoplasmic retention where phosphorylation of YAP1 at S381 and TAZ at S311 allows for additional phosphorylation by CK1 kinase and eventual degradation by the $SCF^{\beta\text{-TrCP}}$-E3 ubiquitin ligase complex. Once retained in the cytoplasm and/or degraded, YAP1/TAZ are unable to translocate to the nucleus to interact with and activate TEAD proteins. In this scenario described above, the Hippo-pathway is considered 'on' because it prevents the active forms of YAP1/TAZ from entering the nucleus. In the scenario where the Hippo-pathway is 'off' where MST1/2 and therefore LATS1/2 and YAP1/TAZ are not phosphorylated, non-phosphorylated (active) YAP1/TAZ are able to enter the nucleus and interact with the TEAD family proteins while encouraging TEAD-mediated transcription (Sarmatsi Emami, 2020).

YAP1 and TAZ tend to be pro-proliferation whereupon interaction with *Transcriptional Enhanced Associated Domain* (TEAD) family proteins, can help transcribe signature genes like *ANKRD1, CYR61, and CTGF*. Downstream gene signatures such as these serve as a sign that YAP1 and/or TAZ are active (Dibaugnies, 2018). Because of the genes signatures, YAP1/TAZ are proposed to be oncogenes because of their pro-proliferative and anti-apoptotic gene signatures, particularly when overexpressed. It is for this reason that the Hippo-pathway is often known as the Hippo-tumor suppressor pathway because of its strong ability to prevent the active forms of YAP1/TAZ.

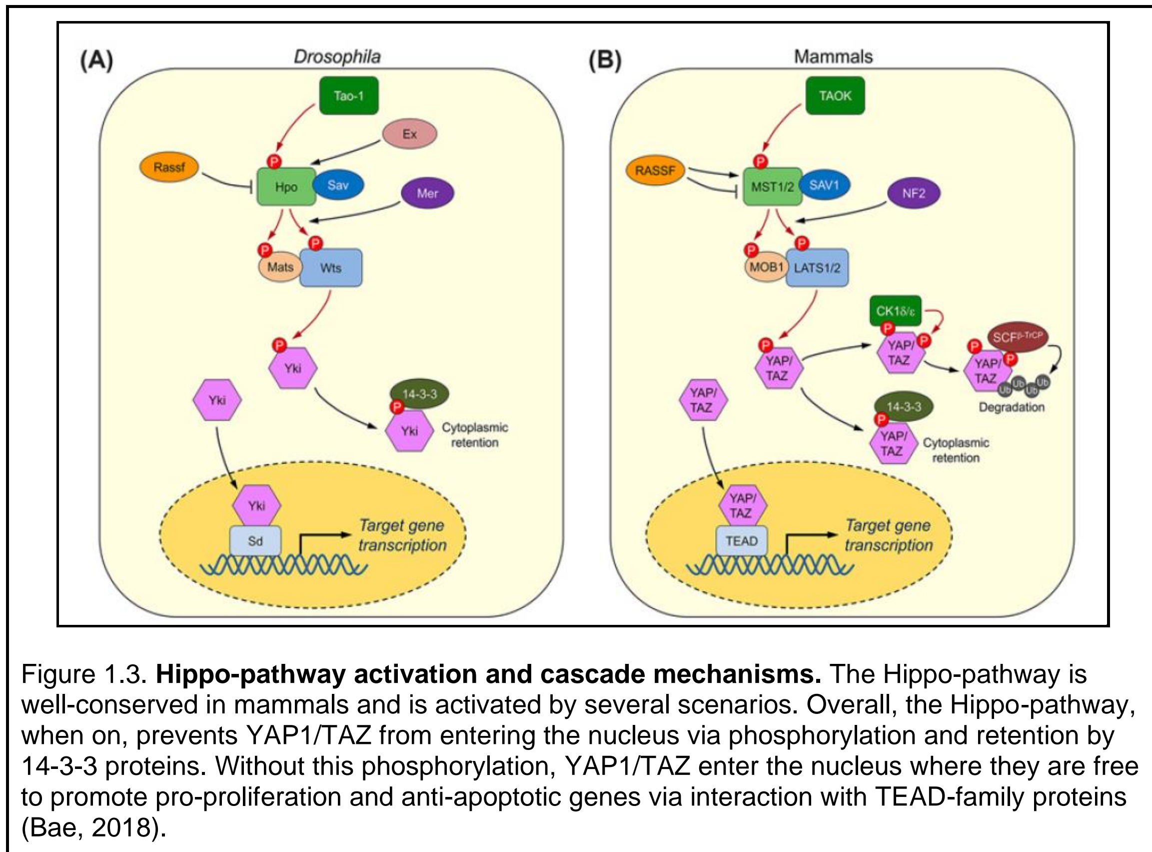

Figure 1.3. **Hippo-pathway activation and cascade mechanisms.** The Hippo-pathway is well-conserved in mammals and is activated by several scenarios. Overall, the Hippo-pathway, when on, prevents YAP1/TAZ from entering the nucleus via phosphorylation and retention by 14-3-3 proteins. Without this phosphorylation, YAP1/TAZ enter the nucleus where they are free to promote pro-proliferation and anti-apoptotic genes via interaction with TEAD-family proteins (Bae, 2018).

Though the core components of the Hippo-pathway are well understood, the overall activation and regulation of the Hippo-pathway is not as clear where significant cross-talk with other pathways is being revealed. Often, however, the Hippo-pathway is activated via cell-sensing and is turned on when cell populations are too confluent, which highlights the role the Hippo-pathway plays in organ size. Supporting this notion, several papers have demonstrated this scenario. E-cadherin, an important component in cell adheren junctions, is known to be at low levels when cell populations are at a low density, but upregulated when cells are at a high density. Complementary to this, the binding of E-cadherin between two or more cells (such as that when cells are at a high density) creates a signal that has been found to bypass the initial MST1/2 core protein and instead interacts directly with the LATS1/2 and YAP1/TAZ portion of the cascade. Here, higher E-cadherin levels resulted in the nuclear exclusion of YAP1 and a higher phosphorylated YAP1 (Kim, 2011 & Sharif, 2015). This makes sense seeing that cells undergo cell-cell contact inhibition at high density. Aligning with this, several studies confirm that the

Hippo-pathway can be a mechano-sensing pathway that is directly related to the cell's cytoskeleton. This observation is logical when one considers the Hippo-pathway's role in organ development regulation where cells must constantly respond to and sense their environment in a mechanistic fashion (i.e. cytoskeletal signaling). In this way, the Hippo-pathway is linked to an altered cell morphology, obstructed cell polarity, and disrupted extracellular matrix. When one considers that tumorigenesis often involves cancer cells not only purposefully dysregulating these pathways but also evading the pro-apoptotic signals that result from the dysregulation, the Hippo-pathway's involvement in these pathways is much more appreciated (Seo, 2018 & Chang, 2020). In relation to this mechano-sensing, several studies have focused around F-actin (the polymerized form of G-actin) which forms the long filamentous actin strands that make up part of the cytoskeleton. MST1/2 has been found to colocalize with F-actin where the disruption of actin filaments leads to the activation of MST1/2, hence activating the Hippo-pathway and preventing cell proliferation and tumorigenesis (Densham, 2009). Latrunculin A, a known disrupter of actin polymerization, inhibits YAP1 nuclear translocation. Activation of the Hippo-pathway as a result of F-actin disruption and mechano-sensing is likely to prevent a build-up of cells and to regulate cell population and organ size, which may be a sign of cancer (Wada, 2011).

Actin is heavily remodeled during cancer progression and invasion (Figure 1.4). Yet, matters are further complicated when cancer cells change type and morphology. Here, epithelial to mesenchymal transition (EMT) plays a key hallmark in cancer progression in which stationary epithelial cells transition to their mesenchymal type which is conducive to an invasive and metastatic cancer phenotype. A contributing factor to this EMT is increased actin polymerization which allows for increased cell motility and invasiveness. Additionally, the knockout of actin-capping proteins (which prevents additional actin polymerization) activates YAP1, implying that increased actin polymerization increases YAP1 activity. This adds complexity to the Hippo-pathway which seems to activate during filamentous actin disruption, yet deactivates during filamentous actin polymerization. These contrasting actions may further explain pathway remodeling during cancer development.

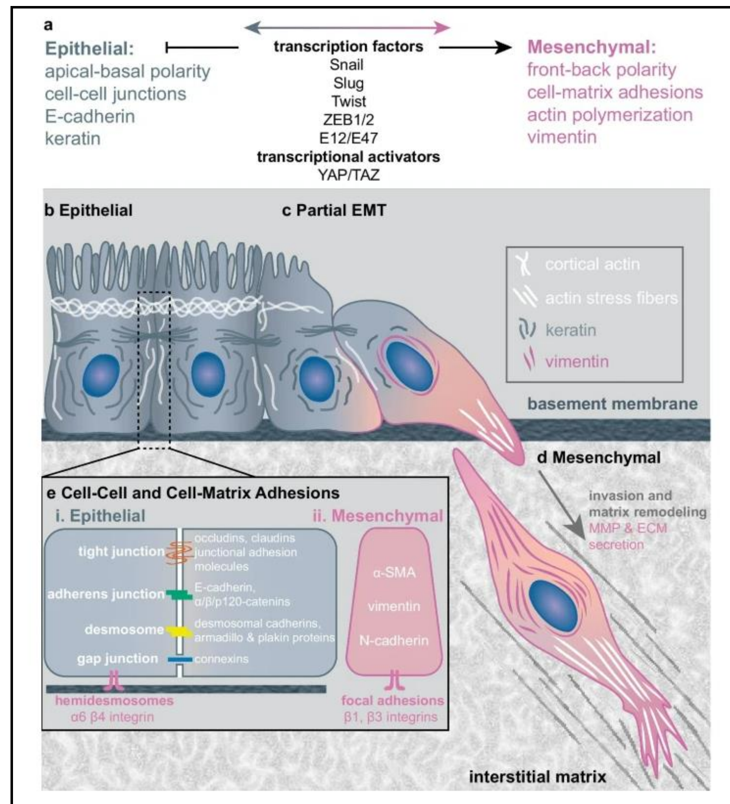

Figure 1.4. **Epithelial to mesenchymal transition and actin remodeling.** A major hallmark of cancer is epithelial to mesenchymal transition (EMT), which allows for cell invasion and metastasis. A large contributing factor to EMT is actin remodeling in which increased stress-fiber polymerization allows for cell motility and is in part caused by a dysregulated Hippo-pathway which is turned off during high actin polymerization (Leggett 2021).

In addition, *Thousand And One Amino Acid Protein Kinase 1* (TAOK1) has been revealed as a potential activator of the Hippo-pathway by phosphorylating MST1/2 where it itself is a STE20-like kinase and also serves as a member of the MAPK pathway. Previously, TAOK1 has been linked to inducing apoptotic conditions upon stresses like DNA-damage, thus acting as tumor-suppressor-like (Zhu, 2020). Therefore, it is logical to speculate TAOK1's potential role in activating the Hippo pathway. In a 2011 report, it was shown that TAOK3 can directly phosphorylate MST1/2, leading to its activation (Boggiano, 2011).

The RASFF protein family (Ras association domain family) is also known to regulate the Hippo-pathway and is thought to be upstream of MST1/2 (Figure 1.4). The RASSF protein family is a well-known regulator of organ development and proliferation where it acts as a tumor

suppressor by using Ras-GTPase signaling to initiate apoptosis and anti-proliferation genes. Therefore, its cross-talk in the Hippo-pathway may enhance its role as a tumor suppressor. Looking at the RASSF5 protein structure, the protein contains its Ras-association domain near its middle and close to its C-terminus, but also has a SARAH domain (Salvador-RASSF-Hippo). It is this SARAH domain that mediates the interaction between RASSF proteins and primarily MST1/2 which also have SARAH domains located at the C-terminus. Though this interaction between RASSF proteins and MST1/2 are well-documented, the effect of this interaction demonstrates to be more variable. There are 10 known RASSF members (1-10). RASSF1A has been closely linked to regulating tissue development/growth and has strong involvement in cancer tumorigenesis. In conjunction with this tumor suppressor activity, RASSF1A and RASSF5 were shown to activate MST1/2, keeping in line with the tumor suppressor notion of the RASSF family. Here, RASSF5 and RASSF1A were both required to mediate the pro-apoptotic function of MST1/2. In contrast, RASSF6 was found to inhibit MST1/2 activity, therefore highlighting the Hippo-pathway complexity (Oceandy, 2019).

The involvement of tumor suppressors within Hippo-pathway activation seems to be a common trend which strengthens the Hippo-pathway as a tumor suppressor pathway. The *Neurofibromin 2* gene encodes for the Merlin protein (or NF2 protein) which also has frequently been cited as a stimulator of the Hippo-pathway. Merlin (NF2) is a tumor suppressor protein. The deletion and/or loss of function of Merlin has been linked to the cancer Neurofibromatosis type 2, a nervous system cancer, though NF2 downregulation has also been linked to breast cancer where its canonical expression inhibits tumorigenesis and proliferation. In association with p53, NF2 reduces the inhibitory effect that the proto-oncogene MDM2 has on p53, where NF2 also compliments p53 to prevent tumorigenesis (Wang, 2020). Hence, the involvement that NF2 has in cancer has been well documented. NF2 involvement within the Hippo-pathway, however, is a more recent discovery that is still being unraveled. In the Hippo-pathway, NF2 seems to act primarily at the plasma membrane and at the cell cortex. Studies show that NF2 may bypass the MST1/2 core Hippo-pathway component and instead directly acts on LATS1/2 by recruiting LATS1/2 to the plasma membrane without affecting MST1/2 enzymatic activity. The recruitment

of LATS1/2 to the plasma membrane by NF2 increased LATS1/2 phosphorylation by MST1/2, which is evidenced by the lack of LATS1/2 phosphorylation in $NF2^{-/-}$ cells (Yin, 2013 & Petrilli, 2016).

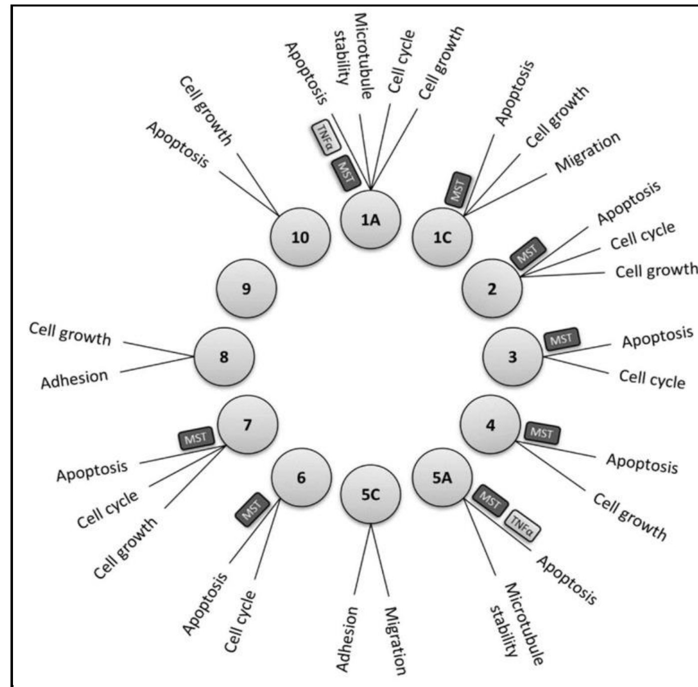

Figure 1.5. **RASSF protein interactions with the Hippo-pathway.** The RASSF proteins are known to be upstream and interact with MST1/2 to activate it. For most RASSF proteins, this is the case, though there are some that provide an antagonistic role against MST1/2 (Oceandy, 2019).

YAP1 and TAZ protein structure, function, role in tumorigenesis, and functional differences

YAP1 and TAZ are the main targets of the Hippo-pathway where the Hippo-pathway works to prevent the nuclear translocation of the two proteins. Because of their proposed role as an oncogene, YAP1 and TAZ have been heavily researched, continue to be a research interest, and are more recently being considered as therapeutic targets for cancer. It was originally thought that YAP1 and TAZ were functionally and structurally redundant proteins, yet further

analysis highlights some key differences in both structure and function (Figure 1.6). YAP1 and TAZ (and other core components of the Hippo-pathway) are highly conserved in eukaryotes with YAP1 first appearing in single cell eukaryotes and TAZ evolving much later. But TAZ intriguingly is only seen in vertebrates. The two proteins have a 60% sequence similarity.

YAP1 is a 65 kDa protein made of 7 exons located on the 11th chromosome. At its N-terminus, YAP1 has a proline rich region and a TEAD-binding domain (TBD) region for binding onto TEAD-transcription factors in the nucleus. In the middle of the YAP1 protein there is a WW domain (YAP1 contains one WW domain, whereas YAP2 contains two WW domains) which allows YAP1 to bind to proteins at PPxY motifs, such as LATS1/2. As such, within this N-terminus portion, there is a phosphodegron at Serine-127 in which LATS1/2 phosphorylates YAP1 for its interaction and cytoplasmic sequestering via 14-3-3 proteins. Hence, these WW domains seem to regulate YAP1 function by controlling its phosphorylation status and subsequent nuclear or cytoplasmic location (Guo, 2020). Intriguingly, breast epithelial cells with non-functioning WW domains had increased transformation and migration (Sudol, 2012). In the middle of the protein, closer to the C-terminus side is a SH3 binding domain that has been shown to enable YAP1 interaction with the SH3 domain of the c-Yes protein, a tyrosine kinase closely related to c-Src and where YAP1 (Yes-associated protein) gets its name. YAP1 has also been shown to interact with c-Src, also via its SH3 binding domain (Di Agostino, 2010). Following the SH3 binding domain is the large Transcriptional Activation Domain (TAD) which takes up the remaining portion of the protein and acts to promote transcription of YAP1 target genes via TEAD interaction. Also within this TAD is another phosphorylation site at Serine-381 that promotes protein degradation .

The WWTR1 protein (TAZ) (50 kDa) is a smaller protein than YAP1, located on chromosome 3, but shares a similar structure with some key differences. At its N-terminus is the TEAD-binding domain, containing a phosphodegron at S89, which corresponds to S127 on YAP1. Following the TBD is a single WW domain, likely presenting the same function as that in YAP1. The remaining portion of the TAZ protein is the TAD, with a phosphorylation site at S311 mediating protein degradation and corresponding to S381 on YAP1. The main structural differences between YAP1 and TAZ are the lack of a proline rich region in TAZ, a single WW

domain in TAZ (which is similar for YAP1 but contrasts YAP2), and the lack of an SH3 domain in TAZ (Plouffe, 2018). These differences in mind, it is logical to find that YAP1 and TAZ share very similar functions in some aspects, such as their ability to promote the transcription of similar genes. But it is also not surprising, given their structural differences, to discover both genes can govern separate mechanisms and phenotypes.

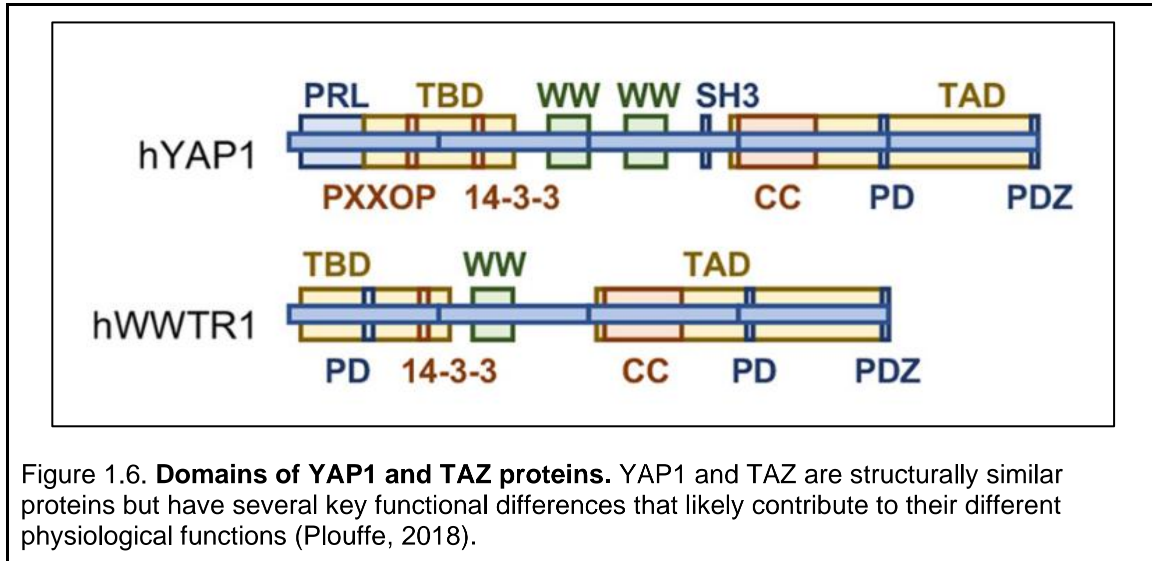

Figure 1.6. **Domains of YAP1 and TAZ proteins.** YAP1 and TAZ are structurally similar proteins but have several key functional differences that likely contribute to their different physiological functions (Plouffe, 2018).

Of note, the Hippo pathway, including the YAP1 and TAZ proteins are often not genetically mutated in cancers. Rather, the entire pathway is instead dysregulated for reasons that are not fully known. This additional complexity suggests that there is more to the underlying function and mechanism of the Hippo-pathway proteins that are not yet understood in addition to cross-talk with other pathways that have not been fully revealed. The functions and roles of YAP1 and TAZ often overlap, but recent research has uncovered that the proteins sometimes have distinct roles in different physiological conditions. This next section will highlight some of these differences. Overall, the YAP1 and TAZ proteins are seen to have a large role in cell growth and proliferation in breast cancer. Intriguingly, the TAZ protein seems to demonstrate a more prominent role in breast cancer, particularly in the triple negative phenotype compared to YAP1. When analyzing breast cancer patient samples, some studies consistently found the Hippo pathway to be dysregulated. But it was found that TNBC patients with higher TAZ levels were

much more likely to develop metastasis and had an overall lower survival, where YAP1 protein levels did not seem to correlate much with TNBC behavior and outcome. In other studies, TAZ positively correlates with TNBC and the cancer stem cell phenotype. In a similar study, it was also found that breast cancer cells with a higher level of active TAZ enabled breast cancer cells to form more and larger mammospheres, suggesting the tumorigenesis capability of TAZ (Li, 2015). TAZ has frequently been linked to endowing breast cancer cells with cancer stem cell (CSC) properties and to initiating tumorigenesis; TAZ therefore seems to play a large role in the beginning and developmental stages of breast cancer. In a landmark study, TAZ was required for stem cell self-renewal and tumor initiation in breast cancer and that cells with high CSC properties had a higher TAZ expression (Cordenonsi, 2011). In several mouse studies, higher TAZ expression meant higher metastasis where TAZ had a predominantly nuclear staining. In xenograft studies, the knock-down of TAZ halted and/or reduced tumor growth where tumors with TAZ overexpression were more resistant to chemotherapy (Bartucci, 2015). TAZ has also been used as a prognosis factor where the higher level of TAZ protein indicates a worse prognosis and higher rate of metastasis. Though TAZ has been cited as a major contributing factor to breast cancer initiation and tumorigenesis, YAP1 has still been shown to play a large role, but perhaps in breast cancer types apart from TNBC. Here, the *YAP1* mRNA levels have positive association with TNBC, but YAP1 protein levels do not correlate at the same level. In contrast, YAP1 protein levels were shown to positively correlate with the luminal subtypes instead. To further highlight some of the differences between YAP1 and TAZ in breast cancer subtypes, both *TAZ* mRNA levels and protein levels were positively correlated with the triple-negative phenotype, but had negative association with the luminal subtypes (Fresques, 2020).

Where TAZ has a larger role in promoting cancer migration and involvement with the extracellular matrix, YAP1 may have a larger role in cell-cycle progression and cell division, potentially explaining the notion that high TAZ expression meant worse prognosis compared to YAP1. In breast cancer, YAP1 cross-talks with glucocorticoid pathways. Glucocorticoids are a class of steroid hormones that are responsible for regulating carbohydrates, proteins, and fats. Glucocorticoids have been used as a treatment for breast cancer because of their ability to

mediate chemotherapy and metastatic breast cancer side-effects. However, the dysregulation of the glucocorticoid receptor has also been implicated as a risk in breast cancer, particularly in association with the YAP1 protein. One study demonstrated that glucocorticoids increased YAP1 levels, but not TAZ levels. In conjunction with this, the increase of YAP1 levels because of glucocorticoids also increased YAP1 target genes like *CYR61* and *CTGF* which are known to increase cell-proliferation and chemoresistance. Likewise, the removal of the glucocorticoid receptor subsequently decreased YAP1 levels and YAP1 association genes, but not TAZ, thus uncovering a key difference in YAP1 axes versus TAZ axes in addition to highlighting YAP1's involvement within the glucocorticoid pathway in cancer (Sorrentino, 2017 & Reggiani, 2021). Continuing with this, others have shown the importance of the mevalonate pathway for YAP1. It was found that adrenergic receptor inhibitors prevented nuclear YAP1 (active YAP1). The inhibitors were specifically statins which are HMG-CoA inhibitors where HMG-CoA reductase helps mevalonic acid which is involved in cholesterol biosynthesis. Intriguingly, the inhibition of the mevalonate pathway saw an increase in cytoplasmic YAP1 (inactive YAP1), suggesting that YAP1 dysregulation may involve cross-talk with several unanticipated pathways (Sorrentino, 2014).

YAP1 and TAZ are often linked together, rather than separately in regards to promoting invasion, metastasis, cell proliferation, and overall cancer development, showing that despite their known differences, their similarity in structure likely results in some overlapping phenotypic qualities. Several studies have demonstrated their combined traits, where both YAP1 and TAZ are seen to be activated in a variety of canonical contexts like disrupted cell polarity, low cell density, wound healing, and stem-cell maintenance (Fresques, 2020). Both have also been linked to several cancer types, including breast, lung, colorectal, liver, gastric, and pancreatic cancer (Zanconato, 2016). A major activator of both YAP1 and TAZ are G-protein coupled receptors. A large component in serum (lysophosphatidic acid) is capable of inhibiting LATS1/2, thus activating YAP1 and TAZ (Yu, 2012). Additionally, Phosphoinositide-3 kinase (PI3K) is closely linked with GPCRs, where the activation of GPCRs leads to the activation of PI3K. Keeping in line with the literature, the inhibition of PI3K resulted in the subsequent blocking of YAP1 nuclear

translocation (Fan, 2013). These and other findings give further evidence of the GPCRs being therapeutic targets in cancer. These findings also highlight just how complex the Hippo/YAP1/TAZ pathway is and how many pathways it interacts with (Figure 1.7).

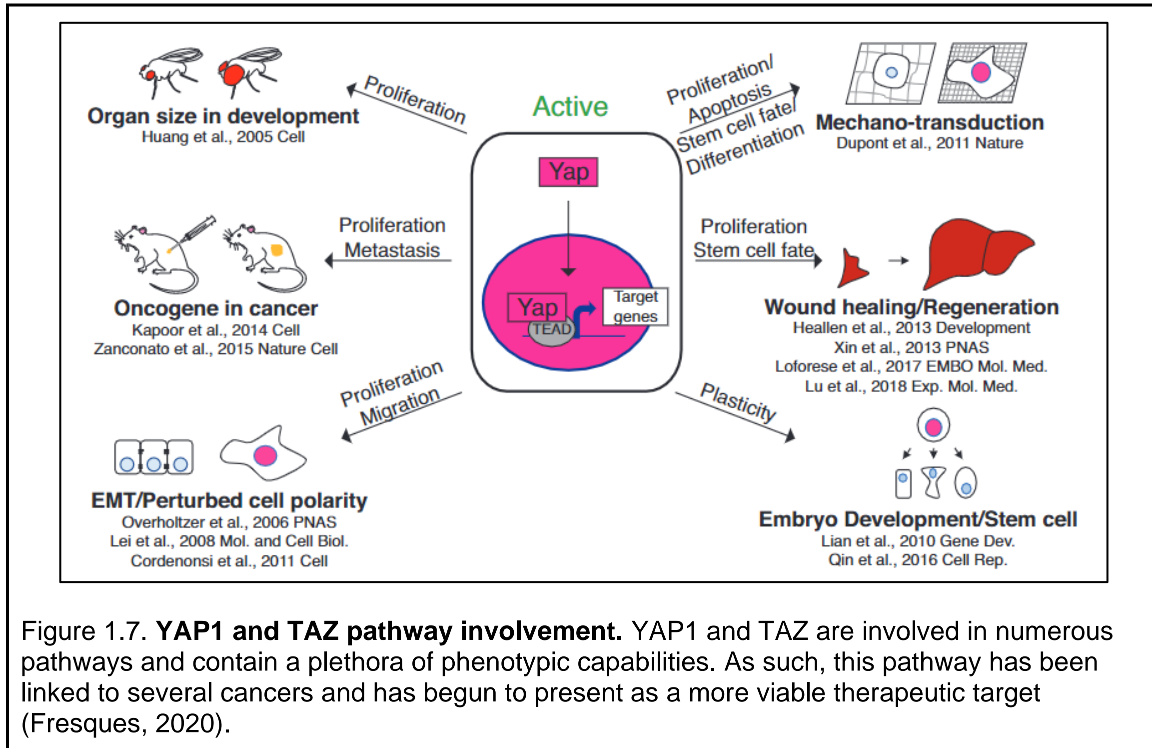

Figure 1.7. **YAP1 and TAZ pathway involvement.** YAP1 and TAZ are involved in numerous pathways and contain a plethora of phenotypic capabilities. As such, this pathway has been linked to several cancers and has begun to present as a more viable therapeutic target (Fresques, 2020).

The dual role of YAP1 and TAZ in cancer.

YAP1 and TAZ have often been proposed as oncogenes because of their roles within the Hippo-pathway, such as transcribing cell-proliferation and survival genes. The overexpression of active YAP1 and TAZ results in cancer metastasis and lower survival, thus adding to their suspicion as an oncogene. YAP1 and TAZ knockdown also result in higher survival and smaller tumor formation. Recently, however, there are studies that show YAP1 and TAZ (though mostly YAP1) present dual function by acting as both an oncogene and a tumor suppressor depending on context, thereby complicating the Hippo-pathway even further. These notions add complexity to the Hippo-pathway especially when one considers trying to use the Hippo-pathway as a therapeutic target. Overall, it is safe to say that the Hippo-pathway is still not fully understood and

therefore warrants further investigation. The previous sections highlighted the pathways and situations involved where YAP1 and TAZ are oncogenes. This section will provide instances where YAP1 and TAZ act as tumor suppressors and what this may mean for cancer biology.

Within the mammary gland, at the center of milk ducts there is the lumen which carries milk and other milk-associated proteins. Surrounding the lumen are luminal epithelial cells, giving the milk duct structure. Surrounding the luminal epithelial cells are a layer of myoepithelial cells that help maintain cell polarity, where myoepithelial cells are also surrounded by the basement membrane. YAP1 has been found to have a higher nuclear expression in normal breast myoepithelial cells, but there was more cytoplasmic YAP1 in the luminal epithelial cells. In this study, YAP1 expression had no correlation to tumor ER, HER2, or LN presence status, suggesting that YAP1 could not serve as a biomarker for breast cancer type or progression. The removal of YAP1 from MDA-MB-231 cells (an invasive breast cancer model for TNBC), resulted in MDA-MB-231 cells becoming much more resistant to apoptosis, suggesting that YAP1 served as a tumor suppressor in this case by making cells more sensitive to apoptosis regimes. The same study also found the removal of YAP1 to increase the potential of migration and invasion in breast cells. Nude mice that removed YAP1 had a larger and faster tumor growth. All of these findings go against YAP1 as an oncogene and instead give evidence of YAP1's tumor suppressing capabilities (Yuan, 2008). Several studies claim and show the dual role of YAP1, demonstrating it is both an oncogene in some of their experiments, yet a tumor suppressor in others. It was shown that some RASSF proteins could induce the interaction of YAP1 with p73. Upon this interaction, cells underwent a higher rate of apoptosis. In addition, YAP1 knock-down in breast cancer cell lines allowed cells to undergo diminished anoikis and increased cell invasion (Jho, 2018).

It was suggested that the identity of YAP1 and TAZ being either a tumor suppressor or oncogene is directly dependent on its binding partner that could regularly switch depending on context. Several studies have highlighted that YAP1 could potentially switch its binding partner from the canonical TEAD family to other transcription factors like p73 and RUNX. The orthodox binding partner of YAP1 and TAZ are the TEAD family proteins which result in the transcription of

cell proliferation and cell survival genes, leading to tumorigenesis. This is also evidenced by the elevation of TEAD proteins seen in breast cancer. YAP1 can evidently also bind to p73 acting as a transcriptional coactivator and inducing apoptosis through BAX. The p73 protein is related to the p53 protein and also acts as a tumor suppressor. Thus YAP1 acts as a tumor suppressor when it is bound to p73 instead of TEAD. Further aligning with the tumor suppressor distinction of YAP1, YAP1 binds to the RUNX protein family, which biologically aligns, given that the RUNX proteins are master regulators of the development pathway. Though this pathway is mostly involved in skeletal development. RUNX2 is an important transcription factor for osteoblast differentiation, but has also been linked to promoting breast cancer tumorigenesis, particularly in ER- cases (Wysokinski, 2015). When bound to YAP1, RUNX2 transcription capabilities are suppressed. The signaling of the Src/Yes tyrosine kinases, however, often dissociates the YAP1-RUNX2 complex, allowing RUNX2 to transcribe its target genes and subsequently induce cancer cell proliferation. The inhibition of the Src/Yes tyrosine kinases stabilizes the YAP1-RUNX2 complex and suppresses cell proliferation, implying that YAP1 is a tumor suppressor when bound to RUNX2 (Kim, 2018).

Recent papers have also suggested that some cancers may benefit from having the YAP1 protein expressed at low levels, deemed as '*YAP1 off cancers'* compared to other cancers that may benefit from having the YAP1 protein expressed at high levels, deemed as '*YAP1 on cancers'.* The authors further suggest that some cancers may actually switch from being a YAP1 on cancer to a YAP1 off cancer, or vice versa, during development if it presents a fitness advantage, thus creating a binary classification of cancers with respect to YAP1 expression. Here, MCF7 breast cells (a non-invasive breast cancer cell line) and MDA-MB-231 (an invasive breast cancer cell line) were both YAP1 on cancers, suggesting that YAP1 plays a potentially important role in these cancers, or at least in these cell lines. In contrast, YAP1 appears to be off, presenting a tumor suppressor-like status in small cell lung cancer, retinoblastomas, and neuroendocrine cancers, including neuroendocrine breast cancer, implying that YAP1 may be on as an oncogene in some breast cancers, but this is not always the case. This provides further

complexity into the Hippo/YAP1 pathway by showing that YAP1 may have dual roles and as such may warrant further investigation before being targeted as therapeutics (Pearson, 2021).

The Hippo-pathway and its involvement with the p53 family and mutant p53.

As this thesis is predominantly focused on RNA-seq data suggesting that mutant p53 proteins result in a shift in the canonical Hippo-pathway as it relates to invasiveness, it would therefore imply that aspects of the Hippo-pathway can interact with p53, members of the p53 family, or mutant p53 (mutp53). The p53 family consists of the proteins p53, p63, and p73. The p63 and p73 proteins are, like p53, also transcription factors that help initiate apoptosis. These two proteins evolved from a common ancestor through gene duplication events and can bind to p53 DNA motifs in addition to promoting p53 target genes. The p63 protein has specifically been noted for its role in skin development and p73 has been noted for its role in nervous system formation. All 3 p53 family proteins work to prevent tumorigenesis in response to DNA damage and other cell stresses, behaving as tumor suppressors. Yet p63 and p73 also have isoforms that harbor different roles. The transactivation (TA) isoforms of p63 and p73 create a full-length p53-like protein, still acting as tumor suppressors. The other isoforms for p63 and p73 are the ΔN isoforms which are capable of binding to and inhibiting the functions of p53, TAp63, and TAp73, thus acting as oncogenes (Furth, 2018) .

Intriguingly, YAP1 is known to bind to p63, p73, and mutp53, but has not been shown to bind to WT p53, begging the question what is its involvement with the p53 pathway (Figure 1.8)? YAP1 and its potential interaction with mutp53 was first discovered when researchers realized that in breast cancer, YAP1 shared the same transcriptome alterations as mutp53, meaning there may have been a potential binding interaction between the two proteins. Specifically, it was found that mutp53 (harboring the p53 mutation R273H and R175H) could bind to YAP1 in addition to complexing with Nuclear Transcription Factor-Gamma (NF-Y), though this NF-Y addition was not necessary for mutp53 binding to YAP1 as demonstrated by the presence of a YAP1-mutp53 complex in NF-Y depleted cells. Regardless, the result of the three protein complex

(Mutp53/YAP1/NF-Y) resulted in the promotion of NF-Y target genes and cell proliferation. Intriguingly, the involvement of statins promoted cytoplasmic YAP1 which disrupted its complex with TEAD and mutp53. This shows that YAP1 may exert a transcriptional role, not only over TEAD, but also over mutp53 in breast cancer (Di Agostino, 2016). This paper only tested a few transcription targets (mainly NF-Y), though it may be intriguing to uncover what other transcriptional regulations may exert over mutp53. It also will be intriguing to discover which mutp53 can interact with YAP1. The *TP53* mutations R273H, R175H, R280K, A193T, R248L, L194F, and P309S have been documented to bind to p53. The oligomerization domain of p53 (residues 338-355) are perhaps responsible for YAP1 binding (Tocci, 2019).

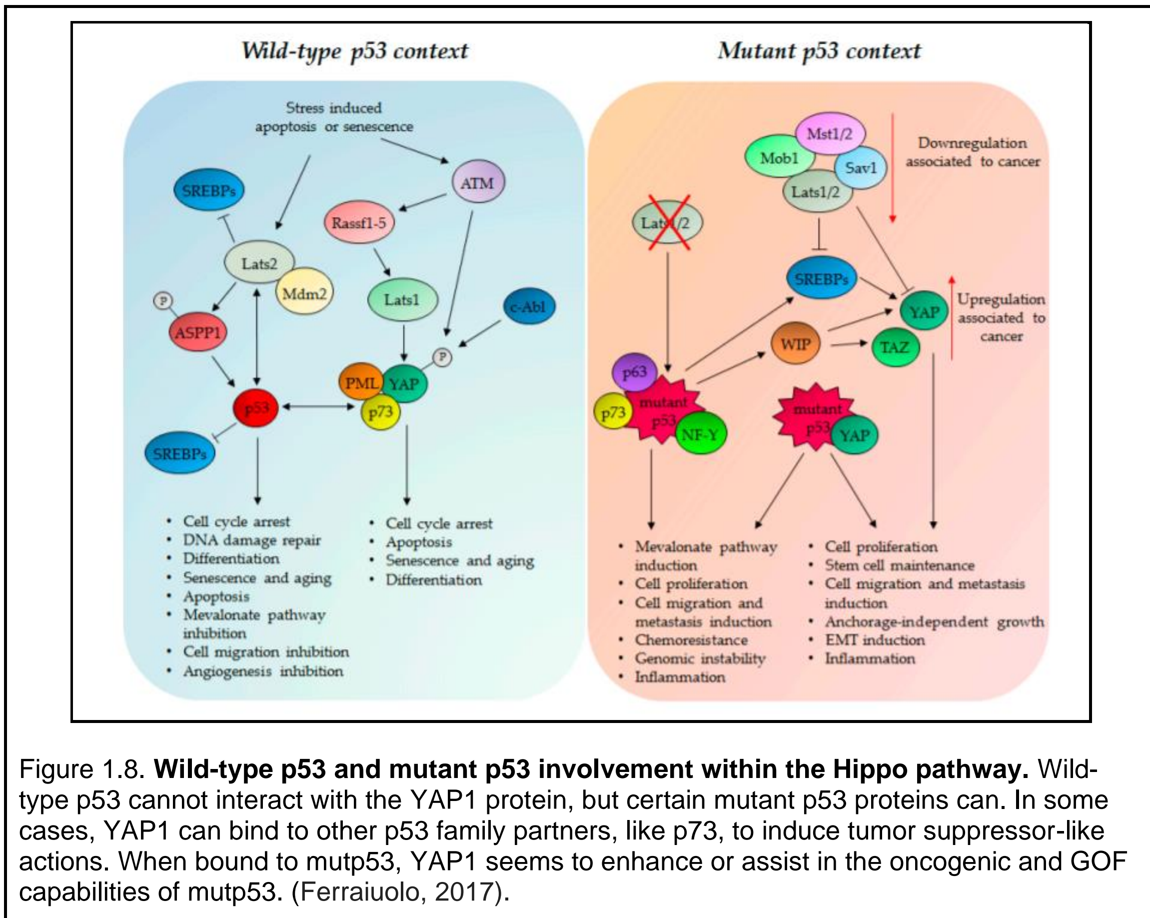

Figure 1.8. **Wild-type p53 and mutant p53 involvement within the Hippo pathway.** Wild-type p53 cannot interact with the YAP1 protein, but certain mutant p53 proteins can. In some cases, YAP1 can bind to other p53 family partners, like p73, to induce tumor suppressor-like actions. When bound to mutp53, YAP1 seems to enhance or assist in the oncogenic and GOF capabilities of mutp53. (Ferraiuolo, 2017).

Considering that YAP1 is a transcription coactivator that binds to and promotes the activity of transcription factors, it may be no surprise that YAP1 can bind to mutp53. What may be unexpected, however, is the complex interaction of mutp53 with both YAP1 and TEAD, forming a

tri-protein complex. Upon interaction with beta-arrestins, YAP1 is able to translocate into the nucleus. After the recruitment of mutp53 (also by beta-arrestin), the mutp53 was found to complex with both YAP1 and TEAD, forming a mutp53/YAP1/TEAD complex still able to transcribe YAP1 target genes like *CYR61* and *CTGF* (Tocci, 2019).

YAP1 involvement with the p53 promoter, rather than the mutp53 protein, has also been well documented, adding to the transcriptional powers YAP1 possesses. Canonically and also hinting at the tumor suppressor capability of YAP1 mentioned in the previous section, YAP1 has been shown to bind to the p53 promoter in liver cancer. This interaction resulted in the p53-mediated induction of apoptosis via p21, caspase3, and inhibition of Bcl-2 (Bai, 2013). Not only can YAP1 bind to the *TP53* promoter and induce *TP53* transcription, but p53 was also shown to bind to the *YAP1* promoter to promote *YAP1* transcription, creating a positive feed-back loop. Additionally, mutp53 was shown to recruit the WASP-interacting protein (WIP) where WIP phosphorylation by AKT2 (which is sustained by mutp53) promoted YAP1 and TAZ activation (Escoll, 2017). A fascinating realization, however, is that much of the current literature does not acknowledge the interaction of TAZ with mutp53 in the same way that YAP1 can interact with mutp53. What this suggests is that YAP1 may be alone in this GOF involvement with mutp53, particularly in their direct interaction. However, the previous study mentioned (Escoll, 2017) does provide evidence that TAZ at least may be part of the altered pathways that come from mutp53.

YAP1 is not the only protein in the Hippo-pathway that has involvement with p53. Wild-type p53 can interact with LATS2 to repress SREBP proteins, keeping in line the tumor suppressor functions of p53 and LATS2 (Aylon, 2016). SREBP proteins are involved in glucose metabolism and are involved in the production of lipids and fatty acids. In breast cancer, the high expression of SREPB1 was shown to correlate with metastasis and poor survival. In this case, the mechanism of action came from over activation of PI3K and KRAS (Wen, 2018). LATS2 evidently seems to play a very large role in SREPB regulation that is independent of YAP1 or TAZ interaction. LATS2 assists in regulating cholesterol synthesis by checking SREPB activity (Aylon, 2016). This is crucial for liver homeostasis. It is logical to find that SREBP levels rise with mutant p53, implying that mutp53 has a GOF that prevents it from interacting with LATS2 and

inducing tumor suppressor actions. Moreover, the mutp53, along with the SREBP dysregulation, is able to sustain the mevalonate pathway which in turn activates YAP1 and TAZ for the transcription of cell proliferation genes, where YAP1 also aids in the transcription of cholesterol metabolism genes (Sorrentino, 2014 & Wang, 2014).

Hypothesis and approach

A major and defining aspect of the YAP1 and TAZ proteins are their post-translational modifications (PTMs). In their non-phosphorylated form, the proteins translocate to the nucleus to transcribe cell survival and proliferation genes, implying that a higher amount of nuclear YAP1 and TAZ leads to higher cell proliferation and survival. Based on our previously established RNA-seq data, we are confident that different p53 mutations exert a phenotypic difference in regards to invasiveness. Here, the p53 mutations R273C and Y220C (highly invasive) are predicted to have larger amounts of YAP1 and TAZ compared to the non-invasive WT-p53 and Y234C. Moreover, our RNA-seq data can only provide predictions about the YAP1 and TAZ PTMs in the respective cell lines, but gives no direct answers. This gives questions about the YAP1 and TAZ proteins in our p53 mutant cell lines that are yet to be answered:

1. Does the predicted YAP1 and TAZ protein level match with the p53-mutant RNA-seq data and are the protein levels indeed correlated with cell invasion?
2. Do the protein levels of the active versions of YAP1 and TAZ (nuclear and non-phosphorylated) follow the same trend with respect to invasiveness?

This thesis provides answers to these questions that hopefully give further insight into how the Hippo-pathway is dysregulated in breast cancer invasion and how it may be used as a therapeutic target in breast cancer.

We hypothesized that 1) the YAP1 and TAZ protein levels would match the RNA-seq data and correlate with mutp53 induced invasiveness; the invasive R273C and Y220C lines (and other invasive cell lines) would have the highest levels of YAP1 and TAZ where the non-invasive cell lines would have the lowest levels of YAP1 and TAZ. We also hypothesized that 2) the active

versions of YAP1 and TAZ would be predominantly higher in the invasive cell lines and lower in the non-invasive cell lines. We approached these hypotheses by using western blot analysis to measure protein levels and western blot fractionation analysis to quantify active and non-active YAP1 and TAZ.

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CHAPTER 2

POST TRANSLATION MODIFICATIONS OF YAP1 AND TAZ AND ITS DEPENDENCE OF P53 MUTATION AND CELL INVASION

Introduction

The Hippo tumor suppressor pathway has increasingly been found to be dysregulated in many cancers. Yet, unlike many dysregulated pathways in cancer that are often the cause of genetic mutations, the Hippo-pathway instead has a very low frequency of genetic mutations within the core components. Overall, deep deletions have only been seen to occur in the LATS1/2 and NF2, though these types of mutations are commonly seen in mesothelioma. Additionally, MST2 and TAZ are seen to have the highest amplification rates among the Hippo pathway components (Wang, 2018). Regardless, these instances have only been documented at occurring in very low levels. Overall, however, the function of the Hippo pathway in cancer often seems to arise at pathway dysregulation involving protein-protein interactions and post translational modifications (PTMs), as opposed to genetic alterations. In this way, some of the most important PTMs in the Hippo pathway are the phosphorylation of YAP1 and TAZ.

Non-phosphorylated YAP1 and TAZ translocate to the nucleus to interact with and activate TEAD-family transcription factors. These activated TEAD-family transcription factors then induce cell survival and proliferation genes, which are often known as YAP1/TAZ signatures. The expression of these signature sets are therefore indicative of active or non-phosphorylated YAP1 and TAZ. The YAP1/TAZ signature gene sets are documented to correlate with poor prognosis in lung and breast cancers in addition to correlating with cell proliferation (Yamaguchi, 2020). Since YAP1 and TAZ are known to promote various hallmarks of cancer, an analysis on the PTMs of YAP1 and TAZ in association with different *TP53* mutations may shed light on how mutant p53 interacts with and dysregulates the Hippo pathway.

Aim

Post translational modification analysis of YAP1 and TAZ in four prevalent p53 missense mutant proteins and 2 controls in non-transformed mammary epithelial cells as it relates to cell invasion.

Approach

Model system of breast epithelial cells to analyze the effects of mutant p53 and hippo-pathway dysregulation

The MCF-10 cell line was originally described in a 1990s study that isolated cells from human mammary fibrocystic tissue that exhibited an immortal phenotype. From this primary cell culture, the authors were able to sub-cultivate the isolated cells into MCF-10F cells that were grown in floating, low calcium concentrations and MCF-10A cells grown at normal calcium levels. Despite its immortal phenotype, MCF-10A cells still demonstrate the properties of normal breast epithelium by demonstrating a lack of tumorigenicity in nude mice, the ability to grow three-dimensional cultures in collagen, growth depending on hormones and growth factors, and the inability to be anchorage independent (Soule, 1990). For this reason, MCF-10A cells represent a non-transformed healthy breast tissue cell line. MCF-10A cells are the most commonly used mammary cell line. Because of their popularity, the cell-line molecular make-up has been thoroughly characterized. Importantly, this line does not contain the p16 and p14 genes, as a result of chromosomal locus depletion, which are important for the amplification of the Myc oncogene. Additionally, MCF-10A cells do not express ER nor PR (Qu, 2015).

Depending on culture conditions, MCF-10A cells can exhibit different properties. When cultured on matrigel, MCF-10A cells form hollow sphereoids and form similar structures when

cultured within matrigel. When cultured in suspension, the cells form mammospheres. Based on two-dimensional culture, MCF-10A cells seem to have a basal cell origin, expressing predominantly basal markers like CK5, but have also been shown to express some luminal markers like E-cadherin. Finally, these breast cells express a wild-type p53 (Tait, 1990). Important to highlight for this study is that MCF-10A cells lack ER, PR, and do not have the over amplification of HER2. For this reason, these cells, as a primary cell line, can serve as a beginning stage of TNBC. Additionally, the expression of wild-type p53 also allows one to add various p53 mutants and measure the effect on phenotype as it relates to a TNBC context. For this reason, we used the MCF-10A cell for this study.

Post translation modification analysis of YAP1 and TAZ

YAP1 and TAZ are co-transcription activators that activate the TEAD-family transcription factors. To achieve this result, YAP1 and TAZ must be non-phosphorylated to be able to translocate into the nucleus. Inactive YAP1 is characterized by phosphorylation at serine-127 where inactive TAZ is characterized by phosphorylation at serine-89; phosphorylation at different sites dictate eventual degradation. For this reason, we used western blot analysis to particularly look at either total YAP1 and TAZ levels where probing was unbiased towards phosphorylation status. We likewise targeted only phosphorylated YAP1 or TAZ where probing was biased towards phosphorylation status. By measuring both total and inactive YAP1 and TAZ, this allowed us to quantify and compare active YAP1 or TAZ to non-active YAP1 or TAZ as it relates to cell invasion and mutant p53 status. Finally, by targeting YAP1 and TAZ in different cellular compartments (nuclear and cytoplasmic), we were able to directly measure the amount of active YAP1 or TAZ protein. Using these methods would provide an overall picture for the YAP1 and TAZ and how they differentiate among different p53 mutant cell lines and cell invasion.

Method

Cell culture

The MCF-10A epithelial cell line was routinely cultured in 150 x 20mm tissue culture plates. Cells were cultured with DMEM/F12 media (ThermoScientific #11320-082) supplemented with 5% horse serum (ThermoScientific #16050-122), insulin (10ug/ml Sigma Aldrich #I9278-5ML), cholera toxin (100ng/ml Sigma Aldrich #C8052-1MG), hydrocortisone (0.5ug/ml Sigma Aldrich #H0888), and human epidermal growth factor (hEGF) (ThermoScientific #PHG0311) in 5% CO_2 37°C humidity incubator. Upon reaching 70-90% confluency, cells were routinely passaged with 1X phosphate buffered saline and trypsin (0.25% in HBSS with 0.2g/ml EDTA) (VWR #16777-166). Cells were also tested for mycoplasma contamination every 2 weeks and when needed.

Production of MCF-10A clones

Overall there were 6 different p53 proteins involved within this study. Ranked from most invasive to least invasive, these p53 mutants were: R273C, Y220C, R273H, wild-type overexpressed (WT-OE), wild-type (WT), and Y234C. The cells harboring p53-OE were created using vectors from DNASU (The center for personalized diagnostics at the Biodesign Institute, ASU). The different *TP53* missense mutations were first inserted into the pLenti4/V5-DEST Gateway destination vector (ThermoScientific #V49810) which was then transformed into *E. coli* cells and expanded into 500 ml cultures. The plasmid was purified from these cultures using Macherey-Nagel DNA Maxi-Preps. Finally, stable cell lines expressing the different p53 proteins were created via lentiviral transduction.

Western Blot

Cells were allowed to grow to 80% confluency on 6 well plates (Greiner #5665-7160) and were subsequently lysed using RIPA Lysis buffer (150mM NaCl, 50mM Tris, 0.1% sodium azide (sigma #S8032, 1% IGEPAL CA-630 (Sigma Aldrich #I3021-50ml), Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (100X) (ThermoScientific #78442), 200uM sodium fluoride (Sigma Aldrich #S6776), and 200uM sodium orthovandate (Sigma Aldrich #450243). Cells were first washed 3 times with cold 1x TBS. RIPA Lysis Buffer was then applied to the cells and cells were scraped. Lysates were incubated on ice for 10 minutes with 5 seconds of vortexing every 2 minutes. Lysates were centrifuged at 10,000 rpm for 10 minutes where supernatant was collected and the pellet discarded. Lysate protein concentration was determined using the Pierce BCA kit (ThermoScientific #23225). Using 5-10ug of lysate, samples were run on 4-20% TGX (BioRad #567-1084 or #567-1095) and blotted on a 0.45um PVDF membrane (GE Healthcare #10600023) using the BioRAD semi-dry transfer system. Membranes were blocked for at least 1 hour and 30 minutes in 5% milk in 1xTBST. Primary antibodies for YAP1 (Cell Signaling Technologies #12395), TAZ (CST #83669), pYAP1 (S127) (CST #13008), and pTAZ (S89) (CST #59971) were all diluted 1:1000 in 5% milk in TBST, respectively. GAPDH primary antibody was used as a loading control (CST #5174). Primary antibodies incubated overnight in 4°C. Primary antibodies were washed away with 3x washes of 1xTBST (5-10 min incubations). Anti-rabbit IgG HRP-linked secondary antibody (CST #7074) was diluted 1:3000 in 5% milk in TBST. Secondary antibody was allowed to incubate for 1 hour at room temperature and was subsequently washed away with 1x TBST (3 washes 5-10 min each). Western blots were imaged on the ImageQuant LAS 4000 from GE Healthcare and bands were quantified using the ImageJ software from the NIH. Student’s t-tests were performed to determine if there was a significant difference between the different cell lines.

Fractionation Western Blots

Cells were allowed to grow to 80% confluency in 150 x 20mm tissue culture plates. Seven million cells were harvested for each line. Cell cytoplasmic and nuclear fractionations were achieved using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (ThermoScientific #78833) according to manufacturer protocols. The Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (100X) (ThermoScientific #78442) was supplied to the kit reagents prior to usage. Either equal volume of lysates or 5ug of lysates were used for western blot analysis. The methodology used for western blots follows the same steps listed above under Western Blot Section. The α/β-tubulin (CST #2148), c-Jun (CST #9165), GAPDH (CST #5174), beta-actin (CST #4970), YAP1 (CST #12395), and TAZ (CST #83669) primary antibodies were diluted 1:1000 in 5% milk in TBST and allowed to incubate overnight at 4°C. Anti-rabbit IgG HRP-linked secondary antibody (CST #7074) was diluted 1:3000 in 5% milk in TBST. Secondary antibody was allowed to incubate for 1 hour at room temperature and was subsequently washed away with 1x TBST (3 washes 5-10 min each). Western blots were imaged on the ImageQuant LAS 4000 from GE Healthcare and bands were quantified using the ImageJ software from the NIH. Student’s t-tests were performed on the protein levels to determine if there was a significant difference between the cell lines.

Results

Stable expression of mutant p53 in MCF10A cell lines

Unlike many other tumor suppressor genes like *RB* which typically have oncogenic mutations in the form of truncations or deletions, *TP53* predominantly has missense mutations in oncogenic settings. This, however, begs the question if all p53 mutants are created equal. Consistently, it has been found that mutant p53 proteins induce differing phenotypes depending

on mutation. As a homo-tetramer transcription factor, the charge and structure of the p53 protein is crucial for its function. In the context of a missense mutation, one amino acid change may result in a protein structure shift and charge shift, resulting in altered DNA/protein-binding capability and therefore phenotype. This overall may endow a cell with GOF properties in the form of cancer hallmarks (Mello, 2013). We therefore designed our study to specifically measure these mutant p53-GOF properties as it relates to YAP1/TAZ mediated cell invasion. To achieve an adequate range in differing phenotype, we analyzed 2 highly invasive p53 mutant cell lines (R273C and Y220C), 2 mildly-invasive cell lines (R273H and WT-OE), and 2 non-invasive cell lines (WT and Y234C). The mutations R273C and R273H are mutations that occur within the DNA-binding domain of p53. These mutations are therefore known as “DNA contact mutations.” The mutations Y220C and Y234C do not occur in the DNA binding domain but instead may affect the structure of the p53 protein. These mutations are therefore known as “Structural Mutations.” The WT and WT-OE proteins have no DNA nor structural mutations. Prior to western blot analysis, the protein levels of mutant p53 were determined via V5-tag primary antibody and p53 primary antibody and were found to have comparable levels to the known breast cancer lines HCC70, MDA-MB-231, MDA-MB-468, AU565, and SKBR3 (Figure 2.1). Notably, the mutant p53 proteins were expressed at much higher levels compared to WT-p53. The WT-OE and WT controls in this experiment are important for demonstrating the capability and difference induced by p53 mutants. Likewise, all protein levels determined by western blot were scored as a fold-change over wild-type p53 over-expressed for comparison.

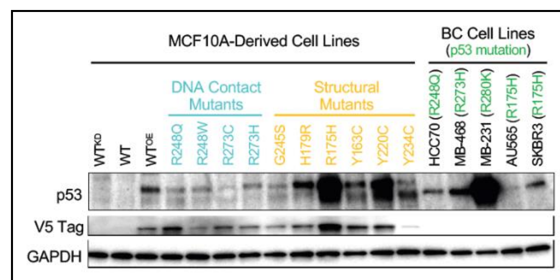


Figure 2.1 **Generation of stable MCF-10A cell lines expressing mutant p53.** MCF-10A cells were transduced with lentivirus containing mutant p53 protein. The mutant p53 proteins have a V5 tag at the C-terminus. V5 tag band is seen for R273C, Y220C, R273H, Y234C, and WT-OE but not WT.

Measure of total YAP1 and TAZ level in MCF-10A cells expressing mutant p53 proteins

The protein level of YAP1 and TAZ are thought to be crucial for cell invasiveness and tumor progression. Generally, breast cancers see elevated *YAP1* and *TAZ* mRNA and protein levels. In TNBC, however, TAZ is thought to have a more dominant role where *TAZ* mRNA and protein elevation was more obvious compared to *YAP1* mRNA and protein levels, particularly regarding cell invasiveness (Diaz-Martin, 2015). It therefore stands to reason that TAZ protein levels may correlate with p53-mutant driven invasiveness. To test this theory, we performed western blots on different MCF-10A cell lines containing the different p53 versions: R273C, Y220C, R273H, WT-OE, WT, and Y234C. MCF-10A cell lines were maintained in DMEM/F12 media containing human epidermal growth factor (hEGF), horse serum, insulin, cholera toxin, and hydrocortisone and lysed upon reaching ~80% confluency. Lysates were blotted on a PVDF membrane and probed with YAP1 and TAZ antibodies where each antibody detected total protein level.

Beginning with YAP1, there was no large visible difference between YAP1 levels and the different cell lines implying that YAP1 protein level is neutral regardless of invasive phenotype. Overall, the Y220C mutant had the largest level of YAP1 protein compared to the rest of the cell lines and intriguingly had the most difference in YAP1 protein level when compared to the R273H mutant (moderately invasive) ($p = 0.103$) (Figure 2.2 and Figure 2.3). T-test p-values confirmed that there was no significant difference of YAP1 levels between any of the cell lines nor when grouped between invasive and non-invasive.

TAZ protein levels across the different cell lines did have some difference, but overall were similar also hinting at overall protein level neutrality despite invasive phenotype (Figure 2.4 and 2.5). The mutants Y234C, R273H, and R273C had similar TAZ protein levels, while the Y220C mutant showed to have the least amount of TAZ protein. Intriguingly, the only significant difference was between Y220C vs WT ($p=0.012$). Contrary to the RNA-seq data, however, the

WT cell line had more TAZ protein than the Y220C cell line. No other significant difference was observed when comparing the different cell lines or different invasive or non-invasive groups. This pattern for TAZ remains similar, though slightly different than that of YAP1.

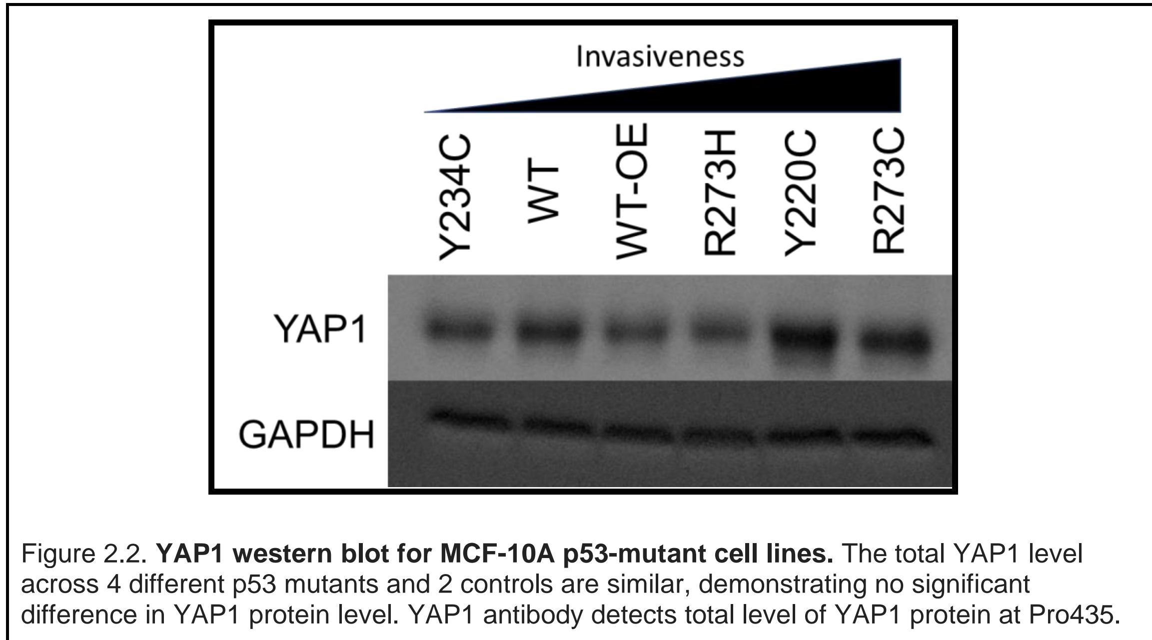
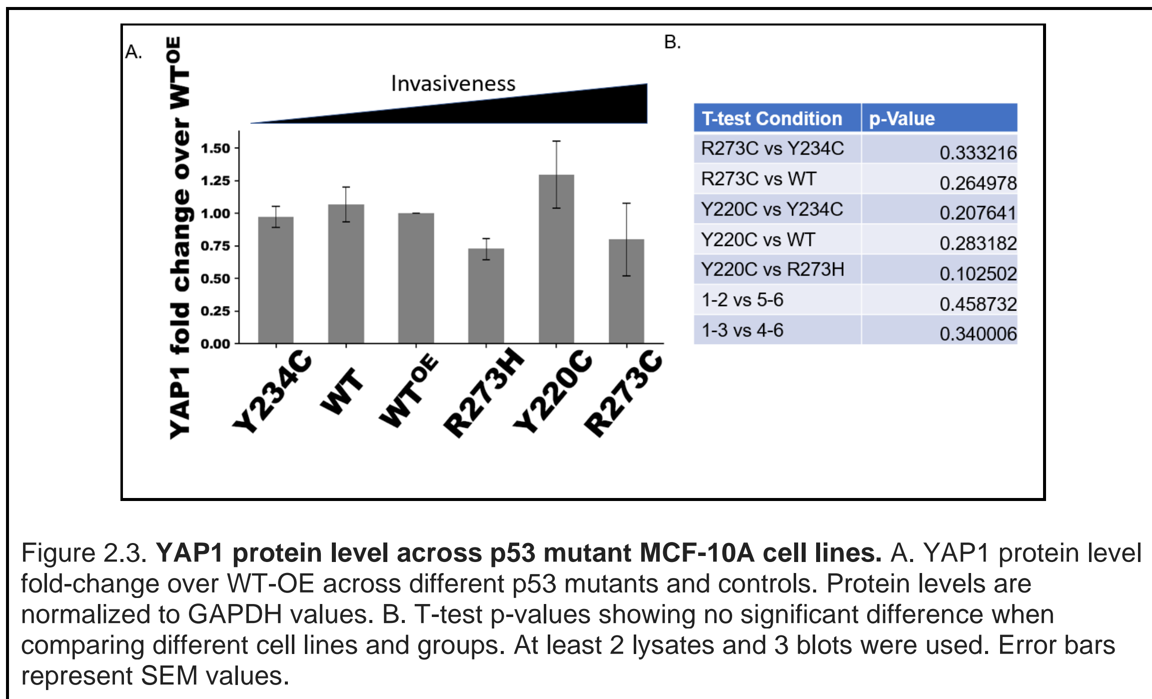


Figure 2.2. **YAP1 western blot for MCF-10A p53-mutant cell lines.** The total YAP1 level across 4 different p53 mutants and 2 controls are similar, demonstrating no significant difference in YAP1 protein level. YAP1 antibody detects total level of YAP1 protein at Pro435.



T-test Condition	p-Value
R273C vs Y234C	0.333216
R273C vs WT	0.264978
Y220C vs Y234C	0.207641
Y220C vs WT	0.283182
Y220C vs R273H	0.102502
1-2 vs 5-6	0.458732
1-3 vs 4-6	0.340006

Figure 2.3. **YAP1 protein level across p53 mutant MCF-10A cell lines.** A. YAP1 protein level fold-change over WT-OE across different p53 mutants and controls. Protein levels are normalized to GAPDH values. B. T-test p-values showing no significant difference when comparing different cell lines and groups. At least 2 lysates and 3 blots were used. Error bars represent SEM values.

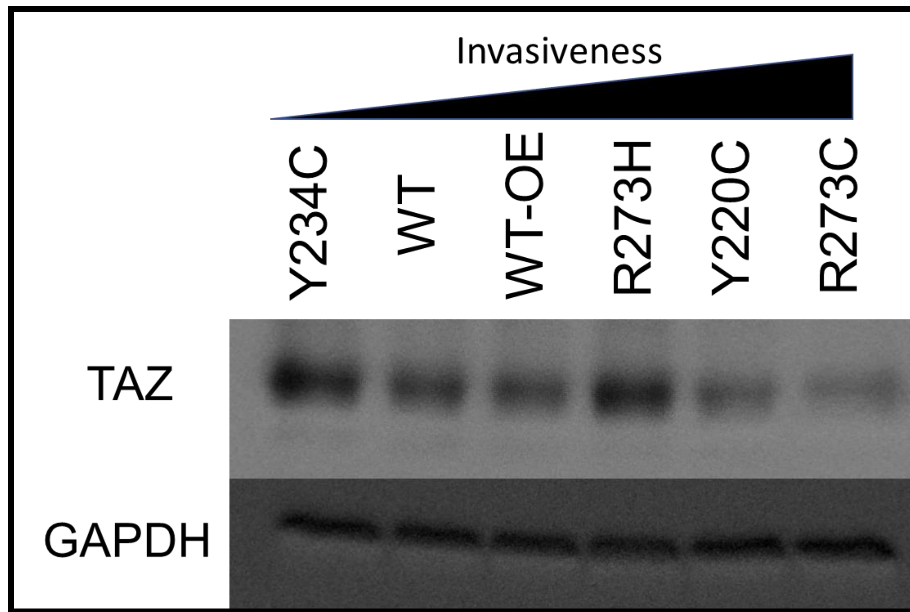
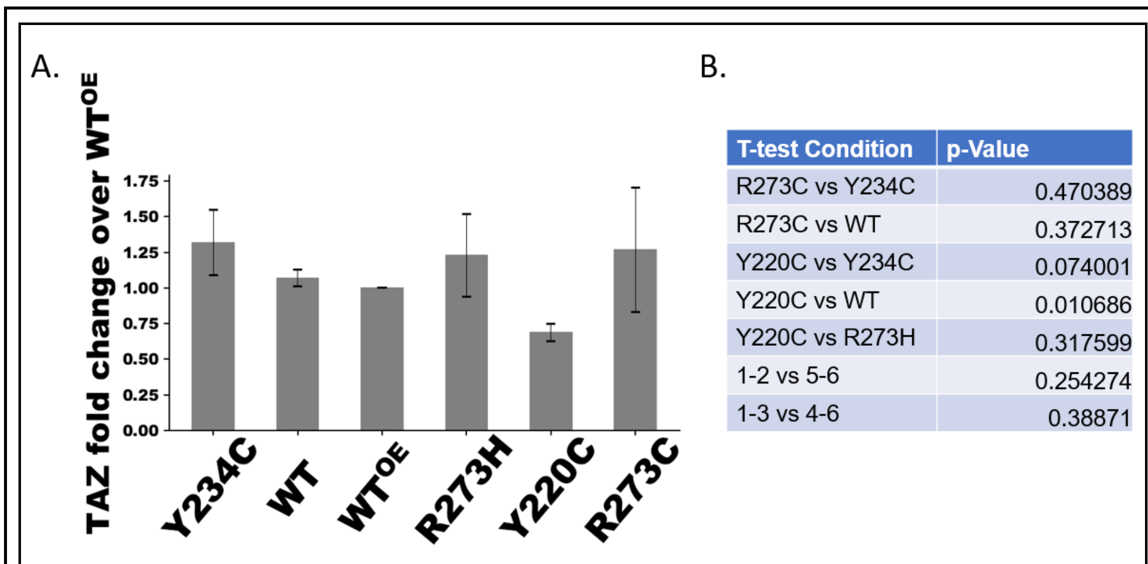


Figure 2.4. **Western blot of TAZ across different p53 mutant MCF-10A cells.** Representative western blot shows total TAZ protein level across 4 p53 mutants and 2 controls. Protein levels seem to vary. TAZ antibody detects total protein level at Pro49.



T-test Condition	p-Value
R273C vs Y234C	0.470389
R273C vs WT	0.372713
Y220C vs Y234C	0.074001
Y220C vs WT	0.010686
Y220C vs R273H	0.317599
1-2 vs 5-6	0.254274
1-3 vs 4-6	0.38871

Figure 2.5. **TAZ protein level across p53 mutant MCF-10A cells.** A. TAZ protein level fold-change over WT-OE across different p53 mutants and controls. Protein levels are normalized to GAPDH. B. T-test p-values showing overall no significant difference across invasive groups, but some difference between Y220C and WT. At least 2 lysates and 3 blots were used. Error bars represent SEM values.

Inactive YAP1 and TAZ across p53 mutant MCF-10A cells

The total level of YAP1 and TAZ are important for cell and cancer behavior, but the phosphorylation status of YAP1 and TAZ are additionally important as it will better demonstrate the suspected protein activity, rather than just analyzing pure protein level. Mediated by LATS1/2, YAP1 and TAZ phosphorylation represent inactive YAP1 and TAZ. When YAP1 and TAZ are phosphorylated (YAP1 at S127 and TAZ at S89), they remain in the cytoplasm. Other phosphorylation sites dictate YAP1 and TAZ degradation. When unphosphorylated, the proteins are able to translocate to the nucleus to help activate TEAD transcription factors and induce cell survival and proliferation genes. Understandably, a loss of YAP1 and TAZ phosphorylation has often been found to result in the increased expression of YAP1 and TAZ target genes, implying that a lower level of YAP1 or TAZ phosphorylation may lead to increased cell invasion (Gill, 2018).

MCF-10A cell lines were maintained in DMEM/F12 media containing human epidermal growth factor (hEGF), horse serum, insulin, cholera toxin, and hydrocortisone and were lysed upon reaching ~80% confluency. Lysates were blotted on a membrane and probed with YAP1 and TAZ antibodies that are specific to phosphorylation of YAP1 at S127 and TAZ at S89. Hence, the antibodies detected inactive YAP1 and TAZ separately.

Interestingly, the pYAP1 levels amongst the 6 cell lines was overall higher in the less invasive cell lines (Y234C and WT) and lower in the more invasive cell lines (R273C, Y220C, R273H, and WT-OE) (p=0.023) (Figure 2.6 and 2.7.) The difference in pYAP1 levels for R273C (highly invasive) and WT (non-invasive) was significant (p=0.031), but intriguingly had no significance when compared to Y234C (least invasive) (p=0.051). Overall, the WT cell line had the highest level of pYAP1. The Y220C mutant had the highest pYAP1 levels amongst the invasive cell lines and was similar to the pYAP1 levels in Y234C. The R273C, R273H, and WT-OE lines all had similar levels of pYAP1. Additionally, when grouping the 2 least invasive cell lines (Y234C and WT) and comparing the pYAP1 values to the two most invasive cell lines (R273C

and Y220C), there was a significant difference (p=0.02), implying that there is overall higher levels of pYAP1 in MCF-10A cells harboring a non-invasive phenotype. In agreement with our results, cytosolic YAP1 expression and nuclear YAP1 expression was seen to positively correlate with invasive breast cancer (Vlug, 2013).

This trend observed in pYAP1 was more obvious when pTAZ levels were analyzed (Figures 2.8 and 2.9). Here, pTAZ levels were highest in the Y234C and WT cell lines and subsequently decreased as invasiveness increased amongst the cell lines. Significant differences in pTAZ levels are between R273C and WT (p=0.047) and between Y220C and WT (p=0.021). It is worth noting that the overall pTAZ levels when comparing invasive trend and non-invasive trend was also significant; the pTAZ levels in the 3 most invasive cell lines were starkly different than that of the 3 least invasive cell lines (p=0.00047). Taken together, these results imply that inactive TAZ displays higher expression in non-invasive p53-mutant cell lines, in comparison to invasive p53-mutant cell lines. Aligning with our results, other studies show that overexpressing LATS1/2 (therefore increasing TAZ phosphorylation) results in less TEAD activity and therefore lower TAZ cell proliferation and migration target genes (Lei, 2008). Moreover, the higher amount of inactive TAZ (such as in Y234C and WT) may result in lower cell migration and invasion.

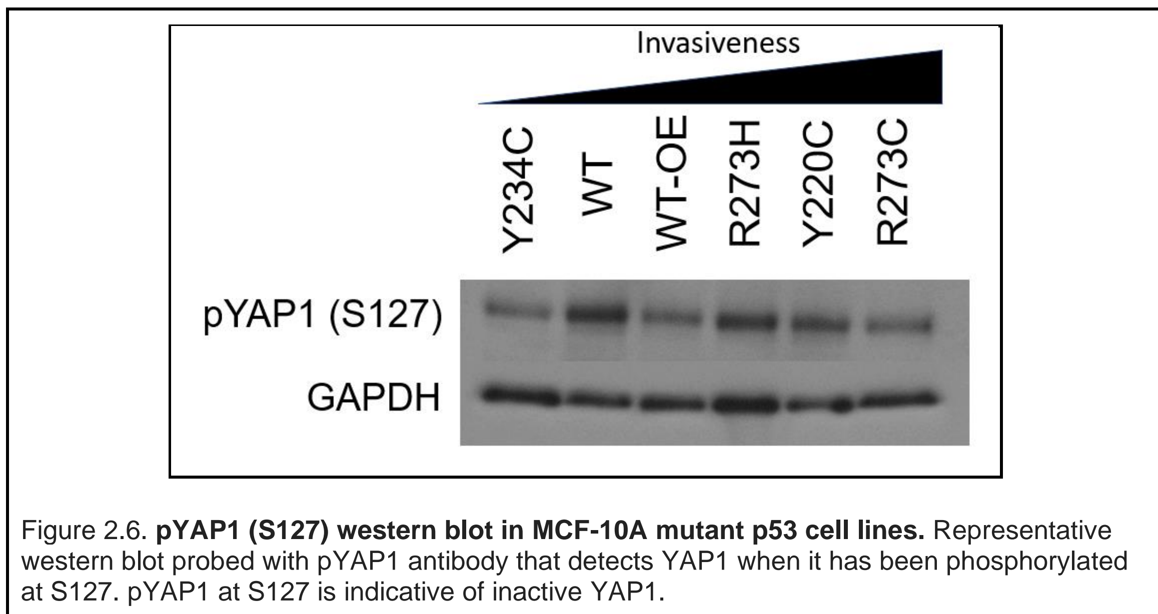
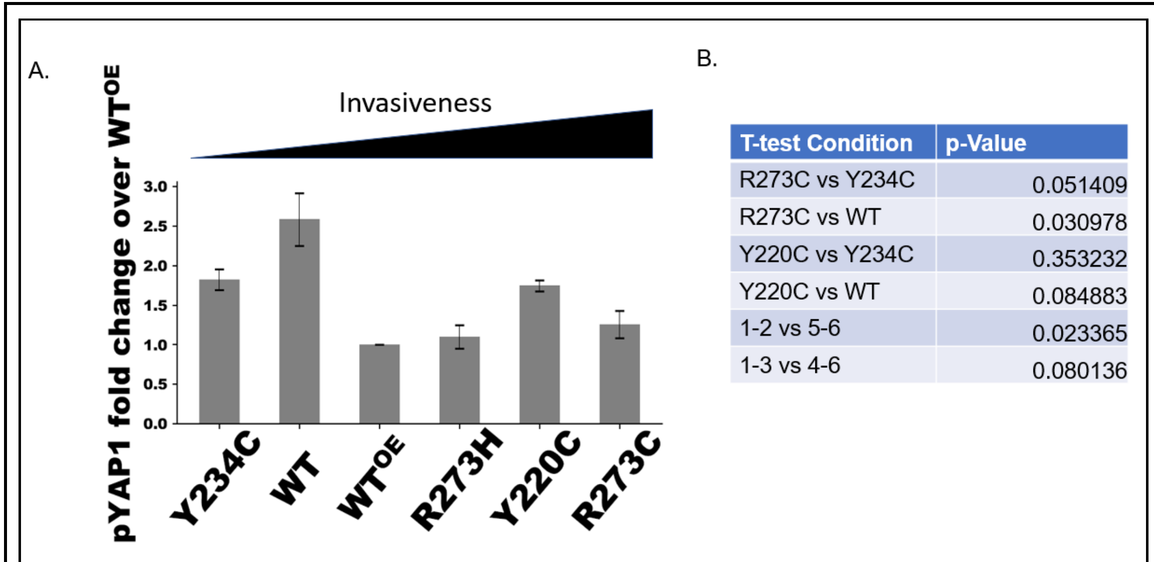


Figure 2.6. **pYAP1 (S127) western blot in MCF-10A mutant p53 cell lines.** Representative western blot probed with pYAP1 antibody that detects YAP1 when it has been phosphorylated at S127. pYAP1 at S127 is indicative of inactive YAP1.



T-test Condition	p-Value
R273C vs Y234C	0.051409
R273C vs WT	0.030978
Y220C vs Y234C	0.353232
Y220C vs WT	0.084883
1-2 vs 5-6	0.023365
1-3 vs 4-6	0.080136

Figure 2.7. **pYAP1 levels in p53 mutant MCF-10A cell lines.** A. Protein level of YAP1 that has been phosphorylated at S127 (inactive YAP1). Values represent the fold-change over WT-OE. At least 2 lysates were used, representing 3 blots. Error bars represent SEM values. B. T-test p-values representing the difference among various cells and groups.

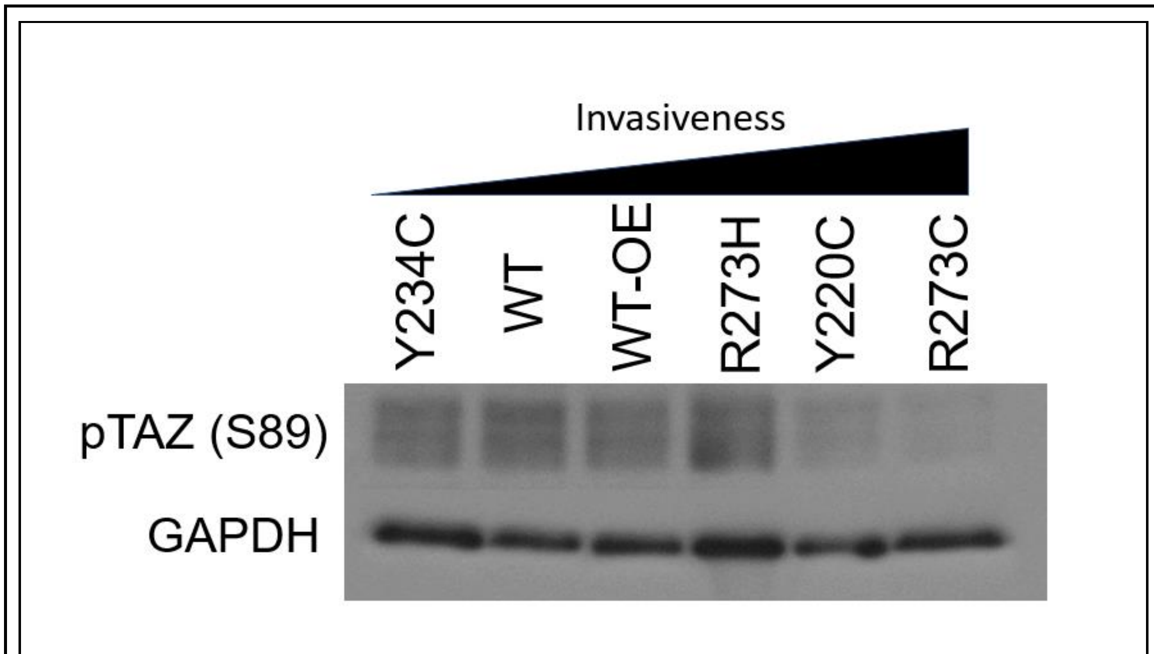
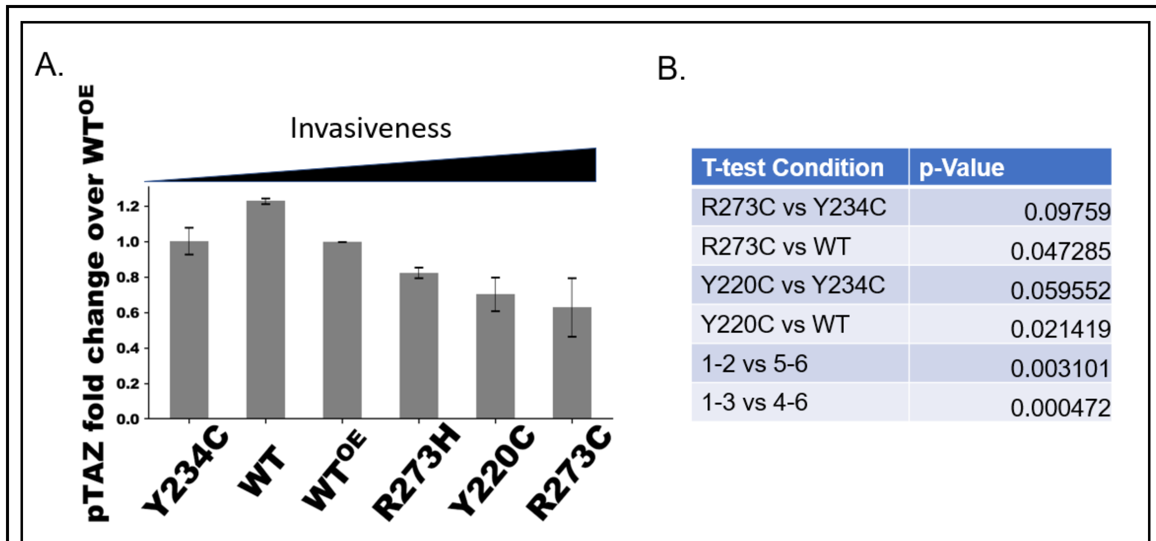


Figure 2.8. **Western blot detecting pTAZ (S89) in mutant p53 MCF-10A cells.** Representative western blot detects pTAZ in 6 p53-mutant MCF-10A cell lines. pTAZ antibody used to probe membrane and detects TAZ protein only when it has been phosphorylated at S89, representing inactive TAZ.



T-test Condition	p-Value
R273C vs Y234C	0.09759
R273C vs WT	0.047285
Y220C vs Y234C	0.059552
Y220C vs WT	0.021419
1-2 vs 5-6	0.003101
1-3 vs 4-6	0.000472

Figure 2.9. **pTAZ levels in p53 mutant MCF-10A cell lines.** A. Protein level of TAZ phosphorylated at S89 (inactivate TAZ). Values represent fold-change over WT-OE and error bars represent the SEM. Protein levels were achieved using at least 2 lysates and over 3 independent experiments. B. T-test p-values representing the pTAZ level difference between various cell lines and groups.

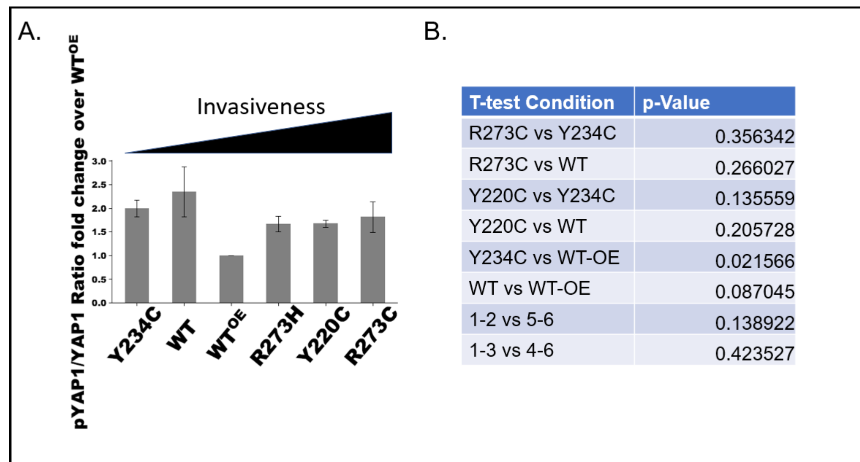
Comparing total YAP1 and TAZ levels to inactive YAP1 and TAZ

Phosphorylated YAP1 and TAZ proteins (at a certain phosphorylation site) represent inactive YAP1 and TAZ. In this case of cell invasion, phosphorylation of both proteins was seen to be overall higher in the non-invasive cell lines and lower in the invasive cell lines. This data, while intriguing, does not compare inactive protein to active protein. It is the ratio between the two that may be more crucial when deciding if YAP1 or TAZ play a crucial role in mutant-p53 mediated invasion. In this way, a mutant cell line may have larger amounts of pTAZ (such as Y234C), but it may also have an even higher amount of active TAZ protein. The cell therefore may have a higher expression of active TAZ protein in which the supposedly large expression of pTAZ is misleading. Measuring the ratio of phosphorylated protein to total protein would give a better analyzation of how much active protein may be present in the different MCF-10A cell lines.

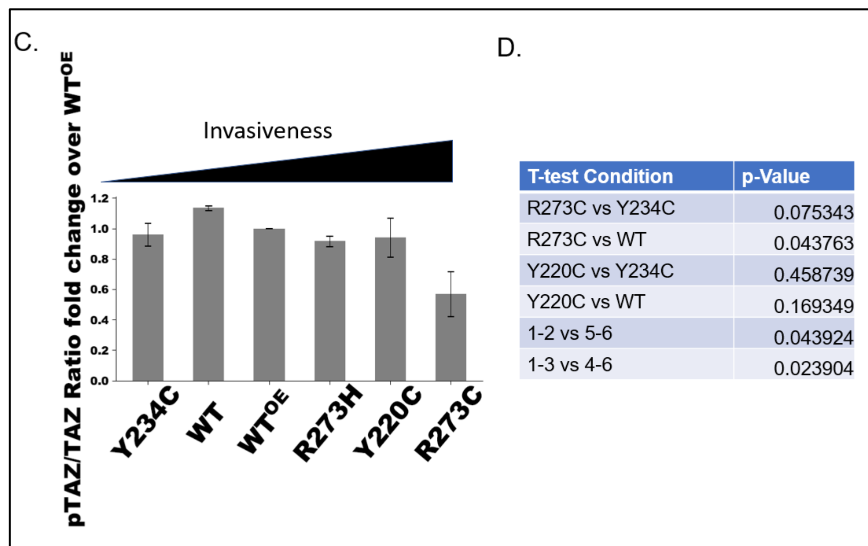
To this end, the pYAP1 and pTAZ levels were compared to the total protein levels to achieve the pYAP1/YAP1 and pTAZ/TAZ ratio (Figure 2.10). All ratios values were represented

as fold-change over WT-OE. Looking first at the pYAP1/YAP1 ratio, the largest ratios were amongst the Y234C and WT cell lines and showed significant differences from WT-OE (p=0.007), showing that inactive YAP1 made up the majority of total YAP1 protein within these cell lines. The WT-OE line had the lowest ratio, suggesting that WT-OE had the least amount of inactive YAP1 out of its total YAP1 protein. Intriguingly, the top 3 invasive cell lines (R273C, Y220C, and R273H) all had similar pYAP1/YAP1 ratios, implying that the degree of inactive YAP1 was the same across these p53 mutants. Additionally, despite some significant difference in the pYAP1 values (Figure 2.6 and 2.7), there was no significant difference between the non-invasive groups and the invasive groups, suggesting no overall trend in regards to pYAP1/YAP1 ratio amongst the different MCF-10A cell lines.

Continuing with the pTAZ/TAZ ratio, the highest ratio was in the WT cell line, like that of the pYAP1/YAP1 ratio. All ratios were represented as fold-change over WT-OE. The lowest ratio was in the R273C cell line. The Y220C and R273H cell lines appear to have similar ratios to WT-OE and Y234C. T-test analysis revealed notable differences between the two most invasive cell lines (R273C and Y220C) and the two least invasive cell lines (WT and Y234C) (p=0.044). Moreover, there was significant contrast when grouping the 3 invasive cell lines (R273C, Y220C, and R273H) and comparing them to the 3 least invasive cell lines (WT-OE, WT, and Y234C) (p=0.024). Individually, the pTAZ/TAZ ratio in R273C was significantly different from the ratio in the WT cell line. Taken together, these results imply a trend where the pTAZ levels in the non-invasive cell lines represent more of the total TAZ protein in these lines when compared to the invasive cell lines. In agreement with our findings, a higher overall inactive TAZ protein has been seen to have lower TEAD activity in addition to lower rates of epithelial to mesenchymal transition, which resulted in lower expression of TAZ target genes (cell proliferation and migration genes such as *CTGF* and *CYR61*) (Rashidian, 2015).



T-test Condition	p-Value
R273C vs Y234C	0.356342
R273C vs WT	0.266027
Y220C vs Y234C	0.135559
Y220C vs WT	0.205728
Y234C vs WT-OE	0.021566
WT vs WT-OE	0.087045
1-2 vs 5-6	0.138922
1-3 vs 4-6	0.423527



T-test Condition	p-Value
R273C vs Y234C	0.075343
R273C vs WT	0.043763
Y220C vs Y234C	0.458739
Y220C vs WT	0.169349
1-2 vs 5-6	0.043924
1-3 vs 4-6	0.023904

Figure 2.10. **pYAP1/YAP1 ratio amongst variable p53 MCF-10A cell lines.** A. pYAP1/YAP1 ratio, shown as fold-change over WT-OE. Error bars represent SEM values. Ratios were achieved using at least 2 lysates and 3 separate blots. B. T-test p-values for pYAP1/YAP1 ratios showing any difference between different cell lines and groups. C. pTAZ/TAZ ratio, shown as fold-change over WT-OE. Error bars represent SEM values. Ratios were achieved using at least 2 lysates and 3 separate blots. D. T-test p-values for pTAZ/TAZ ratios showing any difference between different cell lines and groups.

Quantifying active YAP1 and TAZ protein

Measuring inactive YAP1 and TAZ can provide crucial information and highlight useful trends. The inactive protein, however, is not performing any enzymatic function in the canonical

Hippo/YAP1/TAZ pathway as it relates to cell invasion and proliferation. It is the active YAP1 and TAZ proteins that will perform this task by translocating into the nucleus to activate the TEAD transcription factors and induce cell-survival and invasion genes such as *CTGF* and *CYR61* (Weiler, 2020). Hence looking directly at the active YAP1 and TAZ proteins in the various cell lines will not only validate the previous ratio findings, but will also provide more insight into how active YAP1 and TAZ may facilitate mutant p53-mediated cell invasion, rather than just analyzing inactive protein levels (Fresques, 2020).

To achieve this, MCF-10A cells containing different p53 proteins were lysed. The lysed components were split into nuclear fractions and cytoplasmic fractions. Lysates were transferred to a PVDF membrane and probed with YAP1 and TAZ antibodies to detect total protein within the separate fractions. Because the nuclear fraction is much more concentrated, the raw blot images may be misleading when trying to compare the cytosolic and nuclear fractions. As such, to plot protein levels, 'real' values were back calculated for each of the band signals according to lysate concentration and actin normalization. When YAP1 is phosphorylated at S127 and TAZ at S89, they are both sequestered within the cytoplasm, unable to translocate to the nucleus to activate TEAD family transcription factors. These phosphorylated versions are therefore inactive. When unphosphorylated at these regions, YAP1 and TAZ translocate to the nucleus to activate TEAD family transcription factors. These unphosphorylated versions are therefore active. Hence, any YAP1 or TAZ detected in the cytoplasm represent inactive protein and any YAP1 or TAZ detected in the nucleus represent active protein.

Prior to probing with YAP1 and TAZ antibodies, a control blot was first performed to establish that the different subcellular fractions were separated accurately. Antibodies against tubulin (a cytoplasmic only protein) and c-Jun (a nuclear only protein) were used to establish these controls (Figure 2.11). The blot showed that only the cytoplasmic fractions contained tubulin bands where only nuclear fractions contained c-Jun bands. GAPDH was used as a loading control, but it is worthy to note that the GAPDH signal appeared weakly in some nuclear fractions due to the fact that GAPDH has higher expression in the cytoplasm in comparison to the nucleus (Tristan, 2011).

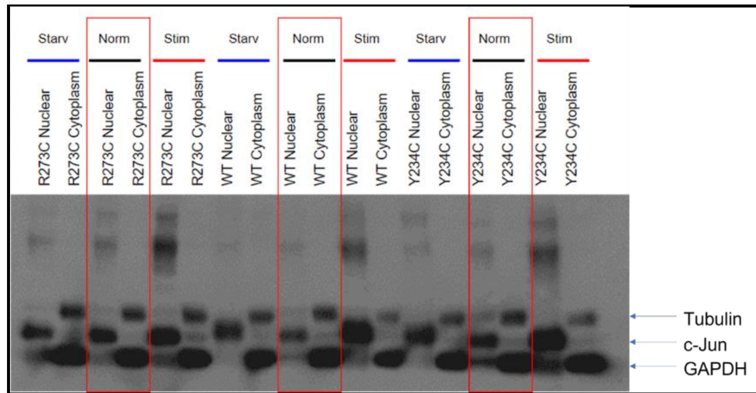


Figure 2.11. **Establishment of subcellular lysate fractions.** Western blot showing different cell fractions in different MCF-10A cell lines. Tubulin (cytoplasm) and c-Jun (nucleus) antibodies used to prove that fractions were created accurately.

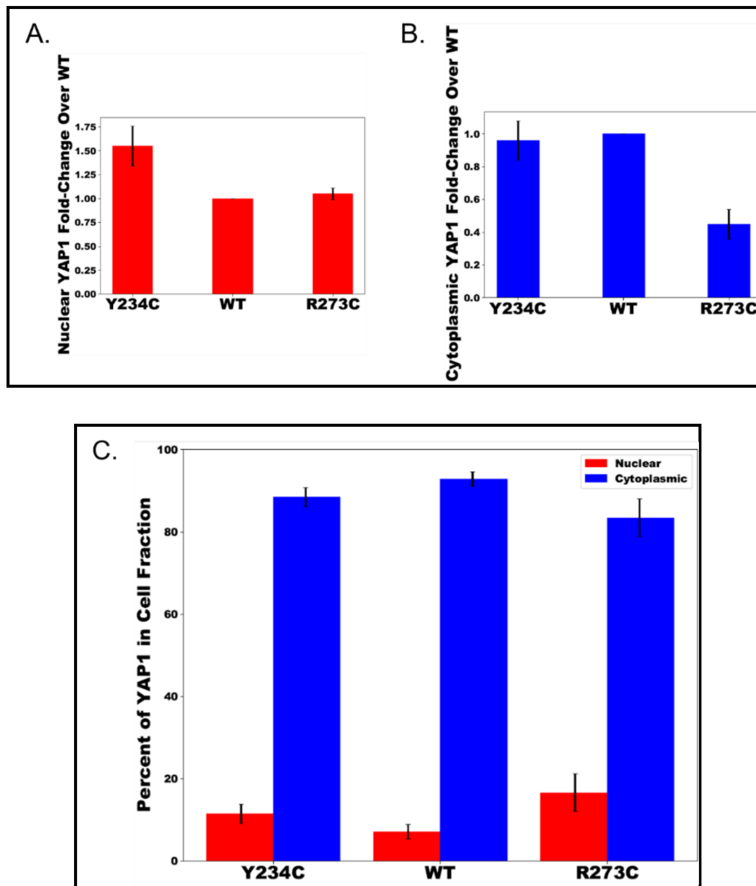


Figure 2.12. **Subcellular fraction expression of YAP1.** Real-values were achieved from western-blot bands and normalized to beta-actin. Values are represented as the fold-change over WT and error bars represent SEM, using 3 separate blots. A. YAP1 expression in nuclear fraction. B. YAP1 expression in cytoplasmic fraction. C. Percent of YAP1 in nucleus and cytoplasm.

Beginning with YAP1, the highest nuclear level of YAP1 occurred in the Y234C cell line (non-invasive) where the nuclear YAP1 expression in WT and R273C were similar (Figure 2.12). Intriguingly, R273C had the lowest cytoplasmic YAP1 expression whereas Y234C and WT had similar cytoplasmic expression levels. Combining the cytoplasmic and nuclear expression levels together as a total protein, however, revealed that R273C had the highest percentage of nuclear YAP1, followed by Y234C, then WT. Despite the contrasting values between the invasive R273C and the non-invasive Y234C and WT, a t-test showed that there was no significant difference between any of the percentages.

Continuing with TAZ, the highest nuclear TAZ expression was in the R273C cell line, followed by Y234C, and then WT (Figure 2.13). R273C had the lowest cytoplasmic TAZ expression, where cytoplasmic TAZ expression intriguingly increased amongst the cell lines as invasiveness decreased. The values between Y234C and WT together were significantly different than R273C, but not individually ($p=0.04$). By analyzing the different fractions as a total lysate, like YAP1, R273C showed to have the highest percent of nuclear TAZ, compared to WT and Y234C.

Comparing TAZ fractionation values to YAP1 values, the active TAZ (nuclear) levels in the invasive R273C were much higher than the non-invasive cell lines, which is a stark contrast to YAP1 where Y234C was seen to have the highest active YAP1 level. It is also worth noting that R273C nuclear TAZ levels were almost an entire fold-change higher than WT; for YAP1, the R273C nuclear TAZ levels were similar to WT and were actually about half a fold-change lower than Y234C. Aligning with the literature, TAZ seems to have a more dominant role in governing triple negative breast cancer invasion. In previous reports, nuclear YAP1 did not seem to have any clinical relevance when analyzing highly invasive triple negative breast cancer tumors (the relevance was concentrated to HER2- breast cancers) (Kim, 2014). In contrast, TAZ is not only highly expressed in triple negative breast cancer in contrast to YAP1 (Chan, 2008), but it importantly is seen to have a higher active (nuclear) expression in triple negative breast cancer (Diaz-Martin, 2014). Overall, our findings demonstrate that active TAZ expression may lead to increased mutant-p53 mediated cell invasion.

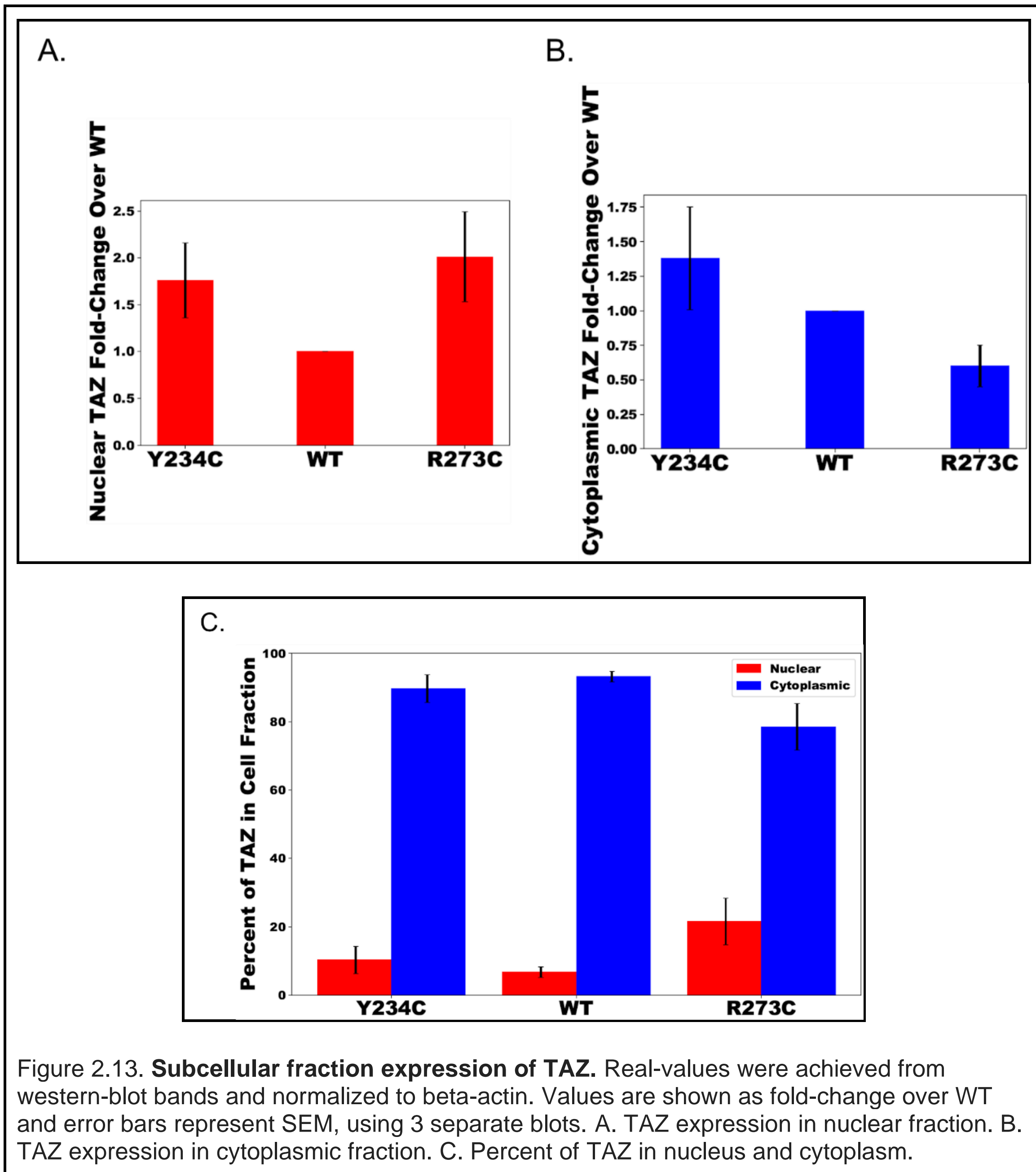


Figure 2.13. **Subcellular fraction expression of TAZ.** Real-values were achieved from western-blot bands and normalized to beta-actin. Values are shown as fold-change over WT and error bars represent SEM, using 3 separate blots. A. TAZ expression in nuclear fraction. B. TAZ expression in cytoplasmic fraction. C. Percent of TAZ in nucleus and cytoplasm.

Conclusion and summary of the results

TP53 is highly mutated in several human cancers, highlighting its prominent role as a tumor suppressor. While several tumor suppressor genes are often truncated or deleted in human

cancers, *TP53* is unique by frequently experiencing missense mutations in human cancers. The resulting full-length, though slightly altered p53 protein, often endows a cell with GOF properties, such as the hallmarks of cancer which actively contributes to cancer progression (Tan, 2015). One of these GOF properties is increased cell invasion which is the ability of cancer cells to navigate through the extracellular matrix and invade neighboring tissues. This allows tumor cells to move and metastasize elsewhere throughout the body. As p53 is deemed the guardian of the genome, it is no wonder that once mutated, the genome becomes dysregulated (Zhang, 2020). In this way, one of the dysregulated pathways in human cancers as a result of a mutant p53 has been the Hippo/YAP1/TAZ pathway (Ferraiuolo, 2017).

The YAP1 and TAZ proteins have been proposed as oncogenes because of their ability to induce the expression of cell survival and proliferation genes. Their dysregulation in breast cancers is well known. Overall YAP1 and TAZ expression and/or YAP1 and TAZ activity is increased in several cancers (Yu, 2015). This expression is often correlated with a poor prognosis. In regards to breast cancer, YAP1 majorly promotes adhesion and angiogenesis whereas TAZ is well known for promoting EMT, invasion, and breast cancer cell self-renewal (Huang, 2012). Additionally, TAZ is preferentially involved in the triple negative phenotype in contrast to YAP1 that is more involved in the luminal cancers (Guo, 2016).

Referencing our previously established RNA-seq data in regards of cell invasion, we selected 6 MCF-10A cell lines each harboring a different p53 protein; our selection created a range of invasion where the R273C and Y220C mutants were most invasive, R273H and WT-OE were moderately invasive, and WT and Y234C were non-invasive. Using these cell lines, we used a western blot approach to detect YAP1 and TAZ proteins in addition to phosphorylated YAP1 and TAZ proteins. We likewise divided our cells into the nuclear and cytoplasmic fractionations to determine YAP1 and TAZ protein levels in these subcellular compartments. Intriguingly, our TAZ data overall matched with the invasion trend, but YAP1 did not. Overall, active TAZ was higher in the most invasive cell lines and inactive TAZ was higher in the least invasive cell lines. For YAP1, inactive YAP1 was highest in the least invasive cell lines, matching the invasion trend and agreeing with previous reports (Diaz-Martin, 2014). However, active YAP1 was highest in the

least invasive Y234C and equal in WT and R273C, controversial to the invasion trend. This nonetheless highlights that TAZ holds more power in triple negative breast cancer than YAP1. Our results further demonstrate the phenotypic difference that mutant p53 proteins have on MCF-10A cells.

Our results match with those found in previous studies and are supported by clinical data. TAZ overall has shown to be elevated in several cancers, not only highlighting TAZ as a potential therapeutic, but also calling attention to its use as a prognosis indicator (Yuen, 2013 & Wang, 2010). In particular, TAZ is overexpressed in ~20% of breast cancer cases, the majority of these being high grade tumors which agrees with our TAZ results that suggest active TAZ is elevated in the more invasive cell lines. Moreover, active TAZ overexpression specifically has increased the invasive and migratory capabilities in MCF-10A cells, further validating our findings (Chan, 2008 & Pece, 2010). Additionally, in HER2+ breast cancers (a relatively aggressive and invasive breast cancer), patients with lower TAZ expression in tumors had a higher pathological complete response compared to patients with higher TAZ expression, implying that TAZ may be linked to cell invasion and response to therapeutics (Vici, 2014).

Impact of the Study

TP53 is mutated in the majority of TNBC cases and often results in heterogeneous phenotypes in breast cancer. As a very aggressive breast cancer, TNBC often has a poor clinical outcome and is typically resistant to many forms of therapeutics. Owing to this degree of complexity, TNBC has few viable therapeutic targets and even fewer successful therapeutic regimes. Therefore, a study such as ours reinforces TAZ's involvement in triple negative breast cancer and as an oncogene. Doing so provides new and supporting evidence for TAZ being a therapeutic target in TNBC.

This study is thorough because several previous reports only reference total protein level, negating the phosphorylation status of YAP1 or TAZ. As demonstrated with our study, protein

level may not tell the entire story. Our results however look at the overall picture by referencing total protein level, inactive protein level, and active protein level in 4 p53 mutants and 2 controls. In doing so, our data further highlights TAZ's important role in triple negative breast cancer cell invasion. Because our active TAZ trend matched with mutant p53 mediated cell invasion, this places TAZ on an axis with mutant p53, an idea that is relatively novel. More studies are needed to further elucidate how TAZ interacts with the mutant p53 network to induce tumor cell invasion.

Limitations of study

This study looks to model TNBC by using MCF-10A cells with a p53 mutation, but our model only contains these few genetic mutations. The rest of the genome is relatively stable which does not model actual tumor cells.

TAZ protein is notoriously an unstable protein with a short half-life. Our study attempted to account for this aspect by repeating experiments several times with different lysates, but it is worth noting that the quick degradation of TAZ protein may be affecting results.

This study only looked at the canonical YAP1 and TAZ roles. In reality, a dysregulation of the Hippo/YAP1/TAZ pathway would likely affect every role of YAP1 and TAZ, not just the canonical roles.

Future Directions

Validating TAZ as a driver in cell invasion

Our study indicates that TAZ may be a driver in p53-mediate cell invasion. To validate these findings, the knock-down of TAZ ought to confirm whether the cell invasion is truly mediated via TAZ. We hypothesize that any knock-down of TAZ ought to reverse any increased cell invasion seen in the p53-mutant cell lines.

Analyzing YAP1 and TAZ enzymatic activity

The active TAZ and YAP1 levels can describe dysregulated intracellular processes in the p53 mutant cell lines. The actual result from active YAP1 and TAZ (activating TEAD-transcription factors), however, is most crucial in trying to understand how a cell is driving its invasion. A measure of TEAD activity and YAP1/TAZ gene signature expression will further validate our findings.

In vivo and clinical analysis of YAP1 and TAZ

Our study is strictly in vitro. A future step is to validate our findings in mouse models along with finding additional co-drivers to cell invasion. Additionally, YAP1 and TAZ IHC analysis of breast tumors will further elucidate the Hippo/YAP1/TAZ pathway involvement in breast cancer as it relates to a clinical setting.

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