LOSS OF PRP4K IS LINKED TO ANOIKIS RESISTANCE AND PARTIAL EPITHELIAL-TO-MESENCHYMAL TRANSITION IN BREAST AND OVARIAN CANCER

by

Livia Clarke

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Abstract

Metastatic disease is the primary cause of cancer-related death, and as a result the metastatic potential of a tumor is an important indicator of patient survival. For tumor cells to metastasize, they must overcome a special kind of programmed cell death known as anoikis, which occurs when cells detach from the extracellular matrix (ECM). Our lab has recently demonstrated that depletion of PRP4K leads to anoikis resistance due to sustained pro-survival signaling. In this thesis, I further characterized pathways promoting anoikis resistance in PRP4K depleted cells. I determined that PRP4K loss could trigger increased expression of the kinase TrkB as well as activation of the PI3K signaling pathway. I also determined that loss of PRP4K could induce partial epithelial-to-mesenchymal transition (EMT). Specifically, I determined that depletion of PRP4K in several cell lines did not alter the expression of epithelial markers such as E-cadherin yet correlated with increased levels of mesenchymal markers such as vimentin, fibronectin and the EMT transcription factor Zeb1. This partial EMT phenotype was associated with altered cell migration in non-transformed mammary epithelial cell lines, and depletion of PRP4K promoted increased migration in the triple negative breast cancer cell line MDA-MB-231. On the other hand, induction of EMT in non-transformed mammary cell lines had different effects on PRP4K expression depending on the method of EMT induction. While some EMT induction methods had no effect on PRP4K protein expression, others resulted in reduced PRP4K expression. One difference between these induction methods was whether YAP signaling was activated, with activation of YAP gene targets being associated with reduced PRP4K expression. This data together supports a role for PRP4K as a tumor suppressor, where PRP4K loss promotes anoikis resistance and correlates with both EMT and increased YAP activity, which in turn increases the metastatic potential of cancer cells.

List of Abbreviations Used

AGO – argonaute

Akt – protein kinase B

Alk – anaplastic lymphoma kinase

AMPK – AMP-activated protein kinase

ANOVA – analysis of variance

ANXA4 – annexin A4

Apaf – apoptosis protease activating factor

APC/C – anaphase-promoting complex

ARID1A – AT-rich interaction domain 1A

AS – alternative splicing

ATM – ataxia telangiectasia mutated

ATP – adenosine triphosphate

Bak – BCL-2 antagonist/killer

Bax – BCL-2-associated X protein

BARD1 – BRCA1 associated RING domain 1

Bcl-2 – B-cell lymphoma 2

Bcl-xl – B-cell lymphoma-extra large

BDNF – brain-derived neurotrophic factor

BH – BCL-2 homology

Bid – BH3 interacting domain death agonist

Bim – BCL-2-like protein 11

BL1 – basal-like subtype 1

BL2 – basal-like subtype 2

BRCA1 – breast cancer type 1 susceptibility gene

BRCA2 – breast cancer type 2 susceptibility gene

BRG1 – SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 4

BSA – bovine serum albumin

CA125 – cancer antigen 25

CAF – cancer-associated fibroblast

CD24 – cluster of differentiation 24

CD44 – cluster of differentiation 44

CDC20 – activator of E3 ubiquitin ligase anaphase-promoting complex

cDNA – complementary DNA

CHEK2 – checkpoint kinase 2

CREB – cAMP response element binding protein

CRISPRi – clustered regulatory interspaced short palindromic repeats interference

CSC – cancer stem cell

CtBP1 – C-terminal binding protein 1

CTGF – connective tissue growth factor

CTNNB1 – catenin beta 1

DAPI – 4’,6-diamidino-2-phenylindole

DGCR8 – DiGeorge syndrome critical region gene 8

DISC – death-inducing signaling complex

Dkk-1 – dickkopf-related protein 1

DMEM – Dulbecco’s Modified Eagle Media

DNA – deoxyribonucleic acid

E-cad – E-cadherin

ECM – extracellular matrix

EDTA – ethylenediaminetetraacetic acid

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

EGTA – ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid

EIF3E – eukaryotic translation initiation factor 3 subunit E

ELK1 – ETS domain-containing protein

EMT – epithelial-to-mesenchymal transition

EMT-TF – epithelial-to-mesenchymal transition transcription factor

ER – estrogen receptor

ERBB3 – Erb-B2 receptor tyrosine kinase 3

ERBB4 – Erb-B2 receptor tyrosine kinase 4

Erlin1 – ER lipid raft associated protein 1

ESRP – epithelial splicing regulatory protein

F12 – Ham's F-12 Nutrient Mixture

FAK – focal adhesion kinase

Fas – TNF receptor superfamily, member 6

FBS – fetal bovine serum

FDA – Food and Drug Administration

FGFR2 – fibroblast growth factor receptor 2

FLNB – filamin B

FOXC1 – forkhead box C1

FOXO1 – forkhead box O1

GSK-3Beta – glycogen synthase kinase 3 beta

HE4 – human epididymis protein 4

HER – human epidermal growth factor receptor

HDAC – histone deacetylase

HGH – human growth hormone

IL – interleukin

IM – immunomodulatory subtype

KD – knock-down

Ki-67 – antigen Ki-67

KKHK box – lysine-histidine rich subdomain

KLF4 – kruppel-like factor 4

KLF13 – kruppel-like factor 13

LAR – luminal androgen receptor subtype

LATS1/2 – large tumor suppressor kinase 1/2

LEF – lymphoid enhancer factor

LKB1 – large kinase B1

M – mesenchymal subtype

MAD1 – mitotic arrest deficient protein 1

MAD2 – mitotic arrest deficient protein 2

MAPK – mitogen-activated protein kinase

Met – tyrosine protein kinase Met

MET – mesenchymal-to-epithelial transition

miRISC – miRNA-induced silencing complex

miRNA – microRNA

MOB1 – MPS one binder kinase activator-like 1

MMP – matrix metalloproteinase

MPS1 – monopolar spindle 1

MRE – miRNA response element

mRNA – messenger RNA

MSL – mesenchymal stem-like subtype

MST1/2 – mammalian STE20-like protein kinase 1/2

Myc – myc proto-oncogene

NADPH – reduced nicotinamide adenine dinucleotide phosphate

N-cad – N-cadherin

NCBI – National Center for Biotechnology Information

N-CoR – nuclear receptor corepressor complex

NF1 – neurofibromin 1

NF2 – neurofibromin-2 (merlin)

NF-kB – nuclear factor kB

NT-4/5 – neurotrophin-4/5

OE – over-expression

OMM – outer mitochondrial membrane

P-Akt – phospho- Akt

PALB2 – partner and localizer of BRCA2

PAM50 – Prosigna Breast Cancer Prognostic Gene Signature Assay

PARP – Poly (ADP-ribose) polymerase

P-bodies – processing bodies

PBS – phosphate buffered saline

PCK – pan-cytokeratin

PDCD4 – programmed cell death 4

P-gP – P-glycoprotein

PI3K – phosphoinositide 3 kinase

PIK3CA – phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha

Poly-HEMA – poly 2-hydroxyethyl methacrylate

PR – progesterone receptor

PRP31 – pre-mRNA processing factor 31

PRP4K – pre-mRNA splicing factor 4 kinase

PRP6 – pre-mRNA processing factor 6

P/S – penicillin/streptomycin

PTEN – phosphatase and tensin homolog

QK1 – protein quaking

qPCR – quantitative PCR

RAB7 – ras-related protein 7

RAD51 – RAD51 recombinase

RB1 – retinoblastoma susceptibility gene

RBFOX1 – RNA binding protein fox-1 homolog 1

RECK – reversion-inducing cysteine-rich protein with Kazal motifs

RhoB – ras homolog gene family, member B

RNA – ribonucleic acid

ROMA – risk of malignancy algorithm

ROS – reactive oxygen species

RS domain – arginine- serine rich subdomain

RTK – receptor tyrosine kinase

RT – reverse transcription

RT-qPCR – quantitative reverse transcription polymerase chain reaction

SAC – spindle assembly checkpoint

SAV1 – protein Salvador homolog 1

SCRIB – Scribble

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

sFRP-1 – secreted frizzled related protein 1

shRNA – short hairpin RNA

siRNA – small interfering RNA

snRNP – small nuclear ribonucleoprotein

Sox2 – SRY (sex determining region Y)-Box 2

SR protein – serine and arginine-rich protein

SRSF1 – serine and arginine rich splicing factor 1

SWAP – suppressor-of-white apricot

TAM – tumor-associated macrophage

TAN – tumor-associated neutrophil

TAZ – transcriptional co-activator with PDZ-binding motif

TBST – tris buffered saline with Tween 20

TCF – T cell factor

TCGA – The Cancer Genome Atlas

TEAD – transcriptional enhancer factor TEF

TF – transcription factor

TGF-Beta – transforming growth factor beta

TIMP3 – tissue inhibitor of metalloproteinase 3

TNBC – triple negative breast cancer

TNFR – tumor necrosis factor receptor

TP53 – tumor protein p53

TPM1 – tropomyosin 1

TRAIL – TNF-related apoptosis inducing ligand

TrkA – tropomyosin receptor kinase A

TrKB – tropomyosin receptor kinase B

TrkC – tropomyosin receptor kinase C

TVS – transvaginal ultrasonography

TWIST1 – twist family BHLH transcription factor 1

UTR – untranslated region

VEGF – vascular endothelial growth factor

VEGF-A – vascular endothelial growth factor A

Vim – vimentin

Wnt-5a – wnt family member 5A

XPO5 – exportin-5

YAP – yes associated protein

Zeb – zinc finger E-box homeobox

Zo1 – zonula occludens protein 1

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Chapter 1: Introduction

1.1 Preface

Pre-mRNA Splicing Factor 4 Kinase (PRP4K) is a dual-specificity kinase with roles in pre-mRNA splicing, transcriptional regulation, the spindle assembly checkpoint, Hippo signaling and anoikis resistance. As well, PRP4K has been determined to be a biomarker of taxane response.

In both breast and ovarian cancers, low PRP4K expression is associated with worse overall survival in comparison to high PRP4K expression (Corkery, et al., 2018; Cho et al., 2018). In my thesis work, I wanted to determine why low PRP4K is linked to a worse prognosis. In order to approach this question, I used various breast and ovarian cancer models. For breast cancer, I used the Luminal A breast cancer cell line, MCF7, because of its previous use in the lab as well as the triple negative breast cancer cell line, MDA-MB-231, because it is the most aggressive subtype of breast cancer. I also used two non-tumorigenic breast epithelial cell lines, HMLE and MCF10A. For an ovarian cancer model, I used the ID8 syngeneic high-grade serous mouse model.

Overall, in my thesis studies I wanted to answer three questions related to the effects of low PRP4K expression. First, I wanted to determine what pathways play a role in the anoikis resistance phenotype associated with PRP4K loss. Next, I wanted to explore the relationship between PRP4K loss, epithelial-to-mesenchymal transition (EMT) and migratory potential. Finally, I wanted to examine the connection between the induction of EMT and PRP4K protein expression.

1.2 Breast and Ovarian Cancer

1.2.1 Breast Cancer

Breast cancer is the third most frequently diagnosed cancer in Canada, accounting for 13% of all cancers (Canadian Cancer Statistics, 2018). In 2017, it was estimated that 1 in 8 Canadian women will develop breast cancer in their lifetime, and 1 in 31 women will pass away from the disease (Public Health Agency of Canada, 2017). Breast cancer is a heterogeneous disease that can be classified into different molecular subtypes with distinct prognoses. For example, actively dividing cells in a tumor are an important marker of cancer prognosis and treatment response. In breast cancer the nucleolar protein antigen Ki-67 is often used to determine the index of proliferating cells in a tumor and to predict responses to neoadjuvant chemotherapy (Inwald, et al., 2013). Beyond markers for cellular proliferation, Perou et al. (Perou, et al., 2000) reported the presence of molecular subtypes in breast cancer that showed differences in the expression of important molecular markers, in aggressiveness (Sorlie, et al., 2001 & Sorlie, et al., 2003) and in response to a specific chemotherapy regimen (Rouzier, et al., 2005). These molecular subtypes are based largely on gene expression profiles linked to the expression of the estrogen and progesterone hormone receptors (ER and PR, respectively) and the human epidermal growth factor receptor 2 (HER2). Each subtype is associated with a different prognosis and clinical outcome.

The Prosigna Breast Cancer Prognostic Gene Signature Assay (PAM50) is a qRT-PCR intrinsic subtyping classifier that was developed by analyzing 189 breast tumor samples. PAM50 measures the expression of 50 genes in a breast cancer tumor to classify the tumor into one of the four molecular subtypes. In addition to subtype classification, PAM50 also provides quantitative values for proliferation, luminal gene expression, and

ER, PR and HER2 expression. The PAM50 test has shown to be a better predictor of patient outcome than other classification methods (Sweeney, et al., 2014).

The different molecular breast cancer subtypes include: luminal A, luminal B, HER2 enriched, normal-like and triple-negative (Dai., et al. 2015), which I discuss in detail below and are summarized in Figure 1.1.

1.2.1.1 Luminal

Luminal tumors are the most common subtype and express the hormone receptors ER and PR. Luminal A tumors are ER+ and PR+, HER2- and express low levels of the tumor proliferation marker Ki-67. Luminal B tumors are ER+ and PR+, but have lower expression of ER. Luminal B tumors are also HER2+ and express high levels of Ki-67 (Dai, et al., 2015). Both luminal A and B tumors have fairly good prognosis, with a 5-year survival rate of 92-96% and 85-92%, respectively (Minnicozzi, 2013). Luminal A and B tumors also respond well to hormone therapies due to the presence of the estrogen receptor. (Dai., et al. 2015). The most common adjuvant hormone therapies for the luminal subtype include the anti-estrogen drug, tamoxifen for both pre- and post-menopausal women and aromatase inhibitors for post-menopausal women (Williams & Harris 2014).

1.2.1.2 HER2-enriched

Like the name suggests, HER2-enriched tumors over-express HER2 and are ER- and PR-. These cancers tend to grow faster than luminal cancers and have a worse prognosis, with a 5-year survival rate of 82-92%, but can be treated with therapies targeting the HER2 receptor. (Minnicozzi, 2013; Dai, et al. 2015). Commonly used HER2

targeted therapy drugs are antibodies that work by attaching to HER2 proteins to prevent the growth of HER2+ breast cancer cells (Canadian Cancer Society). These drugs include Trastuzumab (Herceptin), Pertuzumab (Perjeta) and Trastuzumab emtansine (Kadcyla or T-DM1).

1.2.1.3 Normal-like

Normal-like cancers have gene expression profiles similar to normal breast tissue (Russnes, et al., 2017) and are ER+, PR+ and HER2-. These cancers are similar by histology to luminal A tumors, but exhibit Ki-67 gene expression and high expression of basal epithelial cell-associated genes (Perou, et al., 2000). This subtype is the rarest breast cancer subtype and makes up approximately 8% of lymph node negative breast cancers in one study (Smid, et al., 2008), and have a fairly good prognosis (Dai, et al. 2015).

1.2.1.4 Triple-negative

Triple negative breast cancers (TNBC) have no or little expression of hormone receptors (ER and PR) and HER2, account for 10-20% of breast cancers and tend to occur more often in younger women and in women of African and Hispanic descent (Breastcancer.org & Liu, 2018). TNBC tumors are normally larger, of higher grade, are more aggressive, have a higher risk of relapse, and have the worst prognosis of all the breast cancer subtypes, with a 5-year relative survival rate of 70-80% (Lehmann, 2011; Minnicozzi, 2013). Because of the lack of receptor expression, TNBC cannot be treated with targeted therapies and chemotherapy remains the only treatment option. Response to chemotherapy in TNBC patients is extremely variable mainly because it is such a heterogeneous disease. The most common mutated genes in TNBC are tumor protein p53

(TP53) and phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha (PIK3CA), but many other less common mutations exist, such as: phosphatase and tensin homolog (PTEN), retinoblastoma susceptibility gene (RB1), neurofibromin 1 (NF1), breast cancer type 1 susceptibility gene (BRCA1), breast cancer type 2 susceptibility gene (BRCA2), Erb-B2 receptor tyrosine kinase 3 (ERBB3), Erb-B2 receptor tyrosine kinase 4 (ERBB4), anaplastic lymphoma kinase (ALK), tyrosine protein kinase Met (Met), Myc proto-oncogene (Myc), yes associated protein 1 (YAP1), epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor 2 (FGFR2) (Pareja, 2016; Hiu, 2017). Using 587 TNBC gene expression profiles from 3247 breast cancer patients, Lehmann and colleagues identified 6 TNBC subtypes, each with a unique gene expression signature. The 6 identified subtypes include: two basal-like subtypes (BL1 and BL2), an immunomodulatory subtype (IM), a mesenchymal subtype (M), a mesenchymal stem-like subtype (MSL) and a luminal androgen receptor subtype (LAR). They found that each subtype had a differential response to chemotherapy and targeted therapies (Lehmann, 2011). Because TNBC has such limited treatment options resulting in poor prognosis there has been a major effort in research in this area (Dai, et al., 2015). In my thesis studies, I have primarily focused on luminal A and TNBC cell models.

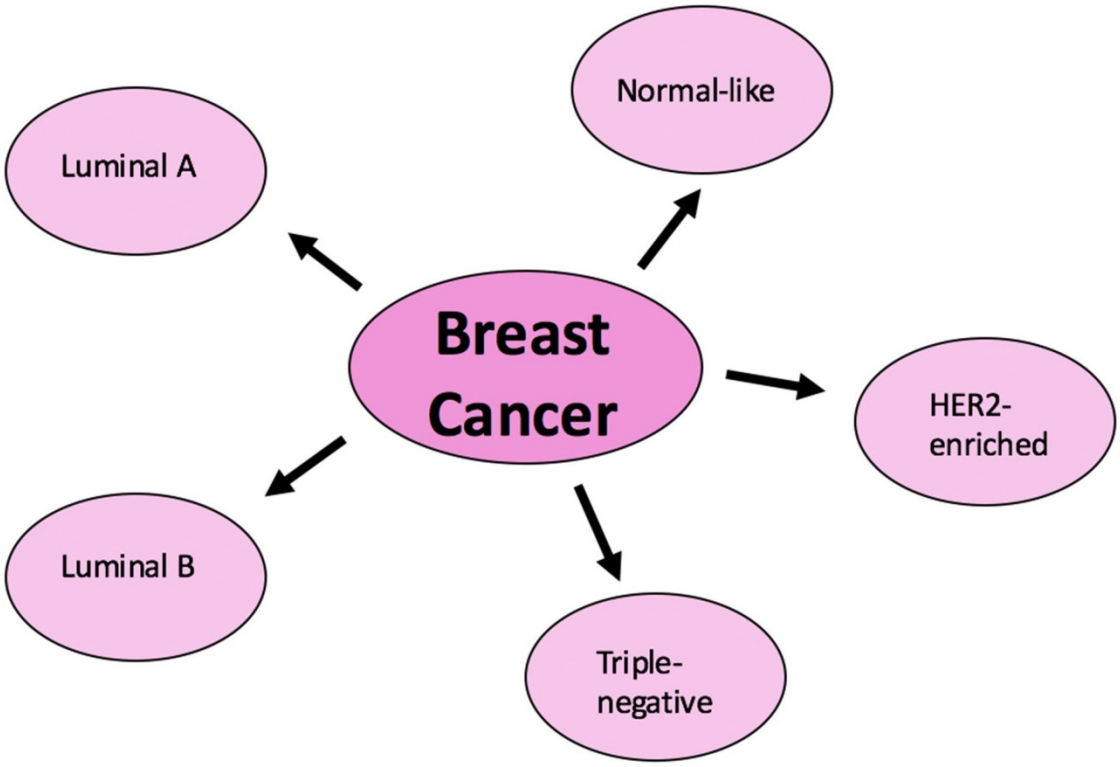

Figure 1.1: Breast cancer classification. Breast cancer is classified into different molecular subtypes, which include Luminal A, Luminal B, triple negative, Her2 enriched, and Normal-like. The various subtypes are distinguished by the expression of the estrogen and progesterone hormone receptors (ER and PR, respectively) and the human epidermal growth factor receptor 2 (HER2). Each subtype is associated with a different prognosis and clinical outcome.

1.2.1.5 BRCA1 and 2 Mutations

Inheriting specific genetic mutations can greatly increase an individual's chances of developing cancer. In terms of both breast and ovarian cancer, BRCA1 and BRCA2 gene mutations are the most well-known heritable genetic mutations. BRCA1 and BRCA2 are tumor suppressor genes that play roles in diverse cellular pathways such as DNA damage repair, cell-cycle arrest, apoptosis, genetic instability, transcriptional activation, and tumorigenesis (Yoshida, & Miki, 2004). Mutations in BRCA1 and BRCA2 predispose women to develop breast and ovarian cancer and to develop it at a younger age (Paul & Paul, 2015). Studies have shown that the lifetime risk of breast cancer for women with a BRCA1 or BRCA2 mutation is 45-80% (King, et al., 2003 & Antoniou, et al., 2003). For ovarian cancer, the lifetime risk is 45-60% and 11-35% for BRCA1 and BRCA2 mutation carriers, respectively (King et al., 2003; Antoniou, et al., 2003; van der Kolk, et al., 2010).

Since BRCA1/2 mutations affect DNA damage repair pathways, cancer cells with these mutations are more susceptible to DNA damaging agents, such as cisplatin and carboplatin. As well, inhibitors of Poly (ADP-ribose) polymerase (PARP), an enzyme involved in base excision repair and the repair of single-stranded breaks are also options in the treatment of breast and ovarian cancers with mutated BRCA1/2 (Paul & Paul, 2015).

1.2.2 Ovarian Cancer

Although ovarian cancer is only the ninth most commonly diagnosed cancer in Canadian women, it has the highest mortality rate of all cancers of the female reproductive system and the worst survival in comparison to all other cancers

(Navaneelan, 2015). Over the last 3 decades, the 5-year survival rate of ovarian cancer has increased from 38% to 46% due to improvements in cytoreductive surgery and chemotherapy treatments (Siegel, Miller, Jemal, 2016), but the cure rate of the disease is still only 30%. The reason for the low cure rate is because 75-80% of patients are diagnosed with advanced stage disease (Stage III or IV) because of the lack of early detection methods. If more patients were diagnosed at an earlier stage (Stage I or II), 70-90% of patients could be cured with existing treatment methods (Yang, et al., 2018).

Ovarian cancer is classified in terms of the ovarian structure the tumor originated from. The three main categories are surface epithelial-stromal tumors, sex cord-stromal tumors and germ cell tumors, which account for about 90%, 7% and 5% of ovarian malignant tumors, respectively. Each category is then further divided into several sub-categories (Chen et al., 2003). Malignant ovarian cancer within the surface epithelial-stromal group is mainly comprised of five histotypes: high-grade serous (70%), endometrioid (10%), clear cell (10%), mucinous (3%), and low-grade serous (<5%) (Reid, et al., 2017) (Figure 1.2). Other studies have separated epithelial ovarian cancer into two distinct groups named type I and type II carcinomas, that differ in genomic variation and prognosis. In general, type I carcinomas develop more slowly and have a fairly stable genomic profile while type II carcinomas tend to be more aggressive (Kurman & Shih, 2016). The two ovarian cancer classification methods are summarized in Figure 1.2.

1.2.2.1 Diagnosis and Treatment of Ovarian Cancer

In ovarian cancer diagnosis, pelvic exams, transvaginal ultrasonography (TVS), abdominal ultrasonography, and exploratory or diagnostic laprosopy can be performed

when assessing a pelvic mass (Doubeni, et al., 2016). The biomarker, cancer antigen 25 (CA125) is often used to predict malignancy as well as response to treatment. CA125 is a heavily glycosylated transmembrane mucin and is overexpressed in approximately 80% of ovarian cancers (Yin & Lloyd, 2001). It can be detected within bodily fluids using double determinant immunoassays (Bast et al., 2005). High levels of CA125 could be indicative of malignant disease, poor response to treatment and residual disease (Yang, et al., 2017). The human epididymis protein 4 (HE4) is another protein whose expression is increased in most ovarian cancers (Chudecka-Glaz et al., 2012). Studies have shown that HE4 can predict recurrent disease earlier than CA125 in some patients and even in patients who do not express CA125 (Yang, et al., 2017). Moore and colleagues developed the risk of malignancy algorithm (ROMA), which evaluates CA125, HE4 and menopausal status, and was approved by the FDA in 2011 to distinguish malignant and benign masses. ROMA showed 95% sensitivity and 75% specificity in malignant ovarian cancer determination (Moore et al., 2009).

Currently, the standard treatment for patients with advanced ovarian cancer involves cytoreductive surgery and platinum-based chemotherapy (Ledermann, et al., 2016). Patients tend to respond well to the initial chemotherapy treatment, but many patients then develop recurrent disease that is resistant to chemotherapy (Yang et al., 2017). Ultimately, ovarian cancer is a heterogeneous disease and more research is needed to develop treatment options that are more individualized to the particular patient and ovarian cancer type.

Ovarian cancer is characterized by a wide variety of gene mutations that could be taken advantage of in terms of treatment. In fact, more than one-fifth of ovarian cancers are hereditary (Toss, et al., 2015). The main hereditary ovarian cancer mutations involve

BRCA 1/2, which were discussed in detail in section 1.2.1.5 and could be treated using PARP inhibitors. Other hereditary ovarian cancers include mutations in TP53, BRCA1 associated RING domain 1 (BARD1), checkpoint kinase 2 (CHEK2), RAD51 recombinase (RAD51) and partner and localizer of BRCA2 (PALB2) (Toss, et al., 2015). High-grade serous ovarian cancers have a high rate of TP53 mutations, making TP53 a potential therapeutic target in this cancer type. Type I epithelial ovarian cancers have a high rate of KRAS and BRAF mutations; therefore, targeting the MAP kinase pathway may be beneficial in this type of ovarian cancer (The Cancer Genome Atlas Research Network, 2011). Furthermore, the VEGF pathway is thought to be a potential therapeutic target as increased expression of VEGF is associated with poor prognosis and resistance to chemotherapy treatments (Rojas et al., 2016). The anti-VEGF-A antibody, bevacizumab, in addition to chemotherapy treatment, was found to increase progression-free survival (PFS) when compared to chemotherapy on its own in patients with recurrent ovarian cancer (Aghajanian, et al., 2012). In order to increase cure rates in patients with ovarian cancer, research has focused on developing better methods to identify the cancer at earlier stages and to not treat each patient's cancer the same, when in reality, ovarian cancer is such a heterogeneous disease that it requires new biomarkers for patient stratification (Kamal, et al., 2018; Montagnana, et al., 2017).

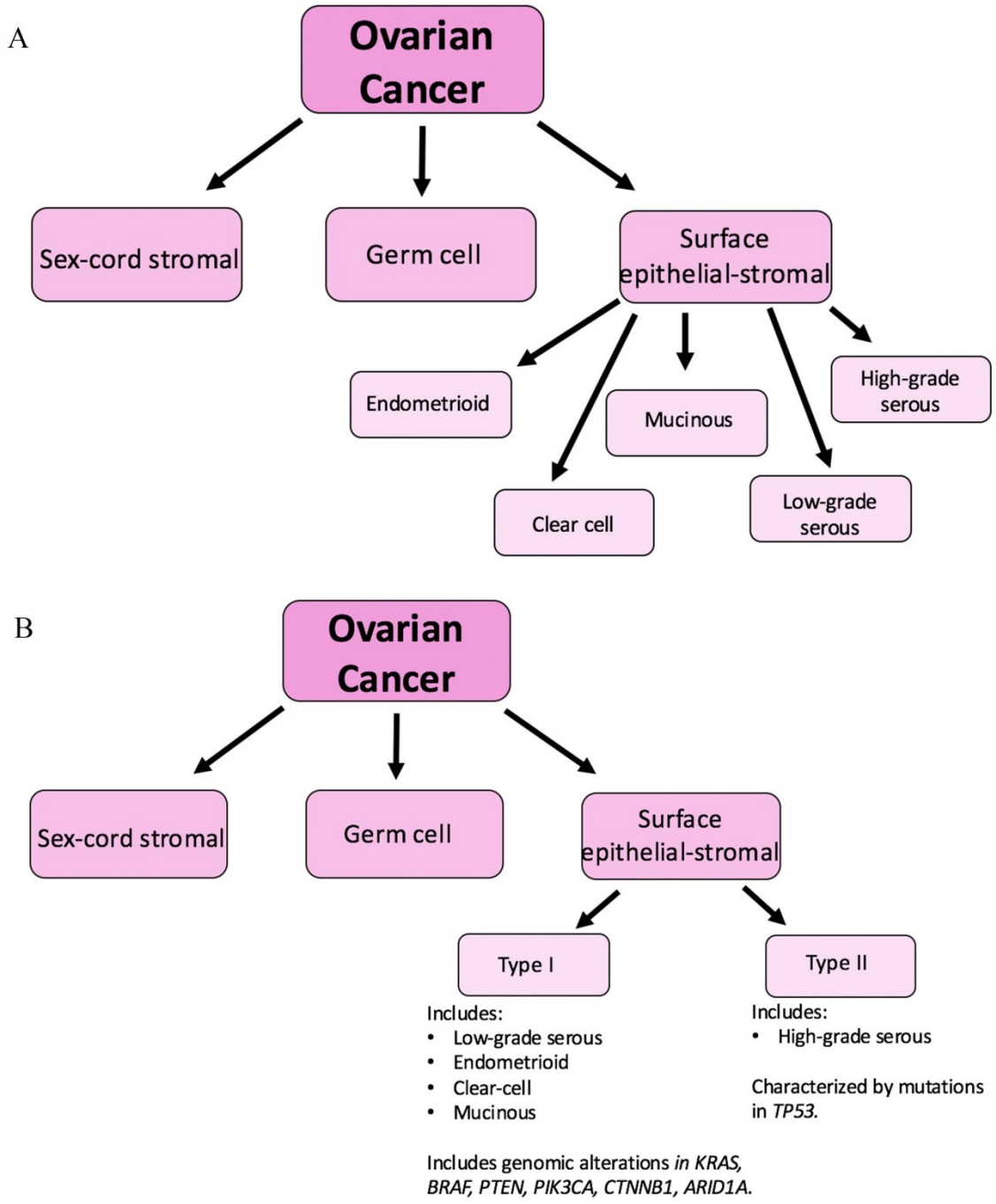

Figure 1.2: Ovarian cancer classification. Ovarian cancer is classified in terms of the ovarian structure the tumor originated from. The three main categories are surface epithelial-stromal tumors, sex cord-stromal tumors and germ cell tumors and each category is further divided into sub-categories. Surface-epithelial-stromal tumors can be classified by two different methods.

1.3 PRP4K- Pre-mRNA Splicing Factor 4 Kinase

1.3.1 Protein Structure

Pre-mRNA splicing factor 4 kinase (PRP4K) is a 150 kD kinase composed of 1007 amino acids with an extended N-terminus with conserved subdomains rich in lysine-histidine (KKHK box) and arginine-serine (RS domain). Both the KKHK box and RS domain are also found in known splicing-associated proteins. PRP4K is a dual-specificity kinase and also contains two evolutionary conserved sequence motifs, MI (DDMFA) and MII (DNWTDAEGYYRV) that are likely involved in substrate recognition (Dellaire et al., 2002). Figure 1.3 depicts the protein structure of PRP4K.

Figure 1.3: PRP4K protein structure. Protein structure of human PRP4K. Adapted from Dellaire, et al., 2002.

1.4 Functions of PRP4K

PRP4K is an essential kinase that is highly conserved between species and is functionally pleiotropic. The many functions attributed to PRP4K are described below and summarized in Figure 1.4.

1.4.1 The Role of PRP4K in Pre-mRNA Splicing and Transcriptional Regulation

PRP4K is localized mainly to the nucleus (excluded from the nucleolus) within splicing speckles and plays a role in the regulation of spliceosomal assembly. More specifically, PRP4K is involved in the phosphorylation of pre-mRNA processing factor 6 (PRP6) and pre-mRNA processing factor 31 (PRP31), which is necessary for tri-small nuclear ribonucleoproteins (snRNP) integration during complex B formation (Dellaire, et al., 2002; Schneider et al., 2010). In addition, PRP4K is found to co-purify with the U5 small nuclear ribonucleic protein (snRNP) and it interacts and phosphorylates the serine and arginine-rich protein (SR protein), serine and arginine rich splicing factor 1 (SRSF1), which is essential in 5' splice site selection during splicing and alternative splicing (Schneider et al, 2010; Dellaire et al, 2002). In addition to several other proteins involved in pre-mRNA splicing such as suppressor-of-white apricot (SWAP) and Pinin, PRP4K also interacts with proteins involved in chromatin remodeling and gene regulation. These include transcription factors KLF13 (kruppel-like factor 13) (Huang et al., 2007) and ELK1 (ETS domain-containing protein) (Huang et al., 2000), BRG1 (chromatin ATPase SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 4) and N-CoR (transcriptional repressor nuclear receptor corepressor complex), which together suggest PRP4K may play a role in co-transcriptional pre-mRNA splicing (Dellaire et al, 2002).

1.4.2 The Role of PRP4K in the Spindle Assembly Checkpoint (SAC)

The spindle assembly checkpoint works to delay mitotic exit until all chromosomes are correctly attached to the mitotic spindle (Lara-Gonzalez, et al., 2012). The checkpoint is activated in response to unattached kinetochores. This results in the inhibition of CDC20 (activator of E3 ubiquitin ligase anaphase-promoting complex (APC/C)) and therefore the prevention of APC/C activation. Because APC/C promotes the transition from metaphase to anaphase, this leads to a delay in mitotic exit. When kinetochores are attached to microtubules correctly, the SAC is turned off, which allows the activation of APC/C. APC/C works to ubiquitinate mitotic proteins to be degraded leading to mitotic exit. This checkpoint requires the recruitment of several proteins in order to function properly. When the checkpoint is not functioning properly, chromosome misseggregation occurs in the cell (Lara-Gonzalez, et al., 2012).

PRP4K appears to play a role in the recruitment of checkpoint proteins (monopolar spindle 1: MPS1, mitotic arrest deficient protein 1: MAD1, mitotic arrest dificent protein 2: MAD2) to the kinetochore following SAC activation (Montembault, et al., 2007). When PRP4K is depleted, these checkpoint proteins fail to be recruited to the kinetochore and cells exhibit chromosome segregation defects. Furthermore, when cells are treated with the microtubule depolymerizing agent nocodazole to trigger the SAC, the SAC is over-ridden by depletion of PRP4K allowing cells to divide in the presence of this compound (Montembault, et al., 2007).

1.4.3 PRP4K as a Biomarker of Taxane Response in Breast and Ovarian Cancer

Taxanes are microtubule-stabilizing agents that bind the β-tubulin subunit of microtubules, preventing their depolymerization and ultimately inhibiting cell division (Weaver, 2014). Taxanes are commonly used to treat both breast and ovarian cancer and so the emergence of taxane resistance has been a major problem in the treatment of these cancers. Cancer cells can develop taxane resistance through several mechanisms. Some of these mechanisms include the up-regulation of the ATP-binding cassette transporter, P-glycoprotein (P-gP), mutations and modifications to microtubules and tubulin, as well as through the alteration of the SAC (Orr, 2003).

The Dellaire laboratory has identified PRP4K as a HER-regulated modulator of taxane sensitivity (Corkery, et al., 2015). Specifically, loss of PRP4K expression results in a decreased sensitivity of cancer cells to the taxane paclitaxel. When normal cells are treated with taxanes like paclitaxel, they undergo mitotic arrest through SAC activation and then apoptosis. However, when PRP4K is knocked-down (KD), instead of undergoing mitotic arrest, these cells undergo mitotic slippage and re-enter the cell cycle without dividing. Therefore, the reason for the decreased taxane sensitivity of PRP4K KD cells is likely due to the impairment of the SAC in these cells (Corkery, et al., 2015); a result consistent with the previous findings of Montembault et al., 2007.

1.4.4 PRP4K and YAP Signaling

In a recently published paper, Cho and colleagues (Cho, et al., 2018) demonstrated that PRP4K plays a role in the Hippo signaling pathway (Figure 1.11), which is involved in organ growth control, stem cell function, regeneration and tumor suppression (Sharif,

et al., 2015). The Hippo pathway negatively regulates YAP to prevent its activation because increased cellular YAP activation promotes cell proliferation, inhibits cell death and is associated with high-grade cancers (Johnson, et al., 2013; Zanconato, et al., 2016). The roles of YAP are summarized in Figure 1.12. Cho et al., found that PRP4K phosphorylates YAP, inhibiting its binding to the transcription factor TEAD (transcriptional enhancer factor TEF), which prevents its nuclear accumulation and activation. Therefore, the downregulation of PRP4K causes increased YAP activation and the activation of its downstream target genes, many of which are involved in pathways that are associated with cancer development (Cho, et al., 2018). A more thorough discussion of Hippo/YAP signaling will occur in section 1.10.

1.4.5 PRP4K and its Role in Anoikis Resistance

Using a zebrafish xenotransplantation model, Corkery et al., (2018) determined that ID8 mouse ovarian carcinoma cells were able to proliferate more robustly *in vivo* when PRP4K expression was knocked down (KD). In these experiments, cancer cells were injected into the yolk sac of the zebrafish, which provided a suspended environment lacking ECM contacts. Growth in such an environment would require cells to grow in "detached conditions" that would otherwise trigger cell death in epithelial cells through a process known as anoikis (Paoli, et al., 2016). Thus, we interpreted these results to indicate that loss of PRP4K promotes anoikis resistance *in vivo*, which was subsequently confirmed *in vitro* by growing cells in detached conditions. A clue as to how loss of PRP4K contributed to anoikis resistance came from our observation that in addition to localizing to splicing speckles in the nucleus, a small proportion of PRP4K localized to regions within the cytoplasm (Corkery et al., 2018). More specifically, PRP4K localized

to endosomes containing ras-related protein 7 (Rab7), a late endosome marker (Feng, et al., 1995). These data led us to examine the endosomal trafficking and degradation of the EGF receptor, and we found that loss of PRP4K promoted EGFR stability leading to activation of pro-survival signaling through the kinase Akt (Corkery et al., 2018). In this thesis, I will present additional details on the role of PRP4K loss in promoting anoikis resistance, and in the next section I will discuss mechanisms of cell death in general.

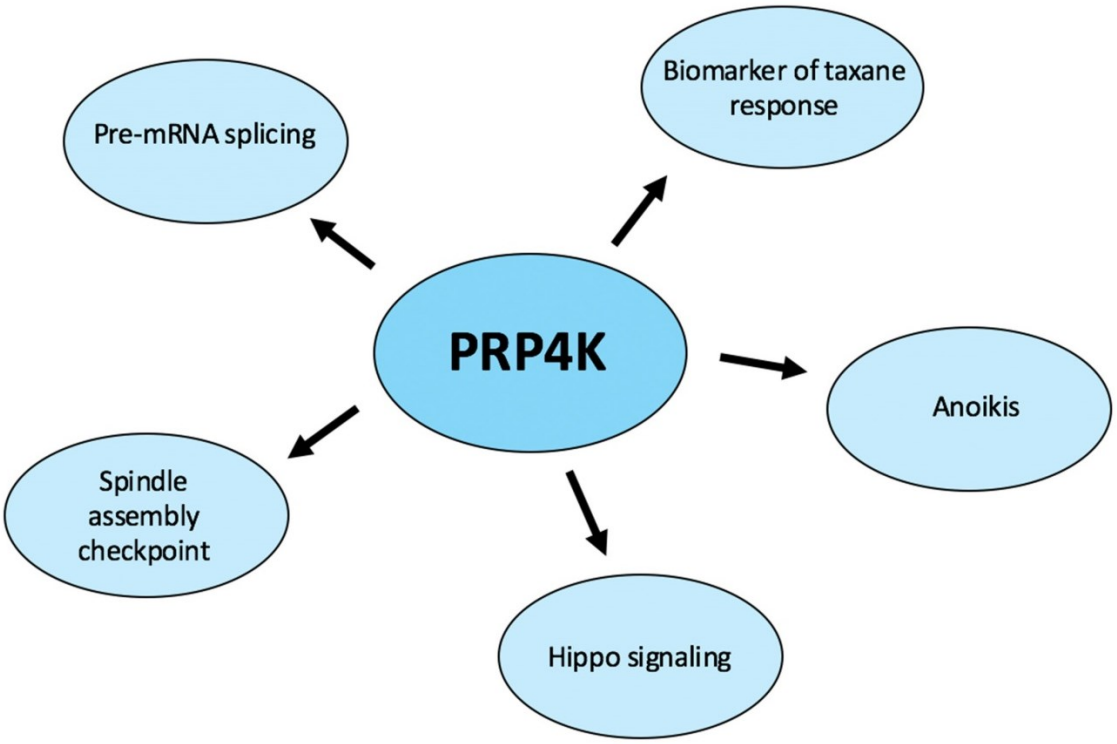

Figure 1.4: Known functions of PRP4K. PRP4K has known roles in pre-mRNA splicing, the spindle assembly checkpoint, Hippo signaling pathways, anoikis and taxane sensitivity.

1.5 Mechanisms of Cellular Death

1.5.1 Overview of Cell Death Pathways

Cells die through a variety of mechanisms. In general, cell death can be an uncontrolled passive process or a controlled active process. These modes of cell death include the passive process of necrosis and the active processes of apoptosis and autophagy (Fietta, et al., 2006).

Necrosis is a disorganized mode of cell death that consists of the permeabilization of the plasma membrane, swelling of the cytosol, enlargement of the endoplasmic reticulum, compaction of the mitochondria and chromatin clumping (Fietta, et al., 2006).

Autophagy is a process of cellular death that involves the degradation of cellular components by lysosomes (Tonekawa, & Thorburn, 2014). It is often activated following starvation to promote the breakdown of substrates and provide energy for the organism. The process consists of the endocytosis of cytoplasmic regions into membrane-bound vacuoles called autophagosomes. These autophagosomes eventually fuse with lysosomes forming an auto-lysosome, where lysosomal enzymes work to degrade the contents (Fietta, et al., 2006).

Apoptosis is an important process in the elimination of aberrant or aged cells and in developmental processes (Fietta, et al., 2006). Apoptosis is a mechanism of programmed cell death that can be triggered within a cell through two caspase-mediated pathways: the extrinsic pathway and the intrinsic pathway (Elmore, 2007). Morphologically, the process of apoptosis is accompanied by cell shrinkage, plasma membrane blebbing, nuclear condensation, and internucleosomal DNA fragmentation. Once the process is complete, the dead cell is packaged into vesicles, which are enveloped and eliminated by neighbouring cells or tissue phagocytes (Ashkenazi., 2014).

Apoptosis has important roles within the body as it is involved in the formation of tissues and organs during development and is also required for several physiological functions of the adult body, including the immune, digestive, endocrine and nervous systems (Ashkenazi., 2014). This process also works to eliminate abnormal cells in the body that might have the potential to develop into cancer (Pistritto, 2016).

1.5.2 Anoikis

Anoikis is a specific type of apoptosis that occurs following epithelial cell detachment from the extracellular matrix (ECM) and is critical in preventing the metastasis of epithelial cancers (Paoli, et al., 2016). Similar to apoptosis, anoikis can also occur through an extrinsic or an intrinsic pathway. Both the intrinsic and extrinsic pathways of anoikis are driven by caspases, which are a subset of the cysteine-dependent aspartate-specific protease family. The two major types of caspases are the initiator caspases and the effector caspases. Both initiator and effector caspases exist in an inactive form and therefore, require activation in order to induce the process of cell death (Paoli, et al., 2016).

The B-cell lymphoma 2 (Bcl-2) family of proteins is a family of proteins that possess specific regions of homology called Bcl-2 homology domains (BH) and have a critical role in the regulation of apoptosis and anoikis (Czabotar, 2014). The Bcl-2 family can be further divided into three groups: (1) the BH3-only proteins that are activated by cellular stress and initiate apoptosis (i.e. following loss of ECM-cell attachment); (2) the pro-survival proteins; and (3) the pro-apoptotic executioner proteins Bax (BCL-2-associated X protein) and Bak (BCL-2 antagonist/killer), which oligomerize and permeabilize the outer mitochondrial membrane (OMM) (Paoli, et al., 2016).

Following their activation, the pro-apoptotic BH3-only proteins promote the activation of cell death through the neutralization of pro-survival proteins and through the activation of the pro-apoptotic executioner proteins Bax and Bak. In addition, pro-survival protein members can interact with and neutralize the pro-apoptotic BH3-only proteins and the pro-apoptotic executioner proteins Bak and Bax to prevent their pro-apoptotic activity (Czabotar, 2014). Therefore, the relative concentrations of these different groups of Bcl-2 family members determines whether or not cell death will be induced (Shamas-Din, 2013). The mechanisms of cell death through anoikis are summarized in Figure 1.5.

1.5.2.1 The Intrinsic Pathway

Following the detachment of a cell from the ECM, the BH3-only pro-apoptotic proteins Bid (BH3 interacting domain death agonist) and Bim (Bcl-2-like protein 11) are activated (Taylor et al., 2008), which promotes the activation of pro-survival executioner proteins Bax and Bak. In normal cells, Bak is located on the mitochondria and Bax resides within the cytosol. Following their activation, Bax is translocated to the outer membrane of the mitochondria (OMM) from the cytosol and oligomerizes with Bak. The newly formed oligomer then perforates and creates a channel within the OMM. This leads to the permeabilization of the mitochondria and the release of cytochrome c. The release of cytochrome c promotes a conformational change in the cofactor apoptosis protease activating factor (Apaf) and ultimately leads to the formation of the initiator caspase-9 activating apoptosome. Activation of the initiator caspase-9 promotes the activation of effector caspase-3, which goes on to cleave critical cellular substrates resulting in the death of the cell (Paoli, et al., 2016).

1.5.2.2 The Extrinsic Pathway

Execution of the extrinsic pathway occurs through ligand binding to members of the tumor necrosis factor receptor (TNFR) superfamily of death receptors (Paoli, et al., 2013). These include the Fas receptor (TNF receptor superfamily, member 6), TNFR1 and the TNF-related apoptosis inducing ligand (TRAIL) receptor-1 and -2. Ligand binding to these receptors promotes the formation of a multiprotein signaling platform called the death-inducing signaling complex (DISC) at the plasma membrane. The formation of the DISC is followed by the activation of signaling processes that lead to the activation of apoptosis, including the activation of the initiator caspase-8, which goes on to cleave and activate effector caspases-3, -6, and -7, ultimately leading to the activation of a downstream proteolysis cascade and cell death (Paoli, et al., 2016; Wajant, 2002).

The extrinsic and intrinsic pathways are connected through caspase-8 and Bid. Activated caspase- 8 within the extrinsic pathway can also cleave and activate the BH3-only protein Bid, which activates the intrinsic pathway through Bax and Bak activation (Valentijn & Gilmore, 2004).

The execution of the extrinsic and intrinsic apoptosis pathways following the detachment of cells from the ECM can occur through various mechanisms. Cell detachment leads to the upregulation of both the Fas receptor and Fas ligand, activating the extrinsic pathway (Rosen et al., 2002). In addition, the rounding of cells that occurs following their detachment from the ECM can place Fas receptors closer together leading to their activation (Muzio, et al., 1998). Finally, the detachment of cells from the ECM also disrupts the cytoskeleton of these cells, promoting the release of pro-apoptotic factors like Bim or death receptors from a sequestered state (Vachon, 2010).

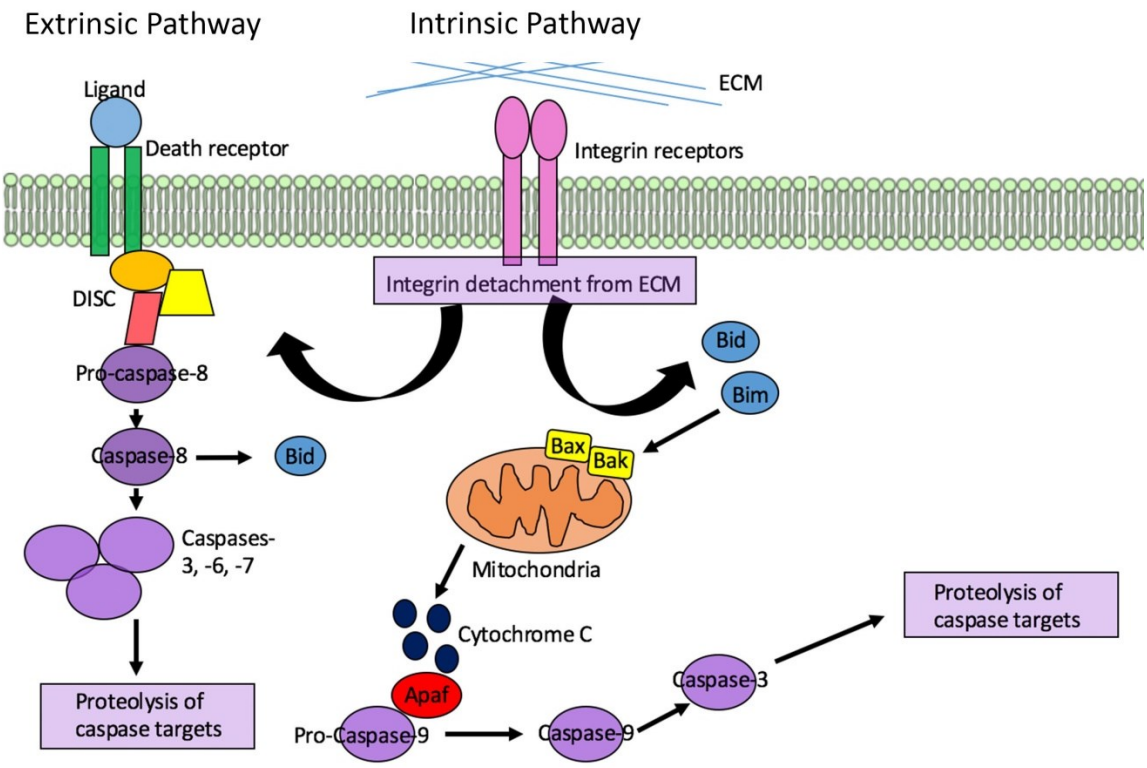

Figure 1.5: Mechanisms of anoikis. Following cell detachment from the ECM, anoikis can occur through an extrinsic or an intrinsic pathway. The extrinsic pathway occurs through ligand binding to members of the tumor necrosis factor receptor (TNFR) superfamily of death receptors. The intrinsic pathway is activated by the up-regulation of pro-apoptotic proteins Bid and Bim, which promotes the activation and oligomerization of pro-survival executioner proteins Bax and Bak.

1.5.3 Anoikis Resistance and Cancer

Many cancers have developed mechanisms to overcome anoikis, which promotes survival of detached tumor cells during their metastasis to secondary sites within the body (Paoli, et al., 2016). Anoikis is regulated by integrin receptors, which bind the ECM in clusters forming focal adhesions (Vachon, 2011). Focal adhesions then provide pro-survival signaling to the cell through the coupling of integrin receptors to various receptor tyrosine kinases (RTK). Thus, loss of cell-ECM detachment leads to the termination of pro-survival signaling and the induction of anoikis (Corkery et al., 2018). Cells in the body should undergo anoikis following detachment from the ECM in order to prevent the colonization of these cells to regions in the body that they do not belong. Cancer cells have developed mechanisms to overcome this type of programmed cell death, allowing them to spread and metastasize (Paoli, et al., 2016).

1.5.4 Mechanisms of Anoikis Resistance

Cancer cells have developed several mechanisms that allow them to overcome anoikis and continue to thrive following their detachment from the ECM (Paoli, et al., 2016). I summarize some of the mechanisms underlying anoikis resistance in the following sections.

1.5.4.1 A Switch in Integrins

Anoikis resistance can arise through the deregulation of integrins and changes in their expression levels. Studies report that normal squamous cells express $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins and low levels of $\alpha v\beta 5$; however, cancer cells are found to express

high levels of αvβ6 (Janes & Watt, 2004). Integrin β6 is expressed in various tissues during development, but is absent in adult normal cells. However, high levels of integrin β6 are found in several types of cancer cells (Breuss, et al., 1995). Certain integrins are also associated with higher levels of pro-survival signaling. For example, β4 integrin overexpression promotes the constitutive activation of PI3K (Bon et al., 2006).

1.5.4.2 Constitutive Activation of Anti-Apoptotic Pathways

There are several signaling pathways that when constitutively activated contribute to anoikis resistance (Paoli, et al., 2016). The PI3K/Akt pathway is one of the most important signaling pathways involved in anoikis resistance. Sustained Akt signaling can occur due to the overexpression or constitutive activation of other receptor tyrosine kinases (Reginato et al., 2003; Douma et al., 2004) as well as through activating Ras mutations that make Ras constitutively active (Derouet, et al., 2007), loss of PTEN function (Vitolo, et al., 2009) due to gene mutation, deletion, or promoter methylation, and through the amplification of Akt genes (Altomare & Testa, 2005). Once activated, Akt promotes cell survival and cell growth through several mechanisms. The activation of Akt can control the activity of transcription factors by direct phosphorylation, which control the expression of pro-apoptotic and anti-apoptotic genes. Specifically, Akt negatively regulates the transcription factors forkhead box protein O1 (FOXO1), forkhead box O3 (FOXO3) and forkhead box protein O4 (FOXO4), which promote the expression of death genes. Akt activates the transcription factors Iκβ kinase (IKK) and cyclic-AMP response element-binding protein (CREB), which upregulate anti-apoptotic gene expression. Akt can also physically phosphorylate pro-apoptotic and anti-apoptotic

proteins to inhibit or promote their function, respectively (Tokunaga et al., 2009). In addition, Akt can negatively regulate the expression of genes that promote cell death, such as Bid and Bim, and phosphorylates procaspase-9 to inhibit the caspase cascade (Tokunaga et al., 2009). Furthermore, Akt can promote the activation of the nuclear factor kB (NF-kB) survival pathway (Romashkova & Makarov, 1999).

Src activity also has a role in anoikis signaling pathways. High levels of Src is associated with a mesenchymal phenotype (Avizienyte & Frame, 2005). As well, increased Src activation promotes increased focal adhesion kinase (FAK) phosphorylation, P13K recruitment and increased activation of Akt. FAK also promotes the activation of the Ras/MAPK pathway, which is also associated with pro-survival (Bouchard et al., 2007). The activation of this pathway is found to affect the regulation of the pro-apoptotic proteins Bim and Bad, ultimately leading to the degradation of Bim; therefore, promoting survival. In addition, the activation of this pathway leads to the upregulation of pro-survival proteins (Giannoni et al., 2009).

The uncontrolled expression of some growth factor receptors is also associated with an anoikis resistant phenotype. One of the most important growth factor receptors involved in anoikis resistance is the tropomyosin receptor kinase B (TrkB) (Geiger & Peeper, 2007). TrkB is often overexpressed in tumors and is also correlated with the increased aggressiveness of tumors as well as chemoresistance (Tabjbakhsh, 2017; Tanaka, 2014). TrkB is known to induce epithelial-to-mesenchymal transition (EMT) through Twist-Snail-Zeb1 and increase the activation of PI3K and MAPK signaling pathways (Tabjbakhsh, 2017; Tanakam, 2014). Therefore, overexpression of TrkB leads to both increased anoikis resistance (Douma et al., 2004) and an EMT phenotype (Smit, 2011). TrkB will be discussed more in detail in section 1.8.

1.5.4.3 Epithelial-to-Mesenchymal Transition (EMT)

Anoikis resistance is associated with the acquisition of a mesenchymal phenotype as many proteins involved in EMT are also able to regulate pro-apoptotic and anti-apoptotic genes. EMT aids cancer cells in becoming more motile and overcoming anoikis (Paoli, et al., 2016). A more in-depth discussion on EMT will be discussed in section 1.7.

1.5.4.4 MicroRNAs

MicroRNAs are small, non-coding RNA molecules involved in the regulation of gene expression. Some microRNAs have been identified with having a role in both EMT and anoikis resistance, by regulating pro-survival pathways (Paoli, et al., 2016). A more in-depth discussion on microRNAs will be discussed in section 1.9.

1.5.4.5 Oxidative Stress and Hypoxia

Continuous exposure to reactive oxygen species (ROS) is found to promote cell survival, proliferation and metastasis (Paoli et al., 2016). Since activated growth factor receptors promote an increase in intracellular ROS levels through the activation of NADPH oxidase and lipoxygenase, the overexpression of growth receptors in cancer cells promote constitutive ROS production (Brown & Grendling, 2009). Sustained ROS levels can promote an anoikis resistant phenotype through the activation of Src kinase and various redox transcription factors that lead to PI3K/Akt activation. As well, hypoxia and ROS production are found to promote EMT in several cancer cell types (Imai et al., 2003).

1.5.4.6 Modulation of Energetic Metabolism

When normal cells detach from the ECM, these cells undergo a change in metabolism where there is a reduction in glucose uptake, glycolytic flux, mitochondrial respiration and the pentose phosphate pathway. These events lead to a reduction in intracellular ATP and NADPH levels, and fatty acid oxidation, as well as an increase in ROS production and apoptosis (Paoli, et al., 2016). The ability to modulate these metabolic pathways promotes anoikis resistance in cells. Following cell detachment in an anoikis-resistant cell, the reduction in glucose uptake activates large kinase B1 (LKB1), which promotes AMP-activated protein kinase (AMPK) activity. AMPK then phosphorylates and inhibits acetyl-coenzyme A carboxylases 1 and 2, the rate-limiting enzymes in fatty-acid synthesis. This results in a decrease in NADPH consumption in fatty-acid synthesis, but increases the generation of NADPH by malic enzyme, an effect that arises indirectly through fatty-acid oxidation (Jeon, Navdeep & Hay, 2012). This process leads to decreased ROS production following cell detachment from the ECM, allowing the cell to escape anoikis (Paoli, et al., 2016).

1.6 Metastasis

Late stage cancers are often associated with metastasis. Metastasis is a process by which cells detach from a primary tumor and spread to distant sites within the body, creating secondary tumors (Alizadeh, et al., 2014). Because metastasis is responsible for ~90% of cancer-related deaths (Gupta & Massague, 2006), a better understanding of the mechanisms of metastasis is essential for better outcomes in patients with late stage cancer. The process of metastasis consists of several steps, which are detailed in Figure 1.6. To begin the process, epithelial cells must detach from surrounding cells within the

primary tumor and invade through the ECM and into the stroma cell layers (Fidler, et al., 2003). The next step is the intravasation of cells into either the lymphatic system or into the circulation, which allows cells to disseminate throughout the body as long as they can survive the various stresses that arise. Once the cell arrests at a distant site within the vasculature, the cancer cell can exit the bloodstream and enter the tissue, which is called extravasation. Once cells have entered a tissue, the cells must adapt to their new microenvironment and begin to proliferate in order to form metastases (Valastyan, 2011).

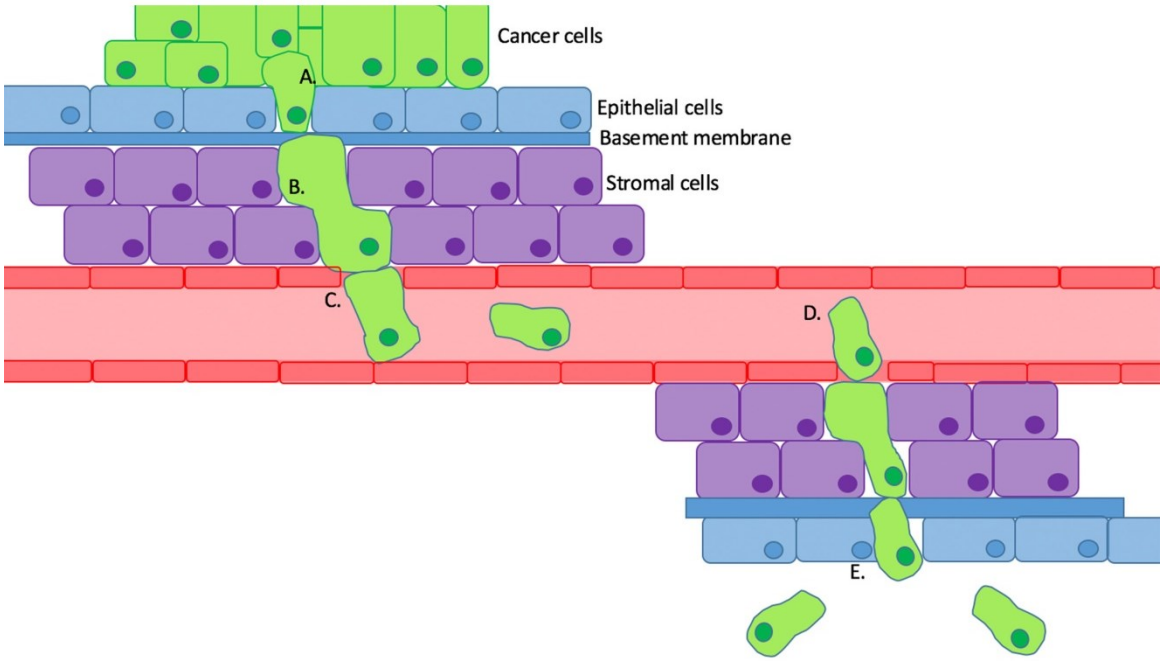

Figure 1.6: The metastatic cascade. (A-B) Epithelial cells first detach from surrounding cells within the primary tumor and invade through the ECM and into the stroma cell layers. **(C)** The next step is the intravasation of cells into either the lymphatic system or into the circulation. **(D)** Once the cell arrests at a distant site within the vasculature, the cancer cell can exit the bloodstream and enter the tissue, which is called extravasation. **(E)** Once cells have entered a tissue, the cells must adapt to their new microenvironment and begin to proliferate in order to form metastases.

1.6.1 Breast Cancer Metastasis

Breast cancers commonly metastasize to bone, lung, liver and brain through the bloodstream or lymphatic system (Patanaphan et al., 1988; Kozlowski et al., 2015). Breast cancer metastasis occurs via the mechanism outlined above (Figure 1.6). In the following section, I will discuss ovarian cancer metastasis in detail. Although some aspects of ovarian cancer metastasis are unique to the location and anatomy of the ovaries, many of the molecular signaling details linking EMT and metastasis are similar between breast and ovarian cancer.

1.6.2 Ovarian Cancer Metastasis

The main mechanism of ovarian cancer metastasis is peritoneal metastasis and differs from the metastatic process of other cancer types as it is a passive metastatic process (Mitra, 2016). The first step in ovarian cancer metastasis is the shedding of cancer cells from the primary tumor into the peritoneal cavity as single cells or as clumps of cells. The process often involves EMT (discussed in detail in section 1.6), aiding in the detachment of cells from each other and from the primary tumor. Once cells have entered the peritoneal fluid, they must develop resistance to anoikis in order to survive detached conditions (Latifi et al., 2012; Ahmed, et al., 2010). The peritoneal fluid moves within the peritoneal cavity upward, toward the head and then downward, toward the feet due to the movement of the diaphragm during respiration and gravity, respectively (Carmignani et al., 2003). The cancer cells move throughout the peritoneal cavity due to the flow of the peritoneal fluid. The role of the peritoneal fluid is to lubricate adjacent organs in the peritoneal cavity and is mostly returned to the circulation through the lymphatic system. However, ovarian cancer is associated with increased expression of vascular endothelial

growth factor (VEGF), leading to increased leakiness of the vasculature. As well, cancer cells can block lymphatic vessels (Ahmed & Stenvers, 2013). Both of these processes help promote the formation of ascites (accumulation of fluid within the abdomen), which helps with the spreading of cancer cells within the peritoneal cavity.

Following survival in the peritoneal fluid, cancer cells must attach to organs in the peritoneal cavity. Ovarian cancer cells often metastasize to the omentum, which covers the bowels (Lengyel, 2010). In order to metastasize the omentum, cancer cells must attach to the mesothelial cells on its surface, which requires the retraction of the mesothelial cells by the cancer cells (Mitra, 2016). Iwanicki and colleagues have shown that the fibronectin receptor helps ovarian cancer cells attach to the fibronectin that is present on the surface of the mesothelial cells and helps with mesothelial cell displacement (Iwanicki, et al., 2011). Increased excretion of matrix metalloproteinases (MMPs) also aid the cancer cells in adhering to the omentum by cleaving fibronectin and vitronectin on the surface of the mesothelium into small fragments, enhancing binding (Kenny et al., 2008). After organ attachment, cancer cells must adapt to their new microenvironment, proliferate and recruit cells to help form the tumor microenvironment (Mitra, 2016).

In addition to peritoneal dissemination, ovarian cancer cells can metastasize to the lymph nodes because the ascites is often drained by the lymphatic system. Furthermore, because the lymphatic system drains into the left subclavian vein through the thoracic duct, ovarian cancer cells can enter the blood through this mechanism (Mitra, 2016).

1.6.3 Role of the Tumor Microenvironment in Cancer Development and Metastasis

Recently, cancer studies have suggested that the tumor microenvironment has a major impact on cancer proliferation and metastasis. More research is being pursued in

order to develop cancer therapies that not only target cancer cells, but the microenvironment that supports their growth and progression. The tumor microenvironment is composed of ECM, myofibroblasts, fibroblasts, adipose cells, immune-inflammatory cells, and the blood and vascular networks (Wang et al., 2017).

In normal tissue, fibroblasts are the main manufacturers of the ECM and become activated during wound repair mechanisms (Li et al., 2007). Fibroblasts within the tumor microenvironment are called cancer-associated fibroblasts (CAFs) (Rodriguez, et al., 2018). CAFs are constitutively activated and play a major role in the development of cancer through ECM remodelling, inducing angiogenesis, recruiting inflammatory cells and inducing the proliferation of cancer cells through the secretion of growth factors and cytokines (Wang, et al., 2018).

Early in cancer development, the immune system is able to eliminate cancer cells. In some people, there is an equilibrium between new tumor proliferation and immune destruction and over time in a subset of people the cancer evolves the ability to escape the immune system, growing larger and eventually metastasizing (Messerschmidt, et al., 2016). Genomic instability allows cancer cells to evolve new properties through DNA mutations, which includes the ability to evade the immune system (Ferguson, et al., 2015). Therefore, the immune system can actually help in cancer development by selecting the dominant cells that can escape destruction and proliferate to form a tumor (Wang, et al., 2018). Many studies have also shown the major role of immune-inflammatory cells in immunosuppression. Some cell types have an innate function of immunosuppression whereas others can be activated with cytokines and chemokines. Immune cells are a major component of the tumor microenvironment and can have pro-tumor or anti-tumor functions, depending on their polarization status (Sounni & Noel,

2013). Immune cells within the tumor microenvironment include monocytes/macrophages, neutrophils, and lymphocytes. Once monocytes are within the tumor, they differentiate into tumor-associated macrophages (TAMs) that are constitutively activated and considered an M2 macrophage. TAMS release factors such as VEGF, hGH (human growth hormone), MMPs, and IL-8 (interleukin-8), which play a role in ECM remodeling, hypoxia, angiogenesis, proliferation and immune escape (Li, et al., 2007). Tumor-associated neutrophils (TANs) are constitutively activated and considered a pro-tumor N2 neutrophil. TANS secrete proteolytic enzymes, such as MMPs, that breakdown the ECM and help in metastasis (Shishir, et al., 2018).

The blood vessels surrounding tumors tend to be leaky, helping in the intravasation of cancer cells into the circulation. As well, increased levels of growth factors such as VEGF secreted from cells in the microenvironment (tumor cells, CAFs and inflammatory cells) promote the angiogenesis process (Wang et al., 2018). Angiogenesis involves the formation of new vasculature, which helps to provide the surrounding tumor with nutrients (Otrock, et al., 2007). Adipose cells help to create a pro-inflammatory environment, which is favorable for tumor growth. Adipose cells can secrete various cytokines, chemokines and hormone-like factors, influencing other cells in the microenvironment (Shishir, et al., 2018).

The ECM is the main support network for cancer cells. The ECM is influenced by all the cytokines and growth factors, that are secreted by surrounding cells. Both density and stiffness of the ECM affect the invasive behaviour of a tumor (Sounni & Noel, 2013). For instance, dense breast tissue is linked to increased risk of breast cancer as well as increased invasion of the breast cancer (Lavental et al., 2009). In addition to increased density and stiffness of the ECM during cancer processes, cancer cell ECM also has

altered structure, composition, and organization, increased proteolytic activity and dysregulated ECM turnover, which all affect tumor progression (Sounni & Noel, 2013).

1.7 Epithelial-to-Mesenchymal Transition (EMT)

1.7.1 EMT Overview

Epithelial-to-mesenchymal transition (EMT) is a process that involves breaking of cell-cell contacts, loss of polarity, cytoskeletal reorganization and release of MMPs in order to degrade the ECM so that cells can invade the tissue and disseminate. EMT begins with epithelial cells that lose epithelial characteristics such as cell-cell adhesion and apical-basal polarity and gain mesenchymal characteristics such as cell individualization, front-rear polarity, motility and invasiveness (Pradella, 2017). This process is involved in several normal physiological processes, which include: embryonic development, embryonic stem cell differentiation, induction of pluripotency, tissue repair, wound healing and stem cell behaviour. In addition, EMT is involved in several pathological conditions, which include: tissue/organ fibrosis, cancer stem cell behaviour and cancer development (Kim., 2017).

The process of EMT involves several changes in the phenotype of epithelial cells undergoing this process. These changes in phenotype include: (1) a morphological change from epithelial cells organized in a cobble-stone monolayer with apical-basal polarity to spindle-like mesenchymal cells with a more dispersed organization; (2) a change in gene expression with a down-regulation of epithelial gene expression and an up-regulation of mesenchymal gene expression; (3) a change in motility from stationary epithelial cells to motile mesenchymal cells; and (4) an increase in the resistance to senescence and apoptosis in mesenchymal cells compared to epithelial cells (Kim., 2017).

Mesenchymal-to-epithelial transition (MET) is the reverse process of EMT and plays an important role in the re-differentiation of cancer cells and in metastatic colonization. In this process, cells lose their migratory and invasive capabilities, increase the expression levels of proteins involved in adhesion processes and undergo apico-basal polarization (Nieto, 2016). The processes of EMT and MET are represented in Figure 1.7.

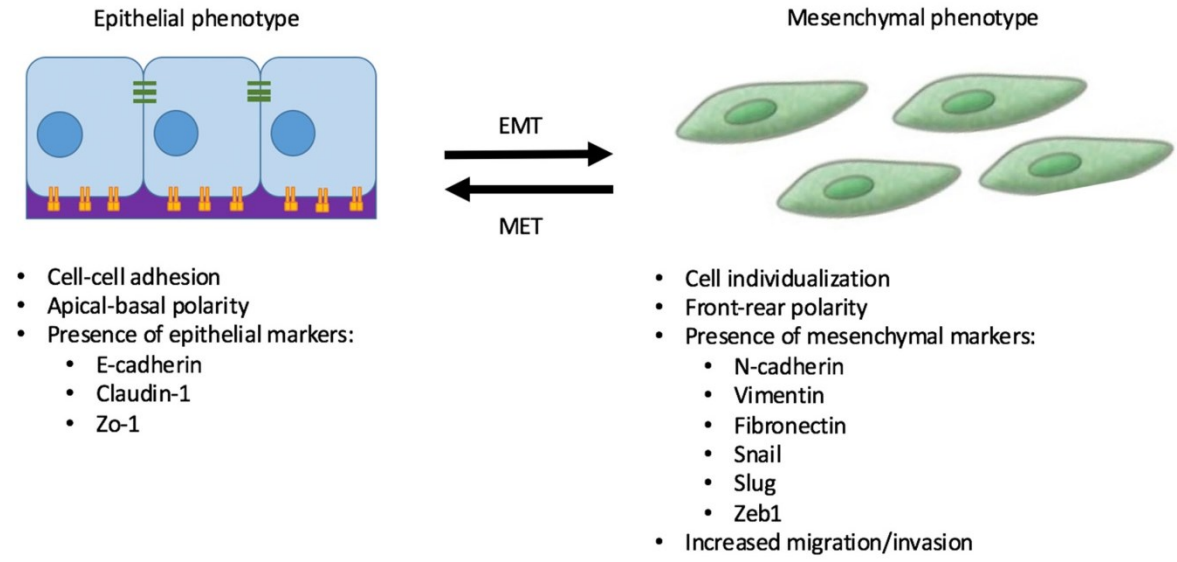

Figure 1.7: Epithelial-to-mesenchymal transition (EMT) overview. EMT is a process where epithelial cells lose epithelial characteristics such as cell-cell adhesion, apical-basal polarity and epithelial markers while gaining mesenchymal characteristics such as cell individualization, front-rear polarity, increased migration and invasion and mesenchymal markers. MET is the reverse process of EMT where mesenchymal cells lose mesenchymal characteristics and gain epithelial characteristics.

1.7.2 Regulation of EMT

The gene expression changes that occur during EMT involve several signaling pathways and require several layers of regulation, including transcriptional, post-transcriptional, translational and post-translational mechanisms (Skovierova, 2018). Below I will further elaborate on the regulatory mechanisms controlling EMT.

1.7.2.1 EMT Signaling Pathways

The EMT process is generally triggered by cytokines that activate key signaling pathways. Several signaling pathways play a role in facilitating the EMT process, which include the receptor tyrosine kinase (RTK) pathway, transforming growth factor beta (TGF-Beta) pathway, the Wnt pathway, the Notch pathway and the Hedgehog family pathway. These pathways respond to various stimuli and ultimately induce the expression of EMT transcription factors that then affect the expression of epithelial and mesenchymal genes (Fabregat, 2016).

1.7.2.2 Transcriptional Pathways Involved in EMT

The expression of transcription factors (TFs) involved in EMT can be induced through various mechanisms including inflammation, metabolic stress and through the activation of signaling pathways (Fabregat, 2016). EMT-TFs regulate the expression of target genes at the transcriptional level, activating mesenchymal gene transcription and repressing epithelial gene transcription. The expression of EMT-TFs is usually activated early in the EMT process and the various TFs often control the expression of each other and act on the same target genes (Liao, 2017). Below, I will summarize key EMT-TFs and their roles in breast and ovarian cancer.

1.7.2.2.1 Snail

Snail can both repress epithelial gene transcription and activate mesenchymal gene transcription through different mechanisms. Snail is encoded by the *SNAI1* gene and binds to the E-box in the promoters of the epithelial proteins E-cadherin, claudin and occludin, recruiting various histone modifiers that repress the transcription of the gene. In addition, Snail interacts with CREB binding protein to prevent the formation of the repressor complex and results in the activation of mesenchymal protein transcription (i.e. fibronectin 1) (Liao, 2017). In breast cancer cells, Smith et al., have shown that the induction of EMT through Snail overexpression increased cell migration and decreased cell adhesion through the activation of p-ERK (Smith et al., 2014). Furthermore, high Snail expression is associated with lymph node involvement, invasion, metastasis, and decreased recurrence-free survival in breast cancers (Cheng, et al., 2001; Blanco et al., 2002; Moody et al., 2005). In ovarian cancer cells, Kurrey and colleagues showed that Snail transfection caused cells to undergo EMT, which increased their invasiveness and motility, suggesting its important role in ovarian cancer metastasis (Kurrey & Babat, 2005). Increased Snail expression has also been associated with clinicopathological tumor staging, lymph node metastasis, tumor recurrence and poor prognosis in ovarian cancer (Kurrey & Babet; 2005; Olmeda et al., 2007; De Cranene & Berx, 2006). Furthermore, Yoshida and colleagues discovered that Snail protein expression increased as ovarian epithelial cells progressed into cancer cells (Yoshida et al., 2009).

1.7.2.2.2 Slug

Slug is encoded by the *SNAI2* gene. Slug binds the corepressor nuclear receptor coreceptor and recruits C-terminal binding protein 1 (CtBP1), which promotes the

repression of E-cadherin. In addition, Slug promotes the repression of epithelial genes by binding the E2-box sequence of the target gene promoter and recruiting CtBP1 and histone deacetylase 1 (HDAC1) to suppress gene expression (Liao, 2017).

In breast cancer, increased Slug expression is associated with reduced chemosensitivity by promoting MMP1 expression (Shen, et al., 2017). Furthermore, researchers have identified a role for Slug in the regulation of the mammary stem cell state (Phillips, et al., 2014; Guo et al., 2012; Nassour, et al., 2012). Specifically, Slug is required for lineage-committed cells to de-differentiate into a stem-like state during tumor initiation (Nassour et al., 2012). As well, high Slug expression is seen in the aggressive basal-like breast tumors, which also express high levels of stemness genes (Storci, et al., 2008). In ovarian cancer tissues, Slug expression is significantly correlated with tumor grade and lymph node metastasis (Gu, et al., 2017). Furthermore, ovarian cancer patients with Slug expression have shorter survival times in comparison to patients who are negative for Slug expression (Gu, et al., 2017).

1.7.2.2.3 Zeb1/2

Zinc finger E-box binding homeobox 1 (Zeb1) also binds to E-boxes within the promoter of E-cadherin, repressing it. Transcriptional repression regulation by Zeb1 often involves the recruitment of CtBP, but can also be independent of CtBP. Zeb1 interacts with Smads involved in the TGF-beta pathway and the transcriptional coactivator p300, which switches Zeb1 from a transcriptional repressor to a transcriptional activator (Liao, 2017).

Zinc finger E-box binding homeobox 2 (Zeb2) is a transcriptional repressor of epithelial genes, E-cadherin and other genes encoding junctional proteins. Zeb2 regulates

gene expression both dependently and independently of the CtBP1 corepressor complex (Liao, 2017). In breast cancer, both mRNA and protein expression of Zeb1/2 are increased in breast cancer tissues when compared to benign breast tissue (Ang, et al., 2016). Additionally, Zeb1 plays a role in breast cancer chemoresistance that is dependent on ataxia telangiectasia mutated (ATM) (Zhang, et al., 2018). The mRNA expression levels of Zeb1/2 are higher in ovarian cancer metastatic lesions when compared to primary ovarian carcinoma tissues (Elloul, et al., 2010; Wu, et al., 2016). Furthermore, Zeb1/2 play a role in ovarian cancer cell migration, invasion, and anchorage-independent cell growth (Prislei, et al., 2015).

1.7.2.2.4 Twist1

Twist family BHLH transcription factor 1 (Twist1) expression in cells induces the EMT process by increasing the expression of fibronectin, vimentin, N-cadherin and by decreasing the expression of E-cadherin. Moreover, Twist1 expression is necessary for metastasis to occur (Liao, 2017). The over-expression of Twist on its own results in the induction of EMT, by promoting a decrease in E-cadherin expression and an increase in the expression of mesenchymal markers (Fabregat, 2016). In breast cancers, Twist1 expression is associated with worse overall survival, larger tumor size, lymph node involvement, higher nuclear grade and positive HER2 expression, all considered poor prognostic factors (Qiao, et al., 2017). Moreover, high Twist expression is associated with the aggressive TNBC (Zhang, et al., 2015). In ovarian cancers, Twist expression predicts poor clinical outcomes due to its role in metastasis, chemoresistance and stemness (Nuti et al., 2014). Twist1 expression levels increase during the progression from a benign tumor, to a borderline tumor and to a malignant tumor (Yoshida et al., 2009). In terms of

chemoresistance, Twist1 expression is linked to taxol and cisplatin resistance in ovarian cancer cells (Wang, et al., 2004; Li et al., 2007). Additionally, Twist1 is a regulator of ovarian cancer stemness, through the regulation of stem cell differentiation through the miRNAS miR-199a and miR-214 (Chen et al., 2008).

1.7.2.2.5 MicroRNAs Involved in EMT

As mentioned earlier, microRNAs are small, non-coding RNA molecules involved in the regulation of gene expression (Khordadmehr, 2018). The expression of various miRNAs regulates the epithelial phenotype and the EMT process. Some microRNAs control the expression of TFs involved in EMT. For example, miR-29b and miR-30a repress Snail1 expression and the miR-200 family and miR-205 repress Zeb1 and Zeb2 expression, reversing the EMT process (Ding, 2014). Other microRNAs target key genes involved in maintaining an epithelial or mesenchymal phenotype. For example, miR-9 represses E-cadherin and miR-194 represses N-cadherin, promoting a mesenchymal and epithelial phenotype, respectively (Ding, 2014). A more in-depth discussion of what microRNAs are and how they work is discussed later on in section 1.9.

1.7.2.3 Regulation of Pre-mRNA Splicing during EMT

Alternative splicing is a mechanism that allows the generation of different mRNA products from the same gene thereby increasing the complexity of the proteome (Baralle, 2017). Dysregulation of this process plays a role in EMT and in the development of cancer. More specifically, mesenchymal cells have been shown to have different splicing patterns than epithelial cells, which occurs because of differences in the expression of splicing factors (Li, 2018).

Epithelial splicing regulatory protein (ESRP) splicing factors are well-studied, key players in the maintenance of an epithelial phenotype, with many transcriptional targets including those involved in cell-cell adhesion, cell motility and cell-matrix adhesion (Ishii, et al., 2014). One of these targets is the fibroblast growth factor receptor 2 (*FGFR2*) pre-mRNA. Alternative splicing of this gene has been discovered in both primary tumors and metastases. Researchers found that the ESRP proteins repress exon IIIc of the gene and increase the inclusion of exon IIIb, which promotes the production of the epithelial isoform of *FGFR2*. When the ESRP proteins are down-regulated, the exon IIIc is included, which promotes the production of the mesenchymal isoform of *FGFR2* (Warzecha, 2009).

The ESRPs proteins also regulate alternative splicing of the Scribble (*SCRIB*) gene, which is a scaffolding protein involved in epithelial cells, that prevents the loss of E-cadherin and zonula occludens protein 1 (Zo-1) from adherens junctions and tight junctions, respectively. A different alternative splicing pattern of *SCRIB* promotes the final protein product to instead allow the loss of E-cadherin and Zo-1 from junctions and promote a motile cell phenotype (Pradella, 2017).

Overall, ESRP proteins play a role in the alternative splicing of genes involved in EMT, including genes with roles in cell polarity and actin cytoskeleton organization. The down-regulation of ESRPs occurs because of the activation TGF-Beta signaling pathways (Horiguchi, et al., 2012). As well, The EMT-TF Zeb1 has been found to inhibit ESRP1 and promotes changes in the alternative splicing of genes and the expression of mesenchymal splice variants (Pradella, 2017).

Li and colleagues discovered that the two RNA binding proteins, protein quaking (QK1) and RNA binding protein fox-1 homolog 1 (RBFOX1), regulate the alternative

splicing of the filamin B gene (*FLNB)*, which regulates EMT in breast cancer. More specifically, over-expression of these two proteins leads to a different alternative splicing pattern in the *FLNB* gene, which affects the binding of the transcription factor forkhead box C1 (FOXC1) and promotes EMT (Li, 2018). Although not the focus of this work, there are many factors involved in the regulation of alternative splicing and it is likely that the integrated effects of all these factors will determine whether a cell exhibits a more epithelial or mesenchymal identity (Pradella 2017).

1.7.2.4 Post-Translational Pathways involved in EMT

Ubiquitination is a post-translational modification where ubiquitin is added to a substrate protein. This process can have various effects on a protein including marking the protein for degradation for the proteasome, affecting the localization or activity of the protein, and affecting its interaction with other proteins (Pickart, 2004).

Ubiquitin-mediated degradation of important EMT TFs, such as Snail1 and Twist1, is another important regulation mechanism in EMT. (Hong et al., 2011; Zhou et al, 2004). In particular, specific F-box proteins have been found to recognize ubiquitin degradation signals on EMT TFs and target them to the proteasome for degradation, which helps in keeping these TFs at low levels in normal cells (Diaz, 2016).

The stability of proteins is also an important factor in EMT regulation. In normal epithelial cells, EMT TFs are expressed at low levels and have short half-lives because of their instability. EMT stimulating factors lead to increased stabilization and longer half-lives of these TFs. Increasing the stability of EMT TFs means that they have more time to activate EMT processes within a cell (Zhou, 2004).

Subcellular localization of EMT TFs also plays a role in the regulation of EMT. In order to be active, TFs must be localized within the nucleus of the cell. Phosphorylation of EMT TFs by specific kinases is one mechanism regulating their cellular localization and therefore activity. For example, Snail localization is regulated by phosphorylation by the kinase glycogen synthase kinase 3 beta (GSK-3Beta) (Zhou, 2004).

1.7.3 Epithelial Proteins and their Functions

Epithelial cells form cell layers through various connections including tight junctions, adherens junctions, desmosomes and gap junctions (Cooper, 2000). As a cell loses its epithelial characteristics and becomes more mesenchymal, the epithelial junctional proteins are degraded and/or re-located, which results in the disruption of the epithelial cell layer (Lamouille, 2014).

1.7.3.1 E-cadherin and Beta-catenin

E-cadherin, encoded by the *CDH1* gene, is an epithelial cell marker and transmembrane protein that connects epithelial cells forming adherens junctions between them. E-cadherin binds beta-catenin through its distal cytoplasmic domain forming an E-cadherin-beta-catenin complex, which then interacts with the actin cytoskeleton through alpha-catenin and its interacting protein, alpha-actinin (Jou, et al., 1995). Loss of E-cadherin is a key step in EMT as this prevents the formation of adherens junctions and helps cells to dissociate from the cell layer. E-cadherin is degraded by proteolytic cleavage or by endocytosis. Following E-cadherin cleavage, beta-catenin is released into the nucleus allowing it to bind the DNA binding protein family lymphoid enhancer factor

(LEF)/T cell factor (TCF), and activating the Wnt signaling pathway and other genes involved in cell proliferation, metastasis and tumorigenesis (Wong, 2018).

1.7.3.2 Claudin and Zonula Occludens 1 Protein (Zo-1)

Tight junctions connect neighbouring cells and are situated at the apicobasal region of epithelial cell membranes (Sawada, et al., 2003). These structures are formed from transmembrane barrier proteins connected to peripheral scaffolding proteins, which are connected to the actin cytoskeleton. Claudin and Zo-1 are both involved in the formation of tight junctions. Claudins are essential transmembrane proteins that interact with PDZ domains of the scaffolding proteins Zo-1, -2, and -3 through the carboxyterminal domain (Soini, 2012). The Zo proteins bind directly to actin and to actin binding proteins including alpha-actinin-4, vinculin, and alpha-catenin (Van Itallie, 2014).

There are twenty-six types of claudins that exist in humans (Soini, 2012). Claudin expression has been found to be decreased in some cancer types and increased in others. In breast cancer, decreased claudin 1, 2 and 7 expression and increased claudin 4 is associated with more aggressive cancer (Kim et al., 2008; Morohashi et al., 2007; Tokes, et al., 2005; Kominsky et al., 2003; Lanigan, et al., 2009).

1.7.4 Mesenchymal Proteins and their Functions

In this section, I will outline the various mesenchymal proteins and their functions, in particular as they relate to the process of EMT and metastasis.

1.7.4.1 N-cadherin

Like E-cadherin, N-cadherin is a transmembrane protein that connects epithelial cells at adherens junctions. N-cadherin is encoded by the *CDH2* gene, and its function is highly dependent on the specific tissue. For example, N-cadherin forms strong interactions between cardiomyocytes of the heart, but forms looser connections in migratory and mesenchymal cells promoting cell invasion and metastasis (Radice, 2013). With EMT, there is often a 'cadherin switch' from E-cadherin to N-cadherin, which is important in the conversion of normal epithelial cells to invasive mesenchymal cells (Priya & Yap, 2015).

In breast cancer, increased N-cadherin expression is associated with increased cell motility, invasion and metastasis (Radice, 2013). It is the interaction of N-cadherin with growth factor receptors that help to promote motility and metastasis in cells (Hazan, et al., 2000). N-cadherin promotes stronger epithelial-endothelial cell interactions, which also promotes invasion and metastasis by helping tumor cells move into and out of blood vessels (Kourtidis, 2017).

1.7.4.2 Fibronectin

Changes in ECM protein expression is an important event that occurs with EMT. Fibronectin is a component of the stroma ECM that is present in breast tumors, but not present in normal tissue. Because its expression is nearly absent from normal tissue, its expression suggests EMT (Park, 2014). High expression of fibronectin is associated with increased tumor malignancy and decreased survival rate of patients with breast cancer (Ioachim, et al., 2002).

1.7.4.3 Vimentin

Various intermediate filament proteins are over-expressed in malignant tissues when compared to normal tissues, playing key roles in EMT progression. Vimentin is an intermediate filament protein that is highly expressed in motile mesenchymal cells and malignant cancers. It is a key mesenchymal marker protein with essential roles in EMT signal transduction pathways, cell migration, invasion and metastasis (Kidd, et al., 2014). Furthermore, vimentin is associated with poor prognosis in breast, prostate, and lung cancer and melanoma (Liu, 2015).

1.7.5 Eukaryotic Translation Initiation Factor 3 Subunit E (EIF3E)

Eukaryotic translation initiation factor 3 subunit E (eIF3e) is a component of the eIF3 multi-subunit translation initiation complex and is mainly involved in ribosome recruitment to mRNA (Gillis & Lewis, 2015). Studies have suggested a role for eIF3e in breast cancer development. Marchetti and colleagues showed that in 21-28% of breast tumors there is a loss of heterozygosity for the eIF3e gene and overall the expression of eIF3e is decreased in 37% of breast tumors (Marchetti, et al., 2001). Gillis et al. showed that reduced eIf3e expression promotes EMT by increasing the expression of the EMT TFs Snail1 and Zeb2, which promotes invasion and migration in breast epithelial cells (Gillis & Lewis, 2015).

1.7.6 Role of EMT in Cancer

Metastasis is responsible for 90% of cancer-related deaths (Pradella, 2017). The EMT process aids in the dissemination of cancer cells from solid tumors so that they can metastasize to secondary regions in the body. The EMT process also provides cancer cells

with many advantages including increased cell survival, stemness and drug resistance, mainly through EMT TFs (Huang 2013).

The EMT process aids cancer cells in overcoming cell death because many of the EMT TFs also act to increase key survival pathway activation, such as the MAPK and PI3K/Akt survival pathways (Pradella, 2017). EMT is also associated with increased stemness and many studies have shown that cells with a mesenchymal phenotype also show greater stem cell character (Fabregat, 2016). Specifically, the EMT-TFs Zeb1, Snail1 and Snail2 all increase the expression of proteins involved in stemness. For example, Zeb1 has been shown to increase the expression of the stem cell proteins SRY-box 2 (Sox2) and kruppel-like 4 (Klf4) (Forte, 2017). As well, over-expression of both Twist and Snail TFs promote a mesenchymal phenotype with stem cell characteristics (high CD44 expression and low CD24 expression) and increased mammosphere formation capacity (Kim, 2017). Both EMT and stemness also play a role in helping cancer cells develop resistance to treatments like chemotherapy (Forte, 2017).

1.7.7 Partial EMT

When studying EMT, many studies only look at the extreme ends of the process, epithelial and mesenchymal states, but intermediate states also exist. Cells do not simply transition from an epithelial state to a mesenchymal state. In reality, EMT is a flexible process, and cells transition through a spectrum of intermediate states. In some cases, a partial EMT state can be the final phenotype in which a cell remains (Nieto, 2016). These intermediate states are often called partial EMT states and are found to be a worse prognosis for cancer patients compared to a complete mesenchymal phenotype due to its

increased plasticity. This increased epithelial/mesenchymal plasticity is highly favorable for metastasis formation (Kim, 2017).

Partial EMT includes a wide range of cellular expression profiles. Many studies identify partial EMT by the existence of both epithelial and mesenchymal markers, but partial EMT can also include cells that have a slight decrease in epithelial markers or characteristics and have not yet gained any mesenchymal markers or characteristics (Nieto, 2016). Huang and researchers studying 43 ovarian cancer cell lines saw that out of all the cell lines with a partial EMT phenotype, only half showed increased N-cadherin expression (Huang, et al., 2013). Researchers Huang et al. identified four EMT subgroups of ovarian cancer cells by looking at the level of expression of E-cadherin (E-cad), pan-cytokeratin (PCK) and vimentin (Vim). The four subgroups included **epithelial** (E-cad positive, PCK positive, Vim negative), **intermediate E** (E-cad positive, PCK-positive, Vim positive), **intermediate M** (E-cad negative, PCK positive, Vim positive), and **mesenchymal** (E-cad negative, PCK negative, Vim positive) (Huang, et al., 2013). Huang et al. determined that the intermediate M cells were the most anoikis-resistant, invasive and migratory and had a higher spheroid-forming ability than fully mesenchymal cells.

Recent studies have found that the co-expression of both an epithelial marker (most often E-cadherin) concurrently with mesenchymal markers is a hallmark of a "partial" EMT cellular phenotype. (Huang, et al., 2013; Yu et al., 2013). Andriani and colleagues identified a partial EMT phenotype in lung cancer cells, where cells co-expressed the mesenchymal marker SNAI2 and the epithelial marker E-cadherin (Andriani, et al., 2015). Lu et al. determined that partial EMT could be identified by the co-expression of the mesenchymal marker Zeb and the epithelial marker E-cadherin (Lu,

et al., 2013). As well, Jeevan et al. identified partial EMT to be the co-expression of the mesenchymal marker vimentin and the epithelial marker E-cadherin in metastatic brain tumor samples (Grigore, et al., 2016).

Triple negative breast cancer (TNBC) is a subtype of breast cancer with very poor outcomes because of the lack of targeted therapies. Studies have shown that in comparison to other breast cancers, TNBC has a higher proportion of cells that express a partial EMT phenotype (Yu, et al., 2013). As well, it has been shown that partial EMT is associated with poor outcomes and aggressiveness in other cancer types. This research indicates that there is an association between cancer aggressiveness and partial EMT (Jolly, et al., 2015).

Partial EMT provides cancer cells with several advantages that increase their tumorigenic potential. First, because cells that have undergone partial EMT possess both epithelial and mesenchymal properties, these cells have both the ability to adhere to each other and still migrate within the body. This allows cells to undergo collective migration, meaning that cells migrate as cell clusters instead of as single cells. These cell clusters are able to leave the bloodstream more effectively, are more resistant to cell death and have a metastatic success rate of 50 times that of individually migrating cells (Jolly, et al., 2015). Secondly, a partial EMT phenotype also gives cells more plasticity- meaning that a cell can more easily switch from one phenotype to another than cells committed to a full epithelial or mesenchymal state (Jolly, et al., 2015). Finally, cells in a partial EMT state also show increased stemness traits and have a better ability to form mammospheres even in comparison to completely mesenchymal cells. In addition, partial EMT is linked to higher drug resistance (Gross-Wilde et al., 2015).

Partial EMT is not solely a cancer-related process. It has also been identified in normal development, wound healing and fibrosis processes and in circulating tumor cells (Nieto, 2016). During development, partial EMT occurs during the branching morphogenesis of the trachea and mammary gland (Nieto, 2016). Figure 1.8 represents the partial EMT phenotype and how it relates to epithelial and mesenchymal phenotypes.

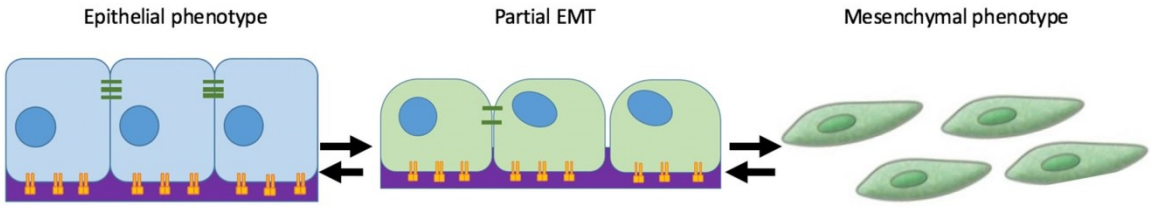

Figure 1.8: Partial epithelial-to-mesenchymal transition (EMT). Cells do not simply transition from an epithelial state to a mesenchymal state. EMT is a flexible process, and cells transition through a spectrum of intermediate states. Often times, a partial EMT state can be the final state of a cell.

1.8 Connection Between Anoikis Resistance and EMT

EMT TFs often promote anoikis resistance through the constitutive activation of certain pro-survival signals. For example, the transcription factors Snail and Zeb inhibit E-cadherin transcription, which promotes anoikis resistance through the activation of the PI3K/Akt pathway (Paoli, et al., 2016).

The tropomyosin receptor kinase B (TrkB) is a member of the Trk family of receptor tyrosine kinases, along with tropomyosin receptor kinase A (TrkA) and tropomyosin receptor kinase C (TrkC) and is a key receptor in both anoikis resistance and EMT pathways. The Trk family plays an important role in neuron development, function, survival and proliferation (Lange, 2018). Neurotrophins are the ligands for the Trk receptors. Neurotrophin-4/5 (NT-4/5) and neurotrophin brain-derived neurotrophic factor (BDNF) are the specific ligands of TrkB, but BDNF is the most well-studied of the two in terms of TrkB signaling pathways (Tabjbakhsh, 2017; Tanakam 2014). The TrkB receptor is a transmembrane receptor formed from three domains: an extracellular ligand binding domain, a transmembrane domain and a cytoplasmic tyrosine kinase domain. The kinase domain is required for its function. Following binding of BDNF to TrkB, receptor dimerization occurs, which results in kinase activation, autophosphorylation of the receptor and the activation of downstream signaling pathways like RAS/MAPK and PI3K/AKT (Tabjbakhsh, 2017; Tanakam 2014).

In addition to its role in the development of the nervous system, TrkB has been found to be over-expressed in a wide range of cancer types, including breast and ovarian cancer (Smit, 2011) and its signaling is linked with angiogenesis, increased tumor progression, invasion, metastasis and resistance to treatment (Tabjbakhsh, 2017; Tanaka, 2014). TrkB was first identified as an anoikis suppressor by Douma and colleagues in a

genome-wide screen for genes involved in anoikis resistance. These researchers found that through the activation of the PI3K signaling pathway, TrkB makes cells become resistant to anoikis and therefore increases their metastatic potential (Douma, et a., 2004). TrkB is also associated with inducing EMT, which promotes the migration and invasion of cells. Smit and colleagues found that E-cadherin and the EMT TFs Twist, Snail and Zeb1 are all required for the tumorigenic effects of TrkB signaling. These researchers developed a model to show how all these factors play a role in TrkB signaling, anoikis resistance, EMT and metastasis (Smit, 2011). The model is illustrated in Figure 1.9.

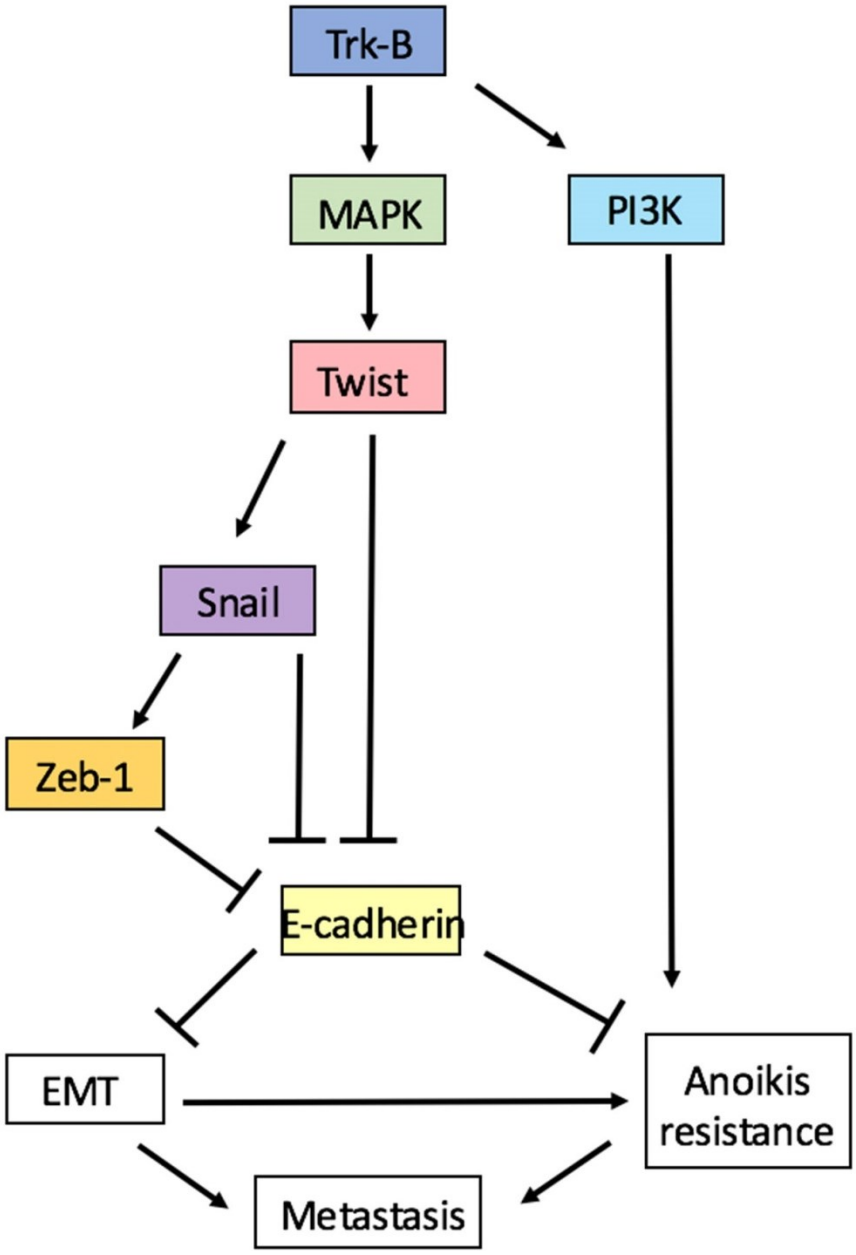

Figure 1.9: A model developed by Smit and colleagues that demonstrates the connections between TrkB signaling, anoikis resistance, EMT and metastasis.

1.9 MicroRNAs

1.9.1 MicroRNA Overview

MicroRNAs (miRNAs) are small (18-24 nucleotides in length) non-coding RNA molecules that interfere with gene expression by targeting mRNA for degradation or repressing its translation. The first miRNA, lin-4, was identified in 1993 in *C.elegans* by Vicor Ambros and colleagues (Lee, et al., 1993; Wightman, 1993), and in 2000, the first human miRNA, let-7, was identified (Pasquinelli, et al., 2000). Today there have been about 2,675 human miRNAs identified (Saliminejad, 2018).

Most often, miRNAs function by binding to the 3' UTR (untranslated region) of mRNA, but interaction with the 5' UTR, coding sequence, and gene promoters has also been shown. MiRNAs play important roles in animal development and in many other biological processes, but the dysregulation of miRNAs is associated with human diseases, such as cancer (O'Brien, 2018).

1.9.2 Biogenesis of MicroRNAs

MiRNAs can be either intragenic (transcribed from introns (mainly) and exons of protein coding genes) or intergenic (transcribed autonomously from protein coding genes). MiRNA biogenesis can occur via two pathways: canonical and non-canonical, but the canonical pathway is the most prominent, and will be the only pathway discussed here (O'Brien, 2018). During canonical biogenesis of miRNAs, pri-miRNAs are transcribed from the genome by polymerase II. These pri-miRNA molecules can be several hundred to a thousand nucleotides in length. Pri-miRNAs are then cleaved into ~70 nucleotide stem-loop structures called pre-miRNAs by a Microprocessor complex consisting of a ribonuclease III enzyme (Drosha) and a RNA binding protein DGCR8 (DiGeorge

syndrome critical region gene 8) (Denli, et al., 2004). DGCR8 identifies specific motifs within the pri-miRNA (Alarcon, et al., 2015), whereas Drosha cleaves the structure, ultimately resulting in pre-miRNA molecules. Drosha cleavage results in a 5'phosphate at one end and a 2-nt 3' overhang at the other end of the pre-miRNA, which is recognized by exportin-5. Following their generation, pre-miRNAs are exported from the nucleus into the cytoplasm by an exportin-5 (XPO5)/RanGTP complex where they are cleaved by the RNase III endonuclease Dicer (Denli, et al., 2004). Once in the cytoplasm, Dicer removes the terminal loop of the pre-miRNA, forming a mature miRNA molecule (Zhang, et al., 2004). The mature miRNA molecule that is formed from the 5' end of the pre-miRNA is called the 5p strand and the molecule that is formed from the 3' end of the pre-miRNA is called the 3p strand. In an ATP-dependent manner, both the 3p and the 5p strand can then be loaded into the Argonaute (AGO) family of proteins. The AGO protein, in complex with its loaded strand (guide strand), forms the miRNA-induced silencing complex (miRISC). The percentage of 3p or 5p strands loaded into the AGO proteins is dependent on the cell type, environment and the stability of the strands (Meijer, et al., 2014; Khvorova, et al., 2003). Often, one strand (3p or 5p) will form a complex with the AGO protein, while the other strand is degraded. Once mature miRNA molecules are loaded onto the AGO proteins, forming the miRISC complex, these complexes together with GW182 proteins can then target complementary mRNA sequences called miRNA response elements (MREs) and regulate gene expression by either mRNA degradation or the inhibition of translation (O'Brien, 2018). This process occurs within processing bodies (P-bodies) and is represented in Figure 1.10 (Lin, 2015).

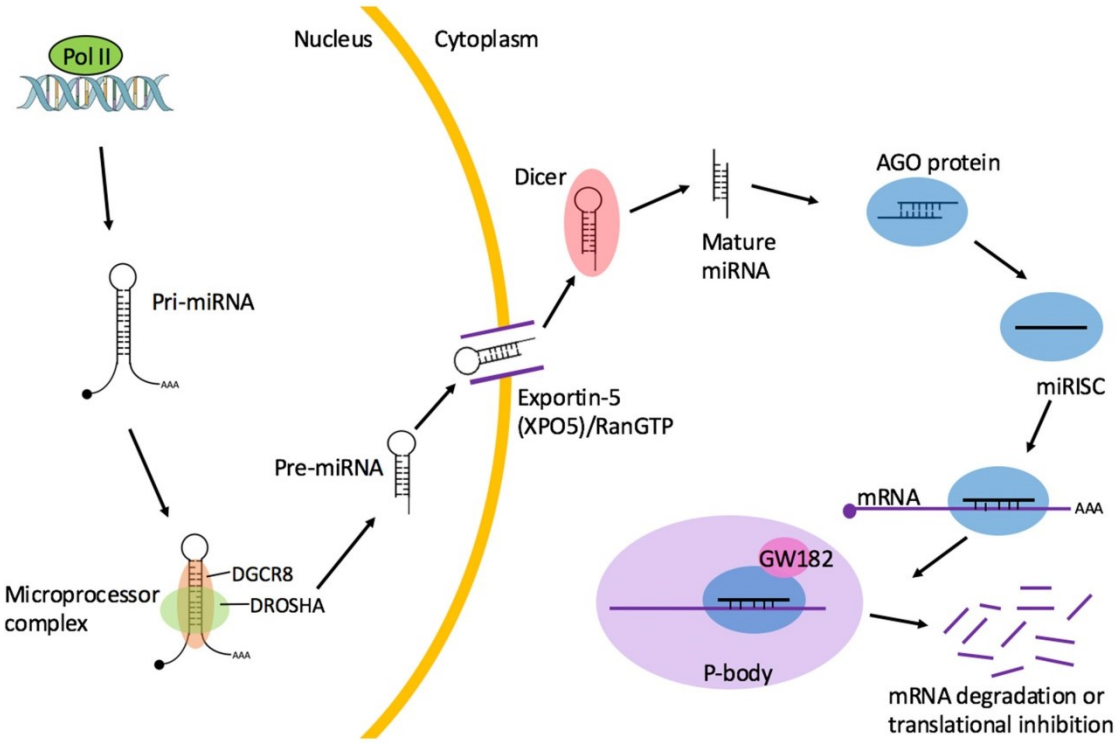

Figure 1.10: The biogenesis of microRNAs. During canonical biogenesis of miRNAs, pri-miRNAs are transcribed from the genome by polymerase II. Pri-miRNAs are then cleaved into structures called pre-miRNAs by a Microprocessor complex. Following their generation, pre-miRNAs are exported from the nucleus into the cytoplasm by an exportin-5 (XPO5)/RanGTP complex where they are cleaved by the RNase III endonuclease Dicer. Once in the cytoplasm, Dicer removes the terminal loop of the pre-miRNA, forming a mature miRNA molecule. Both the 3p and the 5p strand can then be loaded into the Argonaute (AGO) family of proteins. The AGO protein in complex with its loaded strand (guide strand) form the miRNA-induced silencing complex (miRISC). Once mature miRNA molecules are loaded onto the AGO proteins, forming the miRISC complex, these complexes together with GW182 proteins can then target complementary mRNA sequences called miRNA response elements (MREs) and regulate gene expression by either mRNA degradation or the inhibition of translation.

1.9.3 Role of MicroRNAs in Cancer

Although miRNAs play important roles in several key physiological activities, they have also been identified to play a role in the development of cancer acting as either tumor suppressors or oncogenes (Cheng, et al., 2005). Several miRNAs function to repress the expression of tumor-suppressor genes with roles in cell cycle, apoptosis, cell proliferation and differentiation, migration, invasion and metastasis and have been found to be up-regulated in breast cancers. Several miRNAs play a tumor-suppressor role and these miRNAs are often down-regulated in breast cancers (Khordadmehr, 2018).

MicroRNAs exist that play a role in the EMT process through the regulation of EMT transcription factors and in anoikis resistance (Ding, 2014). The miR-200 family of microRNAs (miRs-201, -200b, -200c, -429, and -141) are expressed at a high level in epithelial cells and maintain the epithelial phenotype of cells by interacting with Zeb1 and Zeb2 TFs and repressing their expression. In fact, a double negative feedback loop exists between the miR-200 family and Zeb1/2 (Burk, et al., 2008; Bracken, et al., 2008). MiR-200 binds to the 3'UTRs of Zeb1/2 to repress their expression while Zeb1/2 binds to the promoter of miR-200 to inhibit its transcription (Burk, et al., 2008; Bracken, et al., 2008). Furthermore, the miR-200 family member, miR-200c has been found to inhibit TrkB, a known contributor of anoikis resistance in several cancer types (Howe, et al., 2011). The downregulation of miR-200c leads to the activation of pathways with a role in cell motility, EMT and anoikis resistance (Howe, et al., 2011). As well, microRNAs, miR-155 (Kong, et al., 2008) and miR-30a (Kumarswamy, et al., 2011), are known to play a role in EMT and anoikis resistance (Paoli, 2016).

MiRNAs that stimulate the transition from an epithelial phenotype to a mesenchymal phenotype have also been identified. Specifically, miR-9 (Ma, et al., 2010)

and miR-92a (Chen, et al., 2011) bind and repress the expression of E-cadherin, which promotes EMT and increases cell invasiveness. miR-103/107 down-regulate the expression of miR-200, which maintains the epithelial phenotype (Stinson, et al., 2011; Ding, 2014).

MiRNAs have also been found to play a direct role in cancer metastasis. For example, miR-335 and miR-126 both supress metastasis in breast cancer (Tavazoie, et al., 2008). MiR-21 has been identified as a pro-metastatic miRNA and is associated with several different cancer types (Ding, 2014; Asangani, et al., 2008; Lou, et al., 2010; Cottonham et al., 2010; Liu, et al., 2011).

1.9.4 MicroRNA-21

MicroRNA-21 (miR-21) is positioned on chromosome 17q21.3 and is over-expressed in the majority of cancer types including breast and ovarian cancers. Many studies have identified a role for miR-21 in cancer development, which involves increased cell proliferation, inhibition of apoptosis, and increased invasion and metastasis (Wu, 2017). High miR-21 expression is also correlated with increased disease aggressiveness, increased tumor size, higher stage and grade and poor disease-free survival (Lee, et al., 2011). For example, aggressive TNBC tissue express very high levels of miR-21 (Usman, 2015). Several targets of miR-21 are tumor suppressors, including PTEN, programmed cell death 4 (PDCD4), reversion-inducing cysteine-rich protein with Kazal motifs (RECK), forkhead box O1 (FOXO1), ras homolog gene family, member B (RhoB), tropomyosin 1 (TPM1), tissue inhibitor of metalloproteinase 3 (TIMP3), Spry1/2, and Maspin (Wu, 2017; Hug 2015, Bornachea, 2012).

As well, the over-expression of miR-21 has been linked to resistance to cancer therapies. For example, its over-expression has been associated with resistance to cisplatin in ovarian and gastric cancer cells (Chan, et al., 2014; Yang, et al., 2013). Wu and colleagues showed that increased miR-21, through the PTEN/AKT pathway plays a role in the resistance of breast cancer cells to gemcitabine through the induction of an EMT process (Wu, 2016).

Other studies have also identified a role for miR-21 in EMT. Han and researchers determined that in the TNBC cell line, MDA-MB-231, the knock-down of miR-21 leads to a reversal of the EMT process and cancer stem cell (CSC) phenotype through the inactivation of Akt and Erk1/2 pathways by activating PTEN expression (Han, et al., 2012). Moreover, Zeb1 is thought to be an up-stream regulator of miR-21 (Sahay, et al., 2015). Another study shows that the treatment of cells with TGF-β or Snail over-expression promotes the induction of EMT as well as an increase in miR-21 expression (Bornachea, 2012). In addition, increased miR-21 expression has also been linked to increased metastatic potential (Yan, et al., 2008).

1.10 Hippo Signaling

1.10.1 Hippo Signaling Overview

The Hippo pathway plays a fundamental role in organ growth control, stem cell function, regeneration and tumor suppression (Figure 1.10) (Zhao et al., 2010). The main role of the Hippo pathway is to negatively regulate the activity of yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ). YAP is found to be upregulated in many cancer types and its activation correlates with poor disease

outcome and increased metastatic potential (Sharif, 2015). Activation of YAP/TAZ promotes cell proliferation and inhibits cell death (Johnson, 2013). A summary of the roles of YAP/TAZ are outlined in Figure 1.12. It has been shown that increased amounts of TAZ nuclear staining is associated with high-grade breast cancer (Bartucci, et al., 2015) and that levels of TAZ mRNA and protein is increased in the aggressive TNBC in comparison to other breast cancers (Diaz-Martin, et al., 2015).

The serine/threonine kinases MST1/2 (mammalian STE20-like protein kinase 1/2) in complex with the scaffolding protein, SAV1 (protein Salvador homolog 1), phosphorylate and activate the serine/threonine kinases LATS 1/2 (large tumor suppressor kinase 1/2) and its scaffolding protein MOB1 (mps one binder kinase activator-like 1). LATS 1/2 then phosphorylate YAP/TAZ, inhibiting its binding to the transcription factor TEAD, which prevents the nuclear accumulation and activation of YAP/TAZ. YAP/TAZ are transcriptional coactivators that shuttle between the cytoplasm and the nucleus. Inactivation of the Hippo pathway promotes the nuclear localization of YAP/TAZ. Because YAP/TAZ lack DNA-binding ability, they bind and form complexes with TEAD and other transcription factors in order to activate the gene expression of target genes (Johnson, 2013). The Hippo signaling pathway is represented in Figure 1.11.

The Hippo pathway does not have any specific extracellular signaling peptides or receptors that have been identified, but is regulated by several upstream components and mechanisms that are involved in regulating cell adhesion and cell polarity (Johnson, 2013). Merlin is a tumor suppressor that regulates the Hippo pathway through its activation of the LATS kinase, which prevents nuclear localization of YAP/TAZ (Cooper, 2014).

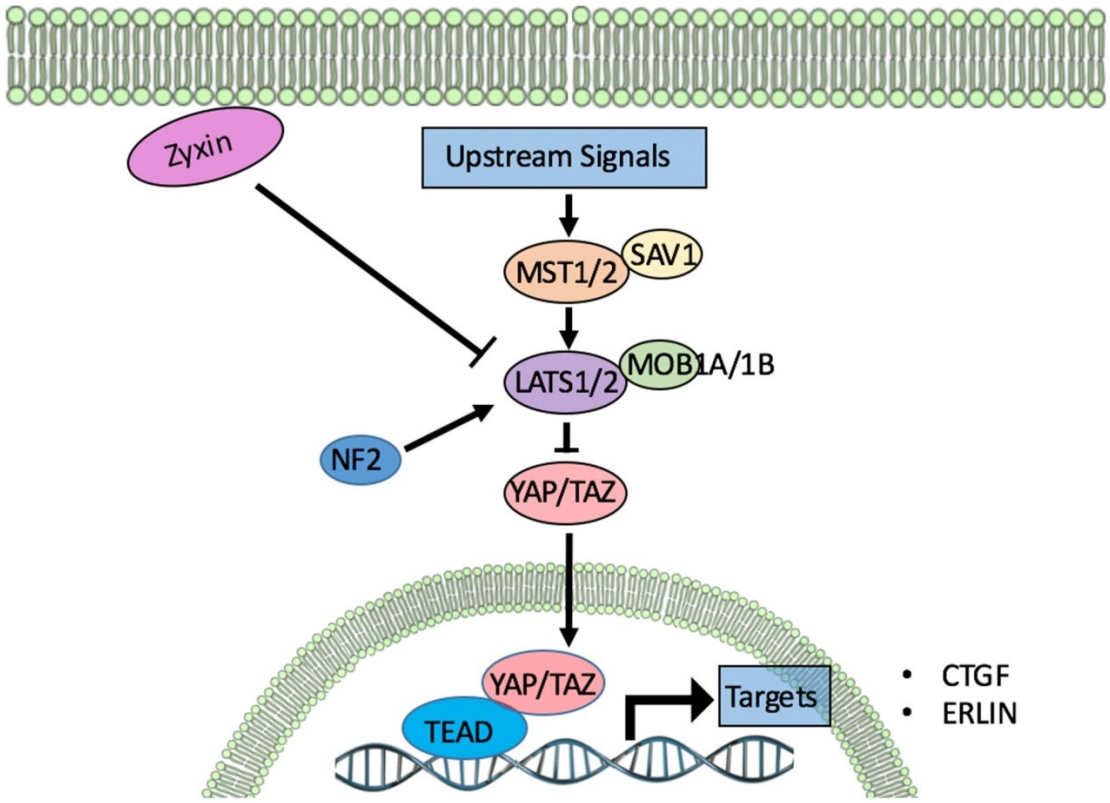

Figure 1.11: Hippo signaling overview. The Hippo signaling pathway is the main regulator of YAP/TAZ activity. The serine/threonine kinases MST1/2 in complex with the scaffolding protein SAV1 phosphorylate and activate the serine/threonine kinases LATS 1/2 and its scaffolding protein MOB1. LATS 1/2 phosphorylate YAP/TAZ, inhibiting its binding to the transcription factor TEAD, which prevents the nuclear accumulation and activation of YAP/TAZ. YAP/TAZ are transcriptional coactivators that shuttle between the cytoplasm and the nucleus and promote the transcription of genes involved in cell proliferation, growth and cell survival.

1.10.2 Role of Hippo Signaling in Cancer

Studies show that the Hippo pathway plays a role in the function of stem cells. For example, the overexpression of TAZ has been shown to increase the ability of normal MCF10A cells to form mammospheres, indicating TAZ over-expression increases the stem cell properties of these cells. In addition, TAZ is over-expressed in ~85% of high-grade human breast cancers (Johnson, Halder, et al., 2014). It is thought that YAP/TAZ activation promotes stemness properties in cells through the inhibition of NOTCH, a tumor suppressor (Totaro 2016).

Researchers found that overexpressing YAP and/or TAZ promotes the acquisition of a mesenchymal phenotype in mammary epithelial cells, suggesting that the Hippo pathway plays an important role in supressing EMT (Lei, et al., 2008). The presence of E-cadherin at adherens junctions reduces the nuclear localization and activity of YAP by activating the Hippo pathway through the MST kinase (Kim, et al., 2011). Another outcome of high expression of YAP/TAZ is chemotherapy resistance. For example, high levels of both YAP and TAZ are associated with drug resistance, including taxol resistance (Lai, et al., 2011). Studies show that high YAP nuclear expression is associated with higher tumor grade in TNBC patients (Diaz-Martin, et al., 2015). As well, Andrade et al., show that the inhibition of YAP increases the radiosensitivity of TNBC cells by inhibiting the EGFR/PI3K/AKT pathway (Andrade, 2017). The various cellular pathways activated by YAP/TAZ signaling are summarized in Figure 1.12.

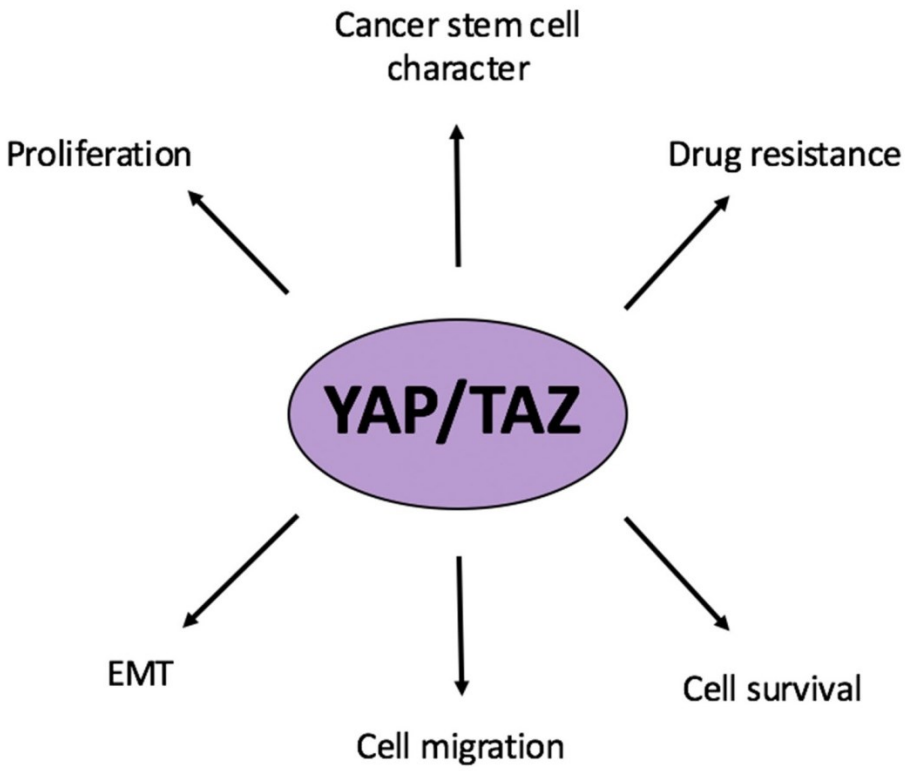

Figure 1.12: YAP/TAZ pathway activation. YAP/TAZ are transcriptional activators of genes involved in proliferation, EMT, cell migration, cell survival, drug resistance and cancer stem cell character.

Chapter 2: Materials and Methods

2.1 Cell Culture

Cell lines used and growth conditions are listed in Table 2.1. All cell lines were grown at 37°C with 5% CO_2. ID8 cells stably expressing GIPZ shRNAs were cultured with the addition of 5 μg/mL puromycin. HeLa, MCF-7 and MDA-MB-231 cells stably expressing TRIPZ shRNAs were cultured using Tetracycline-Free FBS and 2 μg/mL puromycin. MCF10A and HMLE cells stably expressing TRIPZ shRNAs were cultured with the addition of 2 μg/mL and 5 μg/mL puromycin, respectively.

Table 2.1: *In vitro* cell lines and growth conditions.

Cell line	Organism	Tissue	Cell type	Tumorigenic	Growth media
HeLa	Human	Cervix	Epithelial	Yes	DMEM supplemented with 10% FBS, 1% P/S
HMLE	Human	Mammary gland	Epithelial	No	DMEM/F12 supplemented with 5% horse serum, 1% P/S, 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 10 ng/mL EGF
ID8	Mouse	Ovary	Epithelial	Yes	DMEM supplemented with 5% FBS, 1% P/S
MDA-MB-231	Human	Mammary gland: metastatic site	Epithelial	Yes	DMEM supplemented with 10% FBS, 1% P/S
MCF-7	Human	Mammary gland: metastatic site	Epithelial	Yes	DMEM supplemented with 10% FBS, 1% P/S
MCF10A	Human	Mammary gland	Epithelial	No	DMEM/F12 supplemented with 5% horse serum, 1% P/S, 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 10 ng/mL EGF, 1 ng/mL cholera toxin

2.2 shRNA Lentiviral Transduction

To generate the GIPZ and TRIPZ shPRP4K cell lines, GIPZ lentiviral shRNAs (shPRP4K-1=clone: V3LMM_463192, shPRP4K-2=clone: V3LMM_463191, Non-silencing shCtrl=RHS4346) and TRIPZ inducible lentiviral shRNAs (shPRP4K-1=clone: V3THS_383962, shPRP4K-2=clone: V3THS_383960, Non-silencing shCtrl=RHS4743) were purchased from Thermo Scientific. Lentivirus was made by co-transfection of a GIPZ/TRIPZ shRNA with pMPD2.G and psPAX2 packaging vectors into human HEK-293T cells using calcium-phosphate transfection. Early the next morning (max 16 hours later), media was changed on the cells. Forty-eight hours following transfection, the media from the transfected cells was filtered using a 0.45 μm filter and 500 μl was added to the target cell line with 8 μg/mL polybrene for 48 h (first transduction). Following the 48 hours, the target cells were split and then transduced a second time (another 48 hours) with virus, in order to increase the transfection efficiency. Following the transductions, cells were split and allowed to recover in fresh media for 24 hours. To select for infected cells, cells were cultured using 2-5 μg/mL puromycin. The puromycin-resistant cells were then expanded. To induce the expression of the TRIPZ shRNA, 5μg/mL doxycycline was added to cell media for 96 hours, with the media being replaced every 24 hours.

2.3 Twist Retroviral Transduction

To generate the HMLE Twist over-expressing cell lines, retrovirus was made by transfecting pBABE-puro-mTWIST and pBABE-puro plasmids into Pheonix cells using calcium-phosphate transfection. Chloroquine (25 μM) was added to the Pheonix cells 5 minutes before transfection. Early the next morning (maximum 16 hours later), the cells

were washed and the media was changed in order to get rid of any excess chloroquine. Forty-eight hours following transfection, the media from the transfected cells was filtered using a 0.45 μm filter and 500 μl was added to the target cells with 8μg/mL polybrene for 48 h (first transduction). Following the 48 hours, the target cells were split and then transduced a second time (another 48 hours) with virus, in order to increase the transfection efficiency. Following the transductions, cells were split and allowed to recover in fresh media for 24 hours. To select for infected cells, cells were cultured using 2 μg/mL puromycin. The puromycin-resistant cells were then expanded.

2.4 siRNA

On-TARGETplus SMARTpool E-cadherin siRNA and non-targeting control siRNA were purchased from Dharmacon. siRNA experiments were performed using DharmaFECT transfection reagent according to the manufacturer's instructions. Cells were harvested 4 days following transfection for western blot analysis.

2.5 EMT Induction Media

StemXVivo EMT Inducing Media Supplement was purchased from R&D Systems. Cells were plated in 10 cm plates (HMLE- 0.5 x 10^6 cells; MCF10A- 1.5 x 10^6 cells) with 6 ml of normal media. Sixty microliters of 1X StemXVivo EMT Inducing Media Supplement was added to each plate. Three days following cell plating, media was removed and replaced with fresh media and supplement. Five days following the initial plating of cells, plates were harvested for analysis.

2.6 Scratch Assay

Cells were plated so that plates would be 90-100% confluent 5 days after plating. Twenty-four hours after plating the cells, doxycycline was added to the TRIPZ inducible cells. Doxycycline was replaced every 24 hours. Ninety-six hours following doxycycline induction, a scratch was made using a P200 pipette tip. Twenty-four hours before a scratch was made in the cell monolayer, the media was replaced with low-serum media (0.5% serum) in order to prevent cell growth. Scratches were imaged every 4 hours and overall imaging time was dependent on the cell line. The area of the scratch was calculated using ImageJ software.

2.7 Anoikis Assay using Poly-HEMA Coated Plates

A 20 mg/ml solution of poly 2-hydroxyethyl methacrylate (Poly-HEMA) was made by dissolving it in 95% ethanol and stirring at 65°C. Ten cm tissue culture plates were coated with 4 ml of the Poly-HEMA solution and dried at 37°C for 24 hours. In order to look at changes in protein expression under non-adherent conditions, cells were plated on poly-HEMA coated and regular plates for 24 hours and then harvested for western blot analysis.

2.8 Western Blot Analysis

Cells were harvested and lysed in lysis buffer (20mM Tris-HCl ph8, 300 mM KCl, 10% Glycerol, 0.25% Nonidet P-40, 0.5 mM EDTA, 0.5 mM EGTA, 1X protease inhibitors (phenylmethylsulfonyl fluoride (PMSF) and P8340) for 15 minutes on ice. Samples were sheared using a 22-gauge needle and then incubated for 15 minutes on ice.

To isolate the protein, cells were pelleted by centrifugation at 14 800 rpm for 25 minutes at 4°C. Protein concentrations were determined using Bio-Rad Protein Reagent. Samples were mixed 1:1 with 2x sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl ph 6.8) and boiled for 5 minutes. Samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. Acrylamide gels from 7-15% were used, depending on the size of the protein of interest. The membranes were blocked using either 5% milk or bovine serum albumin (BSA) dissolved in Tris-buffered saline with Tween 20 (TBST). The membranes were then incubated in primary antibody over-night at 4°C, with the exception of actin which was incubated at room temperature for 1 hour. The membranes were then washed with TBST 3 times for 5 minutes each and then incubated in secondary antibody at room temperature for 1 hour. The membranes were washed again 3 times for 5 minutes each. Proteins were visualized using BioRad Clarity Western ECL substrate and radiographic film. Table 2.2 outlines all antibodies and dilutions that were used.

Table 2.2: Antibodies and dilutions used for western blot analysis and immunofluorescence.

Antibody	Company (Product number)	WB Dilution (IF)
Akt (pan) (C67E7) Rabbit	Cell signaling (4691)	1:1000
P-Akt (Ser473) (D9E) Rabbit	Cell signaling (4060)	1:1000
Beta-actin	Sigma (A2228)	1:10 000
Beta-catenin (D10A8) Rabbit	Cell signaling (8480)	1:1000
Beta-tubulin	Santa Cruz Biotechnology (sc-9104)	1:2000
Bcl-2 (D17C4)	Cell signaling (3498)	1:1000
Bcl-xL (54H6) Rabbit	Cell signaling (2764)	1:1000
Claudin-1 (D5H1D) Rabbit	Cell signaling (13255)	1:1000
Cleaved caspase 3 (Asp175) (5A1E)	Cell signaling (9664)	1:1000
E-cadherin (24E10) Rabbit	Cell signaling (3195)	1:10 000
EIF3E	Abcam (ab134958)	1:1000
ERK1/2 (137F5) Rabbit	Cell signaling (4695)	1:1000
P-ERK1/2 (Thr202/Tyr201) (E10) Mouse	Cell signaling (9106)	1:1000
Fibronectin	Abcam (ab32419)	1:1000
N-cadherin (13A9)	Santa Cruz Biotechnology (sc-59987)	1:1000
PRP4K Sheep & Rabbit	Dellaire Lab & Novus Biologicals (NBP1-82999)	1:1000 & 1:2000
Slug (C19G7) Rabbit	Cell signaling (9585)	1:1000
Snail (C15D3) Rabbit	Cell signaling (3879)	1:1000
Trk-B (F-1)	Santa Cruz Biotechnology (sc-377218)	1:1000
Twist	Santa Cruz Biotechnology (sc-81417)	1:1000
Vimentin (D21H3) Rabbit	Cell signaling (5741)	1:1000
Yap1 Mouse	Sigma (WH0010413M1)	1:1000 (1:33)
TCF8/Zeb-1 (D80D3) Rabbit	Cell signaling (3396)	1:1000
Zo-1 (D7D12) Rabbit	Cell signaling (8193)	1:1000

2.9 Immunofluorescence

Coverslips were sterilized using 95% EtOH and placed into wells of a 6-well plate. Cells were then plated into the 6-well plates and left to adhere to the coverslips overnight. For the TRIPZ inducible cell lines, 5 μg/mL doxycycline was added to the cells 24 hours after plating and the media was replaced every 24 hours. Ninety-six hours after beginning doxycycline treatment, cells were washed with PBS and then fixed in 3% paraformaldehyde for 30 minutes. Coverslips were washed 3 times in PBS for 5 minutes and then permeabilized using 0.5% TritonX-100 for 5 minutes. Cells were washed again 3 times in PBS for 5 minutes each. Cells were blocked in 5% donkey serum in PBS for 20 minutes and then incubated with primary antibody for 1 hour. Cells were washed 3 times with PBS for 5 minutes each and then incubated with Alexa Fluor secondary antibodies diluted 1:200 in 5% donkey serum in PBS. Coverslips were washed 3 times with PBS for 5 minutes each. The second wash was 10 minutes instead of 5 minutes and 4',6-diamidino-2-phenylindole (DAPI) was added to this wash at a dilution of 1:1000. Coverslips were mounted on frosted glass microscope slides using VECTASHIELD mounting medium. Images were taken using a Zeiss Cell Observer Microscope. Table 2.2 outlines all primary antibodies and dilutions that were used.

2.10 YAP Nucleo:Cytoplasmic Intensity Ratio Determination

MCF10A shCtrl and shEIF3E cells and HMLE shCtrl and Twist OE cells were plated onto sterile coverslips, fixed and immunolabeled as described in section 2.9. Fluorescent images were taken with a Zeiss Cell Observer Microscope under a 40x immersion oil objective lens to compare YAP expression intensities. Thirty-four cells of

each cell type and condition were analyzed. In order to determine the intensity of YAP signal in the nucleus and cytoplasm of single cells, Z-projections of the images were performed. The mean signal intensities were determined using the Slidebook software (Intelligent Imaging Innovation, Boulder, CO). The nuclear: cytoplasmic ratio of YAP expression was calculated for each cell and averages were taken for each cell type and condition.

2.11 RNA Isolation and Quantitative Reverse Transcription PCR (RT-qPCR)

Cell samples were lysed and homogenized using Trizol reagent according to the manufacturer's directions and samples were frozen at -80°C. RNA was isolated using the Ambion PureLink RNA Mini Kit according to the manufacturer's protocol and includes an on-column DNase I digestion. RNA quantity and quality were measured using a Nanodrop 2000 spectrophotometer. Absorbance measurements A260/A280 and A260/A230 with ratios ~ 2.0 were accepted as pure for RNA. One microgram of RNA was reverse-transcribed to cDNA using the BioRad 5X iScript RT supermix kit for RT-qPCR, after which samples were diluted 1:1 with nuclease-free water. Samples without reverse transcriptase were included to confirm no genomic DNA contamination. Quantitative PCR (qPCR) was performed on cDNA samples using the 2X SsoAdvanced Universal SYBR Green Supermix. The BioRad CFX Connect was used to perform the reactions and all experiments were done in triplicate. All primers used were designed using NCBI Primer Blast. All primer sequences are summarized in Table 2.3. Gene expression data was normalized to at least two reference genes and analyzed using the BioRad CFX Maestro Software.

2.12 RNA Isolation and Quantitative Reverse Transcription PCR (RT-PCR) for MicroRNA

RNA was isolated from samples using the Ambion mirVana miRNA isolation kit according to the manufacturer's directions. RNA quantity and quality was measured using a Nanodrop 2000 spectrophotometer. Absorbance measurements A260/A280 and A260/A230 with ratios ~ 2.0 were accepted as pure for RNA. One hundred nanograms of RNA was reverse-transcribed to cDNA using the Taqman microRNA Reverse Transcription kit and primers specific to the 3' and 5' strands of miR-21 according to the manufacturer's directions. U6 was used as a endogenous control and the *C.elegans* miR-39 spike-in control was used as an exogenous control. All data was normalized to these two controls. RT-qPCR was performed according to the Taqman protocol and a BioRad CFX Connect was used to perform the reaction. All experiments were done in triplicate and data was analyzed using the BioRad CFX Maestro Software.

Table 2.3: RT-qPCR primer sequences.

Gene	Forward primer sequence	Reverse primer sequence	Reference ?
B2M	AGGCTATCCAGCGTACTCCA	CGGATGGATGAAACCCAGACA	YES
CCN2	AATGCTGCGAGGAGTGGGT	CGGCTCTAATC ATAGTTGGGTCT	NO
CDH1	GTCAGTTCAGACTCCAGCCC	AAATTCACTCTGCCCAGGACG	NO
CDH2	ATGTGCCGGATAGCGGGAGC	TACACCGTGCCGTCCTCGTC	NO
CLDN1	TTGGGCTTCATTCTCGCCTT	GTCGCCGGCATAGGAGTAAA	NO
CTNNB 1	AAAATGGCAGTGCGTTTAG	TTTGAAGGCAGTCTGTCGTA	NO
ERLIN1	CCCAGTGGACCAGGCTATCA	GACCCCACCACTTGTTCCAC	NO
FN1	CCCACCGTCTCAACATGCTTAG	CTCGGCTTCCTCCATAACAAGT AC	NO
HPRT1	TTGCTTTCCTTGGTCAGGCA	ATCCAACACTTCGTGGGGTC	YES
PRPF4B	CGTTCCCCACTCAGACGTAG	GACCTCTGTCCCTTCTCCGA	NO
PUM1	GGCGTTAGCATGGTGGAGTA	CATCCCTTGGGCCAAATCCT	YES
SNAI1	ACCACTATGCCGCGCTCTT	GGTCGTAGGGCTGCTGGAA	NO
SNAI2	TGTTGCAGTGAGGGCAAGAA	GACCCTGGTTGCTTCAAGGA	NO
TBP	GGCACCACTCCACTGTATCC	GCTGCGGTACAATCCCAGAA	YES
TEAD1	CTGAGTCGCAGTTACCACCA	AGCCTGGAGCCTTTTCAAG	NO
TEAD2	ACATGATGAACAGCGTCCTG	CAGCAGTTCCTGGGTGTCTC	NO
TJP1	GGGACAACAGCATCCTTCCA	ATCACAGTGTGGTAAGCGCA	NO
VIM	TCTACGAGGAGGAGATGCGG	GGTCAAGACGTGCCAGAGAC	NO
ZEB1	GTTCTGCCAACAGTTGGTTT	GCTCAAGACTGTAGTTGATG	NO
ZYX	GCAGAATGTGGCTGTCAACGA AC	TGAAGCAGGCGATGTGGAACA G	NO

Chapter 3: Results

3.1 Knockdown of PRP4K Increases the Expression of TrkB, a Marker of Anoikis Resistance, in ID8 and MCF-7 Cell Lines.

As previously discussed, the Dellaire laboratory has used the zebrafish xenotransplantation model to demonstrate that PRP4K loss is linked to increased anoikis resistance (Corkery et al., 2018). In this work, PRP4K control and knock-down (KD) ID8 mouse ovarian cells were injected into the suspended yolk sac environment of the zebrafish. The KD cells grew two times better, suggesting their increased resistance to anoikis. Furthermore, using *in vitro* spheroid assays, we showed that PRP4K KD increases the anchorage independent growth of ID8 cells as spheroids (Corkery et al., 2018). Using the soft agar colony formation assay, our lab has shown the same anoikis resistance phenotype can be replicated in the MCF-7 breast cancer cell line (Dellaire lab, unpublished). Together, this data demonstrates that loss of PRP4K promotes anoikis resistance in breast and ovarian cancer cells. To further observe the effects of decreased PRP4K expression, I created constitutive mouse (ID8) and inducible human (MCF-7, HeLa, MDA-MB-231, HMLE and MCF10A) cell lines stably expressing a control hairpin (shCtrl) or a hairpin that targets PRP4K (shPRP4K). For each cell line, two independent KD cell lines using different shRNAs were used to account for possible off-target effects of the shRNAs. To determine what pathways play a role in the anoikis resistance phenotype that has been observed with PRP4K KD, ID8 and MCF-7 cell lines were grown in both attached and suspended conditions using Poly-HEMA coated plates. Cells were grown in these conditions for 24 hours and then harvested for western blot analysis.

Following PRP4K KD, PRP4K protein expression was significantly decreased in both the ID8 and MCF-7 cell lines (Figure 3.1 A & B). PRP4K KD in the ID8 cell line led to an increase in TrkB expression (anoikis resistance factor) in attached conditions (Figure 3.1 A). As well, the pro-survival signaling factor Phospho-Akt (P-Akt) was increased with PRP4K KD in the ID8 cell line in comparison to control cells, when grown in attached and suspended conditions. There was no change in Akt and ERK expression (pro-survival signaling factors), but phospho-ERK expression decreased with PRP4K KD in the ID8 cells grown in attached conditions. No changes in Bcl-2, Bcl-xl (B-cell lymphoma-extra large) or cleaved caspase 3 (apoptosis factors) were observed with ID8 PRP4K KD cells grown in attached and suspended conditions.

In the MCF-7 cell line, TrkB expression was increased with PRP4K KD in attached conditions (Figure 3.1 B). No changes in Akt, P-Akt, ERK or P-ERK were seen with PRP4K KD in the MCF-7 cell line. Furthermore, no changes in Bcl-2 and Bcl-xl were observed with MCF-7 PRP4K KD cells grown in attached and suspended conditions. Since TrkB is known to promote anoikis resistance (Douma, et al., 2004), this data supports a role for TrkB in the anoikis resistance phenotype seen after depletion of PRP4K.

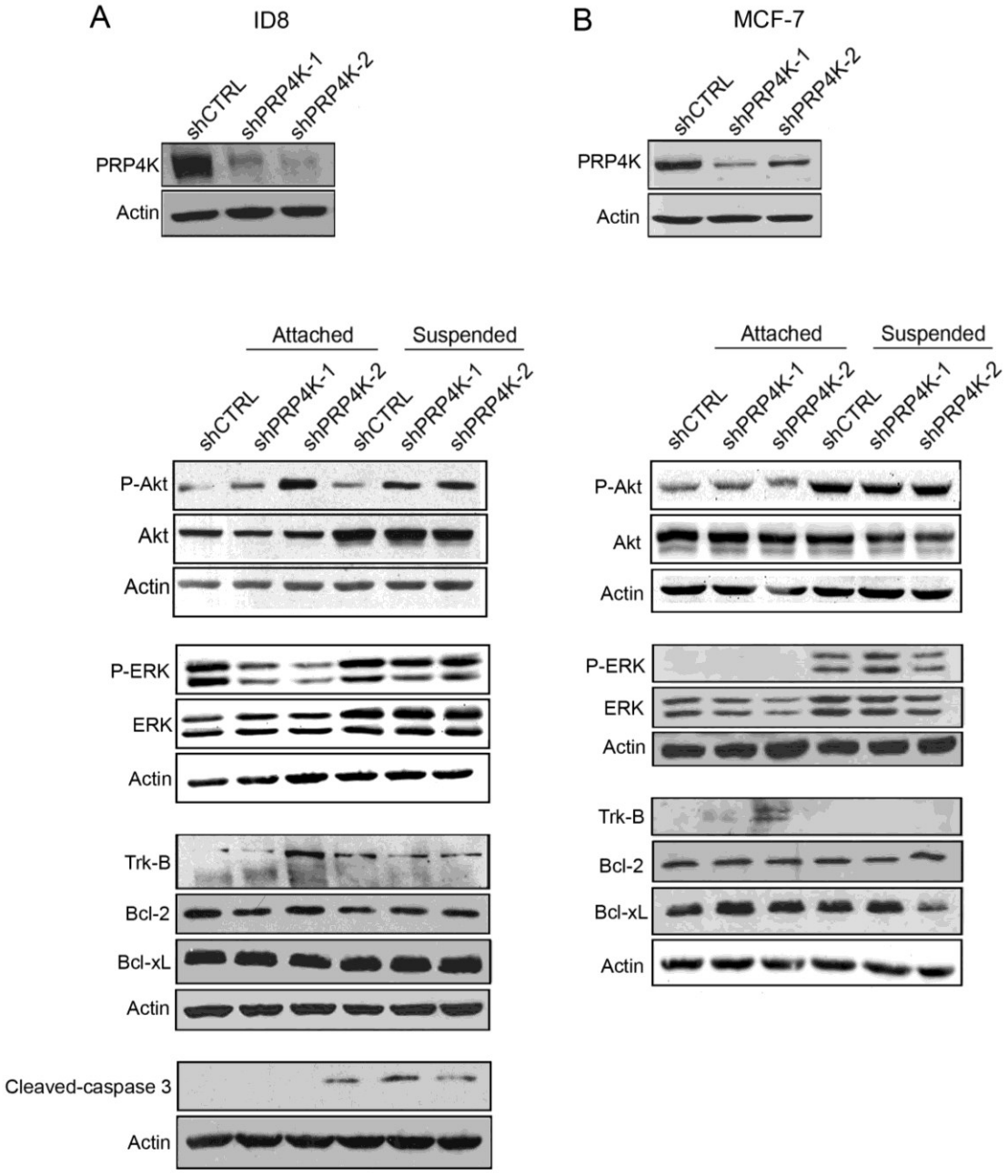

Figure 3.1: Knockdown of PRP4K increases the expression of TrkB, a marker of anoikis resistance, in A) ID8 and B) MCF-7 cell lines. ID8 and MCF-7 shCtrl and shPRP4K cells were grown in attached and suspended conditions for 24 hours. Cell lysates were prepared for western blot analysis in order to detect the expression of proteins involved in pro-survival and anoikis resistance pathways. Experiments were repeated at least two times in order to ensure results were reproducible.

3.2 PRP4K Expression is Decreased while Anoikis Resistance and EMT Marker Expression are Increased in Ovarian Cancer Cells Harvested from the Ascites of Mice

Low cellular PRP4K expression has negative effects when it comes to cancer because it is associated with resistance to cancer therapies and resistance to cell death by anoikis (Corkery et al., 2015; Corkery et al., 2018). In particular, our analysis of ovarian cancer data in The Cancer Genome Atlas (TCGA) data (Cancer Genome Atlas Research Network, 2011) indicates that low PRP4K expression is associated with poor prognosis in this cancer type (Corkery, et al., 2018).

Given the link between low PRP4K expression in human ovarian cancer and poor outcomes, I examined the correlation between PRP4K expression with other markers of aggressiveness in the ID8 syngeneic mouse ovarian carcinoma model. For this experiment, 3 x 10^6 ID8 shCtrl cells were injected intraperitoneally into C57/BL6 mice and these cells were recovered from the ascites of the mice 28 days later (Figure 3.2 A). In order to form ascites, the injected ID8 cells must disseminate. This means that the ID8 cells collected from the ascites fluid should be resistant to anoikis and exhibit more aggressive behaviour than the original ID8 cells that were injected into the mice. I then examined the expression of PRP4K and various EMT (vimentin, fibronectin), apoptosis (Bcl-2), pro-survival signalling (Akt/phospho-Akt), and anoikis-associated factors such as TrkB by western blot.

I observed that the ID8 cells recovered from the ascites fluid of mice displayed lower expression of PRP4K as well as increased TrkB, fibronectin, vimentin, and P-Akt (3 out of 4) expression (Figure 3.2 B). No significant changes in Bcl-2, Akt, ERK and P-ERK were observed. This data demonstrates that low PRP4K is associated with increased cancer aggressiveness, increased anoikis resistance and markers of EMT.

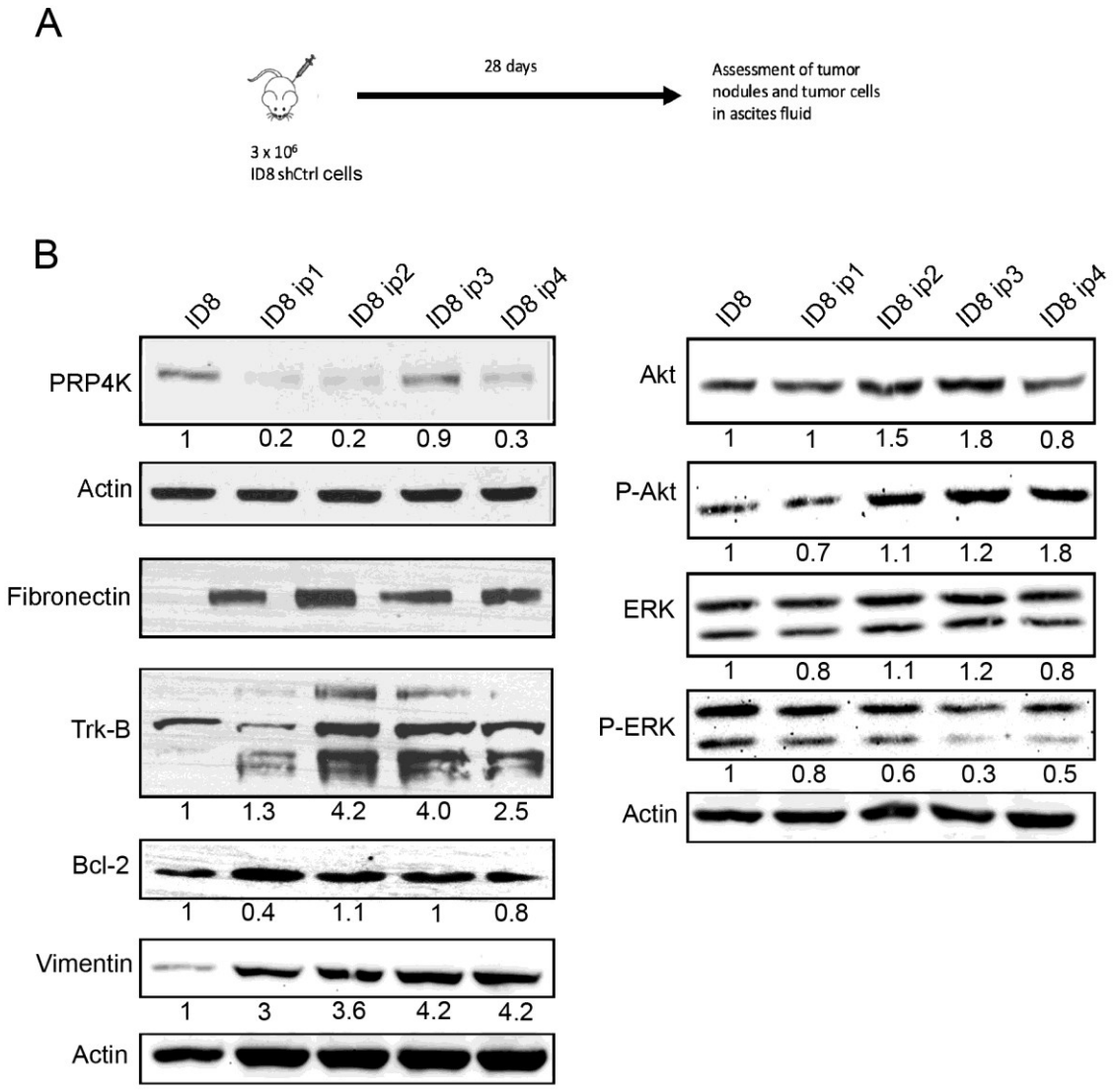

Figure 3.2: PRP4K expression is decreased while anoikis resistance and EMT marker expression are increased in ovarian cancer cells harvested from the ascites of mice. (A) 3 x 10^6 ID8 shCtrl cells were injected intraperitoneally into C57/BL6 mice (n=4) and then recovered from the ascites of the mice 28 days later. **(B)** Cell lysates were prepared and western blot analysis was performed to detect expression of proteins involved in pro-survival pathways, anoikis resistance and EMT. Experiments were repeated at least two times in order to ensure results were reproducible.

3.3 Knockdown of PRP4K Increases Mesenchymal Marker Expression in ID8, MCF-7 and HeLa Cancer Cell Lines

After I discovered that an EMT gene expression profile was correlated with reduced PRP4K expression, I wanted to further explore the relationship between PRP4K and EMT. I performed western blot analysis on various proteins involved in EMT in the mouse ID8, and human MCF-7 and HeLa PRP4K KD cell lines versus control KD. In all three cell lines, PRP4K is significantly decreased by the shRNAs employed compared to control cells, and I found several changes in the expression of EMT markers (Figure 3.3). Although we saw differences between shRNAs used for PRP4K KD, the general trends in gene expression changes were seen across cell lines, tissues and between species (i.e. mouse and human). In summary, vimentin, Zeb1 and fibronectin, all major mesenchymal cell proteins, were often up-regulated with PRP4K KD. More specifically, vimentin was up-regulated in one of the ID8 KD cell lines and one of the HeLa KD cell lines. Zeb1, an EMT transcription factor, was up-regulated in all ID8, MCF-7 and HeLa KD cell lines. Fibronectin was up-regulated in one of the ID8 KD cell lines, both MCF-7 KD cell lines and both HeLa KD cell lines (Figure 3.3). No obvious changes were detected in Zo-1, E-cadherin, slug, snail and beta-catenin expression with PRP4K KD in any cell line (Figure 3.3). Altogether, this data suggests that loss of PRP4K promotes gene expression changes associated with EMT.

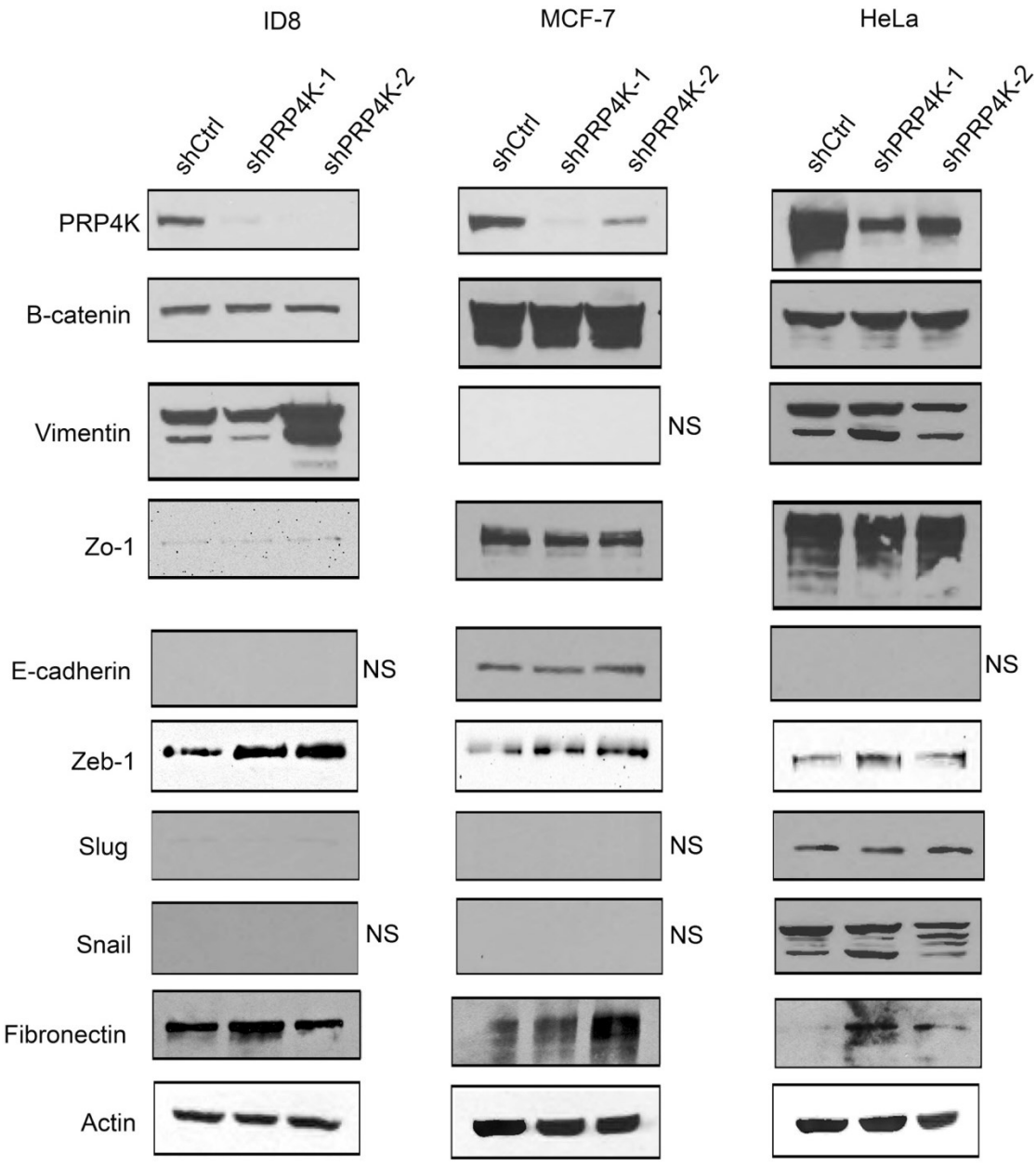

Figure 3.3: Knockdown of PRP4K increases mesenchymal marker expression in ID8, MCF-7 and HeLa cancer cell lines. ID8, MCF-7, and HeLa shCtrl and shPRP4K cells were harvested and cell lysates were prepared for western blot analysis of proteins involved in the EMT process. NS= No signal. Experiments were repeated at least two times in order to ensure results were reproducible.

3.4: Knockdown of PRP4K Increases Mesenchymal Marker Expression in the MDA-MB-231 Cell Line

Furthermore, because of the poor prognosis of TNBC, and the urgency in this area of breast cancer research for new treatment options, I wanted to determine whether the same results could be replicated in a TNBC cell line, MDA-MB-231. Again, I found differences in the expression of EMT proteins with PRP4K KD. Specifically, in the MDA-MB-231 cell line, I found that fibronectin and Zeb1 were up-regulated in both KD cell lines. Slug (SNAI2), an EMT transcription factor, was up-regulated in one KD cell line while Snail (SNAI1), another EMT transcription factor, was upregulated in both KD cell lines. As well, E-cadherin, a major epithelial marker, was down-regulated in both KD cell lines and Claudin1, another epithelial marker, was down-regulated in both KD cell lines (Figure 3.4). The epithelial marker Zo-1 was up-regulated in both KD cell lines while the mesenchymal markers vimentin and N-cadherin were down-regulated in both KD cell lines. The anoikis resistance marker, TrkB, was down-regulated with PRP4K KD. Overall, this data is consistent with the previous data and suggests that PRP4K KD promotes EMT, and that this phenotype can be replicated in a TNBC cell line.

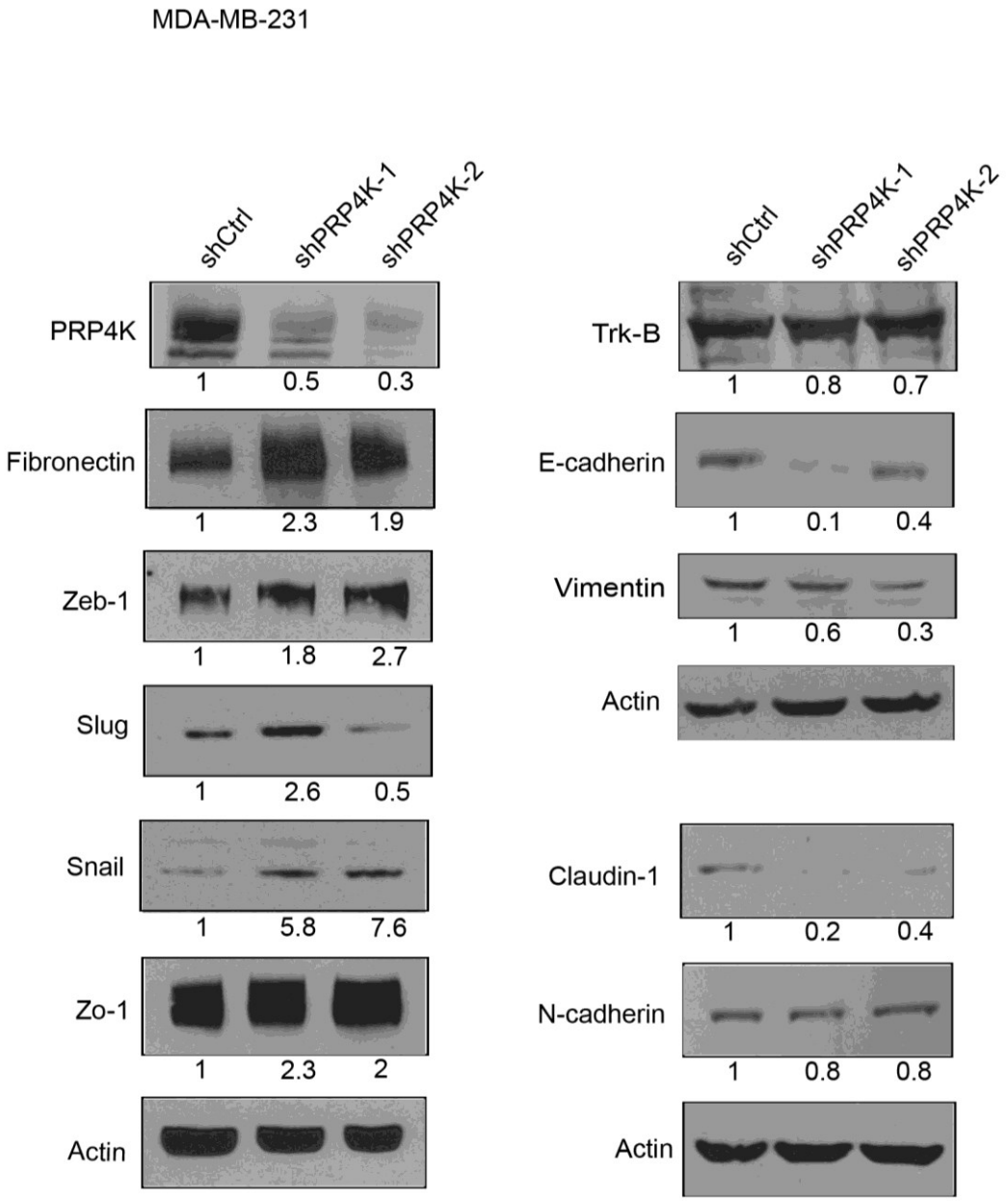

Figure 3.4: Knockdown of PRP4K increases mesenchymal marker expression in the MDA-MB-231 cancer cell line. MDA-MB-231 shCtrl and shPRP4K cells were harvested and cell lysates were prepared for western blot analysis of proteins involved in the EMT process. Experiments were repeated at least two times in order to ensure results were reproducible.

3.5: Knockdown of PRP4K Induces Partial EMT in the HMLE and MCF10A Cell Lines

In my previous studies on the relationship between EMT and PRP4K expression, I used cancer cells that were already either mesenchymal-like (MDA-MB-231), or were transformed (ID8, HeLa, MCF-7); thus, some of the gene expression changes I observed with PRP4K KD may have been more subtle than if I had conducted these experiments in more epithelial-like or non-transformed cell lines. In addition, I wanted to determine if KD of PRP4K was sufficient to induce a full EMT phenotype. Therefore, I examined the same EMT markers via western blot in two non-transformed breast epithelial cell lines. For this, I chose the HMLE and MCF10A cell lines, both of which cannot form tumors in animals and are considered gold-standard epithelial breast cell lines for work on EMT (Qu, 2015; Mani, 2008). To confirm knock-down in my cell lines, I carried out reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) on the HMLE and MCF10A control and KD cells and compared this to PRP4K levels in the MDA-MB-231 cell line to confirm KD of the PRP4K gene *PRPF4B* at the transcript level (Figure 3.5). I then carried out western blot analysis and found that PRP4K expression was decreased at both the protein and transcript level in the HMLE, MCF10A and MDA-MB-231 PRP4K KD cell lines (Figure 3.4-3.6). When I looked at TrkB expression with PRP4K KD, I found that TrkB expression increased in one HMLE knockdown cell line, decreased in the other and decreased in both MCF10A KD cell lines (Figure 3.6). When I carried out an analysis of mesenchymal and epithelial protein markers in the HMLE and MCF10A cell lines, I found very similar results to what I observed after PRP4K KD in the cancer cell lines (Figure 3.6). Fibronectin was up-regulated in both HMLE and MCF10A KD cell lines. Zeb1 was up-regulated in both MCF10A KD cell lines and one HMLE KD cell line, and vimentin was up-regulated in one HMLE KD cell line, but decreased in the MCF10A

PRP4K KD cell lines. Slug was upregulated in both MCF10A KD cell lines, but downregulated in both HMLE KD cell lines. Snail was downregulated in both HMLE and MCF10A cell lines with PRP4K KD. However, E-cadherin was only largely down-regulated in the HMLE KD cell lines, and correlated with increased N-cadherin in one of the HMLE KD cell lines (Figure 3.6). In the MCF10A cell line, N-cadherin expression was slightly decreased with PRP4K KD. Overall, this data is consistent with the pervious data and suggests that PRP4K KD in both cancer and normal cell lines promotes EMT-like changes in gene expression. However, the lack of changes in the epithelial marker E-cadherin in the MCF10A KD cell lines, Zo-1 in both MCF10A KD cell lines and the HMLE KD cell lines and claudin-1 in the HMLE KD cell line and the increase in claudin-1 in the MCF10A KD cell line, suggests that loss of PRP4K induces a partial EMT phenotype.

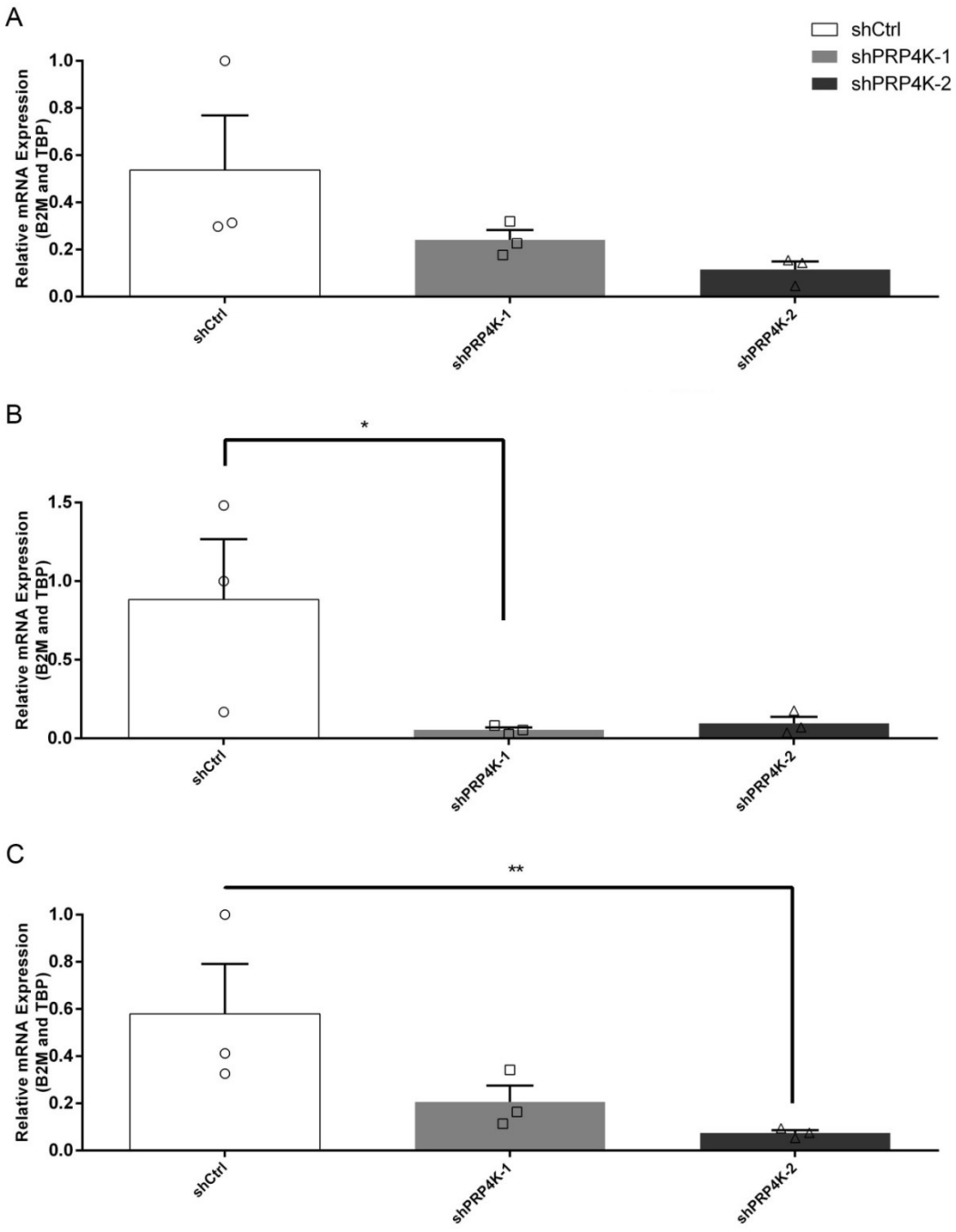

Figure 3.5: PRP4K transcript is down in the A) HMLE, B) MCF10A and C) MDA-MB-231 PRP4K KD cell lines. RNA was isolated from the shCtrl and shPRP4K HMLE, MCF10A and MDA-MB-231 cell lines and then reverse-transcribed to cDNA. Quantitative PCR was performed on the cDNA samples and all data was normalized to at least two reference genes. All experiments were done in triplicate. Error bars= S.E.M. Significance was determined by a one-way ANOVA, *= $p<0.05$, **=$p<0.01$.

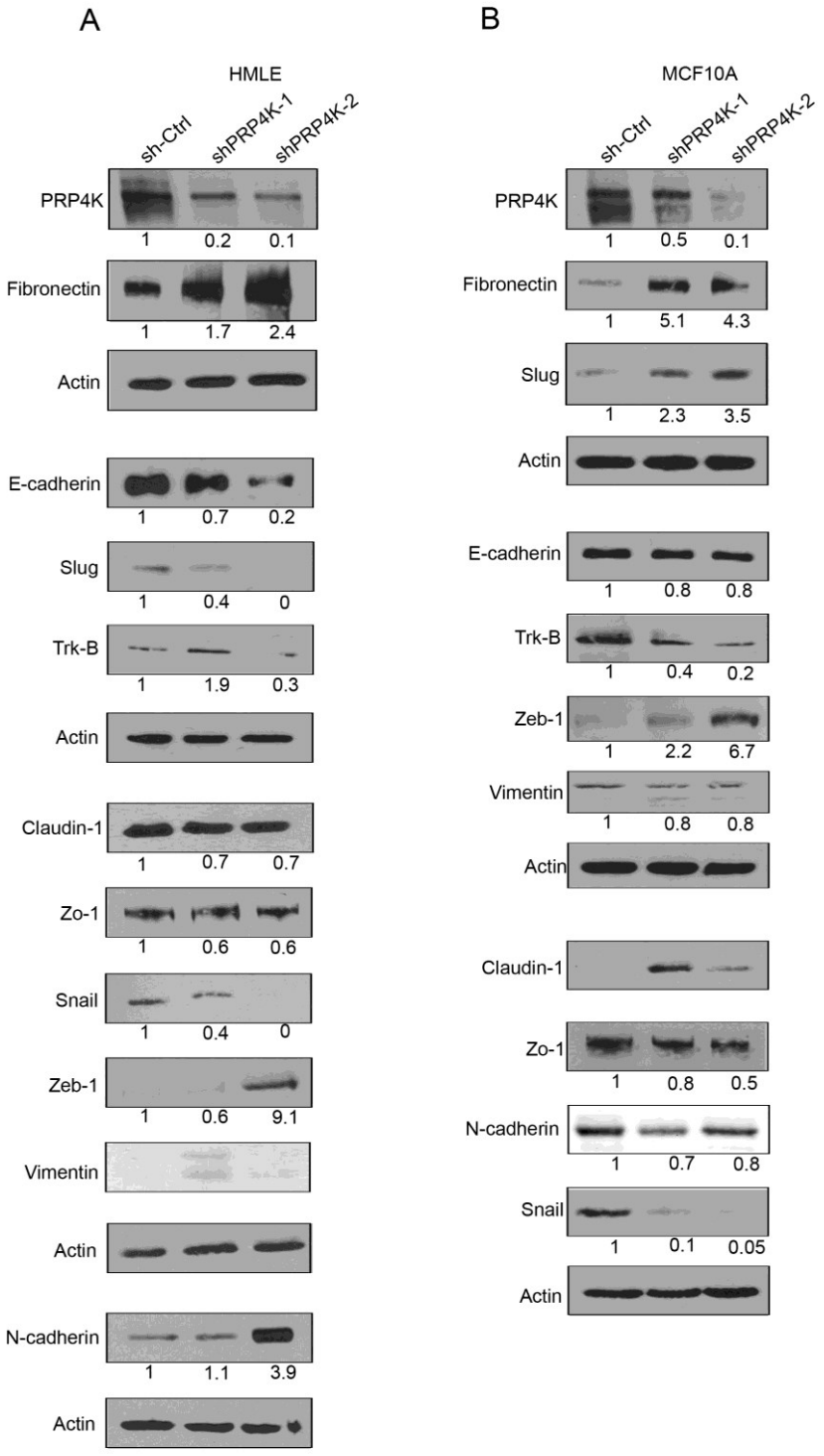

Figure 3.6: Knockdown of PRP4K increases mesenchymal marker expression in the HMLE and MCF10A cell lines. HMLE and MCF10A shCtrl and shPRP4K cells were harvested and cell lysates were prepared for western blot analysis of proteins involved in the EMT process. Experiments were repeated at least two times in order to ensure results were reproducible.

3.6: PRP4K Knockdown Affects Epithelial and Mesenchymal Gene Expression in HMLE, MCF10A and MDA-MB-231 Cell Lines

Since I mainly focused on protein expression changes in my previous experiments and given the role of EMT TFs like Zeb1 and Snail in EMT, I also wanted to look at the transcript expression levels of epithelial and mesenchymal factors to determine whether protein changes correlated with the transcriptional changes of these genes (Figure 3.7). I found that in some cases, protein and transcript expression correlated with each other, but in other cases, transcript and protein expression did not correlate with each other, and sometimes even opposed one another. This data demonstrates that although EMT is occurring with PRP4K KD at the protein level, changes at the transcript level do not necessarily correlate with protein changes. All transcript and protein expression data is summarized in Table 3.1.

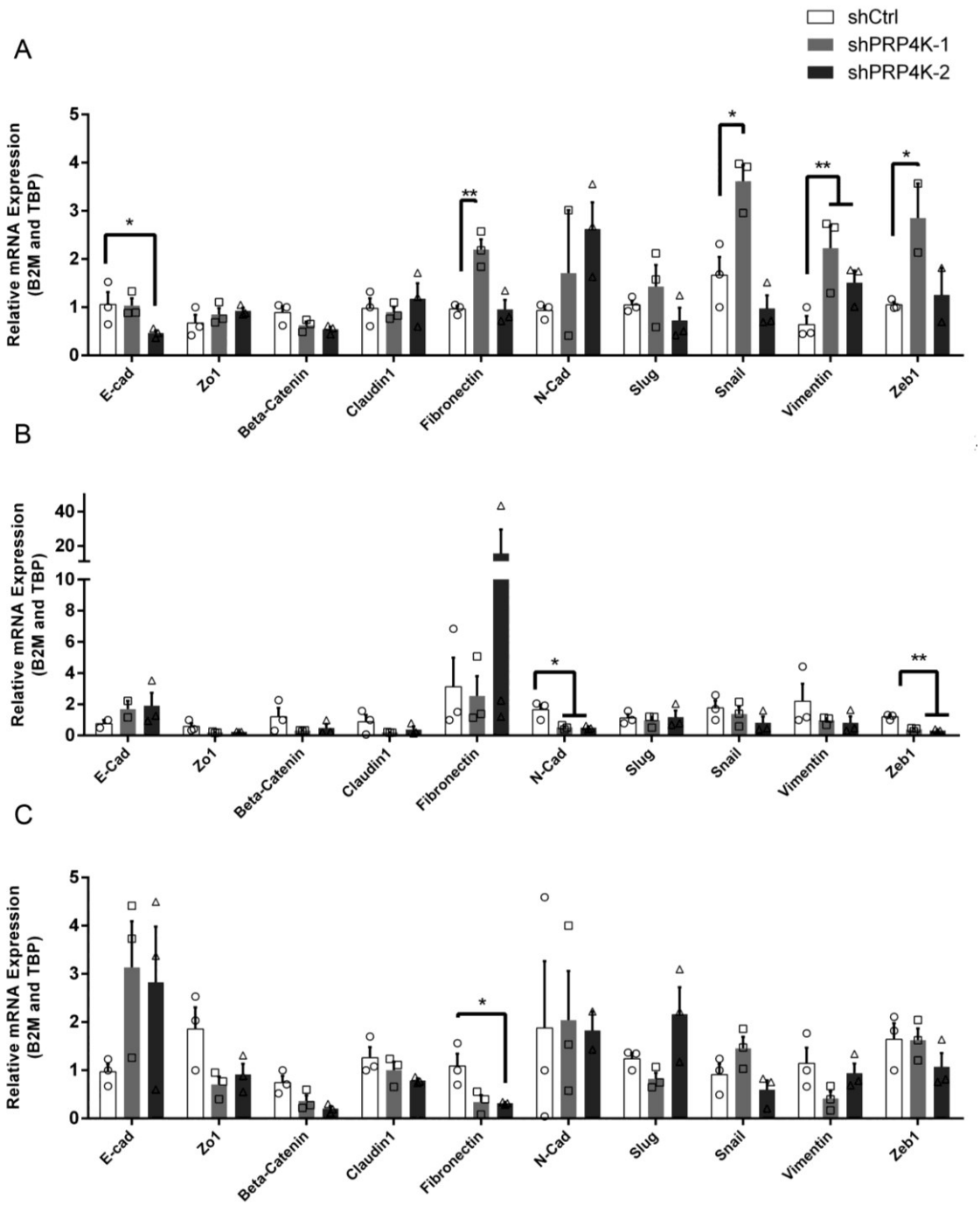

Figure 3.7: PRP4K KD affects epithelial and mesenchymal gene expression in A) HMLE, B) MCF10A and C) MDA-MB-231 cell lines. RNA was isolated from the shCtrl and shPRP4K HMLE, MCF10A and MDA-MB-231 cell lines and then reverse-transcribed to cDNA. Quantitative PCR was performed on the cDNA samples and all data was normalized to at least two reference genes. All experiments were done in triplicate. Error bars= S.E.M. Significance was determined by a one-way ANOVA, *= $p<0.05$, **=$p<0.01$, ***=$p<0.0001$.

Table 3.1: Transcript and protein expression overview in PRP4K KD cell lines. Arrows indicate whether expression increased or decreased with PRP4K KD (horizontal line=no change, N/A=data not available). Each of the two arrows represents a different hairpin.

	HMLE		MCF10A		MDA-MB-231	
	RNA	**Protein**	**RNA**	**Protein**	**RNA**	**Protein**
Beta-catenin	↓ ↓	N/A	↓ ↓	N/A	↓ ↓	N/A
Claudin1	— —	— —	↓ ↓	↑ ↑	↓ ↓	↓ ↓
E-cadherin	— ↓	↓ ↓	N/A	— —	↑ ↑	↓ ↓
Fibronectin	↑ —	↑ ↑	— ↑	↑ ↑	↓ ↓	↑ ↑
N-cadherin	— ↑	— ↑	↓ ↓	— —	↑ ↑	— —
PRP4K	↓ ↓	↓ ↓	↓ ↓	↓ ↓	↓ ↓	↓ ↓
Slug	↑ ↓	↓ ↓	— —	↑ ↑	↓↑	↑ ↓
Snail	↑ ↓	↓ ↓	↓ ↓	↓ ↓	↑↓	↑ ↑
TrkB	N/A	↑ ↓	N/A	↓ ↓	N/A	— —
Vimentin	↑ ↑	↑ ↑	↓ ↓	↑ ↑	↓ ↓	— ↓
Zeb1	↑ —	— ↑	↓ ↓	↑ ↑	—↓	↑ ↑
Zo-1	— —	— —	↓ ↓	— —	↓ ↓	— —

3.7: PRP4K Knockdown Increases the Migration of the MDA-MB-231 Cell Line, Decreases the Migration of the MCF10A Cell Line and has no Effect on the HMLE Cell Line

Following the determination that PRP4K KD results in cells that show increased mesenchymal protein expression and decreased epithelial protein expression, I wanted to determine whether this had an effect on the migratory potential of the cells. In order to observe cellular migration, I performed scratch assays. The inducible HMLE, MCF10A, and MDA-MB-231 control (shCtrl) and KD (shPRP4K) cell lines were treated with doxycycline for four days in order to obtain maximum PRP4K KD and then a scratch was made in the cell monolayer using a P200 pipette tip. By imaging the scratched area over time I was able to determine if PRP4K KD affected the migration of these cell lines. Consistent with the more mesenchymal nature of MDA-MB-231 cells, they exhibited an increase in migration with PRP4K KD. These results were in stark contrast to the migratory behaviour of the HMLE and MCF10A cell lines after PRP4K KD. Whereas migration increased with PRP4K KD in the MDA-MB-231 cell line (Figure 3.8 D, E), it decreased in the MCF10A cells and remained unchanged in the HMLE cell line with PRP4K KD (Figure 3.8 A, B, C). Overall, this data indicates that PRP4K KD does affect the migration of cells, but the effect appears to be dependent on the transformation state of the cell line, with more transformed and mesenchymal-like cells exhibiting increased migration. Thus, I hypothesize that whether migration increases or decreases with PRP4K KD is dependent on whether the cell line is transformed or a normal epithelial cell line. The mechanism for this altered response to PRP4K KD might rely on specific signaling pathways activated in transformed cells that are not activated in normal cells. One of

these pathways is the Hippo/YAP pathway (Zanconato, et al., 2016), which I will address in the next section.

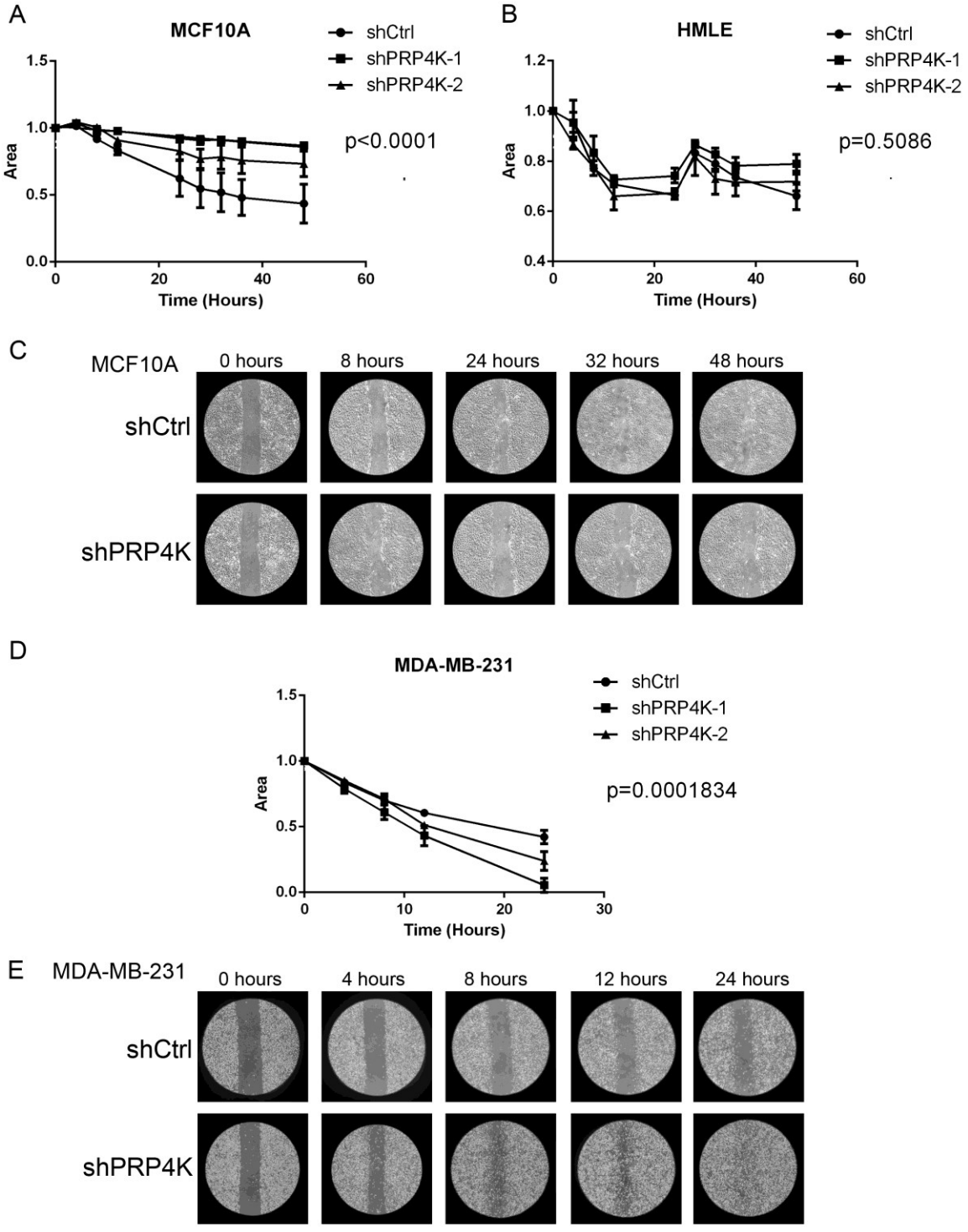

Figure 3.8: PRP4K knockdown increases the migration of the MDA-MB-231 cell line, decreases the migration of the MCF10A cell line and has no effect on the HMLE cell line. Cells were grown to confluency and then a scratch was made using a P200 pipette tip. Scratches were imaged every 4 hours and total imaging time was dependent on the cell line. The area of the scratch was calculated using ImageJ software. Error bars= S.E.M. Significance was determined by a linear regression.

3.8: PRP4K Knockdown does not Increase YAP Signaling Pathways

Following the observations that PRP4K KD promotes an EMT process and affects the migration of cells, I wanted to further look into possible mechanisms for these phenotypes. Cho and colleagues (Cho et al., 2018) recently determined that PRP4K plays a role in the Hippo signaling pathway, which is involved in organ growth control, stem cell function, regeneration and tumor suppression (Johnson, 2013). The Hippo pathway regulates YAP to prevent its activation because increased activation of YAP promotes cell proliferation, inhibits cell death and is associated with high-grade cancers (Zanconato, 2016). Cho et al., found that PRP4K phosphorylates YAP, inhibiting its binding to the transcription factor TEAD, preventing its nuclear accumulation and activation. Therefore, the downregulation of PRP4K causes increased YAP activation and the activation of its downstream target genes, many of which are involved in pathways that are associated with cancer development (Cho, et al., 2018).

Following these observations seen by Cho and colleagues, and given that previous studies indicate that YAP overexpression (OE) promotes a mesenchymal phenotype in mammary epithelial cells (Johnson and Hadler 2015), I hypothesized that the EMT phenotype seen with PRP4K KD could be due to upregulated YAP signaling. I wanted to see whether PRP4K KD affected YAP target gene expression in my hands. I performed RT-qPCR on the HMLE (Figure 3.9 A), MCF10A (Figure 3.9 B) and MDA-MB-231 (Figure 3.9 C) shCtrl and shPRP4K cell lines and looked at major target genes involved in YAP signaling. As well, I looked at overall YAP expression levels by western blot (Figure 3.9 D). Overall, I found that PRP4K KD often led to no change or a decrease in YAP gene expression levels, which was the opposite of what was expected and is not consistent with the previous findings of Cho and colleagues (Figure 3.9) (Cho, et al.,

2018). Additionally, by western blot a significant decrease in YAP protein expression in the MCF10A cell line was seen. A slight increase in YAP expression was detected in the HMLE cell line and a slight decrease was detected in the MDA-MB-231 cell line (Figure 3.9 D). Altogether, this data does not indicate that YAP signaling is the likely mechanism promoting an EMT phenotype associated with PRP4K KD, particularly in non-transformed cells. In fact, the MCF10A data indicates a possible negative feedback loop reducing YAP expression in normal cells in response to PRP4K loss.

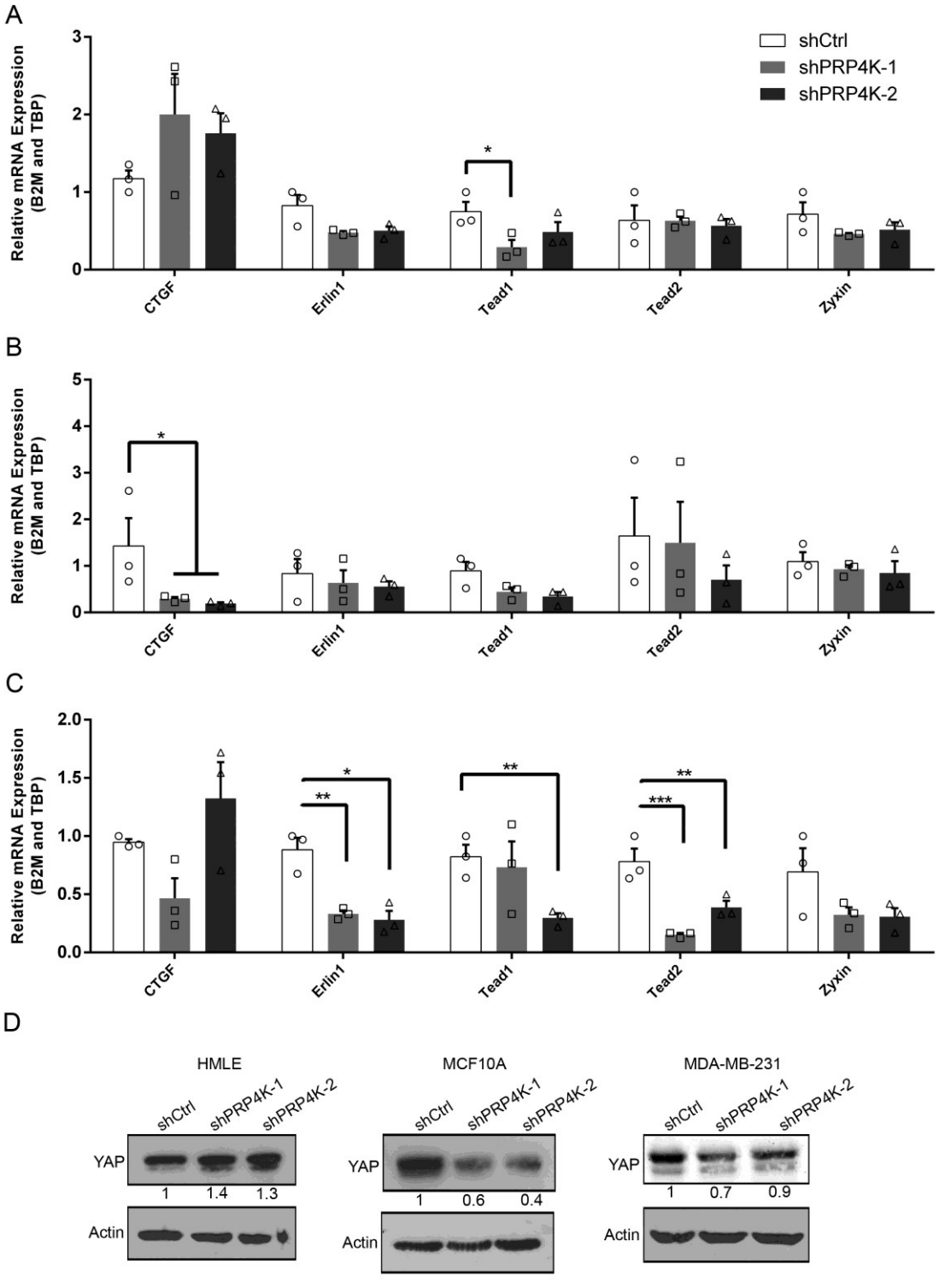
A
Relative mRNA Expression (B2M and TBP)
shCtrl
shPRP4K-1
shPRP4K-2
CTGF
Erlin1
Tead1
Tead2
Zyxin
B
C
D
HMLE
MCF10A
MDA-MB-231
YAP
Actin
1
1.4
1.3
0.6
0.4
0.7
0.9

Figure 3.9: PRP4K KD does not increase YAP signaling pathways. RNA was isolated from the shCtrl and shPRP4K **(A)** HMLE, **(B)** MCF10A and **(C)** MDA-MB-231 cell lines and then reverse-transcribed to cDNA. Quantitative PCR was performed on the cDNA samples and all data was normalized to at least two reference genes. All experiments were done in triplicate. Error bars= S.E.M. Significance was determined by a one-way ANOVA, *= $p<0.05$, **=$p<0.01$, ***=$p<0.0001$. **(D)** HMLE, MCF10A and MDA-MB-231 shCtrl and shPRP4K cells were harvested and cell lysates were prepared for western blot analysis of YAP expression. Experiments were repeated at least two times in order to ensure results were reproducible.

3.9: Relationship Between EMT Induction Methods and Changes in PRP4K Protein Expression

My previous data indicated that when PRP4K is knocked-down in normal epithelial cells a partial EMT process is initiated that affects the migratory potential of a cell. This raised the question of whether PRP4K expression is reduced following the induction of EMT. In order to induce EMT in my cell lines, I used multiple methods. First, I used an EMT inducing media supplement containing Wnt-5a (wnt family member 5A) and TGF-β1, as well as anti-E-cadherin, anti-sFRP-1 (secreted frizzled related protein 1) and anti-Dkk-1 (dickkopf-related protein 1) antibodies. Secondly, I induced EMT by knocking down E-cadherin by siRNA. Thirdly, I obtained MCF10A cells with EIF3E knockdown, which were created by Lewis and colleagues (Gillis, 2013). Previously, they showed that EIF3E KD promotes EMT in the MCF10A cell line (Gillis, 2013). Finally, I stably overexpressed Twist using retrovirus, which has previously shown to induce EMT (Yang, 2016).

3.9.1: The Induction of EMT by EMT Induction Media Decreases PRP4K Protein Expression in the HMLE and MCF10A Cell Lines

The EMT inducing media supplement consists of various factors (described above) that are known to induce EMT in a variety of cell types, including the MCF10A cell line. To induce EMT, the supplement was added to the tissue culture plate directly after cell plating, replaced once on day 3 and cells were harvested for further analysis on day 5. Following treatment with the supplement, both HMLE and MCF10A cells became more spindle-shaped consistent with induction of EMT (Scheel, et al., 2011) (Figure 3.10

A). I performed western blot analysis to examine PRP4K protein expression and found that its expression was decreased in both cell lines as compared to untreated cells (Figure 3.10 B). This data suggests that the induction of EMT can negatively affect PRP4K protein expression.

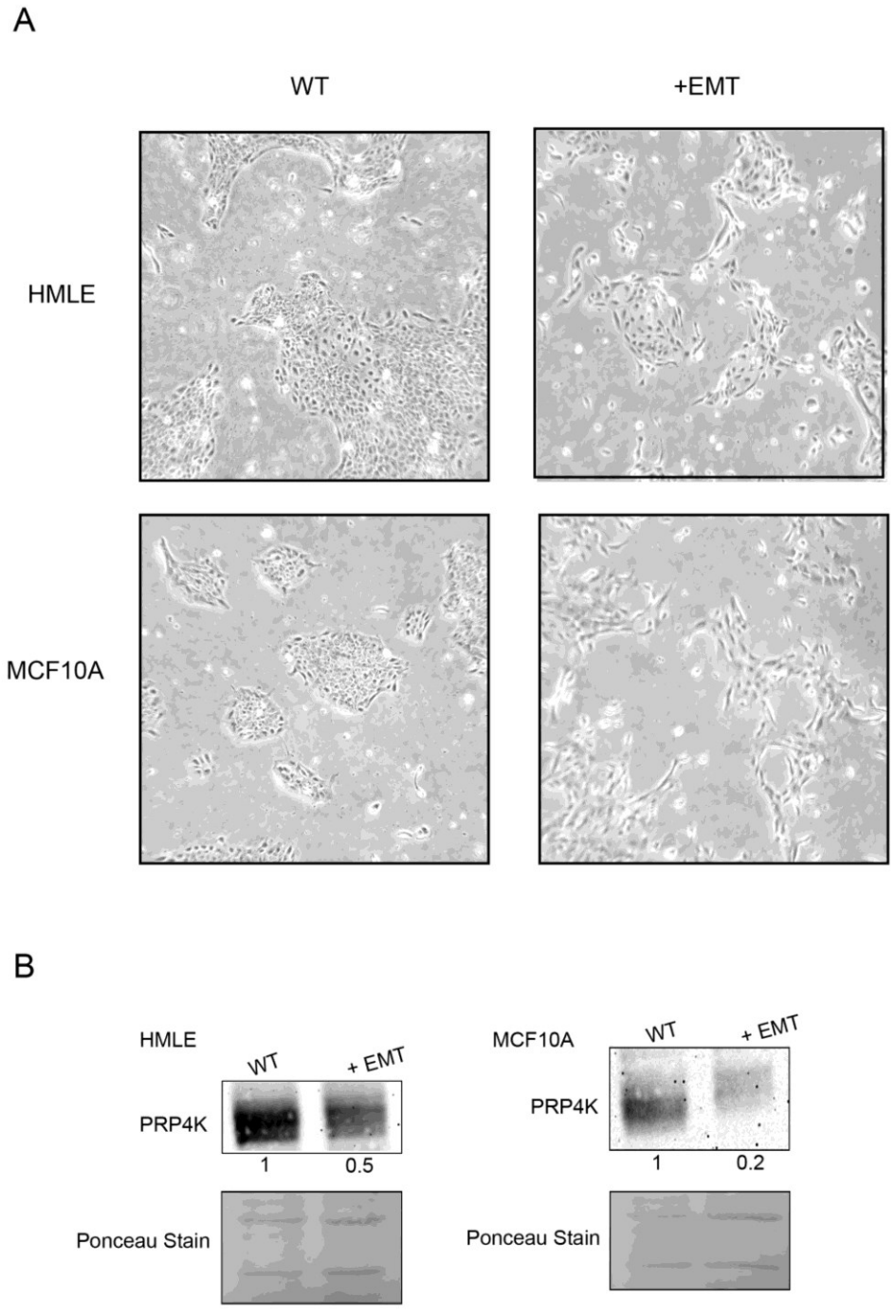

Figure 3.10: The induction of EMT by EMT induction media decreases PRP4K expression in the HMLE and MCF10A cell lines. (A) MCF10A and HMLE cells were imaged using an Olympus CKX41 at 40X magnification. **(B)** MCF10A and HMLE cells were treated with EMT inducing media for 5 days. The cells were harvested and cell lysates were prepared for western blot analysis of PRP4K expression.

3.9.2: The Induction of EMT by E-cadherin Knockdown Decreases PRP4K Protein Expression in the HMLE Cell Line

The next method I used to induce EMT was by knocking down E-cadherin. I treated cells with an siRNA against E-cadherin to knock it down. In comparison to the control siRNA, when cells were treated with a siRNA against E-cadherin, E-cadherin was downregulated, which I show by western blot (Figure 3.11). Although I did not see a difference in PRP4K protein expression in the MCF10A cell line, I did see a slight decrease in PRP4K protein expression in the HMLE cell line following E-cadherin siRNA treatment (Figure 3.11). To summarize, transient knockdown of E-cadherin decreased PRP4K protein expression slightly in the HMLE cell line, but not in the MCF10A cell line.

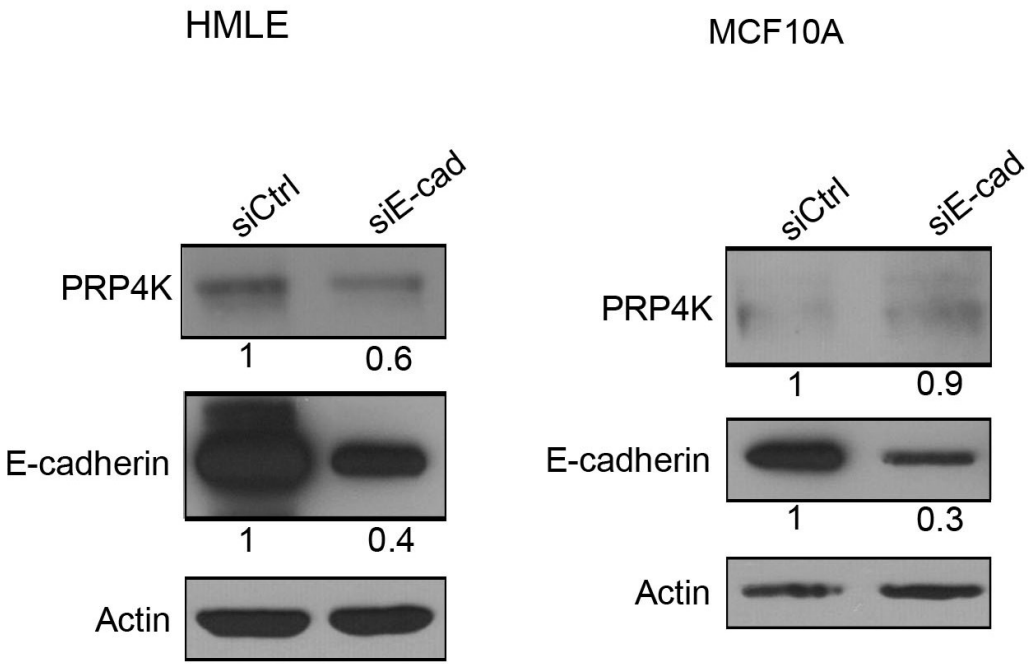

Figure 3.11: The induction of EMT by E-cadherin knockdown decreases PRP4K protein expression in the HMLE cell line. MCF10A and HMLE cells were treated with a siRNA against E-cadherin or a Ctrl siRNA and cultured for 4 days. Cells were then harvested and cell lysates were prepared for western blot analysis of PRP4K and E-cadherin expression.

3.9.3: The Induction of EMT by EIF3E Knockdown Decreases PRP4K Protein Expression, but does not Affect PRP4K Transcript Expression

The third method I used to induce EMT is by knocking down EIF3E. EIF3E KD has previously been shown to induce EMT in the MCF10A cell line (Gillis & Lewis, 2013). I obtained the MCF10A EIF3E control and KD cells from Lewis and colleagues for my experiments. When grown in culture, the MCF10A EIF3E shCtrl cells grow as clusters and the EIF3E KD cells are visually more mesenchymal, growing as single spindle-shaped cells (Figure 3.12 A). I performed western blot analysis on the EIF3E KD cells to look at PRP4K protein expression, which I determined to be down-regulated in comparison to the control cells (Figure 3.12 B). As well, I blotted for various EMT markers in order to confirm that EIF3E KD was promoting EMT, and indeed as previously published, loss of EIF3E increases the expression of mesenchymal markers (Zeb1, N-cadherin, vimentin) and loss of the epithelial marker E-cadherin, indicative of EMT (Figure 3.12 B). Because EMT induction through EIF3E KD led to a decrease in PRP4K protein expression, I wanted to see if this downregulation occurred at the transcript level as well. I performed RT-qPCR to look at PRP4K transcript expression with EIF3E KD and found that its expression was not significantly downregulated (Figure 3.12 C). This data indicates that inducing EMT through EIF3E KD downregulates PRP4K expression in either a post-transcriptional or -translational manner. More specifically, given the role of EIF3E in translation (Gomes-Duarte, et al., 2018), we hypothesize that translation of the PRP4K mRNA may be affected during EMT induction by EIF3E KD.

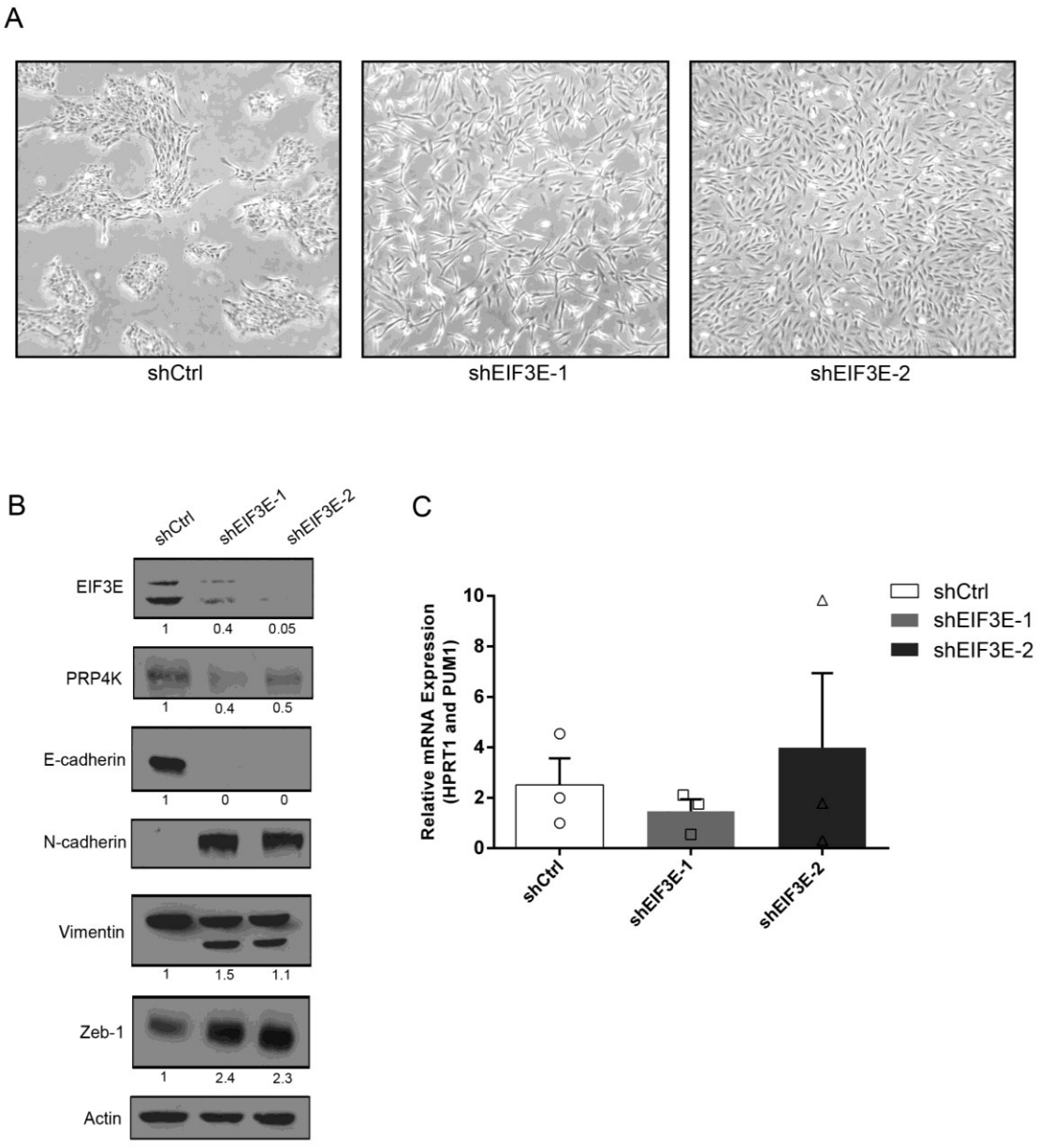

Figure 3.12: The induction of EMT by EIF3E knockdown decreases PRP4K protein expression, but does not affect PRP4K transcript expression. (A) MCF10A shCtrl and shEIF3E cells were imaged using an Olympus CKX41 at 40X magnification. **(B)** MCF10A shCtrl and shEIF3E cells were harvested and cell lysates were prepared for western blot analysis of EIF3E and PRP4K expression as well as of proteins involved in EMT. **(C)** RNA was isolated from the shCtrl and shEIF3E MCF10A cells and then reverse-transcribed to cDNA. Quantitative PCR was performed on the cDNA samples and all data was normalized to at least two reference genes. All RT-qPCR experiments were done in triplicate. Error bars= S.E.M. Significance was determined by a one-way ANOVA.

I also performed RT-qPCR on the EIF3E control and KD cell lines to determine if protein changes in EMT markers seen by western blot were reflected in transcriptional changes (Figure 3.13). I found that indeed, other than PRP4K, transcript and protein changes were positively correlated for both shRNAs used to KD EIF3E.

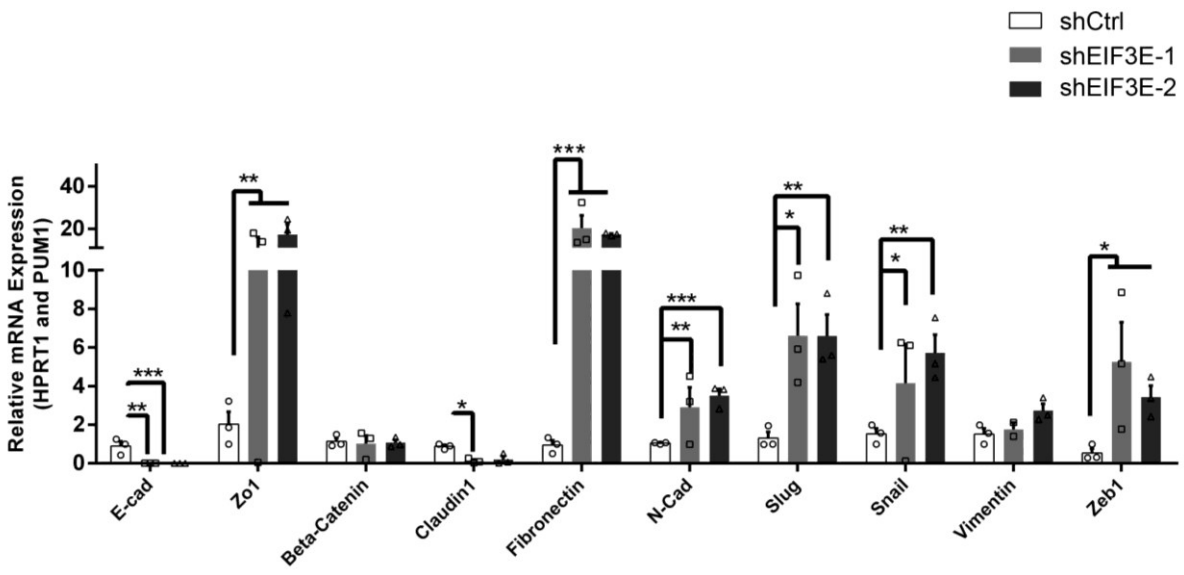

Figure 3.13: EIF3E KD induces EMT at the transcript level. RNA was isolated from the shCtrl and shEIF3E MCF10A cells and then reverse-transcribed to cDNA. Quantitative PCR was performed on the cDNA samples and all data was normalized to at least two reference genes. All RT-qPCR experiments were done in triplicate. Error bars= S.E.M. Significance was determined by a one-way ANOVA, *= $p<0.05$, **=$p<0.01$, ***=$p<0.0001$.

3.9.4: The Induction of EMT by Twist Over-expression does not Affect PRP4K Protein Expression in the HMLE Cell Line

The final method I used to induce EMT was by over-expressing Twist. Twist was over-expressed in HMLE cells using retrovirus. Twist over-expression did not have any effect on PRP4K protein expression when analyzed by western blot (Figure 3.14).

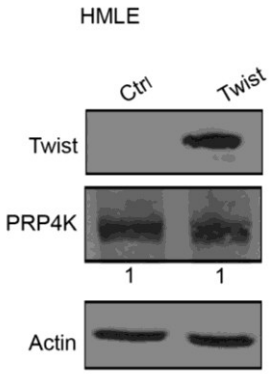

Figure 3.14: The induction of EMT by Twist over-expression does not affect PRP4K protein expression in the HMLE cell line. HMLE Ctrl and Twist OE cells were harvested and cell lysates were prepared for western blot analysis of PRP4K and Twist expression.

In order to confirm that the overexpression of Twist actually does promote EMT, I performed RT-qPCR for EMT genes. N-cadherin, fibronectin, vimentin and Zeb1 gene expression were all increased and E-cadherin gene expression was decreased with Twist over-expression, which is consistent with induction of EMT (Figure 3.15).

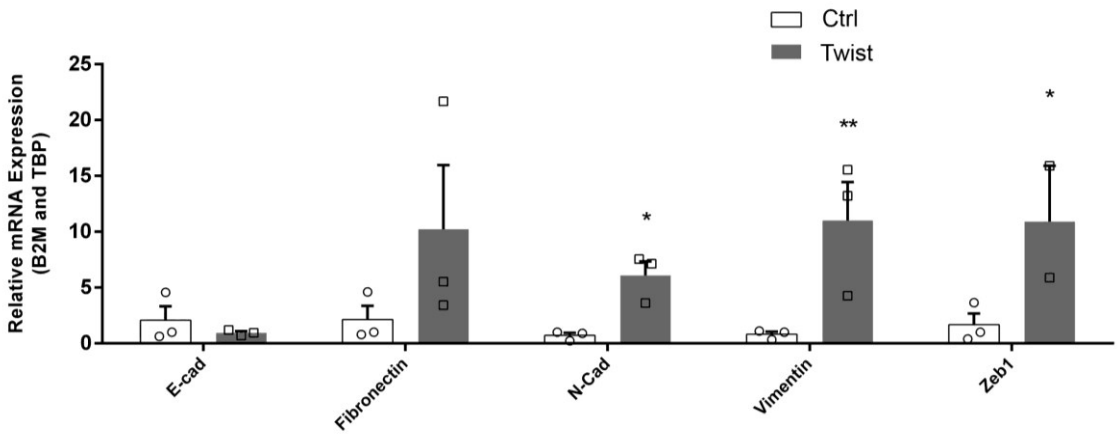

Figure 3.15: Twist over-expression promotes EMT. RNA was isolated from the HMLE Ctrl and Twist OE cells and then reverse-transcribed to cDNA. Quantitative PCR was performed on the cDNA samples and all data was normalized to at least two reference genes. All RT-qPCR experiments were done in triplicate. Error bars= S.E.M. Significance was determined by a t-test, *= $p<0.05$, **=$p<0.01$.

3.10 Inducing EMT by Twist Over-expression and EIF3E Knockdown Decreases MiR-21 Expression

To summarize, I induced EMT in my cell lines using various methods and got inconsistent results. Overall, the EMT inducing media supplement led to a decrease in PRP4K protein expression in both HMLE and MCF10A cell lines (Figure 3.10), E-cadherin KD led to PRP4K downregulation in HMLE cells but not in MCF10A cells (Figure 3.11), EIF3E knockdown in MCF10A cells led to PRP4K protein downregulation (Figure 3.12 and 3.13), and Twist over-expression had no effect on PRP4K expression (Figure 3.14). Because of these inconsistencies, I concluded that there must be a reason why some methods of EMT induction led to changes in PRP4K expression, but others had no effect. In order to look into this further, I chose one EMT inducing method that causes PRP4K downregulation (EIF3E knockdown) and one that does not affect PRP4K expression (Twist over-expression) to do further experiments.

As discussed in section 1.9, microRNAs are single-stranded RNA molecules that can interfere with gene expression and play important physiological roles, but many have also been identified to play a role in cancer development processes (O'Brien, 2018). MiR-21 is a well-studied microRNA that is over-expressed in the majority of cancer types (Ding, 2014). Because miR-21 is commonly dysregulated in cancer, plays a role in EMT processes, and possesses a binding site on the PRP4K 3' UTR, I decided that differences in miR-21 expression might be the reason the various EMT inducing methods affect PRP4K protein expression differently (Ding, 2014; Bornachea, 2012; Dellaire lab, unpublished). Also, our lab has previously shown in several cell lines (MCF-7 breast cancer cells and A549 lung cancer cells) that the over-expression of miR-21 promotes a decrease in PRP4K protein expression (Dellaire lab, unpublished).

To test this hypothesis, I looked at miR-21 expression in control and Twist OE HMLE cells and in control and EIF3E KD MCF10A cells, to see if any differences existed. Because I saw a decrease in PRP4K expression with EIF3E KD compared to control cells (Figure 3.12), I hypothesized that I should see increased miR-21 expression in the EIF3E KD cells when compared to the control cells. Furthermore, because I did not see a change in PRP4K expression with Twist OE compared to control cells (Figure 3.14), I hypothesized that I should not see any change in miR-21 expression. To my surprise, I saw a decrease in miR-21 expression in the EIF3E KD cells and in the Twist OE cells when compared to control cells (Figure 3.16). These results imply that differences in miR-21 expression is not the reason for differential PRP4K expression during EMT-induction by different methods.

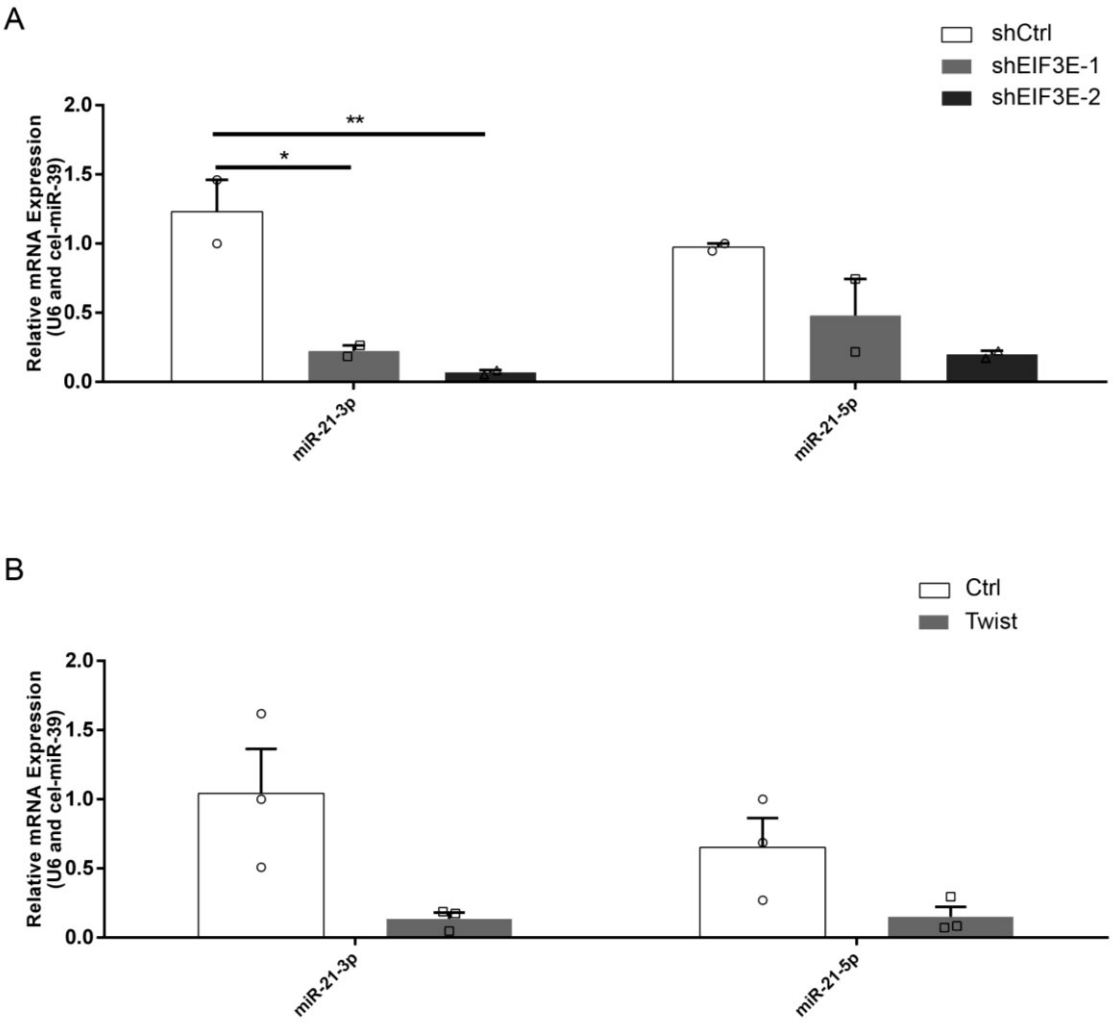

Figure 3.16: Inducing EMT by EIF3E KD and Twist OE decreases miR-21 expression. RNA was isolated from the **(A)** shCtrl and shEIF3E MCF10A cells and the **(B)** HMLE Ctrl and Twist OE cells and then reverse-transcribed to cDNA. Quantitative PCR was performed on the cDNA samples and all data was normalized to the small non-coding RNA, U6 and the *C.elegans* miR-39 exogenous spike in control. All experiments were done in triplicate. Error bars= S.E.M. Significance was determined by a **(A)** one-way ANOVA or a **(B)** t-test, *= $p<0.05$, **=$p<0.01$.

3.11: Changes in PRP4K Protein Expression after Induction of EMT by EIF3E Knockdown Correlates with Increased YAP Target Gene Expression

Because of the recently published paper that suggests PRP4K plays a role in the regulation of YAP activation (Cho, et al., 2018), I next hypothesized that differences in YAP signaling activation might correlate with PRP4K expression levels by different methods of EMT induction. To examine this possibility, I performed RT-qPCR for major YAP targets in both the EIF3E control and KD cell lines and in the Twist control and OE lines. I hypothesized that I would see an increase in YAP target genes in the EIF3E KD cells versus control cells and no difference in the Twist control and OE cells. Of the group of YAP-target genes I evaluated, there was a 25-fold and 10-fold increase in the major YAP target, connective tissue growth factor (CTGF) in the two EIF3E KD cell lines when compared to the control cell line and no change in CTGF gene expression in the Twist control and OE cell lines (Figure 3.17). Although this data indicates that indeed changes in PRP4K expression correlate with YAP activity following induction of EMT by EIF3E KD and not Twist OE, they also raise an intriguing possibility that YAP signaling might also exert an effect on PRP4K protein levels.

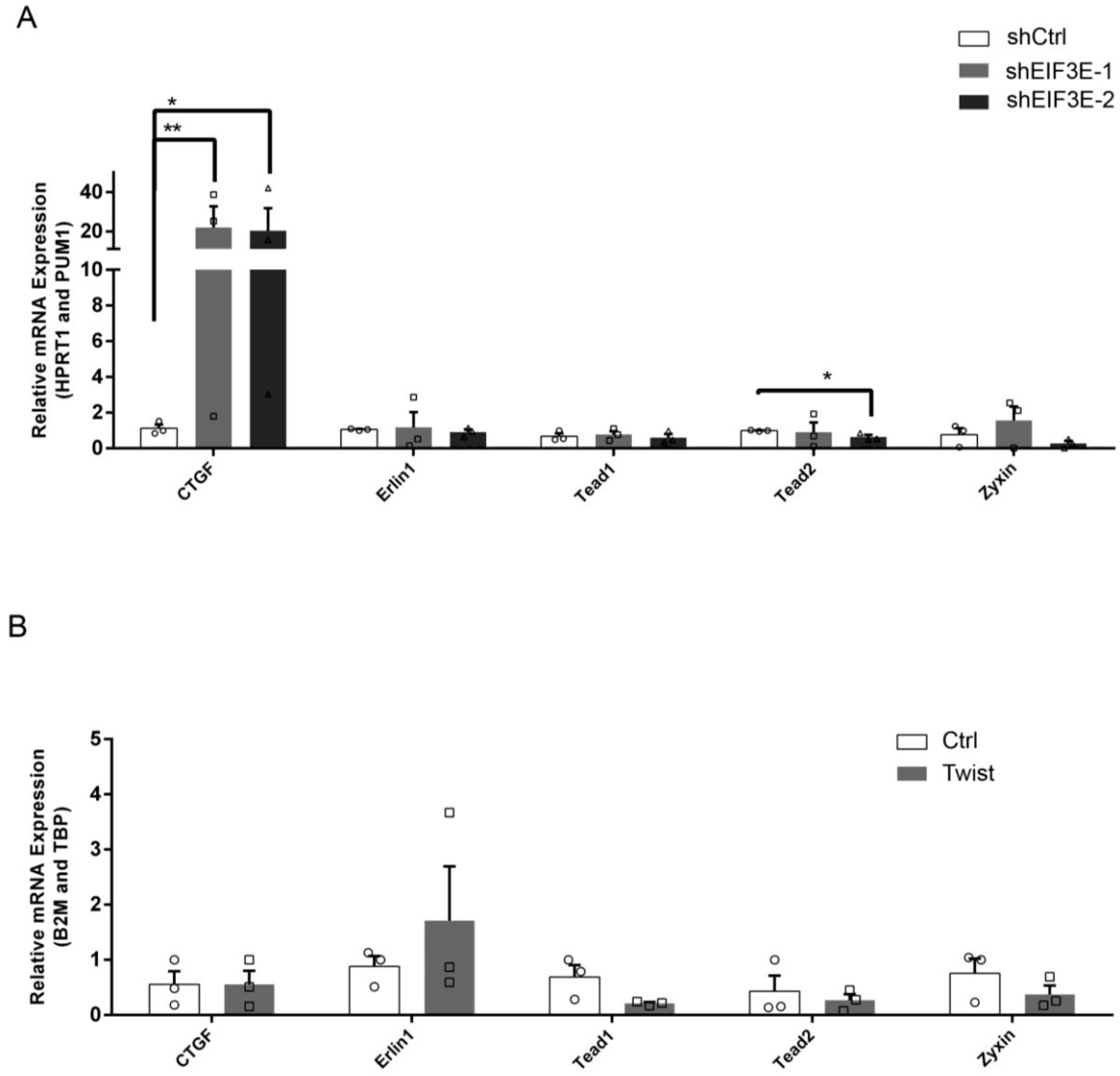

Figure 3.17: A) Inducing EMT by EIF3E KD increases the expression of YAP target genes, while B) Twist OE has no effect on YAP target gene expression. RNA was isolated from the MCF10A shCtrl and shEIF3E and HMLE Ctrl and Twist OE cells and then reverse-transcribed to cDNA. Quantitative PCR was performed on the cDNA samples and all data was normalized to at least two reference genes. All experiments were done in triplicate. Error bars= S.E.M. Significance was determined by a **(A)** one-way ANOVA or **(B)** t-test, *= $p<0.05$, **=$p<0.01$.

3.12: Inducing EMT by EIF3E Knockdown Results in Increased Nucleo-Cytoplasmic Ratio of YAP, while Twist Over-expression has no Effect on YAP Nuclear Localization

Because EIF3E KD was performed in MCF10A cells and Twist OE was performed in HMLE cells, I also wanted to look at YAP expression levels in the two cell lines in order to make sure that the differences seen in YAP signaling was not cell type dependent. To do this, I performed immunofluorescence to compare YAP activation in the two cell lines. I determined the YAP intensity ratio of the nucleus versus the cytoplasm in 34 cells of each the MCF10A EIF3E control and KD cell lines and in the HMLE control and Twist OE cell lines. I found that the YAP ratio values of the MCF10A control and HMLE control cells were not significantly different, indicating that baseline levels of YAP pathway activation are the same between the two cell lines (Figure 3.18 A and D). YAP activation in the MCF10A EIF3E KD lines was significantly upregulated when compared to the MCF10A EIF3E control cells (Figure 3.18 A and B), while there was no difference in YAP activation between the HMLE control and Twist OE cell lines (Figure 3.18 A and C). This was all consistent with the RT-qPCR data and suggests that the reason for the varying effects of different EMT induction methods was due to differences in the activation of YAP signaling.

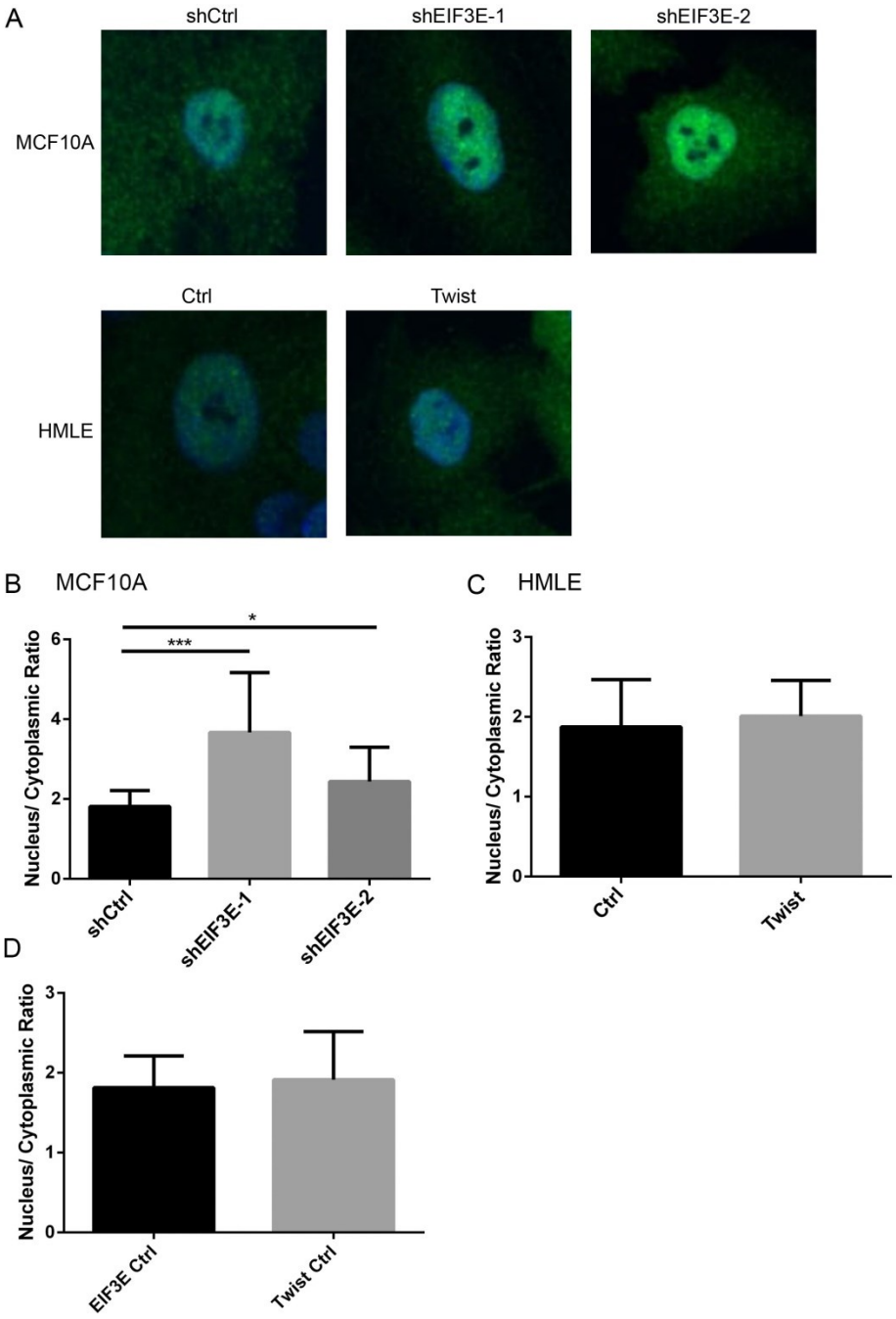

Figure 3.18: Inducing EMT by EIF3E knockdown results in increased nucleo-cytoplasmic ratio of YAP, while Twist over-expression has no effect on YAP nuclear localization. (A) Images were taken using a Zeiss Cell Observer Microscope under a 40x immersion oil objective lens. Green=YAP, Blue=DAPI. **(B)** The intensities of both the cytoplasm and nucleus of 34 cells from each cell type and condition were determined and a nucleus:cytoplasm ratio was calculated. Error bars= S.EM. Significance was determined by a **(B)** one-way ANOVA or **(C-D)** t-test, *= $p<0.05$, ***=$p<0.0001$.

Chapter 4: Discussion

Breast cancer is the leading cause of death among women (Public Health Agency of Canada, 2017). It is a heterogeneous disease that is classified into different molecular subtypes. Those subtypes that over-express specific hormone receptors have a fairly good prognosis because of the ability to treat them with hormone therapies. Other subtypes do not express hormone receptors and lack targeted therapies, resulting in a very poor prognosis. These subtypes include the triple negative subtype (Dai, 2015). Because of the lack of a therapeutic target for this type of breast cancer, they have a very poor prognosis and so the development of new therapies for these types of breast cancer is vital (Deal, 2010). I chose to focus on the triple-negative MDA-MB-231 cell line in my experiments because of its increased aggressiveness and invasiveness and so that my discoveries could contribute to the development of treatments for TNBC.

Low PRP4K expression is linked to negative consequences in terms of cancer. These known consequences include defects in the spindle assembly checkpoint that increase resistance to taxane treatment, anoikis resistance and increased YAP pathway activation (Montembault, et al., 2007; Corkery, et al., 2015; Corkery, et al., 2018; Cho, et al., 2018). As well, using a syngeneic mouse model of ovarian cancer, we demonstrate that low PRP4K expression is associated with increased metastasis (Corkery, et al., 2018). Because metastasis is the leading cause of cancer-related death (about 90%) (Mina & Sledge, 2011), studying the metastatic process and the pathways involved is important in order to find a cure for this horrible disease. My goal was to look more in depth at the anoikis resistance phenotype seen with loss of PRP4K and figure out what pathways are involved.

TrkB plays an important role in the development of the nervous system, but has also been identified as an anoikis suppressor (Douma, et al., 2004). I determined that TrkB was upregulated in cells following PRP4K KD, suggesting that it is playing a role in the anoikis resistance phenotype that is seen with PRP4K KD (Figure 3.1). This data in addition to the published anoikis data from Corkery et al. demonstrates a role for PRP4K in anoikis resistance. Corkery et al. showed using *in vitro* spheroid assays that PRP4K KD increases the anchorage independent growth of cells as spheroids in comparison to control cells. In addition, Corkery creates the perfect *in vivo* anoikis environment by injecting control and PRP4K KD ID8 cells into the yolk sac of the zebrafish. The model is perfect for studying anoikis because there is no ECM for cells to contact and cells end up basically floating in the lipid milieu of the yolk sac. The injected ID8 KD cells grew 2-fold better than the control cells during the same time period. In combination, this data indicates a role for PRP4K in anoikis resistance (Corkery, et al., 2018).

The identification of increased TrkB expression following PRP4K KD and its connection to EMT processes (Smit, 2011) led me to determine whether EMT was occurring with PRP4K KD. A mouse syngeneic ID8 ovarian cancer model was used to look at PRP4K expression in cells forced to adapt to non-adherent growth *in vivo* after peritoneal injection. When control cells were injected into the mice and harvested in the ascites fluid following dissemination, these cells exhibited down-regulated PRP4K and increased TrkB expression. As well, I saw that the mesenchymal proteins fibronectin and vimentin and the anti-apoptotic protein P-Akt were all upregulated (Figure 3.2).

I then examined the impact of PRP4K KD on markers of EMT using the mouse ID8 ovarian cancer cell line, the human MCF-7 breast cancer line, HeLa cervical cancer cell line, MDA-MB-231 TNBC cell line, and two non-transformed “normal” mammary

epithelial cell lines (MCF10A and HMLE). I observed that KD of PRP4K led to changes in EMT markers at the protein level in all cell lines, suggesting that PRP4K KD promotes an EMT-like phenotype (Figure 3.3, 3.4, 3.6). Specifically, the mesenchymal proteins, fibronectin, vimentin and Zeb1 were upregulated with PRP4K KD, while the expression of other mesenchymal markers and the main epithelial marker E-cadherin was left unchanged. This data is consistent with the loss of PRP4K promoting a partial EMT phenotype (Huang, et al., 2013; Yu, et al., 2013). Partial EMT means that epithelial cells maintain some of their epithelial characteristics, such as integrin-mediated cell interactions, while gaining some mesenchymal characteristics. As such, cells that have undergone partial EMT are highly plastic and often exhibit the ability to migrate as clusters, increasing their metastatic potential (Kim, 2017). It is interesting that the EMT TF, Zeb1 increases with PRP4K KD in all cell lines. It is possible that Zeb1 plays an important role in the EMT changes that are seen with PRP4K KD. Further studies should look into Zeb1 expression and how it is linked to PRP4K.

The protein changes seen via western blot in the control and shPRP4K cell lines were not always consistent with transcript changes seen via RT-qPCR (Table 3.1). Many scientists interpret gene expression in terms of protein, but in reality, the correlation between transcript and protein can be as little as 40% (Vogel, 2012). Following transcription, regulatory processes such as post-transcriptional processing and degradation of mRNAs, translational, localization, modification and degradation of proteins all play a role in protein abundance. Overall, transcript does not equal protein, which was amply demonstrated by my protein and transcript datasets (Table 3.1). Thus when studying PRP4K, my work demonstrates that you cannot get the full picture of this

protein's effects by looking solely at transcript expression and it is always important to look at actual protein levels.

One limitation to this study is that in some cases, the two hairpins used to KD PRP4K in a cell line did not show consistent RT-qPCR and western blot results for the genes and proteins evaluated. However, the consistency seen between multiple cell lines across two species (human and mouse) provide increased confidence that the protein/transcript changes seen with loss of PRP4K are real despite the limitation of shRNAs. The shRNA RNA interference technology does have significant off-target effects (Konermann, 2018). Unfortunately, it was not possible to simply knock-out the PRP4K gene *PRPF4B* due to its essentiality for cell division and pre-mRNA splicing (Dellaire et al., 2002; Hart et al., 2015) In the future, the newly developed CasRX system developed by Konermann and colleagues could be used in order to improve gene knockdown experiments and to eliminate off-target effects. The CasRX system was found to outperform both shRNA and CRISPRi, displaying a knockdown of ~96%, in comparison to the 65% knockdown for shRNA and 53% for CRISPRi. Most importantly, the CasRX system showed no significant off-target effects, which is a huge concern and downfall of the currently used techniques to knockdown genes. For example, Konermann et al. show that the KD of annexin A4 (*ANXA4)* by shRNA causes over 900 significant off-target transcript changes; in stark contrast to the CasRX system, which exhibited no significant off-target gene expression changes when used to target the same gene (Konermann, 2018).

Using the scratch assay, I observed that PRP4K KD increases the migration of the MDA-MB-231 cell line, but decreases the migration of the MCF10A cell line and has no effect on the HMLE cell line (Figure 3.8). It is possible that the reason for this difference

in migration seen with PRP4K KD in different cell lines is dependent on whether the cell line is transformed and can form a tumor or has a normal epithelial phenotype. The MDA-MB-231 cells are a claudin-low mesenchymal TNBC line, while both the MCF10A and HMLE are normal mammary epithelial cell lines. Although our data supports a role for PRP4K as a tumor suppressor, simple loss of PRP4K is insufficient to generate an invasive, cancerous cell. Instead, we hypothesize that PRP4K loss is just one "hit" that when combined with other mutations such as over-expression of an oncogene or mutation of other tumor suppressors may generate more aggressive cancers. It is likely that the knockdown of a tumor suppressor, such as Myc or PTEN, or the overexpression of an oncogene, such as HER2, in addition to PRP4K KD could push a non-tumorigenic cell line to become tumorigenic and possess aggressive characteristics. These more aggressive characteristics include taxane and anoikis resistance as well as an increased ability to migrate, with the latter two characteristics combining to greatly increase the metastatic potential of PRP4K low cancers.

In my studies, the differences in migration seen with loss of PRP4K between malignant and normal breast cell lines could be due to intrinsic differences in Hippo signaling between the three cell lines used (Cho, et al., 2018). The Hippo signaling pathway is a conserved developmental pathway controlling tissue growth and organ size, but its dysregulation is involved in many cancer types (Sharif, 2015). The main role of the Hippo pathway is to regulate the activity of YAP/TAZ (Sharif, 2015). Activation of YAP/TAZ promotes cell proliferation and inhibits cell death (Johnson, 2013). Merlin/NF2 is an upstream regulator of the Hippo pathway that through the activation of LATS kinase phosphorylates YAP/TAZ to prevent its nuclear localization and activation of YAP/TAZ-regulated genes (Cooper, 2014). It is possible that we see an increase in

migration with PRP4K KD in the MDA-MB-231 cells because this cell line does not express neurofibromin-2 (NF2) (also known as merlin) (Zanconato, 2015). In this scenario, NF2 loss likely represents the needed "second hit" following PRP4K loss to make a normal cell line more invasive. In such a model, there are a number of possible "second hit" mutations that could affect growth factor and/or Hippo signaling and synergize with PRP4K loss to generate a more invasive and therefore more deadly tumour.

Cho and colleagues recently discovered that PRP4K is involved in the Hippo signaling pathway by phosphorylating YAP/TAZ and preventing its nuclear accumulation and activation (Cho et al., 2018). They observed that the KD of PRP4K by siRNA leads to increased YAP protein expression, migration and invasion in MDA-MB-231 cells. Although I observed increased migration with PRP4K KD in the MDA-MB-231 cells (Figure 3.8), I was not able to replicate the increase in YAP signaling by looking at gene expression of YAP target genes by RT-qPCR. Rather, when I performed RT-qPCR for YAP target genes on my PRP4K KD cell lines, I often saw a decrease in YAP target transcripts (Figure 3.9); which is inconsistent with the findings of Cho et al. I also saw a dramatic decrease in total YAP protein with PRP4K KD in the MCF10A cell line (Figure 3.9 D). The reason for this inconsistency could be due to the fact that Cho et al. used acute knock-down of PRP4K by siRNA, while I used an inducible shRNA system. I consistently used a four-day induction period in order to get the best PRP4K KD within my cells, before performing all my experiments. Although it is not mentioned in the published paper by Cho et al., researchers use cells following siRNA knockdown from 3-7 days following treatment. It is possible that a difference in time the cells were able to adapt to PRP4K KD could lead to differences in results. In both cases, PRP4K was

transiently knocked-down rather than constitutively knocked-down. Differences in YAP signaling may also be due to cell specific effects from genetic drift or specific culture conditions. For example, Hippo signaling through YAP is extremely sensitive to cell density (Zhao, et al., 2007). YAP signaling can be activated following the simple plating of cells at low confluency. Studies show that when cells are plated at low confluency the levels of nuclear YAP/TAZ are increased in comparison to when the same cells are grown at higher densities (Aragona, 2013). Thus, caution must be taken in plating cells at a consistent density when looking at YAP pathway activation.

Following the determination that loss of PRP4K promotes a partial EMT state, I also wanted to determine whether the induction of EMT in cells had any effect on PRP4K expression. I used various methods to induce EMT in cells, but found that different methods of EMT induction had differential effects on PRP4K protein expression. I observed that inducing EMT by using an EMT-induction supplement (Figure 3.10) and through EIF3E KD promoted a decrease in PRP4K protein expression. (Figure 3.12). Furthermore, inducing EMT by E-cadherin KD promoted a decrease in PRP4K protein expression only in the HMLE cell line, yet had no effect on PRP4K protein levels in the MCF10A cell line (Figure 3.11). Finally, inducing EMT by over-expressing Twist in the HMLE cell line had no effect on PRP4K protein expression (Figure 3.14). Thus different methods of inducing EMT, as measured by increased expression of mesenchymal markers and a decrease in expression of E-cadherin, had different effects on PRP4K expression.

Using Twist over-expression (OE) and EIF3E KD as models of inducing EMT, I tested to see if differences in PRP4K protein levels following EMT induction was due to miR-21 expression (Figure 3.16) or YAP signaling (Figure 3.17-18). Despite the 3'UTR of the *PRPF4B* mRNA encoding PRP4K containing a miR-21 binding site, miR-21 levels

were inversely correlated with PRP4K level in control versus EIF3E KD MCF10A and YAP OE HMLE cells; suggesting that miR-21 is not responsible for changes in PRP4K protein levels in these cell models.

In regard to YAP signalling, I did find evidence of differential regulation of YAP signalling between EIF3E KD and Twist OE. Specifically, EIF3E KD significantly increased the expression of the major YAP target gene, CTGF, while Twist OE did not affect CTGF gene expression (Figure 3.17). Second, the nucleus:cytoplasm ratio of YAP protein expression is significantly higher in the EIF3E KD cells compared to control and no significant differences exist between the Twist OE and control cells (Figure 3.18). As well, YAP expression is the same between the two different control cell lines (HMLE and MCF10A) indicating that these results are not due to differences in basal YAP expression between these cell lines (Figure 3.18). Overall, these results support a model by which EMT induction that promotes activation of YAP signaling can also negatively regulate PRP4K protein expression. This raises the intriguing possibility that YAP and PRP4K are part of a regulatory feedback loop and that increased YAP activity may in fact down regulate PRP4K protein expression.

In summary, I determined that PRP4K KD promotes a partial EMT phenotype and that this might also play a role in promoting more aggressive cancers by altering resistance to anoikis and increasing pro-survival signalling via Akt. In addition, I found that KD of PRP4K can have different effects on the migration of cells depending on the cell line. This could be dependent on the status of YAP signaling and/or additional oncogene activation present in transformed cells that have increased migration, like the TNBC cell line MDA-MB-231 versus normal mammary epithelial cells like MCF10A that exhibited reduced migration with PRP4K KD. Finally, I also demonstrated that the

EMT induction method can have different effects on PRP4K expression and that this could be due to differences in YAP activation in the cell line or as a result of the induction method.

Chapter 5: Conclusions and Future Directions

In this thesis, I first determined that PRP4K KD is linked to anoikis resistance through TrkB, partial EMT and in an already transformed tumor cell line can increase cell migration. Furthermore, I found that inducing EMT in cells affects PRP4K expression differently, which is dependent on the EMT induction method and to what extent YAP signaling is activated.

Although I have uncovered more details on the link between PRP4K and EMT, the story is not complete and more research is required to provide mechanistic details of how EMT regulates PRP4K protein levels. My data indicates that PRP4K is likely regulated during EMT post-transcriptionally, and in the context of induction of EMT by EIF3E, a component of the eIF3 translation complex (Hinnebusch, 2006), it is likely that PRP4K is regulated translationally. Thus it will be important to determine how translation of the PRP4K mRNA is affected during EMT using approaches such as translation-state analysis of *PRPF4B* transcript associated with the polyribosome (Vyas, et al., 2009)

It would be interesting to see if the migratory phenotype seen with PRP4K KD in the TNBC cell line, MDA-MB-231, could be replicated in a second TNBC cell line. Perhaps this is a TNBC specific phenotype, or maybe this phenotype is specific to transformed cell lines in general.

Further *in vivo* studies would also be useful in determining the effect of PRP4K KD on cancer development. Specifically, whether loss of PRP4K on its own can induce a non-metastatic cell line to become metastatic or whether a "second hit" mutation is required to generate an aggressive tumor phenotype is an important unanswered question. As well, if simple PRP4K loss is not sufficient to induce metastasis, what oncogenes or

tumour suppressor mutations are necessary? This could be done by sequential mutation of tumor suppressor genes and over-expression of oncogenes in combination in a non-tumorigenic normal cell line like MCF10A.

Finally, a number of questions also remain regarding the relationship between PRP4K and YAP signaling. For example, why does loss of PRP4K not lead to increased YAP pathway activation in all cell lines? Does cell density play a role or additional factors such as NF2 or LATS dysfunction alter how the cell responds to PRP4K loss? Finally, does over-expression of YAP have any effect on PRP4K levels or localization? Answering these questions should help to fill in the blanks that exist in the connections between PRP4K, EMT, YAP and cancer development.

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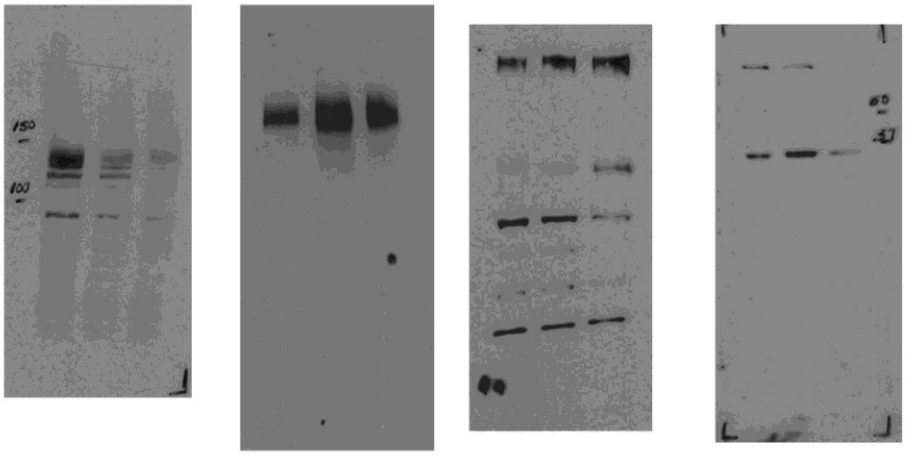
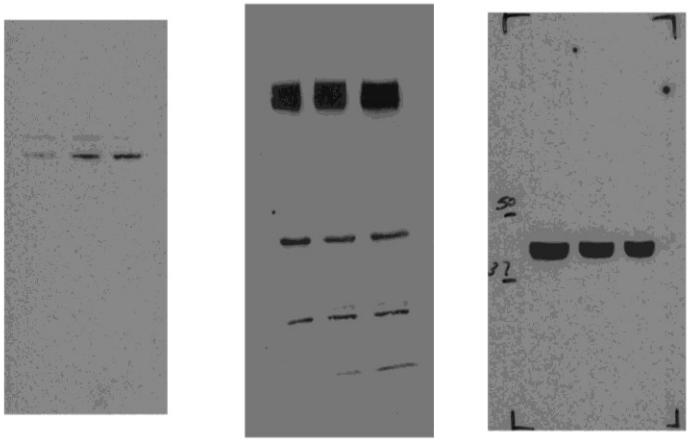
Appendix A Full Western Blots

Figure 3.4: Part 1/2

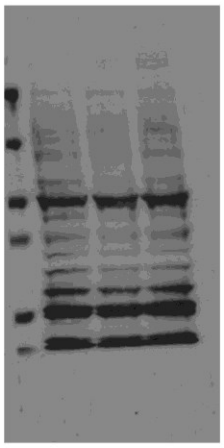


PRP4K | Fibronectin | Zeb1 | Slug

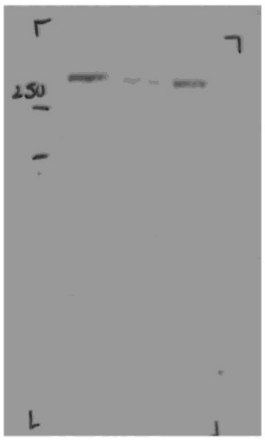


Snail | Zo-1 | Actin

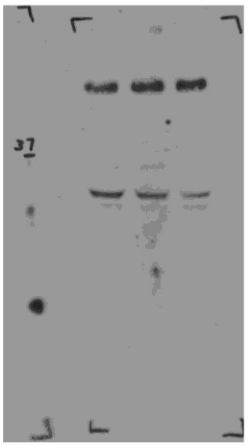
Figure 3.4: Part 2/2



TrkB

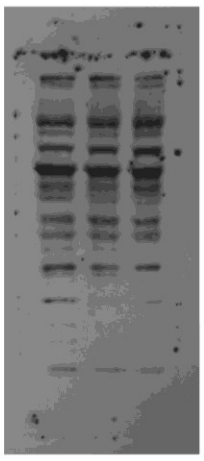


E-cadherin

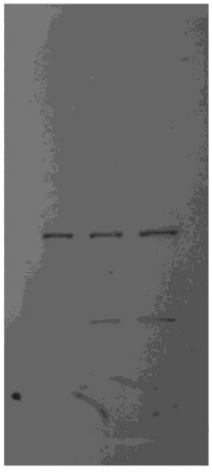


Vimentin

Actin



Claudin-1

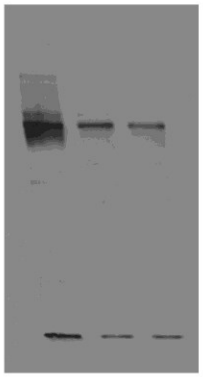


N-cadherin

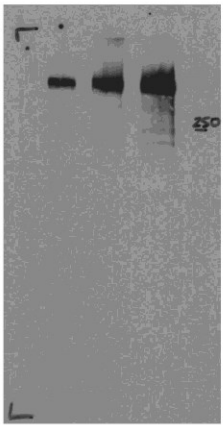
Actin

Figure 3.6: Part 1/4

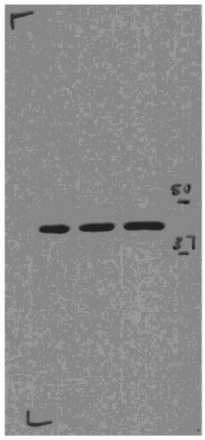
HMLE



PRP4K



Fibronectin



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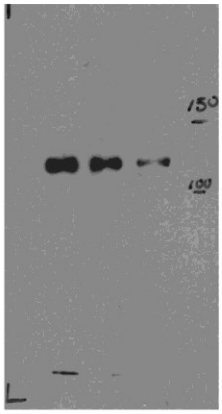
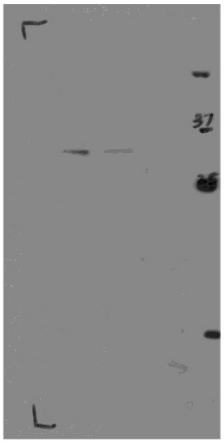
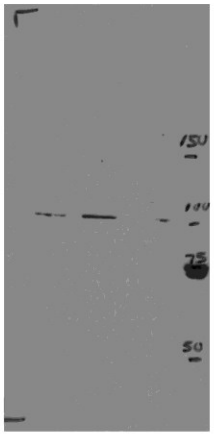


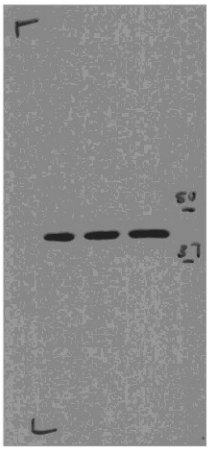
E-cadherin



Slug



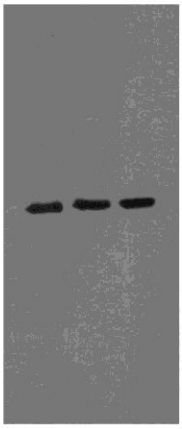
TrkB



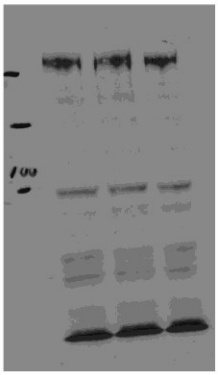
Actin

Figure 3.6: Part 2/4

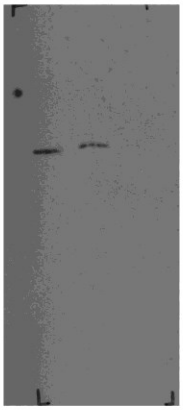
HMLE



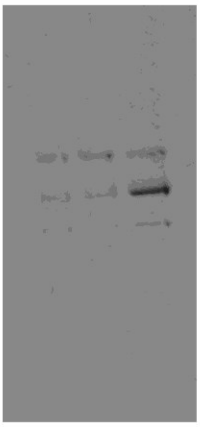
Claudin-1



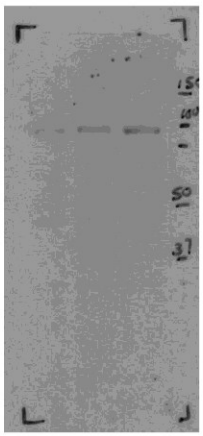
Zo-1



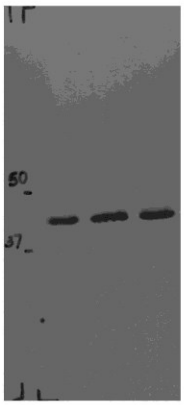
Snail



Zeb-1



Vimentin



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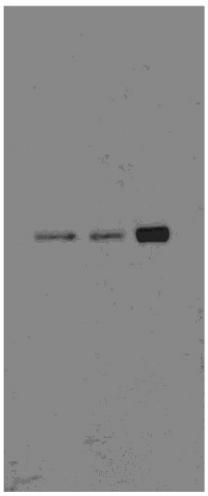
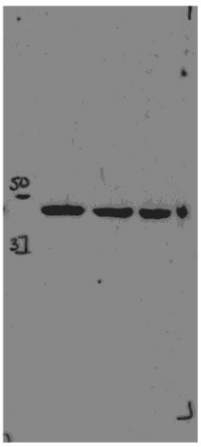
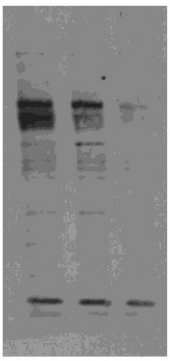


N-cadherin



Actin

Figure 3.6 Part 3/4 MCF10A



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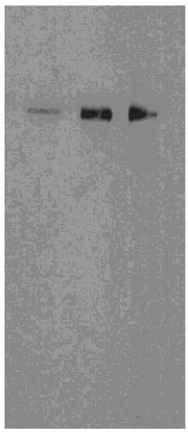

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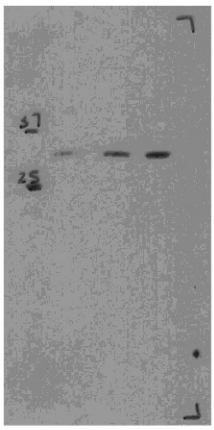

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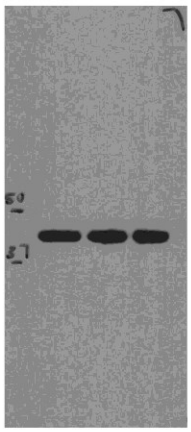
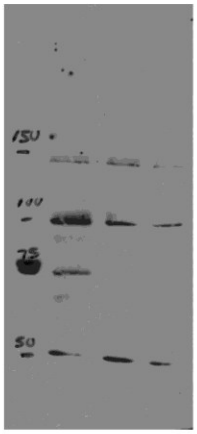


Actin

E-cadherin



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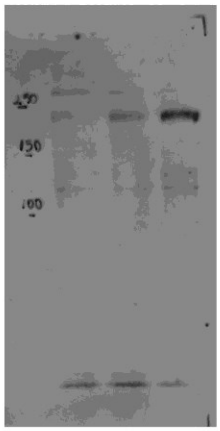
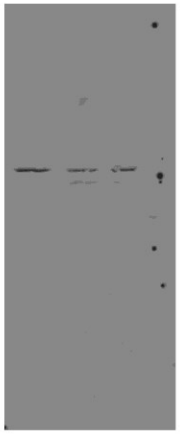
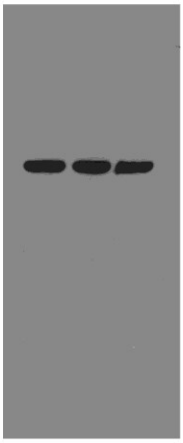


Zeb1



Vimentin

Figure 3.6: Part 4/4 MCF10A



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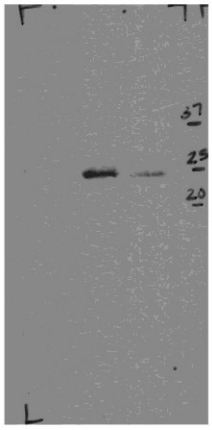
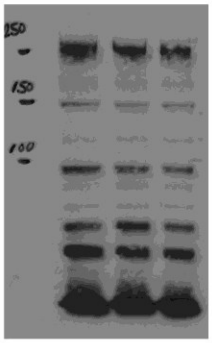
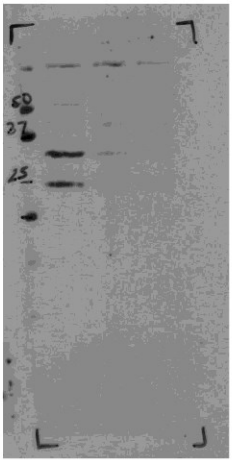


Claudin-1

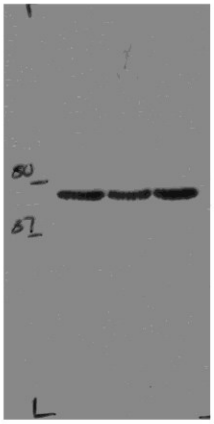


Zo-1

N-cadherin

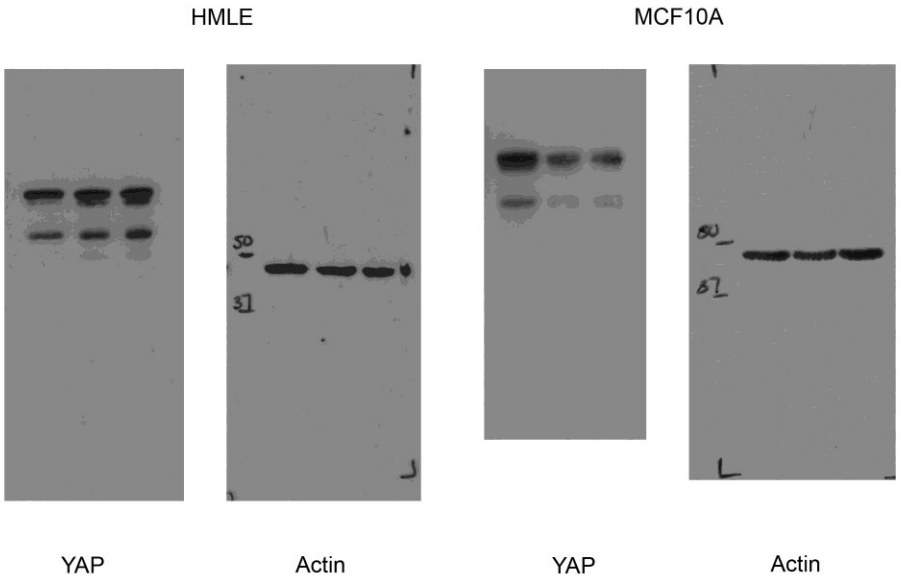


Snail



Actin

Figure 3.9 Part 1/1



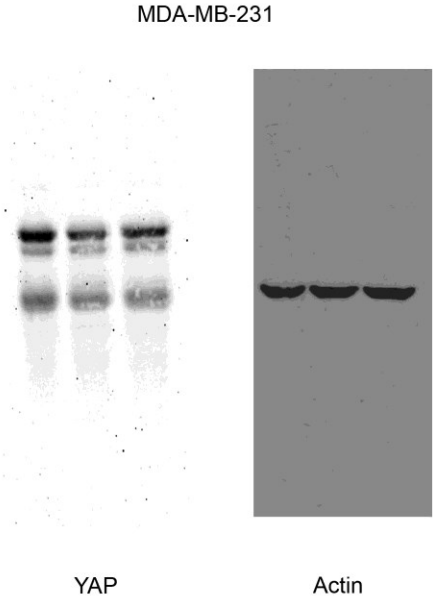


Figure 3.10: Part 1/1

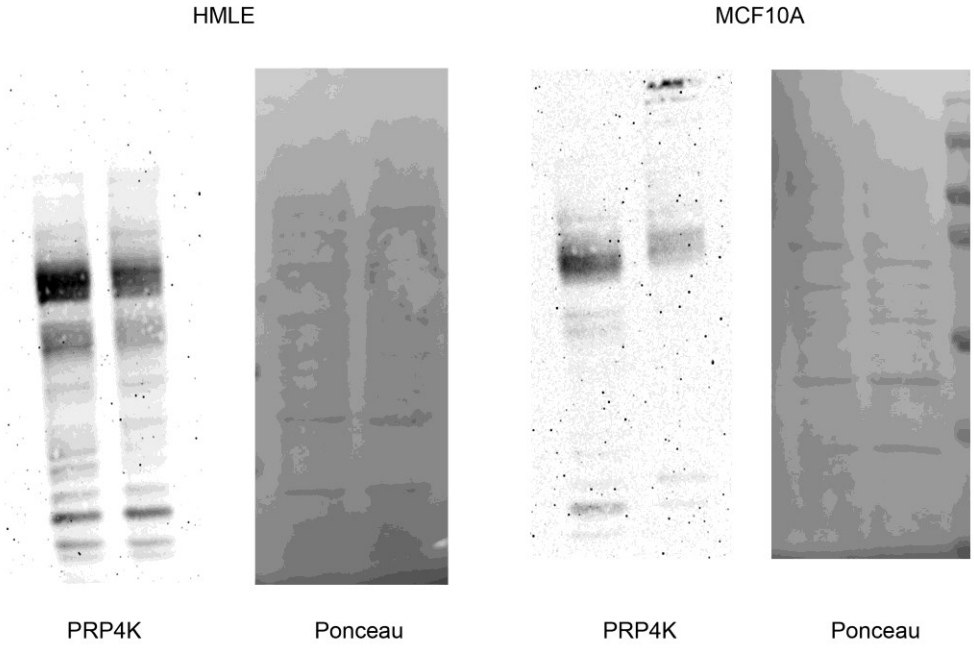


Figure 3.11: Part 1/1

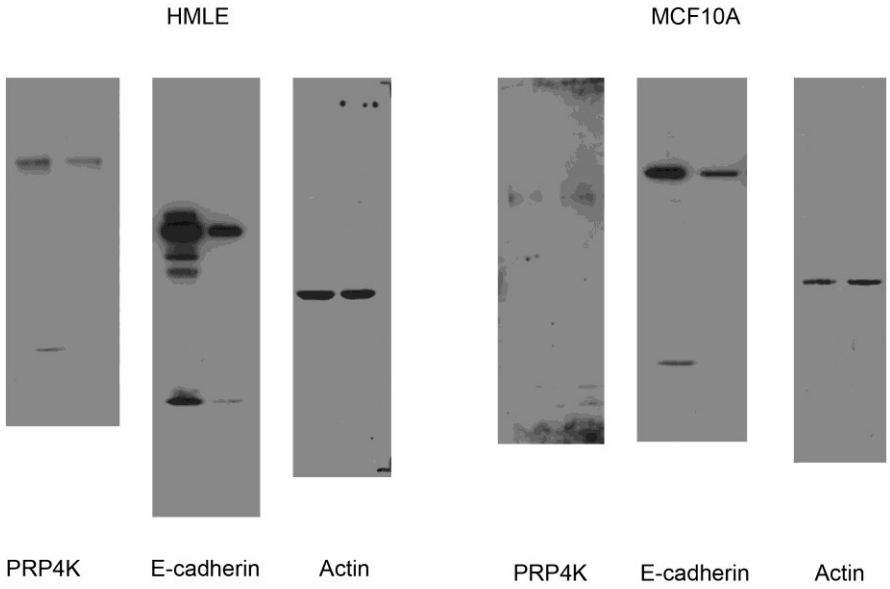
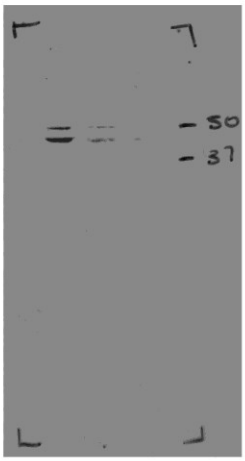
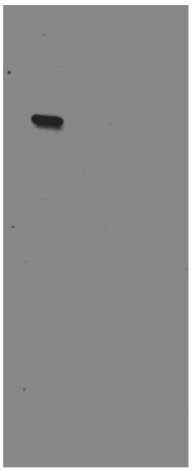


Figure 3.12: Part 1/1

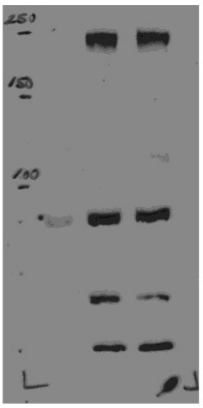


EIF3E

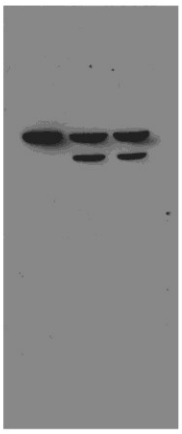
PRP4K



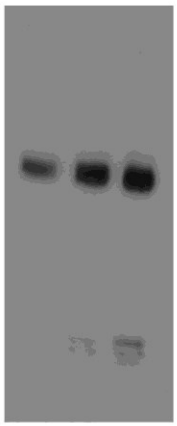
E-cadherin



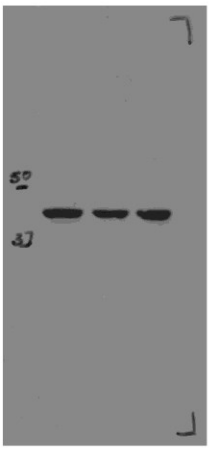
N-cadherin



Vimentin

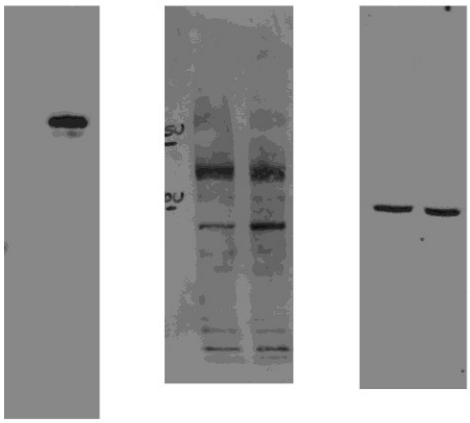


Zeb-1



Actin

Figure 3.14: Part 1/1



Twist PRP4K Actin

Appendix B Copyright Agreements

SPRINGER NATURE

Title: Loss of PRP4K drives anoikis resistance in part by dysregulation of epidermal growth factor receptor endosomal trafficking

Author: D P Corkery, L E Clarke, S Gebremeskel, J Salsman, J Pinder et al.

Publication: Oncogene

Publisher: Springer Nature

Date: Sep 11, 2017

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