LINKING PRP4K CYTOPLASMIC FUNCTIONS TO TUMOUR SUPPRESSION

by

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Abstract

Breast cancer is one of the most common cancers faced by Canadian women today. These carcinomas arise from epithelial cells in mammary tissue, and therefore processes and signaling pathways that regulate tissue homeostasis are important in tumour suppression, including: Hippo-YAP signaling that plays a role in epithelial-to-mesenchymal (EMT) transition; epidermal growth factor receptor (EGFR) signaling; and programmed cell death triggered by cell detachment, a process known as anoikis. In addition, induction of EMT is associated in some cancers with autophagy, or "self-eating", by which organelles and macromolecules are recycled to overcome cellular stress from nutrient and oxygen deprivation. Autophagy is also known to affect cancer cell migration and invasiveness, which are the hallmarks of EMT. PRP4K is a multifunctional protein that is implicated in several cellular processes that play a role in tumour suppression, including pre-mRNA splicing and transcriptional regulation, epidermal growth factor signaling and sensitivity to both anoikis and taxane-based chemotherapy, and more recently loss of PRP4K has been shown to promote YAP signaling. However, how induction of EMT affects PRP4K expression and a possible role for PRP4K in stress-induced autophagy remained to be studied. This thesis focuses on examining the relationship between PRP4K and EMT, as well as PRP4K's role in the cell's response to cellular stress that induces autophagy. Using MDA-MB-231, HMLE and MCF10A cell lines, I demonstrated that loss of PRP4K causes a state of partial EMT, which is associated with cells traits that provide cancers with several advantages including collective cell migration and resistance to anoikis. Additionally, I demonstrated that PRP4K responds to stress by shuttling from the nucleus to the cytoplasm following both nutrient deprivation and oxidative cell stress, as well as inhibition or activation of autophagy using chloroquine and rapamycin (respectively). When PRP4K translocates to the cytoplasm, it does so in a CRM1-dependent manner and colocalizes with markers of autophagy, oxidative stress, and endosomal trafficking. Taken together, this thesis demonstrates a novel potential role of PRP4K in the cellular stress response.

List of Abbreviations Used

AKT: protein kinase B

AMP: Adenosine monophosphate

AMPK: AMP-activated protein kinase

AS: alternative splicing

ATG: autophagy-related genes

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

BSA: bovine serum albumin

CLK: CDC-like kinases

CRM1: chromosomal maintenance 1

DAPI: 4',6-diamidino-2-phenylindole

DCP1: decapping protein 1

DMEM: Dulbecco's Modified Eagle Media

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DDT: dichlorodiphenyltrichloroethane

ECL: enhanced chemiluminescence

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

EMT: epithelial-to-mesenchymal transition

ER: endoplasmic reticulum

FBS: fetal bovine serum

F12: Ham's F-12 Nutrient Mixture

G3BP1: G3BP stress granule assembly factor 1

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER2: human epidermal growth factor receptor 2

HIF: hypoxia-inducible factor

hnRNP: heterogeneous nuclear ribonuclear protein

KD: knock-down

Keap1: Kelch like ECH associated protein 1

KH: lysine-histidine

Lamp 1/2: lysosomal-associated membrane protein ½

LATS1/2: large tumour suppressor 1/2

LC3: light chain 3

MAD1/2/3: mitotic arrest deficient proteins 1, 2 and 3

MPS1: monopolar spindle 1

mRNA: messenger RNA

MST1/2: mammalian STE20-like protein kinase 1

mTOR: mechanistic target of rapamycin

mTORC: mechanistic target of rapamycin complex

N-CoR: transcriptional repressor nuclear receptor corepressor complex

NRF2: nuclear factor erythroid 2-related factor

PAS: pre-autophagosomal structure

PBS: phosphate buffered saline

P-bodies: processing bodies

PI3K: phosphoinositide 3 kinase

PMSF: phenylmethylsulfonyl fluoride

PRP4K: pre-mRNA splicing factor 4 kinase

PRP6: pre-mRNA processing factor 6

PRP28: pre-mRNA processing factor 28

PRP31: pre-mRNA processing factor 31

P/S: penicillin/streptomycin

PTEN: phosphatase and tensin homolog

RAB7: ras-related protein 7

ROS: reactive oxygen species

RS domain: arginine- serine rich subdomain

SAC: spindle assembly checkpoint

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

shRNA: short hairpin RNA

siRNA: small interfering RNA

SNAI1: snail

SNAI2: slug

snRNP: small nuclear ribonucleoprotein

SR protein: serine and arginine-rich protein

SRSF1/2: serine-rich splicing factor 1/2

SRPK: serine-arginine protein kinases

TBST: tris buffered saline with Tween 20

TEAD: transcriptional enhancer factor TEF

TRIM16: tripartite motif containing 16

TrKB: tropomyosin receptor kinase B

Ub: ubiquitin

ULK1: unc-51 like kinase 1

Vim: vimentin

Vps34: vacuolar protein sorting 34

XBP1: X-box binding protein 1

YAP: yes-associated protein 1

Zeb: zinc finger E-box homeobox

Zo1: zonula occludens protein 1

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Chapter 1: Introduction

1.1 Breast cancer development and treatment

1.1.1 Breast cancer

Breast cancer has been among the most common types of cancer exhibited in women (Global Cancer Observatory, 2012). With approximately 1 in 8 Canadian women facing a diagnosis of breast cancer throughout their life, and 13% of cancer deaths in Canadian women, breast cancer disproportionately affects Canadian women in comparison to other cancer types (Government of Canada, 2019). Clinical research has drastically reduced the mortality rate of breast cancer cases; however, a broader understanding of risk factors, development and various treatments are needed to reduce this rate further. Breast cancer is a heterogenous disease and there are multiple subtypes of breast cancer that are classified by their histological pattern (Elston & Ellis, 1991) and often follow similar trends in disease prognosis (Rahka et al., 2010; Strehl et al., 2011). These molecular subtypes include luminal A, luminal B, HER2 enriched, normal-like and triple-negative (Dai et al., 2015).

Luminal subtypes are characterized by their expression of hormone receptors (Perou et al., 2000). They are the most common and tend to have better patient outcomes, in particular, luminal A (Perou et al., 2000; Dai et al., 2015). Luminal A tumours are less likely to relapse and are easier to treat than luminal B tumours (Paik et al., 2004). Normal-like tumours are identical to luminal A tumours in the hormone receptors they express and also tend to have fairly good outcomes (Dai et al., 2015). HER2 enriched tumours over-express HER2, as well as over-express other genes in the amplicon of HER2 and have poor prognosis for patients with a high risk of relapse (Brenton et al.,

2005). Triple-negative tumours are the most heterogenous of all breast cancer subtypes and have the worst prognosis for patients due to their ability to mimic normal basal epithelial cells (Perou et al., 2000). Triple-negative tumours are aggressive and difficult to treat; currently there is no targeted systemic therapy for this type of tumour (Dai et al., 2015). Taken together, breast cancer is a complex disease with multiple subtypes, each with specific outcomes and therapies that affects thousands of women each year. Further research is required to further the progress that has been made in combatting this dreadful disease.

1.1.2 Treatment of Breast Cancer

With the high prevalence of breast cancer in Canadian women and the poor outcomes associated, therapies and treatments for breast cancer are a primary focus in research today. Eligibility for the various treatments available varies with the subtype and stage of the tumour at hand (Morrow et al., 2009; Moo et al., 2018). Currently, breast cancer is diagnosed using physical examinations, magnetic resonance imaging, mammography and ultrasound (National Research Council, 2001; Moo et al., 2018). Currently there are five common treatments available for patients diagnosed with breast cancer: hormone therapy, Herceptin, anthracyclines, taxanes, and combination therapies (Colozza et al., 2007; Yap et al., 2009; Bydoun et al., 2013). In addition to these therapies, discussed in detail below, both immune checkpoint therapy (Borella et al., 2020 Zou et al., 2020) and new precision treatments based on molecular profiling are becoming available including targeting of DNA repair deficient breast cancers with mutations in the

Breast cancer type 1 susceptibility proteins 1 and 2 (BRCA1/2) (McCann & Hurvitz, 2018; Dion et al., 2020).

1.1.2.1 Hormone therapy

Hormone therapies are one of the earliest known targeted therapies to treat breast cancer (Puhalla et al., 2012). Hormone therapies are available to breast cancer patients expressing the estrogen-receptor alpha (ER) and progesterone receptor (PR) and therefore, are not available to patients diagnosed with triple-negative breast cancer. Two common hormone therapies are tamoxifen and raloxifene (Puhalla et al., 2012). Tamoxifen disrupts the transcription of estrogen regulated genes, leading to degradation of the ER protein (DeFriend et al., 1994; Puhalla et al., 2012). Tamoxifen is regularly used to treat luminal cancers, with both luminal A and luminal B responding well to this hormone therapy (Lindstrom et al., 2018).

1.1.2.2 Herceptin

Herceptin, clinically known as trastuzumab is a monoclonal antibody against HER2-overexpressing breast cancer, therefore, it is used to treat HER2 enriched breast cancers (Colomb & Esteva, 2008). The precise mechanism of action for Herceptin is being actively investigated, however it has been proposed that Herceptin may downregulate the HER2 receptor, which would result in less available receptors for binding, or that Herceptin may inhibit a variety of pathways required for cell proliferation, cell signaling or inducing G1 arrest (Colomb & Esteva, 2008). Herceptin was the first monoclonal antibody to be approved in the treatment of cancer, and while

success has been achieved through Herceptin use, patients are still at risk for metastatic disease and combination therapies to use in conjunction with Herceptin are currently under investigation (Slamon et al., 2001; Colomb & Esteva, 2008). To date, patients diagnosed with HER2 enriched breast cancer have poor prognosis and a high risk of relapse (Brenton et al., 2005).

1.1.2.3 Anthracyclines

Among the most well-known form of anti-cancer drugs is anthracyclines, having been used as a treatment for decades against multiple breast cancer subtypes. Typically used in early-stage breast cancer, anthracycline drugs include daunorubicin, doxorubicin, epirubicin and idarubicin and are used to inhibit DNA and RNA synthesis (Sinha & Politi, 1990). They are a class of cytotoxic drugs, which is why their use must be closely monitored, and are sometimes not a viable long-term option (Singal & Iliskovic, 1998; Shah & Gradishar, 2018). Currently, anthracyclines are used when the benefit of breast cancer regression outweighs the toxicity associated with anthracycline use (Shah & Gradishar, 2018). Patients with early stage, non-metastatic cancers may be able to use other therapies, such as taxanes to treat their cancer, minimizing toxicity and unwanted side effects (Shah & Gradishar, 2018).

1.1.2.4 Taxanes

Taxanes are another common form of anti-cancer agent that has proven to be effective in treating early and late-stage breast cancer (Gradishar, 2012; Schwab et al., 2014). Taxanes act to inhibit and disrupt microtubule function by stabilization of

microtubules, thereby disrupting cell division (Katsumata et al., 2009). Taxanes, such as paclitaxel and docetaxel are particularly effective in treating metastatic disease and are among the most commonly used systemic therapies for the treatment of breast cancer (Gradishar, 2012).

1.1.2.5 Combination Therapies

Combination therapies are emerging as a common and effective method for treating cancers, optimizing the use of multiple anti-cancer agents synergistically, while minimizing toxicity has led to better outcomes for breast cancer patients receiving combination therapies (Mokhtari et al., 2017). Combination therapies employ two or more drugs to target multiple pathways and minimize drug resistance, however this type of therapy does not come without disadvantages (Zimmermann et al., 2007; Mokhtari et al., 2017). Such disadvantages include unwanted side effects and drug interactions (Berdeja et al., 2015; Mokhtari et al., 2017). Taken together, the data currently available on combination therapies, as well as other anti-cancer therapies indicates that the field of cancer biology and treatments to this horrid disease still have substantially more to discover.

1.2 EMT and YAP signaling and their role in cancer

A loss of contact inhibition is a common phenomenon experienced by cancer cells (Abercrombie, 1979). Contact inhibition, or an abrupt cell cycle arrest limiting tissue growth, is a normal response that occurs in tissues when individual cells make contact (Eagle & Levine, 1967). One of the signaling pathways implicated in contact inhibition

and tissue homeostasis is the Hippo pathway, which is characterized by activation two core kinases, mammalian STE20-like protein kinase 1 and 2 (MST1/2) and large tumour suppressor 1 and 2 (LATS1/2) (Park et al., 2018). In epithelial tissues, tight junctions, adherens junctions and cadherin complexes all contribute to the activation of MST and LATS kinases (reviewed in Gumbiner & Kim, 2014). Once Hippo signaling is activated, one of the key effector proteins phosphorylated is the transcription factor known as the Yes-associated protein (YAP) which is responsible for the growth of tissues and organs by promoting cell proliferation and inhibiting cell death (Huang et al., 2005; Zhao et al., 2007; Johnson & Halder, 2014; Piccolo et al., 2014). Specifically, both MST1/2 and LATS1/2 are capable of phosphorylating and inhibiting YAP (Park et al., 2018). While YAP are responsible for tissue repair and regeneration, it also has roles in inhibiting cell death and promoting proliferation, which ultimately leads to the development of cancer (Cottini et al., 2014; Johnson & Halder, 2014). Phosphorylated YAP is either held in the cytoplasm or shuttled to the nucleus where it is subjected to autophagic degradation (Park et al., 2018). Additionally, YAP has been shown to influence epithelial-to-mesenchymal transition (EMT) (Cheng et al., 2020). Specifically, YAP has been found to interact with Snail and Slug, Zeb-1 and N-cadherin (Tang & Weiss, 2017; Zhang et al., 2018; Feldker et al., 2020). Snail and Slug function as transcriptional repressors and mesenchymal markers and are involved in the progression of EMT; recently it was discovered that Snail and Slug interact with the YAP/TAZ pathway, forming a complex that effects stem cell growth and function (Tang & Weiss, 2017). It was also found that YAP interacts with the mesenchymal marker Zeb-1 to activate tumour promoting genes and repress epithelial genes (Feldker et al., 2020). Lastly, it has been shown that overexpression of YAP increases the protein level of N-cadherin (Zhang et al., 2018). Because of the role of YAP

in oncogenesis and EMT, further investigation is required but the data indicates that it may be a useful target for cancer therapies.

Epithelial to mesenchymal transition (EMT) is a process in which epithelial cells acquire a mesenchymal phenotype (Thiery, 2002; Kalluri, et al., 2003). This process is important in the progression of cancer and development of metastasis. EMT occurs during embryonic development and is characterized by loss of cell-cell adhesions, a shift from apical-basal polarity to front-rear polarity, as well as change in cellular shape (Pradella, et al., 2017; Clarke et al., 2019). EMT is a flexible, reversible process with several intermediate states, which fall along a spectrum of partial EMT (Kim et al., 2017; Aiello, et al., 2018). Essentially, rather than cells exhibiting strictly epithelial or mesenchymal properties alone, partial EMT allows for cells to exist in an intermediate state, falling within the broad spectrum of exhibiting both partially epithelial and partially mesenchymal morphology (Shibue & Weinberg, 2017). Partial EMT is involved heavily in metastasis of cancer cells, however; it does not appear to be heavily involved in tumour development (Kalluri & Weinberg, 2009). Partial EMT phenotypes consist of intermediate states between epithelial and mesenchymal polarity, morphology, and often maintenance of epithelial gene expression (e.g. E-cadherin) (Kim et al., 2017; Aiello, et al., 2018). These partial EMT states lend several advantages to cancer cell growth and development, including collective migration, in which cells migrate as clusters, higher plasticity, as well as increased stemness traits and drug resistance, compared to strictly epithelial or mesenchymal cells (Kim et al., 2017; Aiello, et al., 2018). These advantages allow EMT to drive metastasis and acquire destructive traits that assist in the development of malignant cancers (Kudo-Saito et al., 2009; Tsai et al., 2012). Specifically, it has been shown that following K27-linked poly ubiquitination,

phosphatase and tensin homolog (PTEN) is capable of promoting partial EMT through the stabilization of mesenchymal markers in renal epithelial cells (Li et al., 2019). These findings demonstrate a potential role for the modification of tumour suppressor genes in the development of partial EMT.

1.2.1 Epithelial Proteins

There are three main epithelial proteins whose expression is reduced during EMT; E-cadherin, Zo-1 and Claudin-1 (Ohkubo & Ozawa, 2004). Each protein has distinct functions but are all vital to the maintenance of an epithelial phenotype. E-cadherin falls into a class of transmembrane glycoproteins known as cadherins. Cadherins are involved in the maintenance of cell-cell adhesions via a calcium dependent manner (Pokutta, et al., 1994; Gumbiner, 2005). E-cadherin is also involved in the migration and morphology of cells during embryogenesis and a loss of E-cadherin is the most common indicator of EMT (Medici et al., 2008; Van Roy & Berx, 2008). During EMT, E-cadherin is decreased, and a mesenchymal protein, N-cadherin is increased (Araki et al., 2011; Wang et al., 2015). This decrease in E-cadherin levels causes the dissolution of cell-cell adhesions, thereby leading to greater metastatic potential in the case of cancer cells. Claudins, such as claudin-1 are a family of proteins that assist in the formation of tight junctions, maintain polarity and have roles in the differentiation and growth of cells (Morita et al., 1999; Morin, 2005). Interestingly, over expression of claudin-1 has been shown to induce EMT without changes in cell morphology (Suh et al., 2012). These findings demonstrate the role claudin-1 has as a marker for EMT. In addition to E-cadherin and claudin-1, zonula occludens protein-1 (Zo-1), also known as tight junction

protein-1 is a protein that localizes at cell-cell adhesion membrane complexes and is involved in EMT (Polette et al., 2007). Specifically, Zo-1 relocalizes from the cytoplasm to the nucleus during EMT; the exact reasoning is still under investigation, but it appears that Zo-1 is a molecule involved in the shuttling process (Polette et al., 2007).

1.2.2 Mesenchymal Proteins

N-cadherin is a member of the cadherin family and is involved in maintaining the structure of tissue, similarly to E-cadherin (Kim et al., 2000; Nakajima et al., 2004). N-cadherin, however, has been found to promote motility and invasion during EMT which is consistent with a mesenchymal phenotype, deeming it a mesenchymal marker (Nieman et al., 1999). Additionally, it has been shown that members of the Snail family, including Snail (SNAI1) and Slug (SNAI2), as well as Zeb-1 are E-cadherin transcriptional repressors, capable of promoting EMT in certain cancers (Peinado et al., 2004; Medici et al., 2008). Vimentin is an intermediate filament that can be classified as a mesenchymal marker (Coulombe & Wong, 2004; Zeisberg & Neilson, 2009). It is responsible for maintaining cell integrity and has been shown to be associated with invasiveness and poor prognosis in cancer (Coulombe & Wong, 2004; Satelli & Li, 2011). Vimentin has roles in promoting EMT by upregulating slug expression and downregulating E-cadherin expression, as well as inducing cancer malignancy, demonstrating a crucial role for EMT progression in cancer cells (Liu et al., 2015). Similarly to vimentin, fibronectin is a mesenchymal marker involved in EMT. Fibronectin is not expressed in non-cancerous breast tissue; however, during cancer, in particular metastatic breast cancer fibronectin is often overexpressed, giving rise to the capacity of fibronectin to act as a marker of

prognosis in breast cancer (Ioachim et al., 2002; Bae et al., 2013; Zhou et al., 2015; Li et al., 2017). Lastly, TrkB is an important marker for EMT, capable of downregulating E-cadherin in several types of cancer (Kupferman et al., 2010). TrkB has been shown to increase migratory capability and has been found to regulate EMT (Smit et al., 2009). Its roles in migration, invasion and EMT demonstrate its importance as a mesenchymal marker in various cancers (Kupferman et al., 2010).

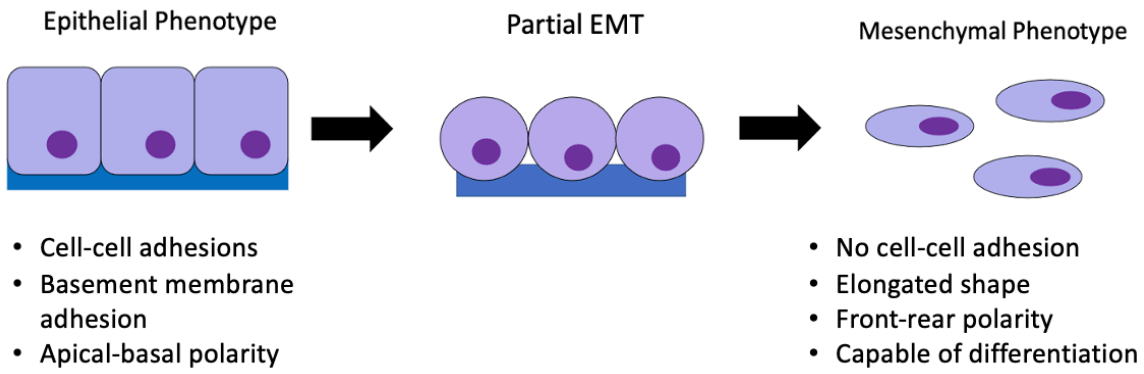


Figure 1.1: The process of epithelial-to-mesenchymal transition. When undergoing EMT, cells are found to lose cell-cell adhesions, display changes in morphology and polarity and experience a change in epithelial and mesenchymal proteins present.

1.3 Autophagy and its role in cancer progression and treatment responses

The term autophagy represents an umbrella term for three distinct degradative systems known as macro-, micro- and chaperone-mediated autophagy, which together are capable of recycling or degrading damaged organelles and biomolecules to maintain cellular homeostasis. Macroautophagy involves the formation of a double membraned autophagosome, whereas microautophagy and chaperone-mediated autophagy operate via the direct engulfment by the lysosomal membrane and the chaperone dependent selection of cytosolic components, respectively (Mizushima, 2007). All three subtypes of autophagy are activated by cellular stresses such as nutrient deprivation, hypoxia, mitochondrial damage, heat stress and intracellular pathogens (Kroemer et al., 2010; Dokladny et al., 2015). Autophagy is an intracellular degradation system that transports cytosolic contents to the lysosome (Mizushima, 2007; Rubinsztein et al., 2011; Sarkar, 2013). The autophagy process is initiated by activation of the AMP activated protein kinase (AMPK) in response to various cellular stresses such as nutrient deprivation, heat stress, mitochondrial damage and mTOR inhibition (Kroemer et al., 2010; Kim et al., 2011; Dokladny et al., 2015). These early signaling events trigger the formation of the phagophore (or isolation membrane), the structure that proceeds the autophagosome. The phagophore elongates to surround the cytosolic contents that have been selected for degradation. Formation of the phagophore requires not only a signaling event, but both the serine/threonine protein kinase unc-51 like kinase 1 (ULK1) complex and four autophagy related genes*, Atg5, Atg7, Atg10* and *Atg12*, commonly known as a "ubiquitin-like conjugation system" (Rusten & Stenmark, 2009; Rabinowitz & White, 2010; Sarkar, 2013; Zachari & Ganley, 2017; Xin et al., 2018). The ULK1 complex recruits the PI3K/VPS34 complex and translocates to autophagy initiation sites upon induction

(Zachari & Ganley, 2017). In combination, these complexes result in the formation of a phagophore.

Phagophore formation may begin via the pre-autophagosomal structure (PAS), a structure which has been well characterized in yeast; however, has not yet been observed in mammals (Mizushima, 2007). The phagophore can originate from various organelles, such as the Golgi, endoplasmic reticulum (ER), mitochondria or plasma membrane (Tooze & Yoshimori, 2010). Regardless of its place of origin, the phagophore is a double membraned structure that develops through a large macromolecular complex containing the class III phosphoinositide 3-kinase (PI3K) vacuolar protein sorting 34 (*Vps34*) (Rubinsztein et al., 2011; Sarkar, 2013). Full development of the phagophore is completed due to the recruitment and function of LC3 (light chain 3), which is responsible for membrane tethering and hemifusion. Such processes are required for the transition of a phagophore into an autophagosome (Sarkar, 2013), leading to full sequestration of the cytosolic contents that have been selected for degradation (Mizushima, 2007). The autophagosome then fuses with a lysosome (creating an autophagolysosome), where the concentration of acidic hydrolytic enzymes are high and rapid degradation can occur (Mizushima, 2007).

Induction of autophagy occurs following cellular stress, such as essential nutrient starvation in yeast and mammalian organisms (Takeshige et al. 1992; Mizushima, 2007; Rubinsztein et al., 2011; Sarkar, 2013). Regulation of autophagy follows one of two pathways, which differ based on their dependence on the mechanistic target of rapamycin (mTOR). mTOR negatively regulates autophagy, mediates protein translation and cell growth (Mizushima, 2007; Rubinsztein et al., 2011; Sarkar, 2013). mTOR ultimately regulates autophagy through two complexes, mTORC1 (mTOR complex 1), and

mTORC2 (mTOR complex 2) (Sarkar, 2013). mTORC1 is currently the best understood pathway, and its inhibition during nutrient starvation or treatment with rapamycin can activate autophagy (Sarkar, 2013). mTOR-independent regulation of autophagy can occur via inositol signaling, changes in intracellular calcium and elevation of cyclic AMP (Sarkar, 2013). Together, the induction and regulation of autophagy results from several cellular stresses and therefore, can serve as an investigation tool for various fields, such as therapeutic treatments for cancer and neurodegeneration.

Autophagy is both pro and anti- tumorigenic and therefore, has been debated as a potential cancer treatment (Rosenfeldt & Ryan, 2011; Gewirtz, 2014). On one hand, autophagy has been shown to contribute to the expression of tumour suppressor genes, which can suppress cancer initiation through autophagy induction via mTOR and AMPK inhibition (Comel et al., 2014; Yun & Lee, 2018). On the other hand, some anticancer drugs can regulate autophagy, such as oncogenes that are activated by mTOR, class 1 PI3K and AKT that could suppress autophagy and therefore increase the development of cancer (Choi et al., 2013; Yun & Lee, 2018). Due to the ongoing uncertainty of whether autophagy could be a beneficial or detrimental process in the development of cancer, further research is required. In this respect, in this thesis I begin to address this knowledge gap by investigating the novel cytoplasmic role of PRP4K in autophagy that may contribute to its role as a tumour suppressor.

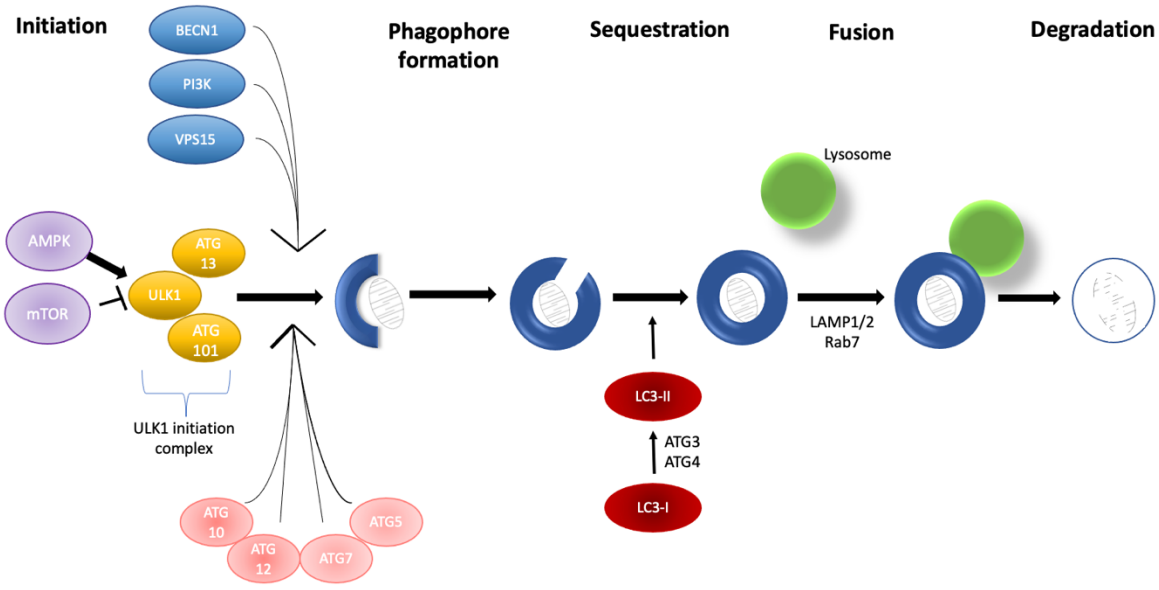


Figure 1.2: A model of the autophagy pathway. Initiation of autophagy begins with AMPK activation or inhibition of mTOR signaling. The ULK1 initiation complex, four autophagy related genes and the Beclin1 complex then assists with the formation of a phagophore. Phagophore closure is completed via the action of LC3, which is followed by fusion with the lysosome and subsequent degradation of the damaged cytosolic contents.

1.4 Cellular stress pathways and their role in cancer development and treatment responses

Due to the resilient nature of cancer cells, capable of evading apoptotic elimination, overcoming senescence, and a plethora of other challenges, it is not surprising that cancer cells must face and combat cell stress along the way. Two obvious stresses that nearly all cancers would see are nutrient deprivation and hypoxia. Due to the rapid growth of tumours and limited vascularization within the core of the tumour, cancer cells are often forced to survive with little to no oxygen or nutrients (Robichaud & Sonenberg, 2017). They do this by developing mechanisms to combat stress, such as controlling protein translation, activating nutrient reserves through autophagy, or activating hypoxia-inducible factor (HIF), a mechanism where cells sense oxygen depletion and gene expression in controlled in such a way to maintain long-term survival (Bellot et al., 2009; Efeyan et al.,2015). For example, cancer cells have been shown to alter their proteome through altered protein translation in response to cell stress, such as hypoxia, nutrient deprivation and oxidative stress (Robichaud & Sonenberg, 2017). Mechanistically, translational control under these stress states involves both mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) which are inhibited and activated (respectively) in response to these stresses (Reiling & Sabatini, 2006; Schneider et al., 2008). MTOR signaling regulates initiation of translation via protein substrates such as eIF4E binding proteins (4E-BP1) and the ribosomal protein S6 kinase (S6K1) (Ma et al., 2009; Showkat et al., 2014). 4E-BP1 is a protein that binds to eIF4E, forming a complex that prevents association with eIF4G and subsequently inhibits protein translation (Hay & Sonenberg, 2004; Showkat et al., 2014). Phosphorylated by mTOR, S6K1 phosphorylates a multitude of substrates responsible for protein synthesis, cell survival, transcription and splicing (Wang et al., 2001; Hannan et

al., 2003; Fang et al., 2006; Showkat et al., 2014). S6K1 is particularly important during nutrient deprivation due to its effects on insulin signaling. Insulin receptor substrate 1 (IRS1) is phosphorylated by S6K1, which provides negative feedback regulation of insulin (Zhang et al., 2008). In contrast, AMPK senses nutrient or oxidative stress and then in turn inhibits mTOR via direct phosphorylation of regulator associated protein of mTOR (Raptor) and indirect phosphorylation of tuberous sclerosis complex 2 (TSC2) (Agarwal et al. 2015). Raptor is a binding partner to mTOR and is required for mTOR signaling (Oshiro et al., 2004). TCS2 is a tumour suppressor gene responsible for the integration of signals from various pathways, including AKT and AMPK (Huang & Manning, 2008), as well as monitoring the incoming growth factor signals and relaying that information to ensure that mTORC1 is properly controlled (Huang & Manning, 2008). AMPK is also responsible for turning on the pathways that generate ATP and turning off pathways that consume ATP when the cell is in a state of nutrient stress (Hardie et al., 2012). In addition, mTOR is an inhibitor of autophagy initiation via phosphorylation of ULK 1 kinase (discussed in detail below), and thus mTOR and AMPK are important regulators of autophagy (Robichaud & Sonenberg, 2017).

Because of the activation and inhibition of autophagy by varying mechanisms is used so widely as a strategy by cancer cells to combat cell stress, four stresses that uniquely activate or inhibit autophagy were used in this study. These include chloroquine and rapamycin, an inhibitor of lysosomal fusion within the autophagy pathway and a direct activator of autophagy via mTOR inhibition (respectively); and the physiological stresses that many cancers face; nutrient deprivation and oxidative stress were the models used to induce cell stress and alter autophagy status.

1.4.1 Chloroquine

Chloroquine is a commonly known anti-malarial drug that has been under investigation for its uses in cancer therapy (O'Neill, et al., 1998). Chloroquine is an autophagy inhibitor, that works to block the fusion between autophagosomes and lysosomes (Poole & Ohkuma, 1981; Klionsky et al., 2016; Mauthe et al., 2018). Because lysosomes are filled with hydrolytic enzymes, chloroquine works by raising the intralysosomal pH, which in turn inhibits the binding of the highly acidic lysosome (Poole & Ohkuma, 1981; Mauthe et al., 2018). By doing so, chloroquine inhibits autophagic flux, a process that is necessary for the execution of macroautophagy (Mauthe et al., 2018). Additionally, chloroquine has been shown to reduce endosomal growth factor receptor degradation (Mauthe et al., 2018). This indicates that while chloroquine has roles in autophagy, it has further functions in endosomal trafficking that have yet to be discovered.

While the effects of chloroquine on the autophagy pathway are well studied, the effect of chloroquine on endosomal compartments, such as the endoplasmic reticulum (ER) are lacking. In 2015, it was shown by Johnson and colleagues that chloroquine promotes ER stress, as well as cell death in cells with an overactivation of the mTORC1 pathway. Additionally, it was shown that when used in combination with the antireoviral drug, nelfinavir, chloroquine was capable of activating the splicing factor, X-box binding protein 1 (XBP1), indicating that chloroquine may have a direct effect on the outcomes of splicing, potentially in an autophagy-dependent, or independent manner (Johnson et al., 2015).

1.4.2 Rapamycin

Rapamycin was originally used as both an anti-fungal and immunosuppressive drug, until its function within the autophagy pathway and its effects on the mammalian target of rapamycin (mTOR) were discovered (Heitman et al., 1991). mTOR is a member of the phosphatidylinositol 3-kinase (PI3K) family and is a regulator of nutrient signaling that functions to suppress autophagy under normal physiological conditions (Schmelzle & Hall, 2000; Saxton & Sabatini, 2017). Both rapamycin and nutrient deprivation directly inhibit mTOR, initiating the autophagy pathway (Schmelzle & Hall, 2000; Kim et al., 2002). There are two pathways that mTOR may be inhibited: mTOR complex 1 (mTORC1), which is sensitive to rapamycin and is responsible for initiation of autophagy under nutrient deprivation conditions. mTOR complex 2 (mTORC2) is insensitive to rapamycin and is thought to act as a negative regulator of autophagy (Kunz et al., 1993; Kim et al., 2002; Jung et al., 2010).

In addition to rapamycin's capability of initiating autophagy, it is also capable of enhancing the kinase activity of Atg1, an autophagy related gene not only required for autophagy initiation and progression, but in combination with Atg13, is required for cytoplasm to vacuole targeting (Kamada et al., 2000). Because of the roles rapamycin plays in the initiation and progression of autophagy, it was hypothesized that rapamycin may be a stress that is capable of inducing the appearance of cytoplasmic PRP4K and subsequent colocalization with markers of autophagy and endosomal trafficking.

1.4.3 Nutrient Deprivation

One stress in particular that many cancers face is nutrient deprivation. Nutrient deprivation is commonly observed in several types of cancer, as the tumour grows and changes, access to nutrients may not be possible for certain cancer cells depending on location and morphology of cells within the tumour environment (Dang & Semenza, 1999). Nutrient deprivation is a main initiator of the autophagy pathway via mTOR inhibition (Shang et al., 2011). While nutrient deprivation is responsible for inhibiting mTOR, it does so in a different manner than classic autophagy initiator discussed previously, rapamycin. Nutrient deprivation inhibits mTOR through the Unc-51-like kinase 1 (ULK1) complex (Chan et al., 2007). As mentioned previously, the ULK1 complex recruits the PI3K/VPS34 complex and translocates to autophagy initiation sites upon induction, where a phagophore is then formed (Zachari & Ganley, 2017). Interestingly, during nutrient deprivation, it has been shown that ULK1 is dephosphorylated, allowing it to associate with AMPK, a kinase responsible for monitoring energy levels throughout the cell (Shang et al., 2010). Therefore, ULK/AMPK association can prepare the cell to rapidly induce autophagy when required.

Due to the stress that occurs when cells are deprived of serum, it was hypothesized that serum deprivation may activate the autophagy pathway and cause PRP4K cytoplasmic translocation and subsequent colocalization with markers of autophagy or endosomal trafficking.

1.4.4 Oxidative stress

Hypoxia or oxygen deprivation in cells is another mechanism that can be used to induce autophagy. Unlike rapamycin and nutrient deprivation, hypoxia has been shown to induce autophagy in a hypoxia-inducible factor (HIF)-dependent manner, in both normal cells and cancer cells (Bellot et al., 2009). HIF is rapidly activated when oxygen levels are depleted and is responsible for controlling gene expression to maintain short-term and long-term cell survival during hypoxia (Schofield & Ratcliffe, 2004). Because hypoxia is known to initiate autophagy via a distinct mechanism, investigating the effects it had on the cytoplasmic translocation of PRP4K and colocalization with markers of autophagy was a primary focus of this study. Additionally, it has been shown that hypoxic conditions are capable of promoting EMT in multiple types of cancer, including ovarian cancer, marked by loss of E-cadherin expression and increased expression of SNAIL (Imai et al., 2003). Because of this, investigating the role of hypoxia on PRP4K cytoplasmic shuttling in cancer cell lines was important, because it provided a direct link between the previous work of the Dellaire lab on epithelial-to-mesenchymal transition and my study on the cytoplasmic functions of PRP4K.

In addition to HIF-signaling, the nuclear factor erythroid 2-related factor 2 (NRF2) also regulates transcriptional programs involved in the cell's response to hypoxia and oxidative stress (He et al., 2020). NRF2 is a tightly regulated process that is involved in maintaining cellular homeostasis when faced with conditions of oxidative stress (He et al., 2020). The main E3 ubiquitin ligase complex involved in the regulation of NRF2 is the Kelch-like-ECH-associated protein 1 (KEAP1) (He et al., 2020). KEAP1 forms an E3 ubiquitin ligase complex with Cullin 3 (CUL3) and RING box protein (RBX1), which binds and maintains low levels of NRF2 during unstressed conditions through

ubiquitination. However, under oxidative stress, cysteines of KEAP1 react with reactive oxygen species (ROS) which inhibits the ubiquitination of NRF2, allowing it to escape KEAP1 mediated degradation and accumulate in the nucleus (Baird et al., 2013; He et al., 2020). It has been shown that cancer cells often use the activation of NRF2 to promote cell survival, and mutations in KEAP1 or NRF2 have been detected in multiple types of human cancer (Kensler & Wabasayashi, 2010; Shibata et al., 2008; Ohta et al., 2008; Baird et al., 2013). Interestingly, it was shown that the adaptor of autophagy, p62 is capable of activating NRF2 by binding to the NRF2-binding site on KEAP1 (Komatsu et al., 2010), which indicates that overproduction of p62 or impairment of the autophagy pathway disrupts the KEAP1-NRF2 interaction and likely subsequent protection against oxidative stress (Komatsu et al., 2010). Additionally, it was recently shown that a regulator of autophagy, Tripartite Motif Containing 16 (TRIM16) positively regulates the p62-NRF2-KEAP1 complex under oxidative stress (Jena et al., 2018). Specifically, TRIM16 overexpression causes an increase in p62 levels, and increases p62-NRF2 interaction through displacing KEAP1 (Jena et al., 2018). Taken together, this data indicates that the p62-NRF2-KEAP1-TRIM16 complex is a key player in the cellular response to oxidative stress and the role of this complex in autophagic degradation following oxidative stress requires further research.

1.5 PRP4K is a functionally pleiotropic tumour suppressor

1.5 Pre-mRNA splicing factor 4 kinase

Pre-mRNA splicing factor 4 kinase (PRP4K), also known as PRPF4B was first characterized as a kinase required for pre-mRNA splicing in fungi and mammals (Alahari

et al., 1993; Schneider et al., 2010; Lutzelberger & Kaufer, 2012). PRP4K is an essential serine/threonine kinase, first identified by Norbert Kaufer and colleagues as the gene *prp4* in *Schizosaccharomyces pombe* (Alahari et al., 1993), and later characterized as a U5 small nuclear ribonucleoprotein (snRNP)-associated protein responsible for activation of the spliceosome in mammalian cells (Dellaire et al, 2002; Schneider et al., 2010).

The structure of PRP4K is highly conserved between fission yeast and mammals. The kinase is composed of 1007 amino acids and weighs 150 kilodaltons (Dellaire et al., 2002). It is composed of a C-terminus with a dual specificity kinase family, as well as an N-terminus that contains lysine-histidine (KH) or arginine-serine (RS) subdomains (Dellaire et al., 2002) (Figure 1.3). The RS domain is particularly important in PRP4K's ability to act as a splicing kinase, as it has been shown that the RS domains of SR proteins are capable of interacting with the RS domains of other splicing factors (Wu & Maniatis, 1993). Additionally, it has been shown that several splicing proteins contain these evolutionarily conserved RS domains within their protein structure, which indicates that they are an integral component of splicing associated proteins (Boucher et al., 2001). RS domains also have functions in splicing activation and spliceosomal assembly and activity (Graveley, 2000; Shen et a., 2004; Shepard & Hertel, 2009). In addition to its KH and RS subdomains, PRP4K also contains MI and MII, two evolutionarily conserved structures involved in substrate recognition (Dellaire et al., 2002).

PRP4K is involved in not only pre-mRNA splicing, but also a host of other cellular functions including: regulation of the spindle assembly checkpoint (Montembault et al., 2007); transcriptional regulation (Huang et al., 2000; Dellaire et al., 2002; Huang et al., 2007); as a biomarker and modifier of taxane sensitivity in cancer (Corkery et al., 2015); Hippo signaling (Cho et al., 2018); anoikis sensitivity (Corkery et al., 2018),

epithelial-to-mesenchymal transition (EMT), a process that has implications in cancer metastasis (Clarke et al., 2020; in review); and is currently being investigated for other cellular roles that impact tumour suppression including EGFR endosomal trafficking (Corkery et al., 2018) and autophagy.

Figure 1.3: The Protein Structure of PRP4K. Human PRP4K protein structure. PRP4K weighs 150 kilodaltons and is composed of 1007 amino acids. It contains several protein motifs and domains including a lysine histidine-rich region containing a "KKHK" repeat motif, an arginine/serine-rich domain (RS), two evolutionarily conserved motifs called MI and MII, and a dual-specificity kinase domain. Adapted from Dellaire et al., 2002.

1.5.1 PRP4K is Involved in Pre-mRNA Splicing and Transcriptional Regulation

As a member of the U5 snRNP, one major function of PRP4K is in spliceosomal assembly. SnRNPs are required for proper assembly of a catalytically active spliceosome (Staley & Guthrie, 1998; Corkery et al., 2015). The spliceosome, a multimegadalton ribonucleoprotein (RNP) complex is composed of U1 and U2 small nuclear ribonucleic proteins (snRNPs), as well as the U4/U6/U5 tri-snRNP (Will & Luhrmann, 2011). Both the 5' splice site and the 3' splice site are located within the spliceosome (Will & Luhrmann, 2011). The spliceosome is responsible for removing introns (non-coding sequences) and joining exons (coding sequences), creating opportunities for protein diversity (Will & Luhrmann, 2011). This removal and joining process is known as RNA splicing (Will & Luhrmann, 2011). The spliceosome also acts as the site of pre-mRNA splicing, in which a pre-cursor mRNA molecule is processed into a mature mRNA molecule. During this process, a 3' poly-A tail is added to the nucleotide, which is responsible for increasing the stability of the RNA strand and a 5' cap is added on the 5' end of the nucleotide. The 5' cap functions to protect the nucleotide from degradation, allowing it to undergo translation (Jiao et al., 2013).

Why mRNA splicing evolved is not well understood; however, it may provide selective advantages for some organisms by allowing for adaptive expansion of the proteome by alternative splicing, a process in which various mRNA molecules can be created from one gene (Birzele et al., 2008). During alternative splicing, introns are removed, and exons are ligated, much like what occurs in canonical pre-mRNA splicing. However, the differential joining or skipping of exons within the RNA molecule creates diversity of possible mRNAs from the same gene and these alternative isoforms can have

vastly different functions and allow adaptive changes to the proteome that may confer a survival benefit (Birzele et al., 2008).

Both pre-mRNA processing factor 31 (PRP31), which is involved in U4/U6 association, as well as pre-mRNA processing factor 6 (PRP6), which is associated with U5, are phosphorylated by PRP4K (Schneider et al., 2010). This phosphorylation is required for the integration of snRNPs into the spliceosome (Dellaire et al., 2002; Schneider et al., 2010). Along with its role in phosphorylating PRP31 and PRP6, PRP4K is involved in the phosphorylation of SR proteins, which are involved in alternative splicing or mRNA (Dellaire et al., 2002; Long & Caceres, 2009; Schneider et al., 2010). As mentioned previously, PRP4K also possesses KH and RS domains, which have also been found in other splicing-associated proteins (Dellaire et al., 2002). Taken together, this data strongly indicates that one of the most highly conserved functions of PRP4K is its role in pre-mRNA splicing. This begs the question how a splicing kinase can be involved in so many additional cellular functions. One possibility is that PRP4K may play a role more directly in transcriptional regulation. For example, PRP4K was shown to interact with and/or phosphorylate transcription factors such as Elk1 and KLF13, as well as proteins involved in chromatin remodeling and nuclear hormone regulation (Huang et al., 2000; Dellaire et al., 2002; Huang et al., 2007). Specifically, Dellaire et al (2002) identified 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) as interacting proteins and isolated PRP4K from HeLa cervical cancer cells in a deacetylase complex with BRG1 and N-CoR (Dellaire et al., 2002). Another possibility is that PRP4K may play a role in other cellular processes

outside the nucleus, either in the cytoplasm or during mitosis when the nuclear envelope breaks down, releasing its contents.

1.5.2 Regulation of the spindle assembly checkpoint by PRP4K

The spindle assembly checkpoint (SAC) is a checkpoint in cell division that delays mitotic exit until chromosomes are properly attached to the mitotic spindle, preventing defects in chromosomal segregation (Reider et al., 1994; Reider & Salmon, 1998). Several checkpoint proteins are required for this process, including mitotic arrest deficient proteins 1, 2 and 3 (MAD1/2/3) and monopolar spindle 1 (MPS1) (Li and Murray, 1991; Weiss and Winey, 1996). Interestingly, it was found that PRP4K is part of the family of SAC regulatory genes, and that it reduces mitotic duration (Montembault et al., 2007). As a SAC regulatory gene, PRP4K is required for proper recruitment and functioning of MAD1 and 2, and MPS1 (Montembault et al., 2007). The study performed by Montembault et al., (2007) demonstrated that when PRP4K levels are decreased, defects in chromosomal segregation can be observed, and the data indicates that PRP4K is required for SAC function (Montembault et al., 2007). Consistent with a role for PRP4K in SAC function, Corkery et al., (2015) demonstrated that when PRP4K is depleted, the checkpoint response to the taxane microtubule poison paclitaxel results in mitotic slippage and survival of cancer cells, providing a mechanism for the association of PRP4K with taxane sensitivity (discussed in more detail in section 1.5.3).

1.5.3 PRP4K is a biomarker and modifier of taxane sensitivity

Taxanes are a class of anticancer drugs consisting of, but not limited to docetaxel, paclitaxel and cabazitaxel that are used to treat breast and ovarian cancers, (Huizing et al., 1995). They do so by binding the beta-tubulin subunit of assembled microtubules, which disrupts microtubule function, leading to cell death (Weaver, 2014; McGrogan et al., 2008). Due to the fact that taxanes are such a common anticancer agent, developing a drug resistance to taxanes is a concern in regard to effective cancer treatment. In particular, overexpression of the HER2 gene has been shown to be predictive for the taxane response, with patients with higher HER2 levels exhibiting poor survival outcomes (Joensuu et al., 2003; Corkery et al., 2015). In 2015, Corkery et al. identified PRP4K as a HER2 regulated gene in breast and ovarian cancer (Corkery et al., 2015). Specifically, the Dellaire lab found that PRP4K was a HER2 regulated modulator of taxane sensitivity, as PPR4K loss reduced the sensitivity of cancer cells to paclitaxel (Corkery et al., 2015). This decreased sensitivity is likely due to disruption of the SAC by PRP4K, which opened the door to further investigation into the role of PRP4K in tumorigenesis.

1.5.4 Loss of PRP4K induces anoikis resistance

Anoikis is a mechanism of cell death induced by detachment from the extracellular matrix (ECM) which prevents cells from differentiating and proliferating when in inappropriate locations within the tissue (Frisch & Francis, 1994). In 2018, Corkery et al., demonstrated that PRP4K has roles in anoikis sensitivity. Specifically, a loss of PRP4K was shown to be a regulator of anoikis sensitivity. In this study PPR4K expression was knocked down in mouse ID8 ovarian carcinoma cells which demonstrated

more robust proliferation than cells with stable PRP4K levels (Corkery et al., 2018). Additionally, Corkery et al, (2018) found that the nuclear protein, PRP4K was observed to be localized to the cytoplasm following cellular stress with chloroquine. In this cytoplasmic response, PRP4K colocalized with a marker of the late endosome, the ras-related protein 7 (Rab7), and was adjacent to the lysosomal marker, LAMP2 (Corkery et al., 2018). This data led to the investigation of endosomal trafficking, in which Corkery et al., demonstrated that PRP4K loss led to the stability of the EGFR, impairing EGFR degradation which is imperative for the initiation of anoikis (Reginato et al., 2003; Corkery et al., 2018). This study also provided the rationale for my investigation into the role of PRP4K in the autophagy pathway which will be discussed in a later section.

1.5.5 Regulation of YAP by PRP4K

In 2018, Cho and colleagues demonstrated that PRP4K regulates Hippo signaling by phosphorylating and inhibiting YAP nuclear activity (Cho et al., 2018). Specifically, PRP4K phosphorylates YAP preventing its binding to transcriptional enhancer factor TEF-1 (TEAD1) and promoting the export of YAP to the cytoplasm (Cho et al., 2018). It was also shown in breast cancer cells that PRP4K inhibited proliferation and invasiveness, and high expression is correlated with good prognosis in patients with triple-negative breast cancer (Cho et al., 2018). This result is intriguing that both increased YAP activity and cancer cell invasion and metastasis are associated with EMT (Son & Moon, 2010; Cheng et al., 2020), suggesting there could be an inter-relationship between PRP4K expression and EMT.

1.5.6 PRP4K is negatively regulated during epithelial-to-mesenchymal transition

In 2019, Clarke et al., demonstrated that PRP4K is negatively regulated by EMT. Specifically, when PRP4K levels were depleted in mammary epithelial cells, partial EMT was induced (Clarke et al., 2019). It was also shown that when PRP4K protein levels were depleted, there were varying effects on cell migration, but an invasive effect on tumourigenic and non-transformed cell lines were seen (Clarke et al., 2019). Additionally, while depletion of PRP4K levels induced partial EMT, induction of EMT was found to negatively regulate PRP4K, decreasing its protein expression and indicating that PRP4K is regulated by post-transcriptional mechanisms or at the protein level (Clarke et al., 2019). Taken together, the data shows that PRP4K was shown to function as a haploinsufficient tumour suppressor that was negatively regulated by EMT.

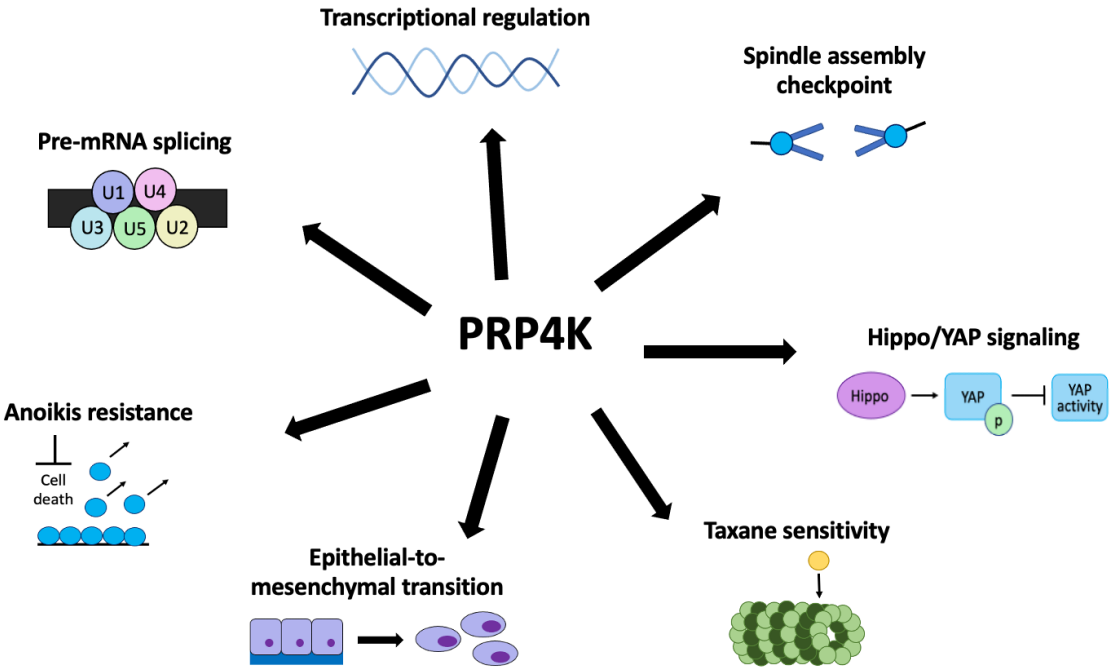


Figure 1.4: The various functions of PRP4K. PRP4K is multifunctional protein and has been shown to be involved in pre-mRNA splicing, transcriptional regulation, the spindle assembly checkpoint, YAP signaling, taxane sensitivity, anoikis resistance and epithelial-to-mesenchymal transition.

1.6 Rationale and Hypothesis

In 2018 Corkery and colleagues demonstrated that the nuclear kinase, PRP4K localized to the cytoplasm of cells that had been treated with chloroquine diphosphate salt (Corkery et al., 2018). As discussed previously, chloroquine is an inhibitor of autophagy. In addition to the novel observation of cytoplasmic PPR4K, Corkery and colleagues also showed that PRP4K colocalized with p62- a marker and adaptor of autophagy, as well as Rab7, a marker of the late endosome. This data indicates that PRP4K may be associating with the autophagy pathway or may be involved in endosomal trafficking. In addition, work by Livia Clarke published as a pre-print in BioRxiv (Clarke et al., 2019) indicated that PRP4K loss induces partial EMT, which is a cellular phenotype associated with aggressive cancer (Figure 1.1) (Aggarwal et al., 2021). For these reasons, I hypothesized that loss of PRP4K promotes aggressive phenotypes in cancer by triggering partial-EMT and dysregulating endosomal trafficking. Therefore, in the first part of my thesis, I explored the relationship between EMT-induction and PRP4K regulation. Since endosomal machinery is also involved in autophagy (Numrich & Ungermann, 2014; Cotelli et al., 2021), and autophagy is induced by a number of cellular stresses experienced by cancer cells such as nutrient and oxidative stress (Lozy & Karantza, 2012; Filomeni et al., 2015), I also hypothesized that PRP4K may play a role in the response to autophagy-inducing cellular stress. Therefore, in the second part of my thesis I examined how nutrient-deprivation and oxidative stress regulates PRP4K cellular trafficking and began to characterize the relationship between PRP4K and autophagy machinery during cell stress. Together these studies further characterize the regulation of PRP4K during EMT and the novel cytoplasmic role of PRP4K in the cells' response to stress, which

together contribute to new knowledge of the possible tumour suppressive functions of this kinase.

Chapter 2: Materials and Methods

2.1 Cell Culture

All cell lines were grown at 37°C with 5% CO_2. HeLa wild type, HeLa Clover-PRP4K and MDA-MB-231 cell-lines were grown in DMEM supplemented with 10% FBS, 1% P/S. HMLE cells were cultured in DMEM/F12, supplemented with 5% horse serum, 1% P/S, 0.5µg/mL hydrocortisone, 10 µg/mL insulin and 10 ng/mL EGF. MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 1% P/S, 0.5µg/mL hydrocortisone, 10µg/mL insulin, 10ng/mL EGF, 1ng/mL cholera toxin. Additionally, a concentration of 2µg/mL of puromycin was cultured with the MDA-MB-231, HMLE and MCF10A cells stably expressing TRIPZ shRNAs. All cell lines used were human.

Table 2.1: *In vitro* cell lines with corresponding growth conditions

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

2.2 shRNA Lentiviral Transduction

TRIPZ shPRP4K cell lines were generated using TRIPZ inducible lentiviral shRNAs (shPRP4K-1=clone: V3THS_383962, shPRP4K-2=clone: V3THS_383960, Non-silencing shCtrl=RHS4743) purchased from Thermo Scientific. Lentivirus was made by co-transfection of TRIPZ shRNA with pMDPD2.G and psPAX2 packaging vectors into human HEK-239T cells via calcium phosphate transfection. Sixteen hours post transfection, media was changed on the cells. Forty-eight hours following transfection, the media was harvested and filtered using a 0.45μm filter. The first transduction was performed by adding 500μl of filtered media with 8μg/mL polybrene to the target cell line for 48 hours. Following the 48-hour incubation, cells were then split and transduced for 48 hours once more. Cells were then placed into fresh media for recovery for 24 hours. Following the recovery period, cells were puromycin selected using 2μg/mL puromycin. TRIPZ shRNA expression was induced using 5μg/mL doxycycline every 24 hours for 96 hours.

2.3 Subcellular Fractionation

Cells were harvested and lysed in fractionation buffer (0.5mM EDTA, 0.5mM EGTA, 0.19$MgCl_2$, 1M KCl, 4.77g HEPES, 1L water, 1mM DTT, 1X protease inhibitors (PMSF and P8340)) for 15 minutes on ice. Cells were passed through a 27-gauge needle 10 times to sheer the sample and incubated on ice for 20 minutes. The sample was fractionated by centrifugation at 3000 rpm for 5 minutes at 4℃ to isolate the protein. The supernatant was extracted, placed in a new tube and centrifuged at 8000rpm for 5 minutes. The nuclear pellet was washed with 500μL fractionation buffer and the pellet

was suspended with a 1mL pipette 10 times. The pellet was then passed through a 25-gauge needle 10 times and centrifuged at 300rmp for 10 minutes. The supernatant was discarded, the pellet was resuspended in TBS with 0.1% SDS and was sonicated for 3 seconds on a setting of 2-continuous.

2.4 Induction of Cellular Stress

Fifty micromolar chloroquine, 1μm rapamycin, 200μm $CoCl_2$, and media with 0.05% serum were used for the induction of cellular stress. Chloroquine was dissolved in distilled water, rapamycin in DMSO and $CoCl_2$ were dissolved in DMEM with 10% FBS and 1% P/S. All stresses and their corresponding solvents were filter sterilized using a 0.2-micron filter and added to cells in 10mL of appropriate media for varying time points.

2.5 Western Blot Analysis

Cells were harvested and lysed in lysis buffer (20mM Tris-HCl ph8, 300mM KCl, 10% Glycerol, 0.25% Nonidet P-40, 0.5mM EDTA, 0.5mM EGTA, 1X protease inhibitors (phenylmethylsulfonyl fluoride (PMSF) and P8340)) for 15 minutes on ice. Cells were passed through a 22-gauge needle to shear the sample and set on ice for 15 minutes. Cells were pelleted by centrifugation at 14 800 rpm for 25 minutes at 4℃ to isolate the protein. Protein concentrations were determined using Bio-Rad Protein Reagent. Samples were mixed with 1:1 with 2x sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.04% bromophenol blue, 0.125M 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 a nitrocellulose membrane. Depending on

protein size, 7-15% acrylamide gels were used. The membranes were blocked using either 5% milk or bovine serum albumin (BSA) dissolved in Tris-buffered saline with Tween 20 (TBST). The nitrocellulose membranes were incubated in primary antibody for 1 hour minimum at 4℃. The membranes were then washed 3 times for 5 minutes each with TBST and incubated in secondary antibody at room temperature for 1 hour. The membranes were then washed 3 times for 5 minutes each again with TBST. Proteins were visualized using BioRad Clarity Western ECL substrate and radiographic film.

Table 2.2: Antibodies and dilutions for western blot.

Antibody	Company (product Number)	Dilution
PRP4K (Rabbit)	Novus Biologicals (NBP1-82999)	1:2500
Tubulin (Mouse)	Sigma (T0198)	1:1000
E-cadherin (Rabbit)	Cell Signalling (3195)	1:1000
N-cadherin (Rabbit)	Cell Signalling (13116)	1:1000
Vimentin (Rabbit)	Cell Signalling (5741)	1:1000
Zo-1 (Rabbit)	Cell Signalling (8193)	1:1000
Zeb-1 (Rabbit)	Cell Signalling (3396)	1:1000
Fibronectin (Rabbit)	Abcam (ab32419)	1:1000
Snail (Rabbit)	Cell Signalling (3879)	1:1000
Slug (Rabbit)	Cell Signalling (9585)	1:1000
Claudin-1 (Rabbit)	Cell Signalling (13255)	1:1000
Trk-B (Mouse)	Santa Cruz Biotechnology (sc-377218)	1:1000
Lamin B (Rabbit)	Invitrogen (PA5-19468)	1:5000
P62 (Rabbit)	Cell Signalling (D10E10)	1:1000
LC3 (Rabbit)	Thermofisher (32254)	1:1500
Keap1 (Rabbit)	Cell Signalling (D6B12)	1:1000
TRIM16 (Rabbit)	Bethyl (A301-159A-M)	1:2500
TRIM29 (Rabbit)	Abcam (ab108627)	1:1000

2.6 Immunofluorescence

Coverslips were plated into 6-well plates with adequate cell number and left to adhere for 24 hours. Cells were then treated with cellular stress for appropriate time period. Following completion of the stress, media was aspirated, and cells were washed with PBS. They were then fixed with 2% paraformaldehyde for 30 minutes and permeabilized with 0.5% TritonX-100 for 5 minutes. Coverslips were then washed with PBS three times for 5 minutes and placed into BSA (2g albumin in 40mL PBS) for 20 minutes and placed in primary antibody to incubate for one hour. Primary antibody incubations were performed in a humidity chamber. Coverslips were then washed 3 times with PBS and placed into fluorescent secondary antibody for 30 minutes in a dark humidity chamber. Coverslips were then washed 3 times with PBS; the second wash contained 4',6-diamidino-2-phenylindole (DAPI) in a 1:1000 dilution for 10 minutes. Coverslips were then mounted to glass microscope slides using Dako mounting medium. Images were captured using a Zeiss Cell Observer Microscope.

Table 2.3: Antibodies and dilutions for immunofluorescence microscopy.

Antibody	Company (product Number)	Dilution
PRP4K (Rabbit)	Novus Biologicals (NBP1-82999)	1:250
LC3 (Rabbit)	Invitrogen (PA5-32254)	1:200
P62 (Rabbit)	Cell Signalling (D10E10)	1:400
Rab7 (Rabbit)	Cell Signalling (D95F2)	1:100
SRPK1 (Mouse)	Abcam (ab58002)	1:100
K48 Ub (Rabbit)	Millipore (05-1307)	1:400
P62 (Mouse)	Cell Signalling (D5L7G)	1:400
Keap1 (Rabbit)	Cell Signalling (D6B12)	1:400
NRF2 (Rabbit)	Cell Signalling (D1Z9C)	1:400
TRIM16 (Rabbit)	Bethyl (A301-159A-M)	1:400
G3BP1	BD Transduction (611126)	1:400

Chapter 3: Results

3.1 Loss of PRP4K is linked to partial epithelial-to-mesenchymal transition

Among PRP4K's several functions, a potential role in regulating EMT has recently been discovered (Clarke et al., 2020 BioRxiv). Specifically, Clarke et al., demonstrated that depletion of PRP4K by small hairpin RNA (shRNA) appeared to induce partial EMT, a state that is marked by simultaneous expression of epithelial (e.g. E-cadherin) and mesenchymal markers (e.g. vimentin or fibronectin) that lends advantages to cancer cells for survival and metastasis (Kim et al., 2017; Aiello, et al., 2018). To further characterize the relationship between loss of PRP4K and EMT, a tetracycline-inducible knockdown approach was used to knockdown levels of PRP4K in HMLE, MDA-MB-231 and MCF10A mammary epithelial cells. Following PRP4K knockdown by inducible-shRNA, various epithelial and mesenchymal markers were used to determine the degree to which EMT was occurring (Figure 3.1.1), where in non-transformed mammary epithelial cell lines like MCF10A and HMLE, E-cadherin expression was maintained despite induction of mesenchymal markers such as fibronectin, vimentin and slug. Additionally, to characterize the basal levels of PRP4K in the three cell lines used in Figure 3.1.1, Western blot analysis was performed on HMLE, MDA-MB-231 and MCF10A cells. It was determined that basal PRP4K levels were not significantly different (p value > 0.05, student's t-test, n=3). between cell lines (Figure 3.1.2). Taken together, this data demonstrates that changes in PRP4K expression plays a crucial role in the progression of EMT, and furthermore provides a possible mechanistic reason for why a loss of PRP4K results in worse outcomes for breast and ovarian cancer patients (Cho et al., 2018; Corkery et al., 2018).

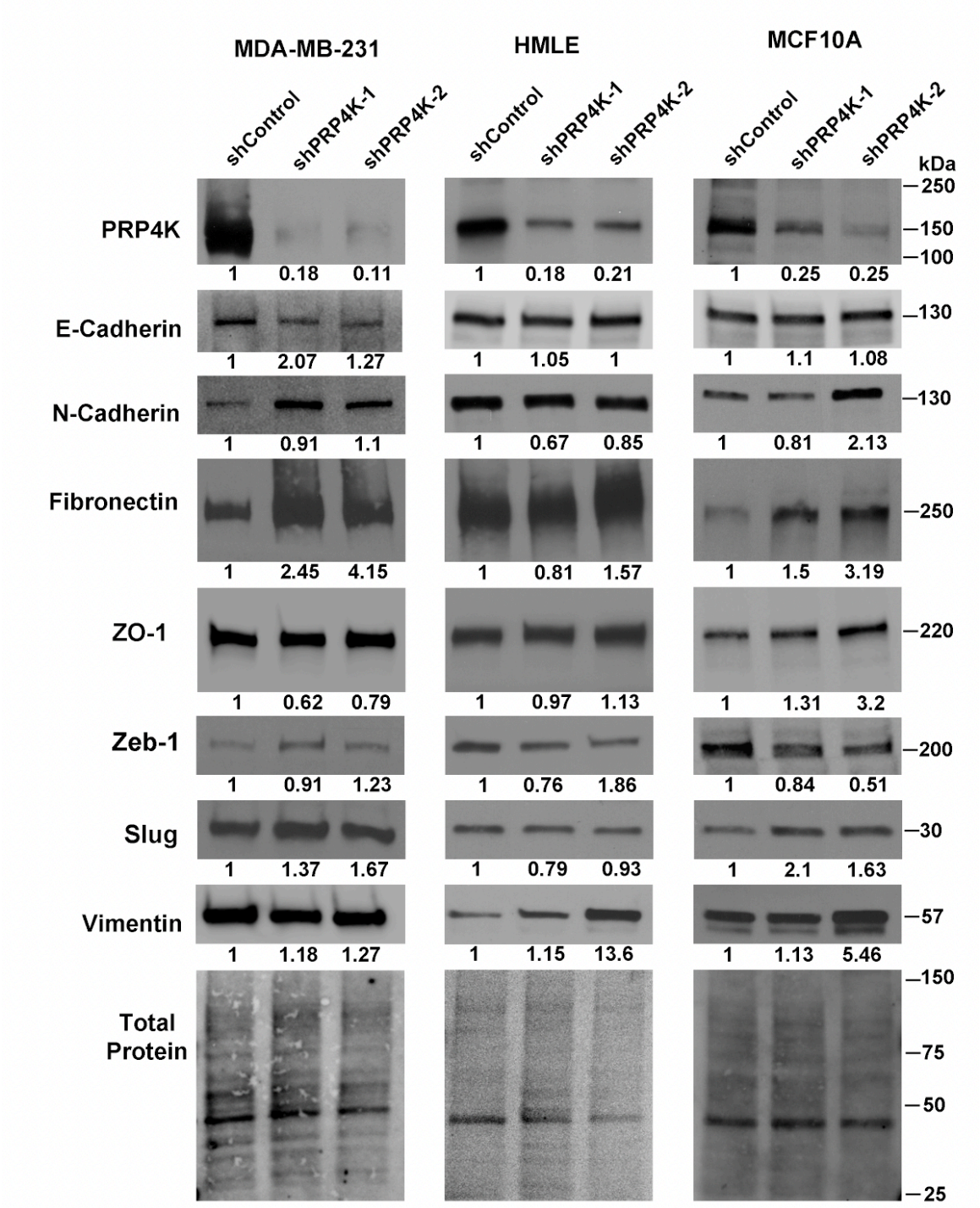


Figure 3.1.1: A loss of PRP4K promotes partial epithelial-to-mesenchymal transition in MDA-MB-231, HMLE and MCF10A breast cancer cell lines. A loss of epithelial proteins and an increase in mesenchymal proteins following PRP4K knockdown demonstrates partial epithelial to mesenchymal transition in MDA-MB-231, HMLE and MCF10A cell lines. Total protein is normalized by using the Mini-PROTEAN TGX stain-free gels' capacity to visualize tryptophan residues in protein after UV induction. Total protein staining was normalized from 25 to 150kDa. *n*=3.

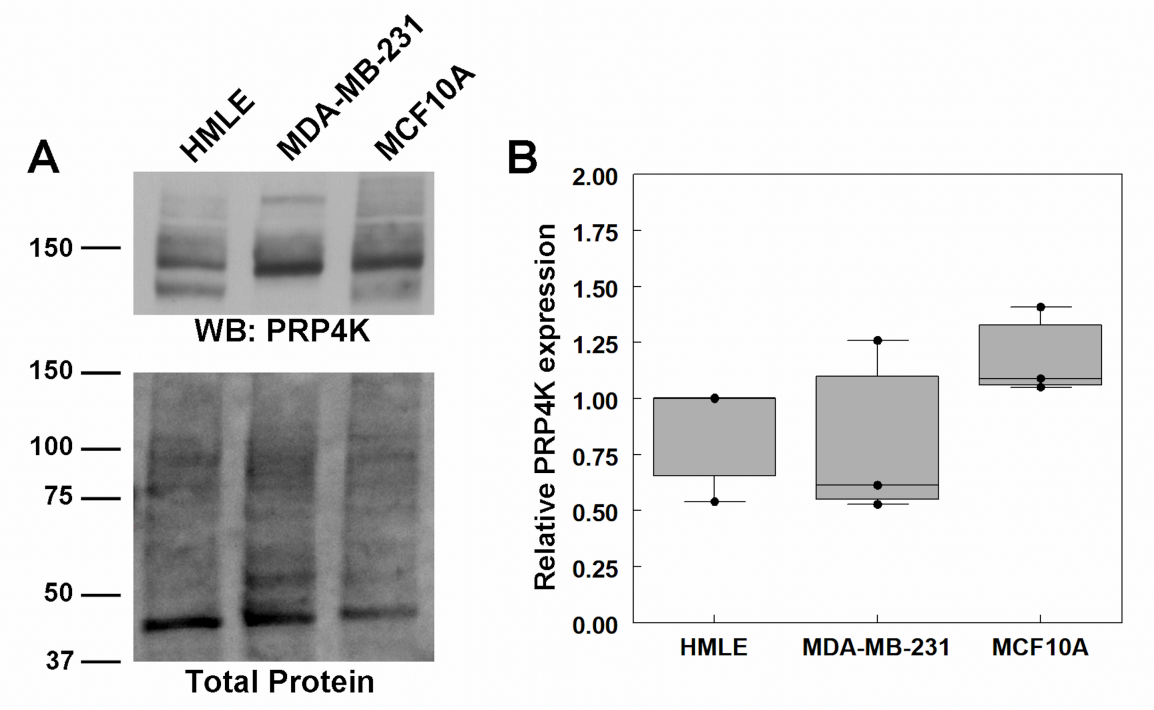


Figure 3.1.2: PRP4K protein levels in HMLE, MDA-MB-231 and MCF10A cell lines are not significantly different. *(A)* Western Blot analysis showing PRP4K levels in HMLE, MDA-MB-231 and MCF10A cell lines. Total protein is visualized by using the Mini-PROTEAN TGX stain-free gels' capacity to visualize tryptophan residues in protein after UV induction. Total protein staining was normalized from 25 to 150kDa. *(B)* Box and whisker plot of normalized PRP4K expression levels relative to total protein in the indicated cell lines. *n*=3.

3.2 PRP4K shuttles to the cytoplasm under cellular stress and interacts with the markers of autophagy, p62, LC3 and Rab7.

As mentioned previously, the Dellaire laboratory demonstrated that under chloroquine treatment, PRP4K shuttles from the nucleus to the cytoplasm and colocalizes with p62 and Rab7 in HeLa Clover-PRP4K cells (Corkery et al., 2018). This is quite novel in comparison to what is known about the shuttling of other splicing factors that tend to localize to stress granules (Twyffels et al., 2011) rather than colocalization with markers of autophagy and the late endosome as seen for PRP4K. Thus, this data indicates that PRP4K may be involved in autophagy pathways. An mClover HeLa cell line created by CRISPR-medicated knock-in approach of mClover green fluorescent protein at the endogenous PRPF4B locus (Corkery et al., 2018) was used to enable facile and specific detection of PRP4K in fixed and living cells by fluorescence microscopy. To further observe how PRP4K reacts to the stress response, four treatments were used in HeLa clover cells; chloroquine, rapamycin, nutrient deprivation and oxidative stress, all which induce or inhibit autophagy as part of the cell's response to these stresses. PRP4K colocalization with various markers of autophagy was observed using immunofluorescence microscopy. PRP4K was found to colocalize with the markers of autophagy (p62 and LC3B) and the late endosome (Rab7) to varying degrees in response to chloroquine (Figure 3.2.1), inhibition of mTOR by rapamycin (Figure 3.2.4), nutrient deprivation (Figure 3.2.6) and oxidative stress induced by $CoCl_2$ (Figure 3.2.8) (summarized in Table 3.1). The cytoplasmic shuttling of PRP4K coincided with increased PRP4K levels in the cytoplasm as ascertained by subcellular fractionation and Western blot analysis (Figure 3.2.3). In addition, total protein levels of PRP4K were determined over time in presence of each stress and. In response to chloroquine, PRP4K protein

levels did not drop overtime but fluctuated with p62 levels, rising by 50% at 10 h post-treatment and returning to near normal levels by 16 h; whereas LC3B levels progressively increased overtime (Figure 3.2.2). In response to rapamycin, PRP4K levels paralleled p62 and LC3B levels and progressively dropped over time (Figure 3.2.5), consistent with increased autophagic flux and potentially autophagic degradation of PRP4K. In response to nutrient deprivation, PRP4K protein levels fluctuated but did not decrease with p62 or LC3B levels overtime (Figure 3.2.7), suggesting in this context PRP4K was subject to autophagic degradation. Finally, in response to oxidative stress induced by $CoCl_2$ treatment, Western blot analysis of PRP4K appeared to indicate complete loss of PRP4K expression (Figure 3.2.9). We believe this is a technical issue with solubility of PRP4K after $CoCl_2$ treatment as we noticed a pink precipitate in lysates, and these results are inconsistent with the immunofluorescence analysis of PRP4K localization and expression shown in Figure 3.2.8, which clearly shows the PRP4K is still expressed although the levels may be lower than prior to $CoCl_2$ treatment.

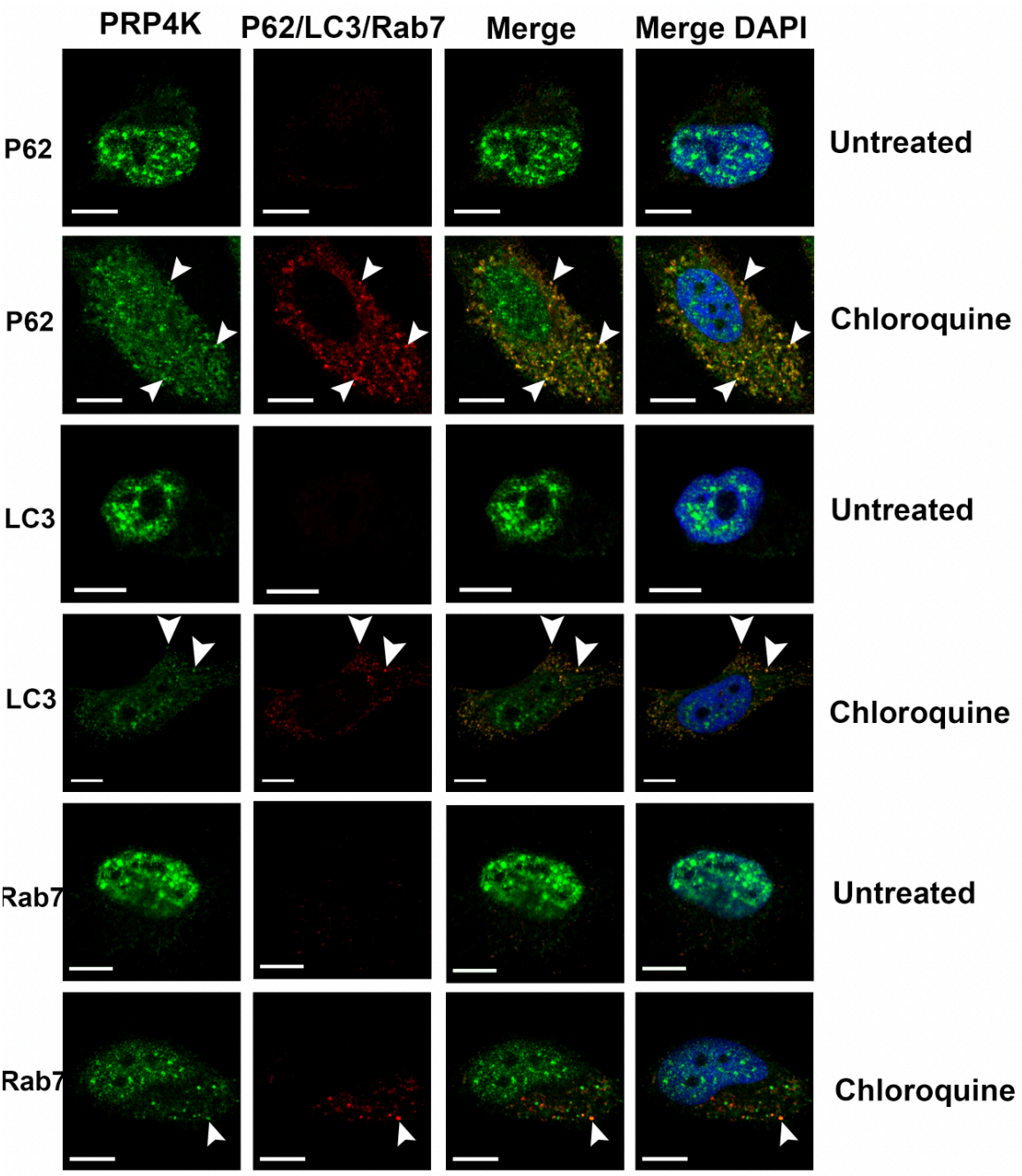


Figure 3.2.1: PRP4K shuttles to cytoplasmic puncta containing p62, LC3 and Rab7 following the inhibition of lysosomal fusion. HeLa cells expressing PRP4K tagged endogenously with Clover green fluorescent protein (HeLa Clover-PRP4K) were treated with or without (untreated) 50 µm chloroquine for 16 h before being fixed and processed for the immunofluorescence detection of PRP4K (green) or p62, LC3 or Rab7 (red, as indicated). DNA was stained with DAPI (blue). Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

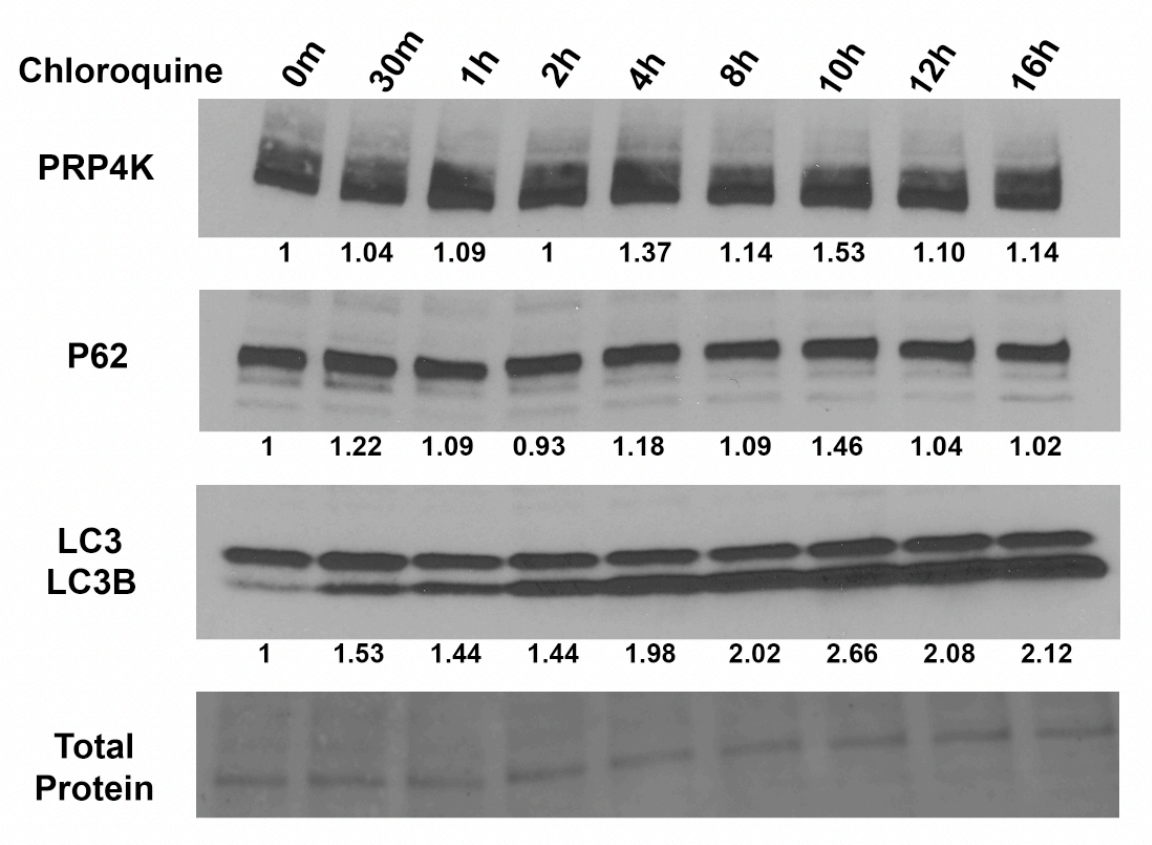


Figure 3.2.2: PRP4K protein levels are not altered following inhibition of lysosomal fusion. PRP4K and p62 levels are stable over time (0-16 hours) following 50 μm chloroquine treatment by western blot in HeLa-Clover-PRP4K cells. LC3 levels increase over time (following chloroquine treatment in HeLa cells). Densitometry measurements are indicated below the respective bands and compared to the control (0m) sample as ratios. Total protein is normalized by using the Mini-PROTEAN TGX stain-free gels' capacity to visualize tryptophan residues in protein after UV induction.

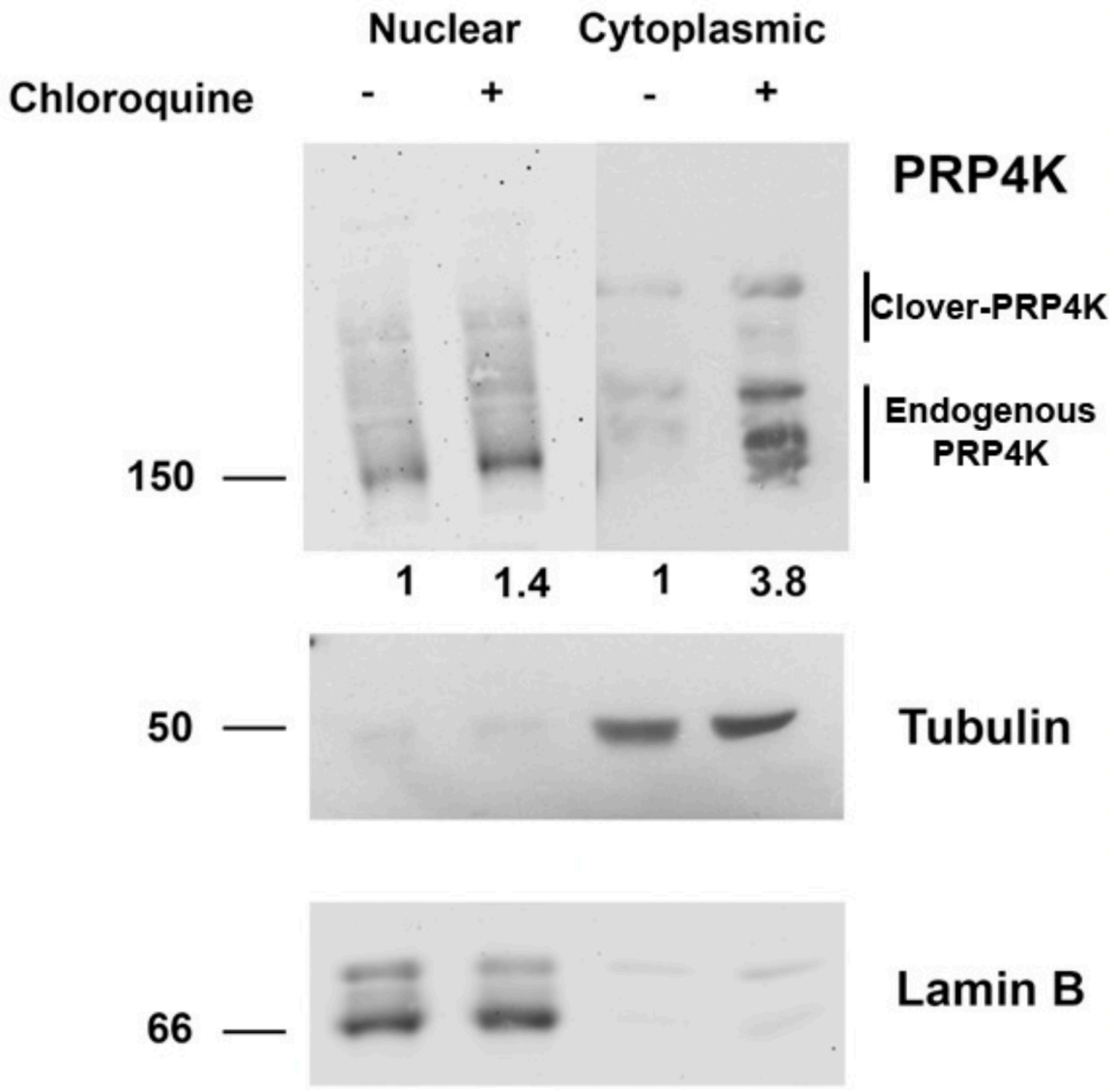


Figure 3.2.3: PRP4K levels are increased in the cytoplasm of fractionated chloroquine treated HeLa Clover-PRP4K cells. HeLa Clover-PRP4K cells expressing both Clover-PRP4K and endogenous PRP4K were treated with 50 μm chloroquine for 16 h and subjected to subcellular fraction and Western blot analysis for PRP4K levels in the nucleus and cytoplasm. Tubulin and Lamin B are protein markers for nuclear and cytoplasmic fractions, respectively. Position of Clover-PRP4K and PRP4K are indicated on the Western blot. Densitometry measurements are indicated below the respective bands and compared to the control (0m) sample as ratios.

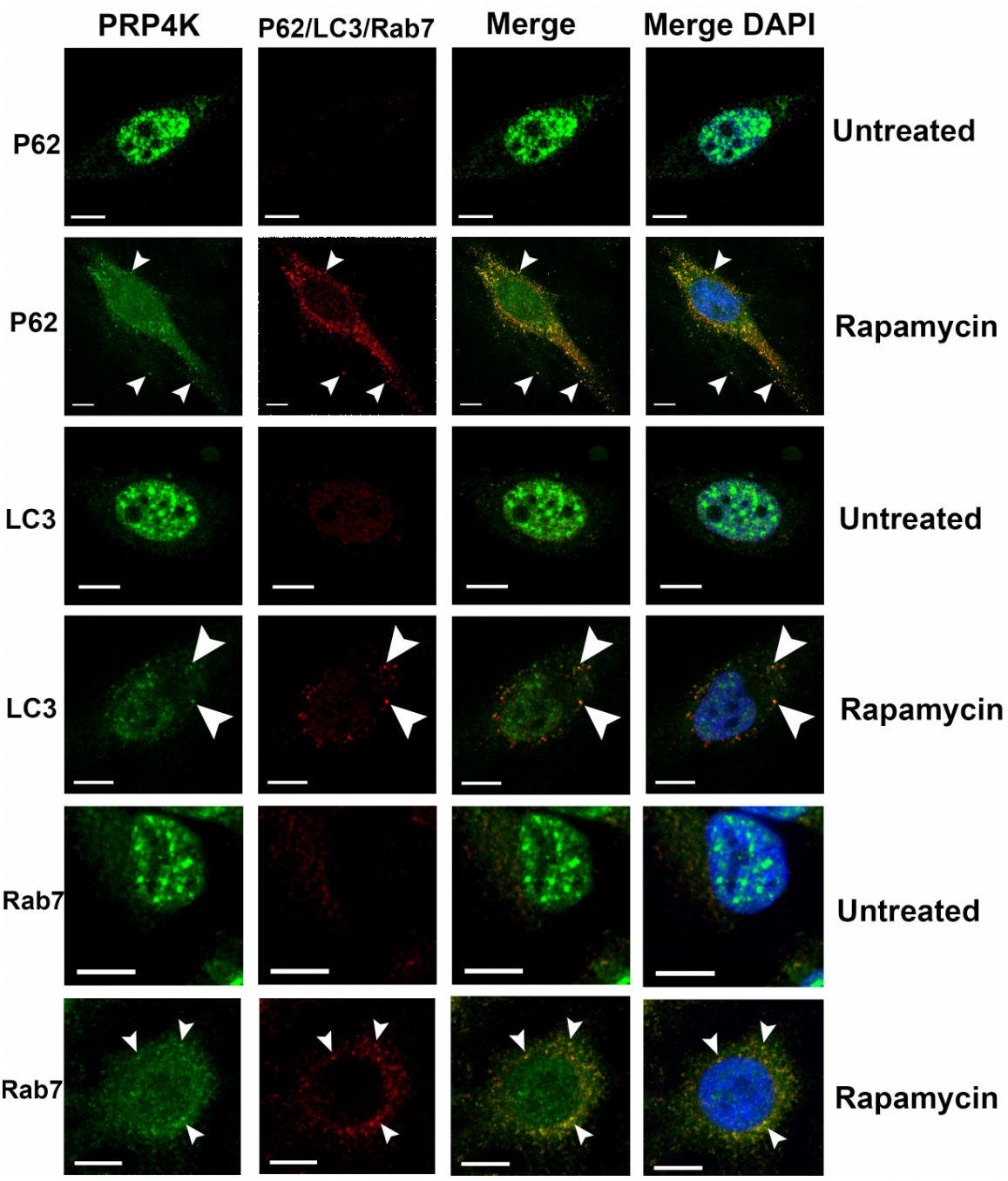


Figure 3.2.4: PRP4K shuttles to cytoplasmic puncta containing p62, LC3 and Rab7 following the initiation of autophagy by mTOR inhibition. HeLa Clover-PRP4K cells following 1 μM rapamycin treatment (initiator of autophagy) compared to untreated cells. Cells were fixed and processed for the immunofluorescence detection of PRP4K (green) or p62, LC3 or Rab7 (red, as indicated). DNA was stained with DAPI (blue). Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 μm.

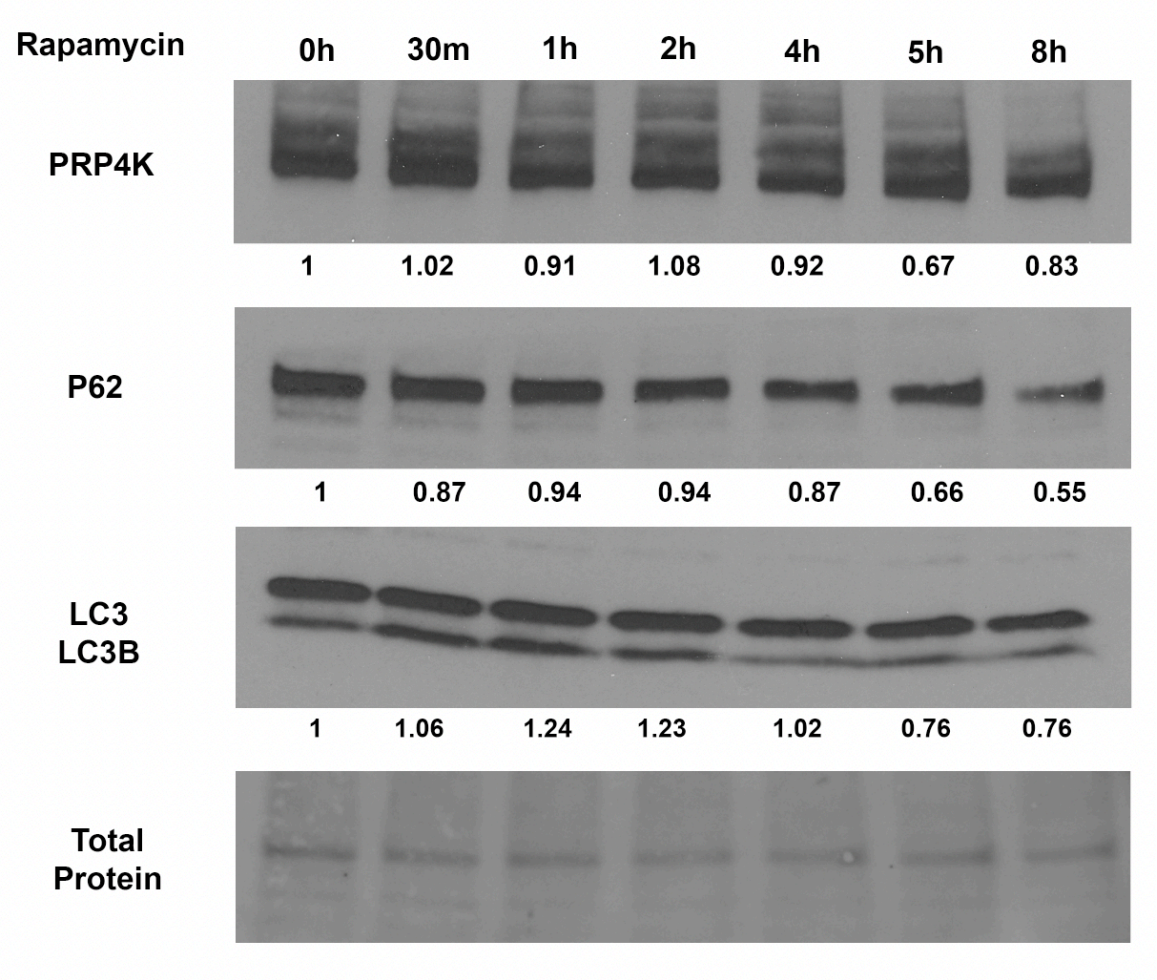


Figure 3.2.5: PRP4K protein levels are decreased following autophagy initiation by mTOR inhibition. PRP4K levels slightly decrease over time (0-8 hours) following 1 μM rapamycin treatment by Western blot. P62 and LC3 levels drop over time (0-8 hours) following rapamycin treatment in HeLa cells. Densitometry measurements are indicated below the respective bands and compared to the control (0m) sample as ratios. Total protein is normalized by using the Mini-PROTEAN TGX stain-free gels' capacity to visualize tryptophan residues in protein after UV induction.

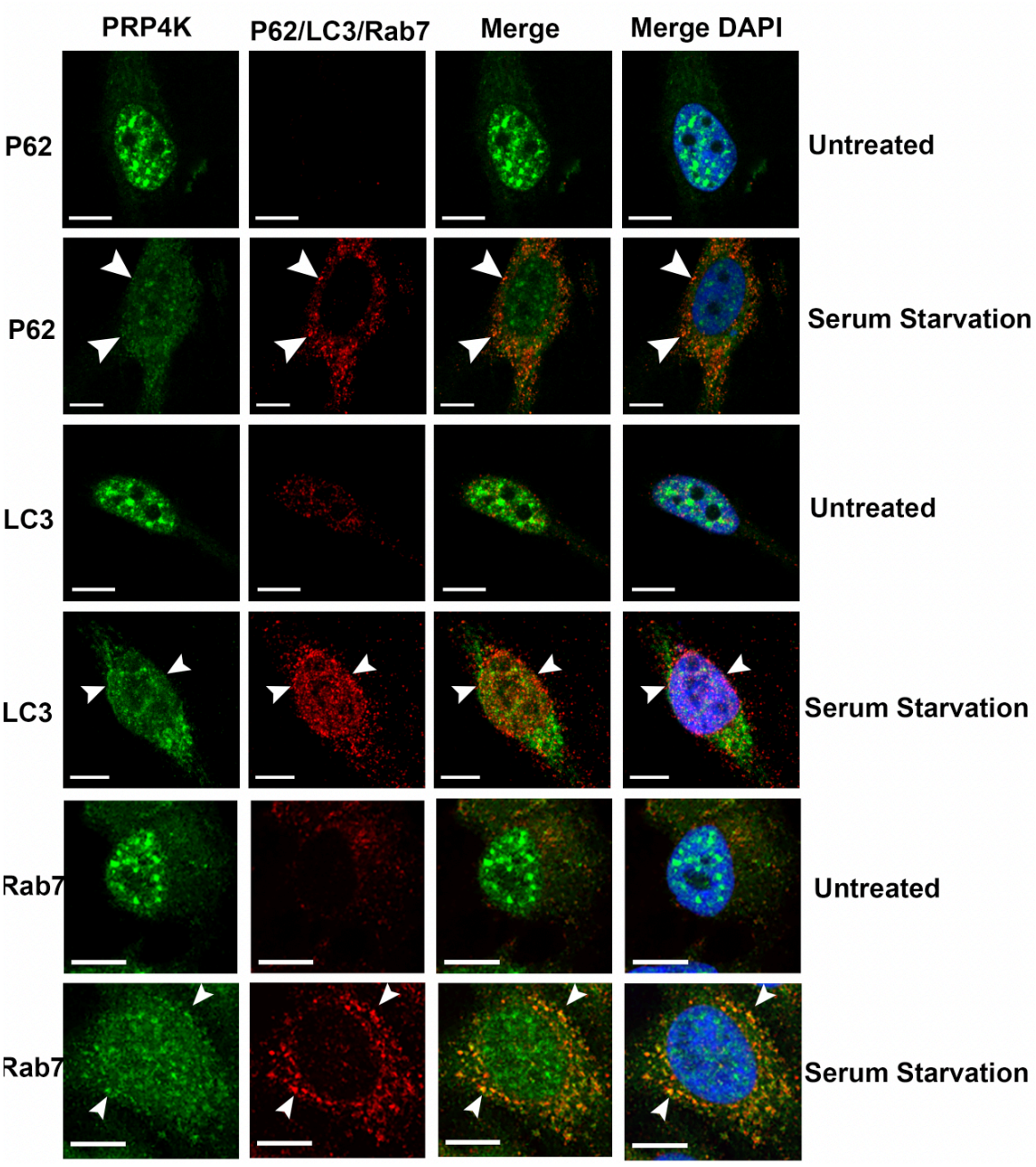


Figure 3.2.6: PRP4K shuttles to cytoplasmic puncta containing p62, LC3 and Rab7 following the initiation of autophagy by nutrient deprivation. HeLa Clover-PRP4K cells following 16 h nutrient deprivation (initiator of autophagy) compared to untreated cells. Cells were fixed and processed for the immunofluorescence detection of PRP4K (green) or p62, LC3 or Rab7 (red, as indicated). DNA was stained with DAPI (blue). Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

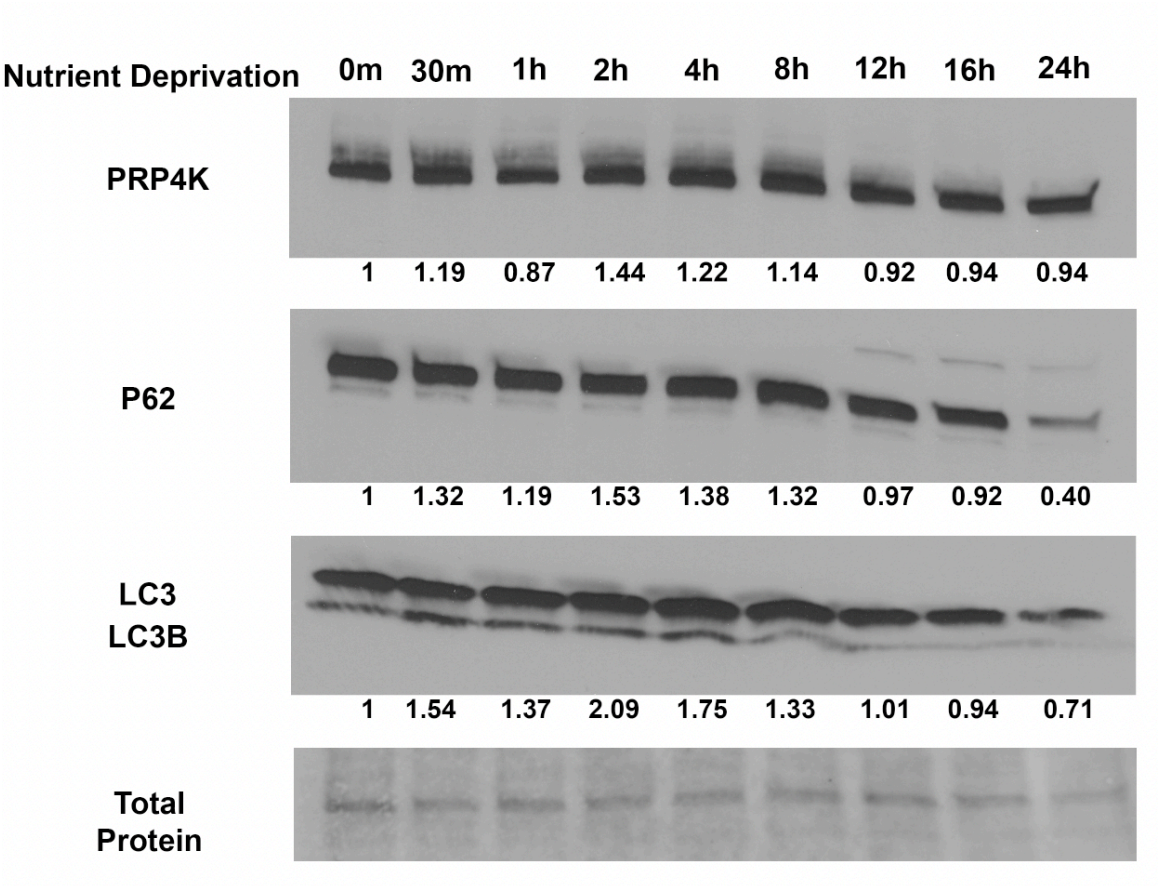


Figure 3.2.7: PRP4K protein levels are not altered following autophagy initiation by nutrient deprivation. PRP4K levels are stable over time (0-24 hours) following nutrient deprivation by western blot. P62 levels and LC3 levels decrease over time (0-24 hours) following nutrient deprivation in HeLa wild type cells. Densitometry measurements are indicated below the respective bands and compared to the control (0m) sample as ratios. Total protein is normalized by using the Mini-PROTEAN TGX stain-free gels' capacity to visualize tryptophan residues in protein after UV induction.

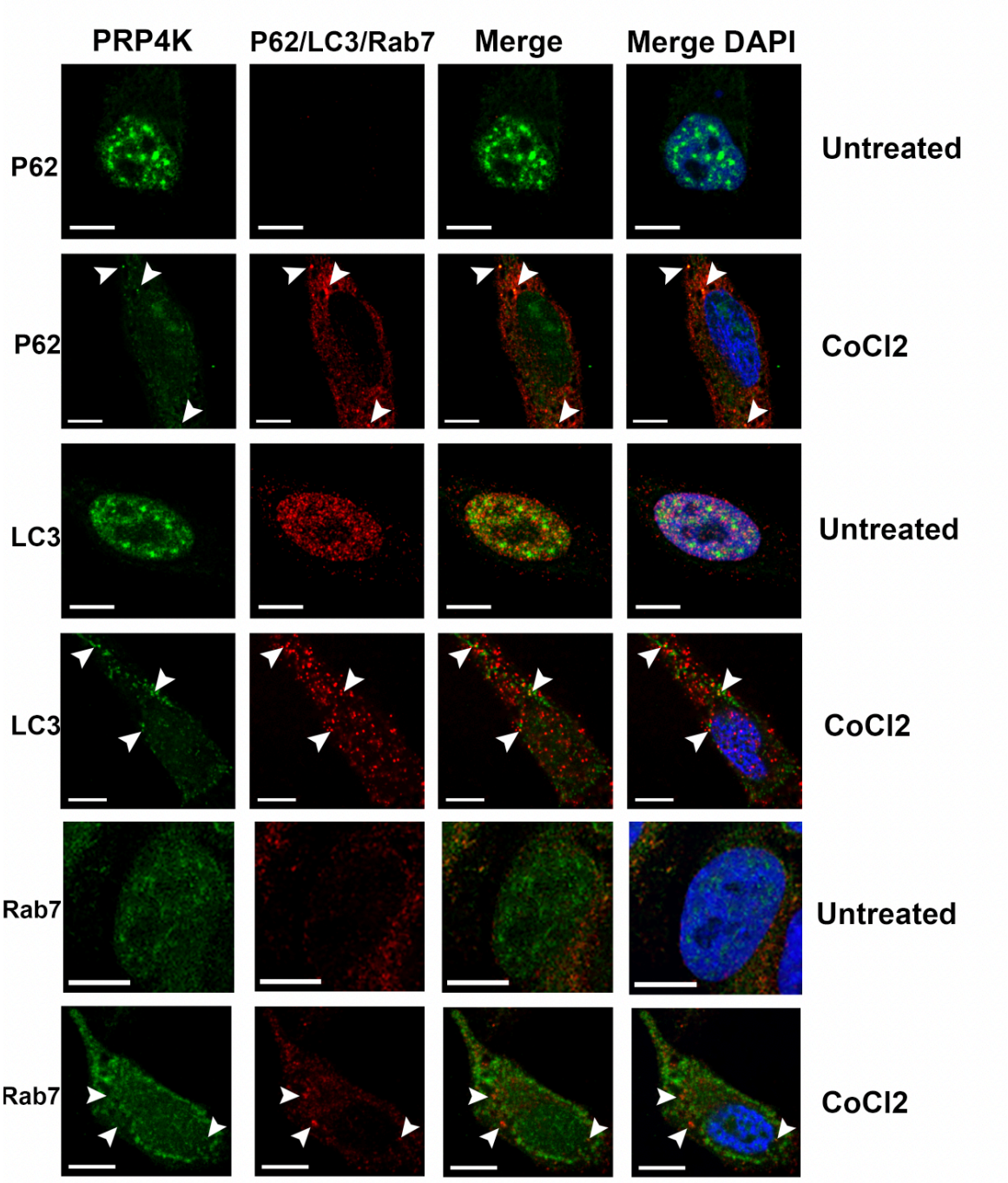


Figure 3.2.8: PRP4K shuttles to cytoplasmic puncta containing p62, LC3 and Rab7 following the initiation of autophagy by oxidative stress. HeLa Clover-PRP4K cells were treated with or without (untreated) 200 µM cobalt chloride for 4 h to induce oxidative stress before processing for the immunofluorescence detection of p62, LC3 and Rab7 (red). PRP4K can be seen in green, and DNA was stained with DAPI (blue). Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

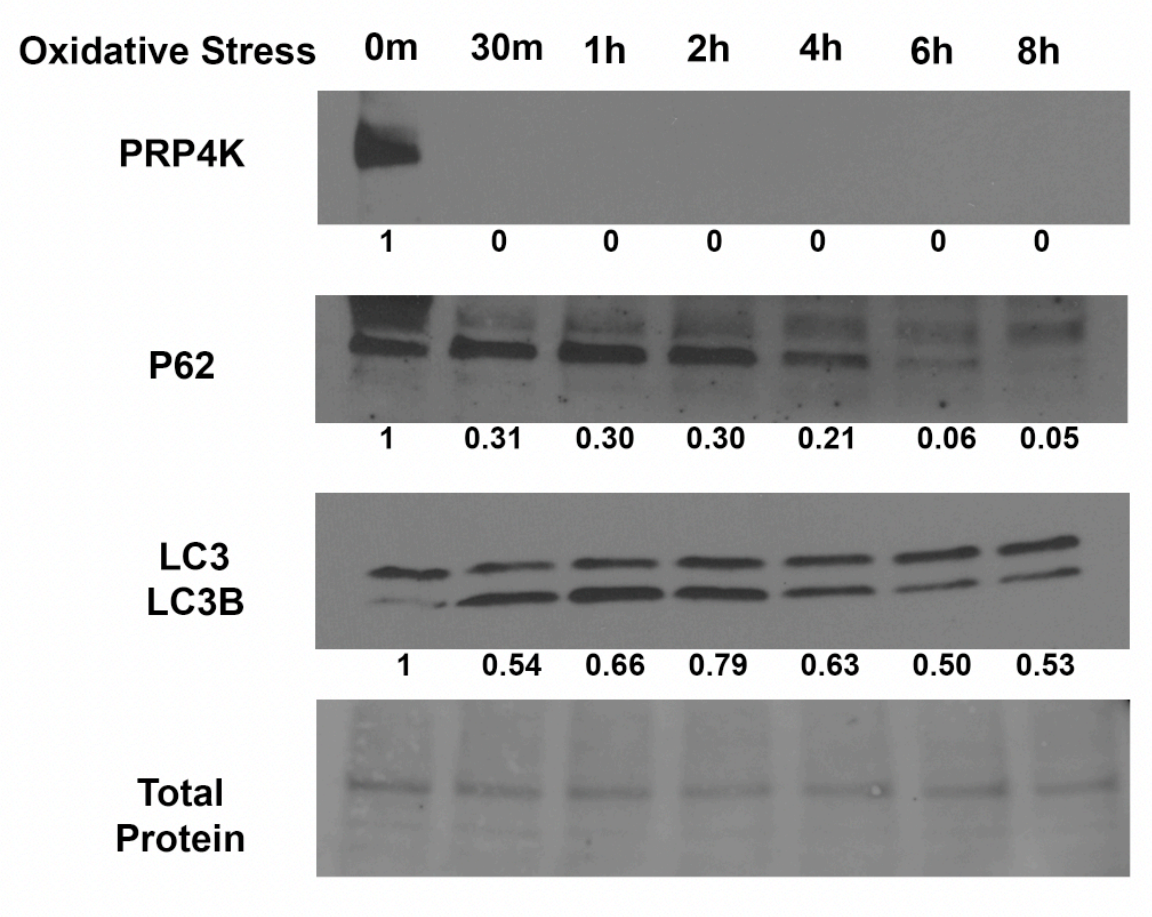


Figure 3.2.9: PRP4K protein levels are drastically decreased following autophagy initiation by oxidative stress. PRP4K levels decrease over time (0-8 hours) following oxidative stress induced by 200 μM $CoCl_2$by western blot. P62 levels and LC3 levels decrease over time (0-8 hours) following oxidative stress by western blot. in HeLa wild type Densitometry measurements are indicated below the respective bands and compared to the control (0m) sample as ratios. Total protein is normalized by using the Mini-PROTEAN TGX stain-free gels' capacity to visualize tryptophan residues in protein after UV induction.
*PRP4K levels may be being precipitated out due to cobalt chloride treatment.

Table 3.1: PRP4K cytoplasmic colocalization and protein levels following stress.

Treatment	Cytoplasmic PRP4K	PRP4K Levels	PRP4K-p62 colocalization	PRP4K-LC3 colocalization	PRP4K-Rab7 colocalization
Chloroquine	Present	Stable	2+	2+	1+
Rapamycin	Present	Stable	2+	1+	2+
Nutrient Deprivation	Present	Stable	2+	1+	2+
Oxidative Stress	Present	Decreasing	1+	2+	1+

3.3 PRP4K colocalizes with Lamp1 following chloroquine and rapamycin treatment in HeLa cells

Since PRP4K colocalizes with p62 after cellular stress, a known component of the autophagosome, we wanted to determine if PRP4K colocalized to other autophagy compartments such as the lysosome. Lysosomal-associated membrane protein 1 (Lamp1) is a major component of the lysosome, deeming it a marker of the lysosome (Luzio et al., 2014). Due to the autophagosome-lysosome fusion process in the autophagy pathway, I hypothesized that Lamp1 and PRP4K may colocalize in a subset of puncta representing autophagolysosomes. In untreated, nutrient deprived and oxidatively stressed cells, PRP4K did not colocalize with Lamp1 in the cytoplasm (Figure 3.6). However, in chloroquine treated and rapamycin treated cells, there was minor colocalization between Lamp1 and PRP4K (Figure 3.3); data consistent with this kinase being associated with the autophagosomes up until fusion with the lysosome containing Lamp1, after which PRP4K signal is presumably lost as it is degraded in the autophagolysosome. As such it is expected that PRP4K colocalization with Lamp1 might be observed in the short-lived autophagolysosomes if autophagic degradation is inhibited by chloroquine, or in cells with increased numbers of autophagolysosomes due to increased autophagic flux in cells treated with rapamycin.

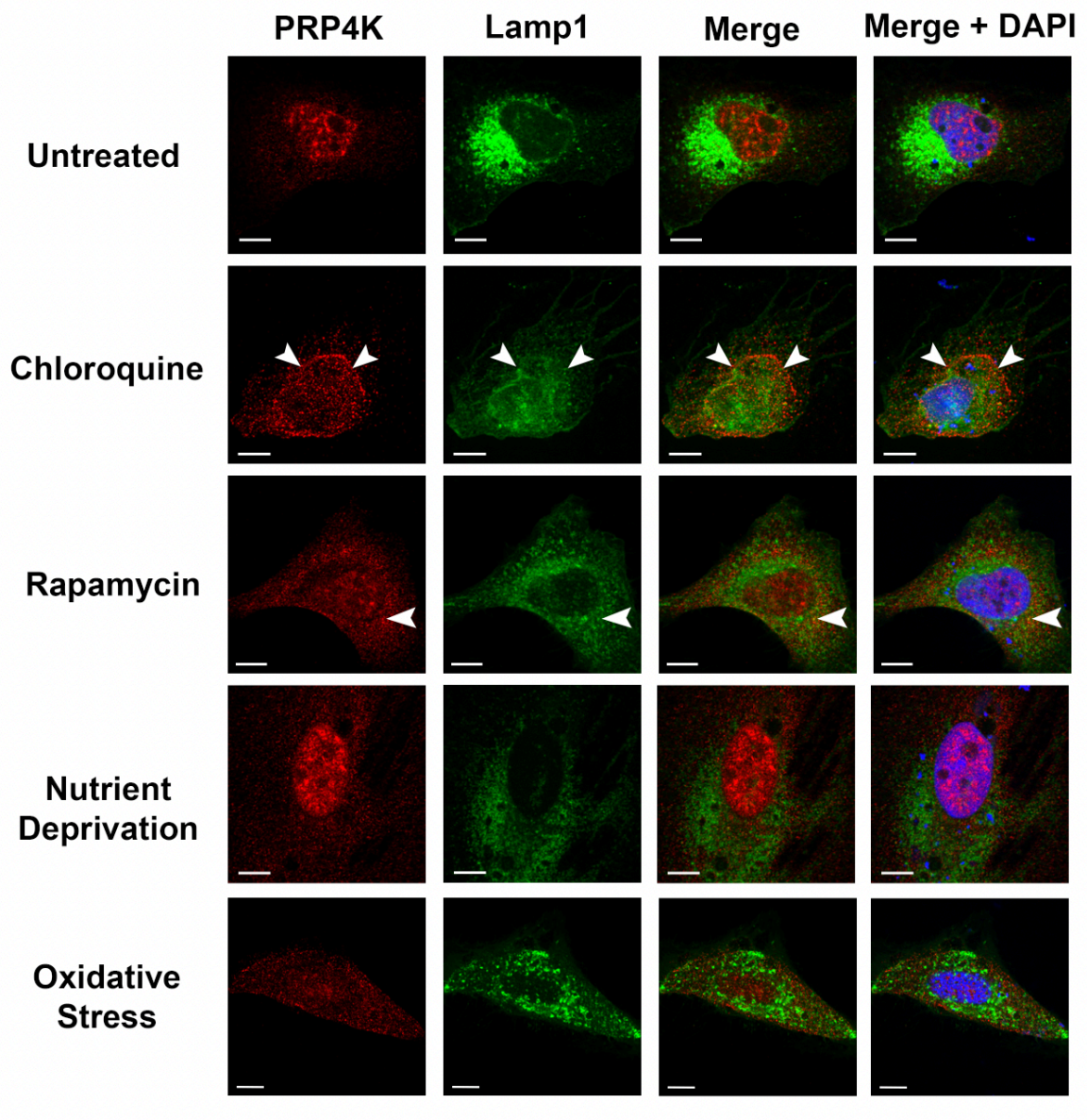
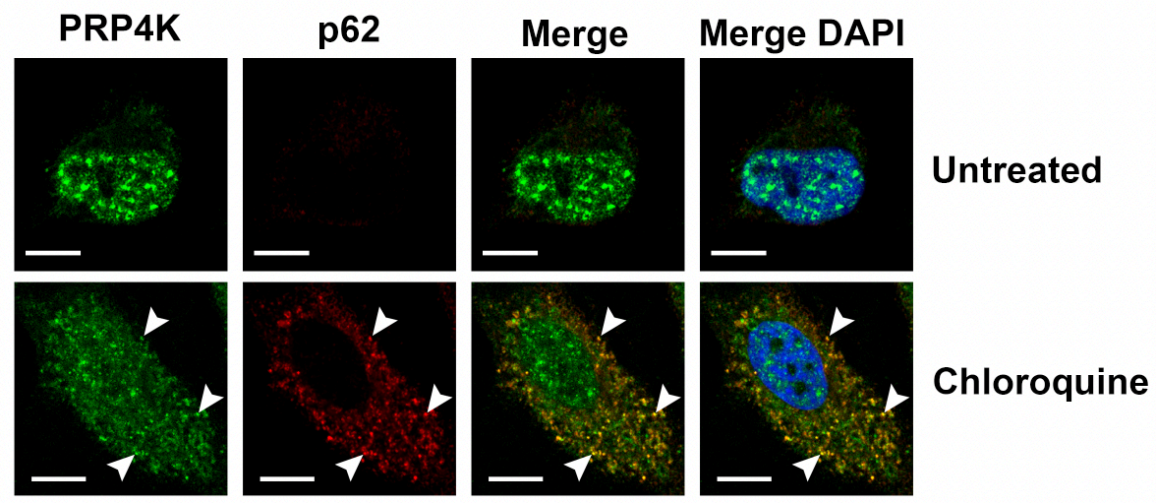


Figure 3.3: PRP4K shuttles to lysosomes following chloroquine and rapamycin treatment. HeLa cells were transfected with 200ng of Lamp1-GFP plasmid DNA. 48 h post-transfection, cells were treated with 50 µm chloroquine, 1 µM rapamycin, 16 h nutrient deprivation or 200 µM cobalt chloride and imaged. PRP4K was stained red, Lamp1 green, and DNA was stained blue (DAPI). Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

3.4 PRP4K Shuttles from the Nucleus to the Cytoplasm in a CRM1 dependent manner following cellular stress.

Chromosomal maintenance 1 (CRM1) (also known as Exportin 1 (XPO1)) is a major nuclear export protein responsible for the nuclear to cytoplasmic shuttling of various proteins (Castanotto et al., 2009). Recently it has been shown that CRM1 dependent shuttling is prevalent for numerous cancer-associated proteins that require nuclear exit (Castanotto et al., 2009; Turner et al., 2012). Due to the nuclear to cytoplasmic shuttling nature of PRP4K under stress, I hypothesized that PRP4K may be shuttling in a CRM1 dependent manner. One way to test the CRM1-dependence of nucleo-cytoplasmic shuttling is by using the small molecule leptomycin B that competitively binds to CRM1 inhibiting the binding of CRM1 to Topo IIα, a process that is required to facilitate nuclear export of various proteins. When HeLa cells are treated with chloroquine, we observe shuttling of PRP4K into the cytoplasm (Figure 3.4.1A). However, when cells were treated with both chloroquine and leptomycin B, the nuclear to cytoplasmic shuttling pattern of PRP4K was blocked, and PRP4K was strictly nuclear (Figure 3.4.1B). In contrast, treatment with leptomycin B alone had no effect on PRP4K localization as no shuttling behaviour was exhibited, which is consistent with our previous findings of no nuclear to cytoplasmic shuttling when no stress is induced (Figure 3.4.1). Thus, these data lead to a model whereby PRP4K nucleo-cytoplasmic shuttling in response to stress, such as chloroquine requires CRM1 (summarized in Figure 3.4.2)

A



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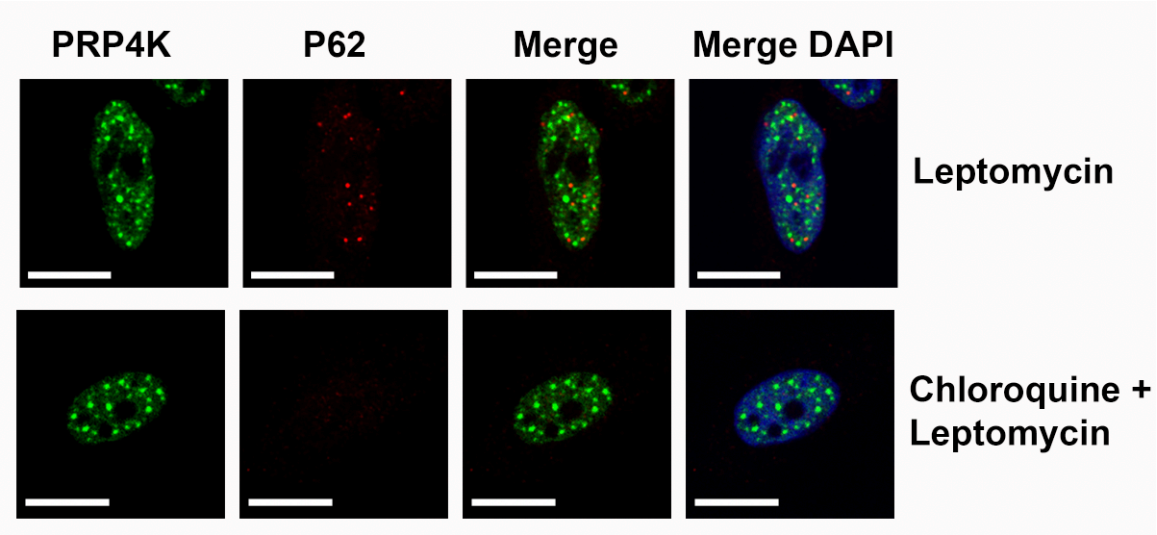

Figure 3.4.1: PRP4K relies on CRM1 for nuclear-cytoplasmic translocation after stress. (*A*) Images obtained from Figure 3.2.1 demonstrating PRP4K (green) cytoplasmic shuttling and colocalization with p62 (red) following 50 µm chloroquine treatment. Images captured on Prime95 confocal spinning disk. (*B*) PRP4K cytoplasmic shuttling is inhibited by the 20nM of the CRM1 inhibitor, leptomycin B. Images captured on Evolve confocal microscope. DNA was stained blue (DAPI). Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

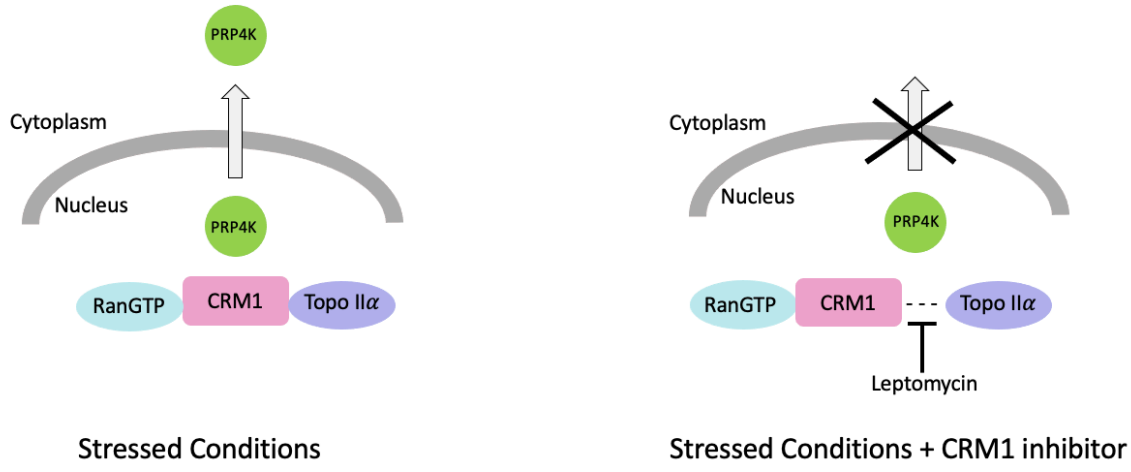

Figure 3.4.2: Model describing the CRM1-dependent mechanism of PRP4K nuclear-cytoplasmic translocation after stress. Mechanism of CRM1 inhibition by leptomycin B. CRM1 binds to RanGTP and Topo IIα to facilitate nuclear export shuttling under normal conditions. When a CRM1 inhibitor is introduced, the binding of CRM1 to Topo IIα is inhibited, ceasing the nuclear export signal.

3.5 Stress-induced shuttling of splicing kinase SRPK1.

Serine/arginine rich protein kinases (SRPKs) are another family of kinases involved phosphorylating splicing factors for the purpose of regulating alternative splicing (Chan & Ye, 2013; Corkery et al., 2015). Similar to PRP4K, SRPKs contain an SR domain in addition to an RNA binding motif (Zahler et al., 1992; Gui et al., 1994). There are three members of the family of SRPKs; SRPK1, SRPK2 and SRPK3 (Gui et al., 1994; Wang et al, 1998). SRPK1 is responsible for the phosphorylation of SR proteins *in vivo*, and it has shown that high levels of SRPK1 can inhibit splicing (Gui et al., 1994). Given the related function of both SRPK1 and PRP4K in the phosphorylation of SR protein splicing factors (Corkery et al., 2015), we sought to determine if SRPK1 was also capable of nucleo-cytoplasmic shuttling in response to cell stress. As seen in Figure 3.5, both PRP4K and SRPK1 are primarily nuclear in untreated HeLa cells. Following various stresses used to examine PRP4K shuttling, SRPK1 and PRP4K both appear primarily cytoplasmic (Figure 3.5) but do not colocalize. This data suggests that both splicing kinases, PRP4K and SRPK1 exhibit nucleo-cytoplasmic shuttling following cellular stress but that they localize to different cytoplasmic compartments.

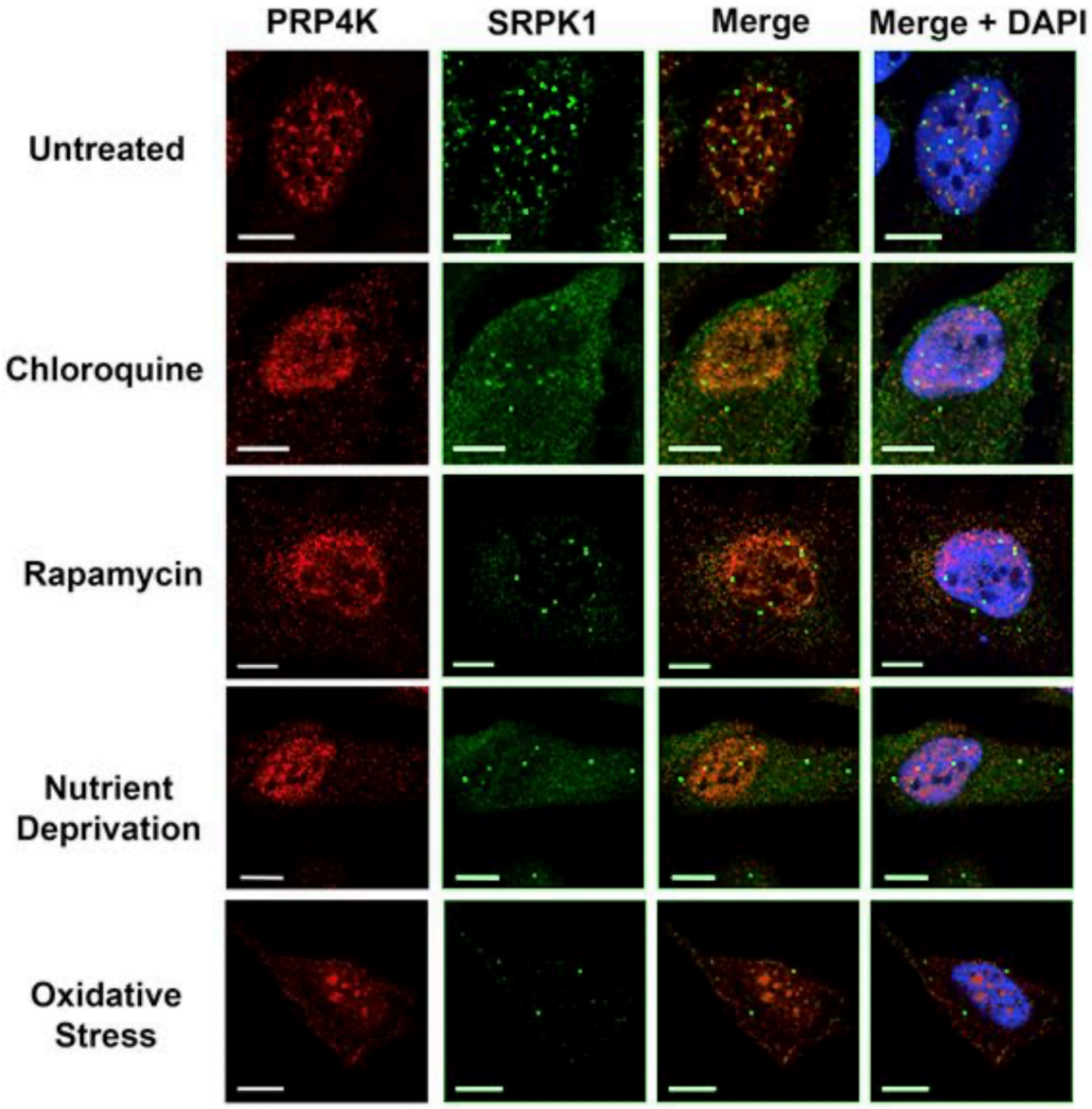

Figure 3.5: PRP4K and SRPK1 co-translocate to the cytoplasm under cell stress. HeLa cells were treated with 50 µm chloroquine, 1 µM rapamycin, 16 h nutrient deprivation or 200 µM cobalt chloride to induce cell stress before fixing and processing for immunofluorescence localization of PRP4K (red), SRPK1 (green). DNA was stained blue (DAPI). Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

3.6 PRP4K colocalizes with G3BP1 following cellular stress.

G3BP Stress Granule Assembly Factor 1 (G3BP1) is a gene responsible for the formation of cytoplasmic puncta called stress granules that are induced during cell stress such as hypoxia, nutrient deprivation and chemotherapy treatment and have a pro-survival function in cancer cells through sequestration of mRNAs and pro-apoptotic proteins to allow cells to recover from stress (Aulas et al., 2020). Due to PRP4K's shuttling nature following many of the same cellular stresses, I hypothesized that PRP4K might colocalize with stress granules containing G3BP1. I examined the localization of G3BP1 and PRP4K under various cellular stresses (Figure 3.6). As expected G3BP1 and PRP4K did not colocalize to any extent in untreated cells but I found that a minority of cytoplasmic puncta of G3BP1 did colocalize with PRP4K after treatment with chloroquine or rapamycin, or after stress by nutrient deprivation (Figure 3.6). In contrast, and unexpectedly PRP4K did not colocalize with G3BP1 in cells treated with $CoCl_2$ to induce oxidative stress despite the formation of prominent stress-granules containing G3BP1 (Figure 3.6). This data suggests that stress conditions caused by chloroquine, rapamycin, and nutrient deprivation cause PRP4K to translocate to stress granules.

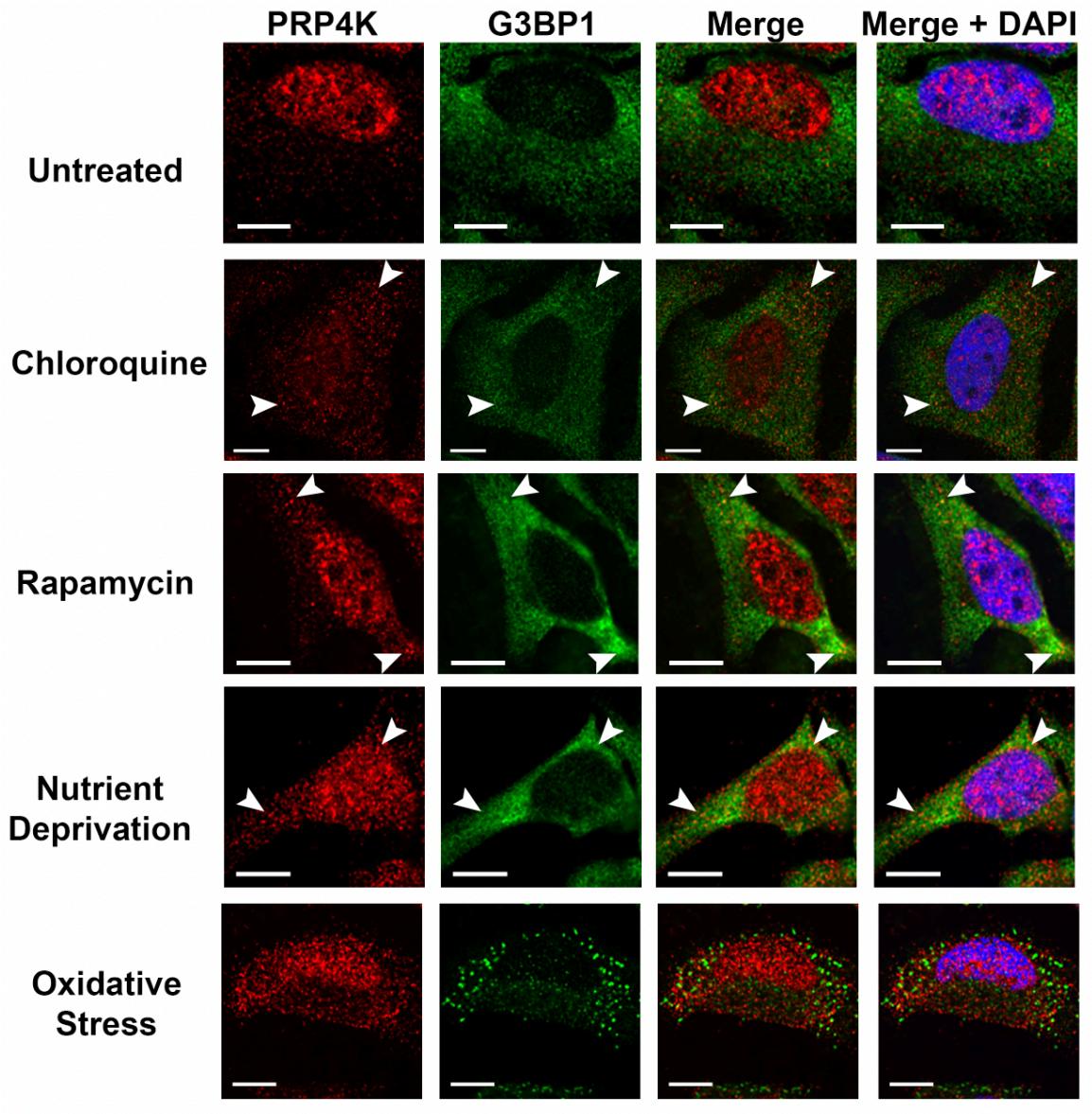

Figure 3.6: PRP4K shuttles to stress granules following chloroquine, rapamycin and nutrient deprivation. HeLa cells were treated with 50 µm chloroquine, 1 µM rapamycin, 16 h nutrient deprivation or 200 µM cobalt chloride to induce cell stress before fixing and processing for immunofluorescence localization of PRP4K (red), G3BP1 (green). DNA was stained blue (DAPI). Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

3.7 PRP4K does not colocalize with DCP1 under any stress conditions in HeLa cells.

Another structure formed after cellular stress are processing bodies (P-bodies), which are enriched in the decapping protein 1 (DCP1), which plays a role in mRNA decapping in the cytoplasm by activating decapping protein 2 (DCP2) (Eulalio et al., 2007; Franks & Lykke-Andersen, J., 2008). P-bodies are similar to stress granules such as G3BP1 in their protein components and their ability to be induced by cellular stress (Stoecklin & Kedersha, 2012; Luo et al, 2018). However, P-bodies are specifically sites of mRNA decay and their function in the cellular stress pathway is still being actively studied, which made it an interesting structure to investigate the relationship with PRP4K. As shown in Figure 3.7, PRP4K does not colocalize with DCP1 in untreated cells or following any stressed conditions. This data suggests that PRP4K is not involved in the formation of P-bodies in the cell.

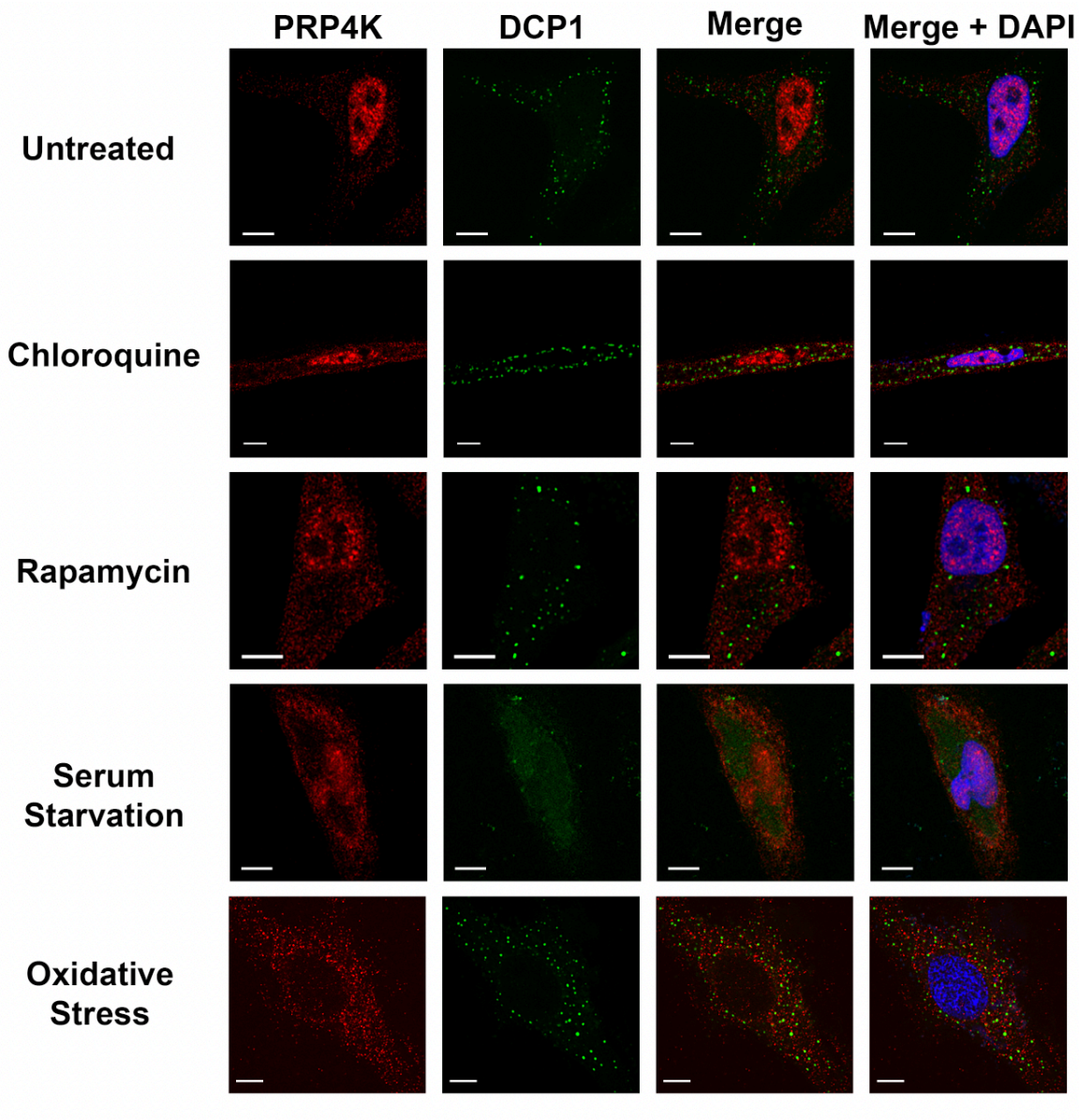

Figure 3.7: PRP4K does not shuttle to P-bodies following induction of cellular stress. HeLa cells were transfected with 200ng of DCP1-GFP plasmid DNA. 48 h post-transfection, cells were treated with 50 µm chloroquine, 1 µM rapamycin, 16 h nutrient deprivation or 200 µM cobalt chloride and imaged. PRP4K was stained red, DCP1 green, and DNA was stained blue (DAPI). Scale bar: 10 µm.

3.8 PRP4K colocalizes with Parkin following various cellular treatments in HeLa cells.

Parkin is an E3 ubiquitin ligase which in combination with PINK1 is involved in mitochondrial quality control (Narendra et al., 2012). Parkin activation has been shown to be essential in the mitophagy pathway, as Parkin is responsible for clearing damaged mitochondria through autophagy (Seirafi et al., 2015). Because of the involvement of Parkin in clearing damaged mitochondria, we sought to investigate whether PRP4K colocalized with Parkin in HeLa cells after stress. Indeed, under all stress treatments, PRP4K and Parkin colocalized in the cytoplasm but did not colocalize in untreated cells (Figure 3.8). In particular, oxidative stress via treatment with $CoCl_2$, which is known to damage mitochondria (Wang et al., 2000) elicited the colocalization response between PRP4K and Parkin with all puncta in precise colocalization. This data suggests that PRP4K may also play a role in mitophagy, particularly after oxidative stress.

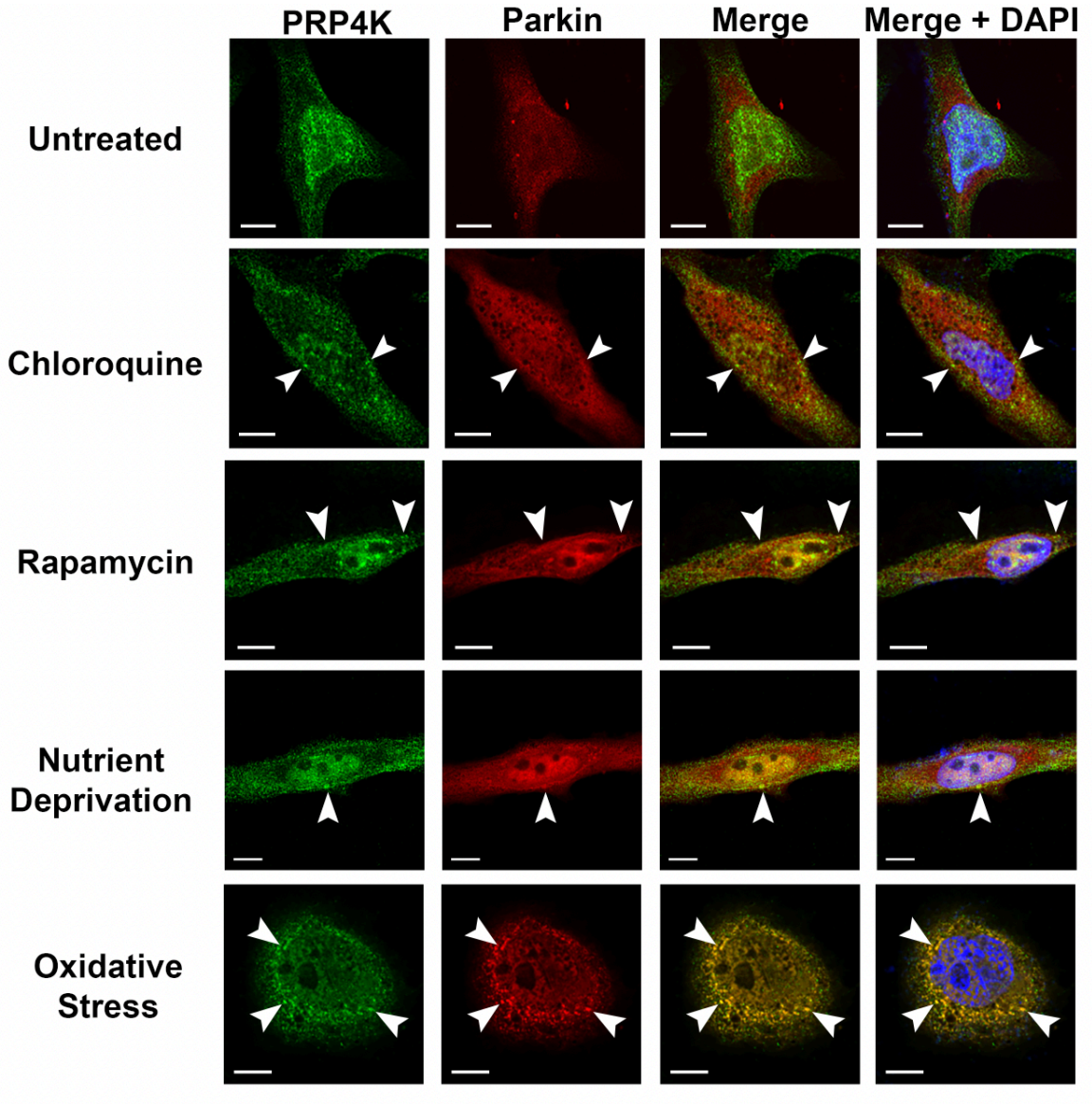

Figure 3.8: PRP4K shuttles to mitochondria following various cell treatments. HeLa cells were transfected with 200ng of mCherry-Parkin plasmid DNA. 48 h post-transfection, cells were treated with 50 µm chloroquine, 1 µM rapamycin, 16 h nutrient deprivation or 200 µM cobalt chloride and imaged. PRP4K was stained green, Parkin red, and DNA was stained blue (DAPI). Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

3.9 PRP4K, TRIM16, Keap1 and p62 colocalize in the cytoplasm of oxidatively treated HeLa cells.

A key transcription factor in the cells response to oxidative stress is the Nuclear factor erythroid 2-related factor (NRF2) (He et al., 2020). NRF2 is sequestered in the cytoplasm under normal conditions by interacting with the Kelch Like ECH Associated Protein 1 (KEAP1) and the Cullin 3 (CUL3) E3 ligase, which together facilitate the ubiquitination and proteasomal degradation of NRF2. Upon oxidative stress, the tripartite motif containing 16 (TRIM16) protein and p62 form a complex with KEAP1 (Jena et al., 2018), triggering the autophagic degradation of KEAP1, which releases NRF2 so that it can translocate to the nucleus (Figure 3.9.1). Once in the nucleus, NRF2 can induce the transcription of a host of genes involved in antioxidant pathways like glutathione synthetases and mitigating reactive oxygen species (ROS) such as superoxide dismutase 1 (SOD1) and catalase (He et al., 2020). Our recent findings of PRP4K colocalization with p62 in autophagosomes in the cytoplasm following oxidative stress (Figure 3.2.8) lead me to hypothesize that PRP4K may be involved in the regulation of the NRF2-Keap1 complex. It was found that PRP4K strongly colocalizes with TRIM16 in the cytoplasm of oxidatively stressed HeLa cells (Figure 3.9.2; panel A). PRP4K, TRIM16 and p62 were then observed for colocalization following oxidative stress. PRP4K, TRIM16 and p62 all colocalize in the cytoplasm following oxidative stress (Figure 3.9.2; panel B). This experiment was then performed to observe KEAP1, PRP4K and p62 cytoplasmic localization. KEAP1, PRP4K and p62 were also found to colocalize in the cytoplasm following oxidative stress (Figure 3.9.3). We also examined the localization of NRF2 relative to PRP4K following oxidative stress (Figure 3.9.4). Interestingly, PRP4K and NRF2 exhibited no colocalization following oxidative stress (Figure 3.9.4). Taken

together, this data suggests that PRP4K may be involved in regulation of the NRF2-Keap1 complex via p62 interaction with TRIM16 to promote the autophagic degradation of KEAP1. Additionally, the effects of PRP4K knockdown on TRIM16 and TRIM29 levels in MCF10A cells were investigated to determine whether PRP4K was vital for the KEAP1-NRF2 pathway. It was found that in PRP4K knockdown cells, levels of TRIM16 were increased by two-fold in comparison to the control hairpin (Figure 3.9.4). TRIM29 levels remained stable. This data opens the avenue for further investigation into varying PRP4K levels and the effect on TRIM16, Keap1 and p62 levels within in the cell.

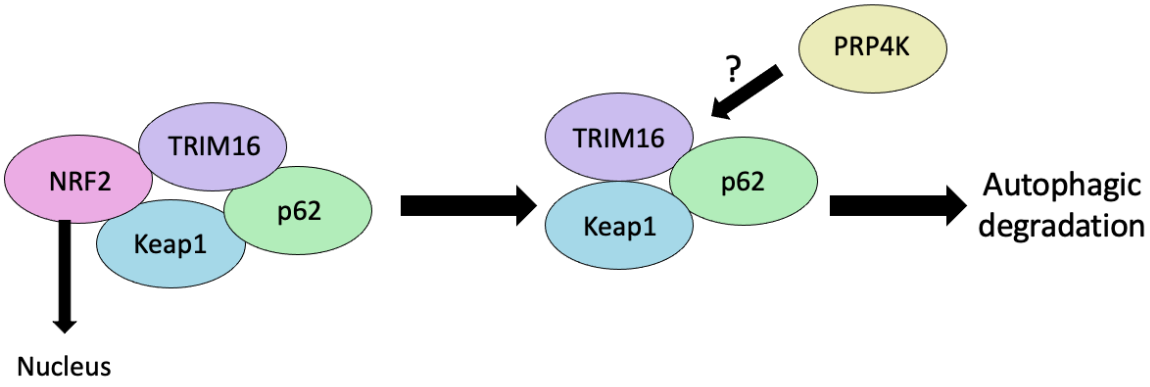

Figure 3.9.1: Model describing the Keap1-NRF2 complex and the potential role for PRP4K in the complex. TRIM16, Keap1, p62 and NRF2 form a complex known as the Keap1-NRF2 complex. Following oxidative stress, NRF2 exits the complex and goes to the nucleus, where TRIM16, Keap1 and p62 are involved with the autophagosome and subsequent autophagic degradation. The data presented in this study suggest a role for PRP4K in this complex, specifically at the autophagosome.

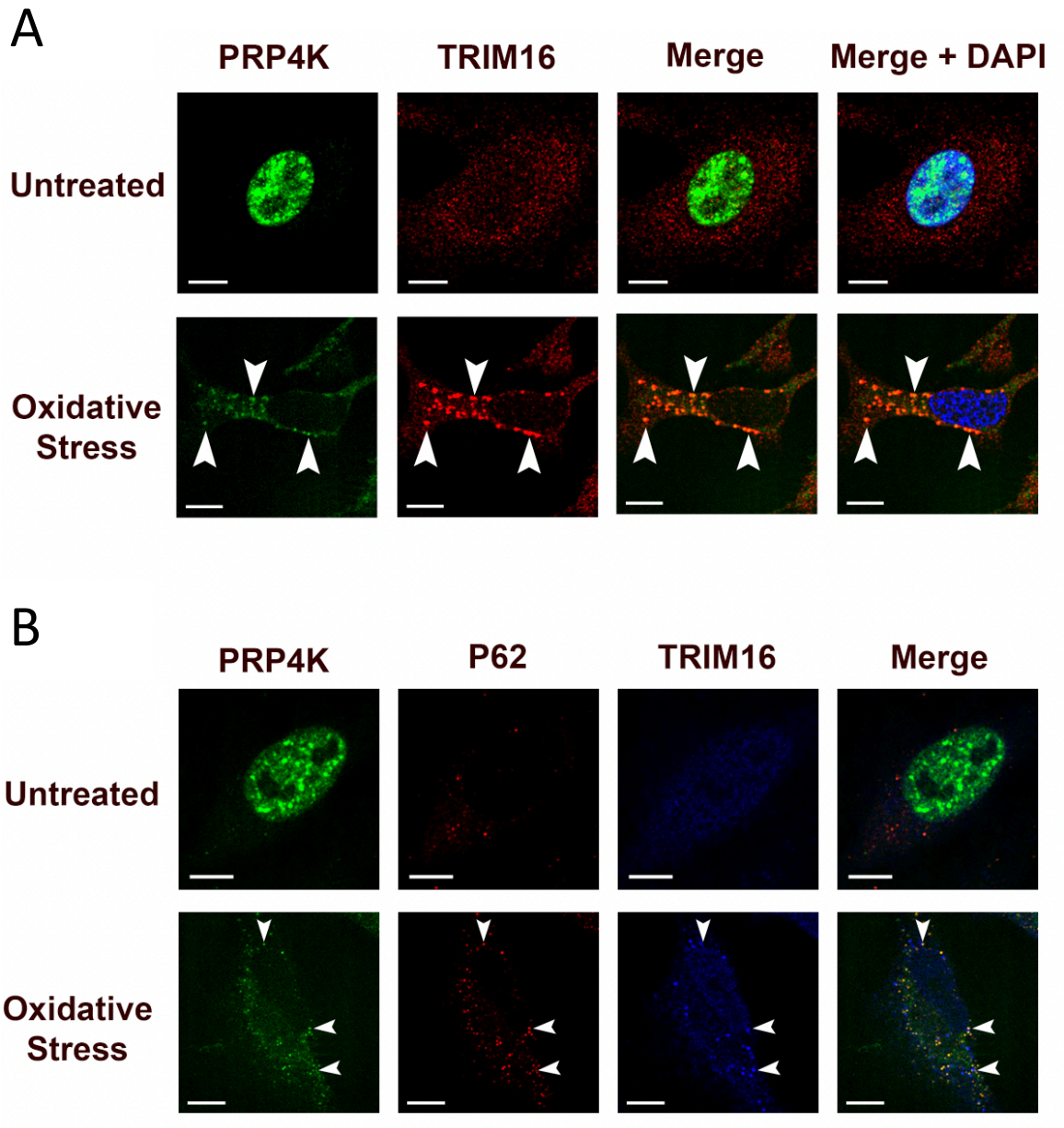

Figure 3.9.2: Cytoplasmic PRP4K colocalizes with P62 and TRIM16 following oxidative stress. *(A)* PRP4K (green) colocalizes with TRIM16 (red) in the cytoplasm following 200 µM cobalt chloride to induce oxidative stress in HeLa Clover-PRP4K cells. DNA was stained blue (DAPI). *(B)* Cytoplasmic PRP4K (green), TRIM16 (blue) and p62 (red) colocalize following 200 µM cobalt chloride to induce oxidative stress in HeLa Clover-PRP4K cells. All images obtained on the Evolve confocal microscope. Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

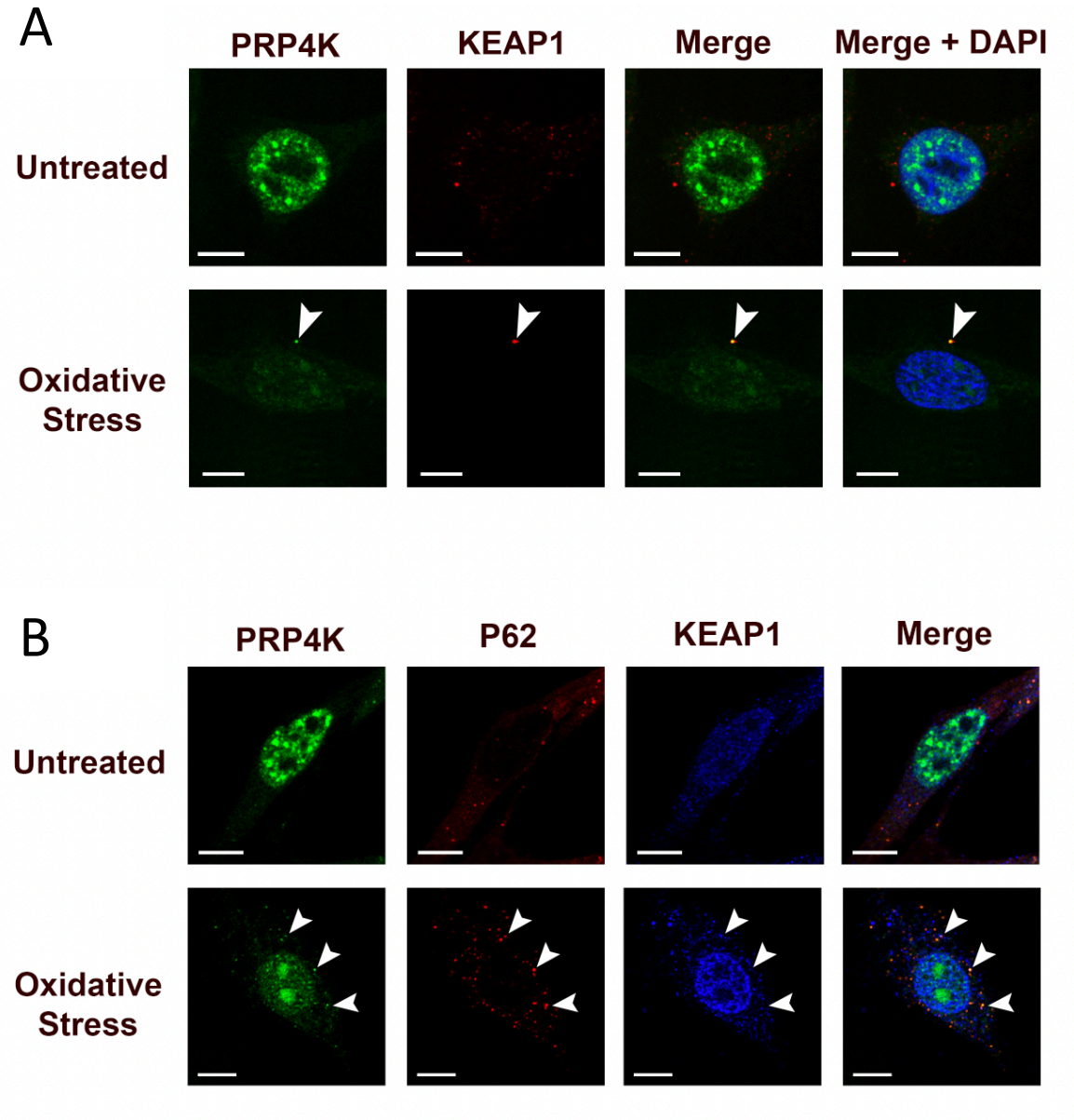

Figure 3.9.3: Cytoplasmic PRP4K colocalizes with P62 and KEAP1 following oxidative stress. (*A*) PRP4K (green) colocalizes with KEAP1 (red) in the cytoplasm following 200 µM cobalt chloride to induce oxidative stress in HeLa Clover-PRP4K cells. DNA was stained blue (DAPI). (*B*) Cytoplasmic PRP4K (green), KEAP1 (blue) and p62 (red) colocalize following 200 µM cobalt chloride to induce oxidative stress. All images obtained on the Evolve confocal microscope. Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

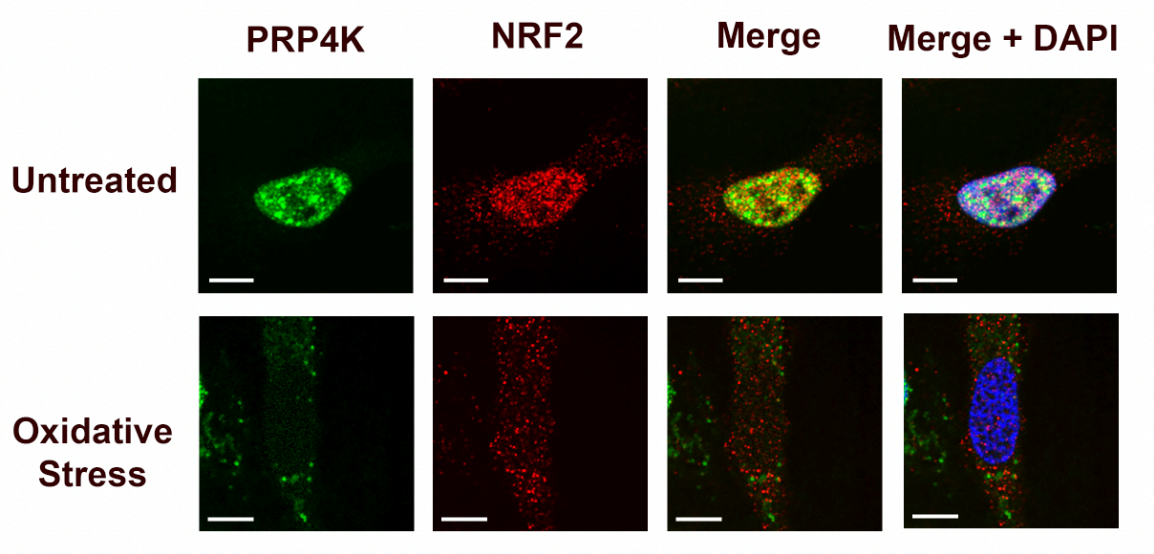

Figure 3.9.4: Cytoplasmic PRP4K does not colocalize with NRF2 following oxidative stress. PRP4K (green) does not colocalize with NRF2 (red) in the cytoplasm following 200 µM cobalt chloride to induce oxidative stress in HeLa Clover-PRP4K cells. DNA was stained blue (DAPI). Images obtained on the Evolve confocal microscope. Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

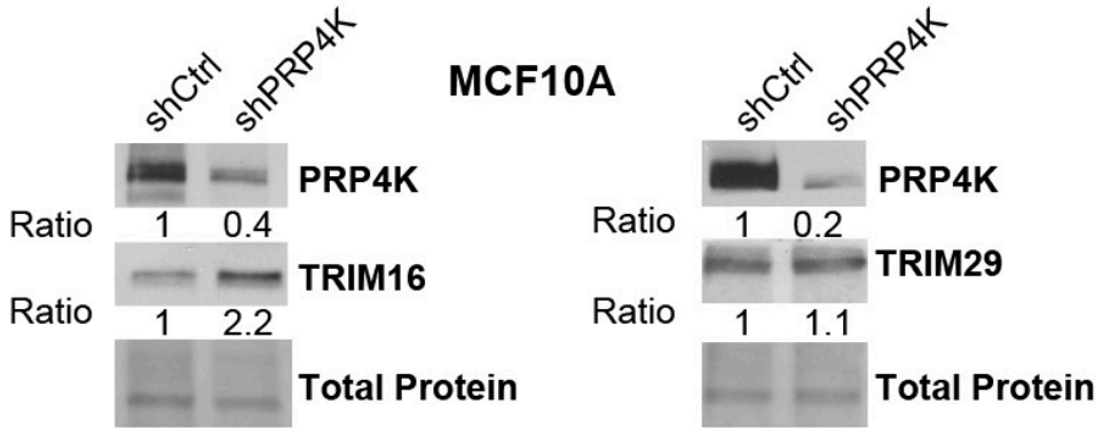

Figure 3.9.5: TRIM16 protein levels are increased and TRIM29 levels are not altered in PRP4K knockdown MCF10A cells. TRIM16 protein levels increase by 2-fold when PRP4K levels are knocked down. Densitometry measurements are indicated below the respective bands and compared to the control sample as ratios. Total protein is normalized by using the Mini-PROTEAN TGX stain-free gels' capacity to visualize tryptophan residues in protein after UV induction.

3.10 PRP4K colocalizes with the marker of ubiquitin, K48 Ub in the cytoplasm of oxidatively treated HeLa cells.

Ubiquitination is a type of post translational modification. Specifically, K48 linked poly-ubiquitination has the capacity to target a protein for proteasomal degradation (Meyer & Rape, 2014). It has been shown previously that the presence of TRIM16 decreases K48-linked poly-ubiquitination and increases K63-linked poly-ubiquitination of NRF2 (Jena et al., 2018). This could be due to TRIM16 mediated degradation of KEAP1, which causes subsequent ubiquitination and degradation of NRF2 (Jena et al., 2018). Additionally, it has been shown that ubiquitin has the capacity to act as a signal for autophagy or lysosomal degradation (Komander & Rape, 2012; Randow & Youle, 2014). Because of the influence of K48 on the KEAP1-NRF2 complex and its involvement in the autophagy pathway, PRP4K and K48 Ub colocalization were observed in oxidatively stressed HeLa cells. Interestingly, PRP4K and K48 strongly colocalized in the cytoplasm of oxidatively stressed cells (Figure 3.10). This data further confirms that PRP4K is involved in the KEAP1-NRF2 pathway and may play a vital role in the degradation of target proteins.

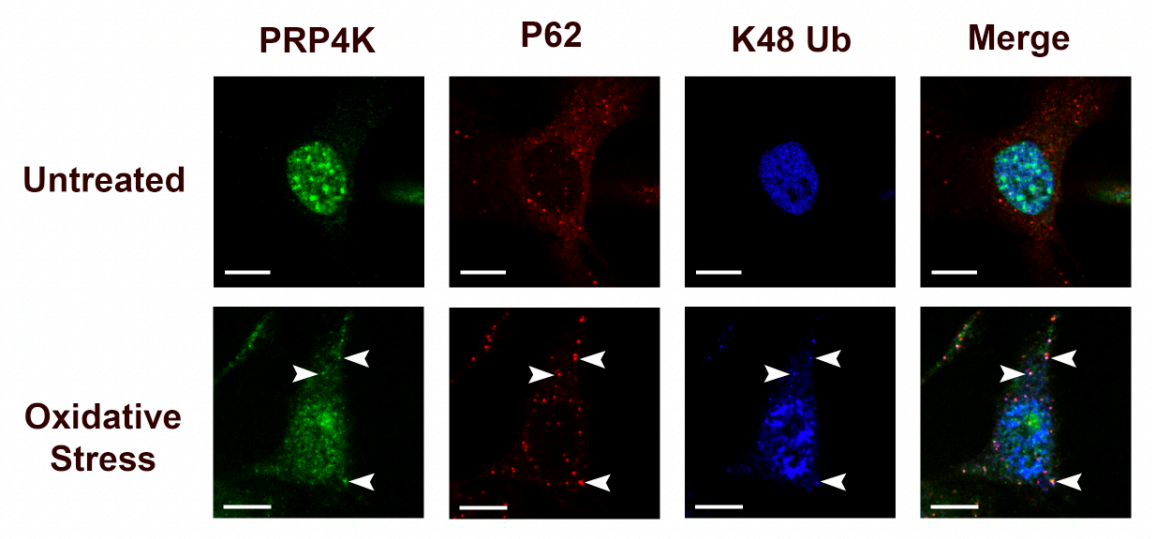

Figure 3.10: Cytoplasmic PRP4K colocalizes with P62 and K48 Ub following oxidative stress. PRP4K (green) colocalizes with p62 (red) and K48 (blue) in the cytoplasm following 200 µM cobalt chloride to induce oxidative stress in HeLa Clover-PRP4K cells. DNA was stained blue (DAPI). Images obtained on the Evolve confocal microscope. Arrows indicate areas of strong colocalization between two proteins. Scale bar: 10 µm.

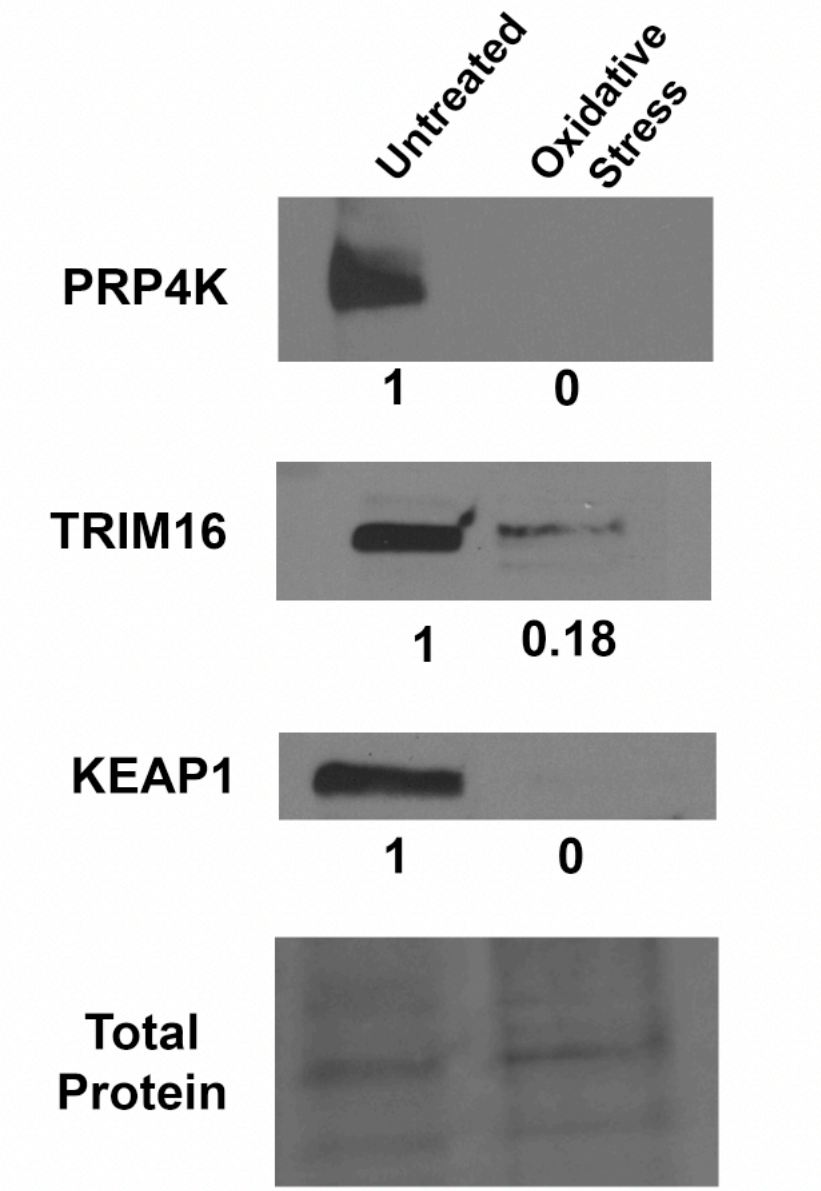

Figure 3.11: PRP4K, TRIM16 and KEAP1 protein levels are altered following oxidative stress in HeLa cells. PRP4K image obtained from Figure 3.2.9 to illustrate the decrease in PRP4K protein levels following 200 µM oxidative stress. TRIM16 protein levels are substantially decreased following 200 µM oxidative stress. KEAP1 banding patterns are altered following 200 µM oxidative stress. Densitometry measurements are indicated below the respective bands and compared to the control sample as ratios. Total protein is normalized by using the Mini-PROTEAN TGX stain-free gels' capacity to visualize tryptophan residues in protein after UV induction.
*Total protein for PRP4K omitted from this figure, shown in Figure 3.2.9.

Chapter 4: Discussion

Breast cancer is among the most common types of cancer exhibited in women and ovarian cancer (Global Cancer Observatory, 2012). Because of the high prevalence coupled with poor outcomes, investigating breast cancer therapies and mechanisms of tumour suppression are of utmost importance.

PRP4K is a multifunctional protein involved in various cellular processes, and previous work from the Dellaire laboratory and others have found that PRP4K expression is positively correlated with better treatment responses and patient outcomes in breast and ovarian cancer (Corkery et al., 2015 and 2018, Cho et al., 2018). Broadly, these data support a role for PRP4K as a tumour suppressor and treatment modifier, as loss of PRP4K in breast and ovarian cancer cell lines induced taxane therapy resistance and promoted metastasis in a mouse model of ovarian cancer (Corkery, et al., 2015; Corkery, et al., 2018; Clarke et al., 2019). Since loss of PRP4K expression was linked to increased metastasis, which is driven in part by EMT, I examined the relationship between PRP4K loss by shRNA knockdown and EMT in the triple negative breast cancer cell line MDA-MB-231 and normal mammary epithelial cell lines MCF10A and HMLE (Figure 3.1.1). Although the level of PRP4K was not drastically different between the cell lines (Figure 3.1.2), I found that PRP4K depletion by shRNA resulted in a "partial EMT" state in the mammary epithelial cell lines characterized by maintenance of epithelial markers like E-cadherin but upregulation of mesenchymal markers such as fibronectin, vimentin and slug (Figure 3.1.1).

Recently, the Hippo-Yap pathway has been shown to play a role in EMT, specifically through regulating the expression of various epithelial and mesenchymal

markers (Cheng et al., 2020). It was shown that overexpression of YAP causes downregulation of E-cadherin and upregulation of Slug, which serve as epithelial and mesenchymal markers in EMT, respectively (Cheng et al., 2020). Additionally, overexpression of YAP was shown to promote migration and invasion of cancer cells, while knockdown of YAP was shown to inhibit cancer cell migration and invasion (Park et al., 2019; Cheng et al., 2020). It was also demonstrated that YAP is capable of inducing EMT through the control of E-cadherin expression by the formation of a complex with Wilms Tumour 1 (WT1) (Park et al., 2019). WT1 has previously been identified as a regulator of MET, the reverse process of EMT, and the YAP-WT1 interaction was shown to be involved in cell dissemination, a characteristic phenotype exhibited in EMT (Hohenstein & Hastie, 2006). Taken together, the downregulation of E-cadherin and upregulation of Slug during EMT demonstrated in these studies are consistent with our findings of partial EMT induced by PRP4K knockdown. These studies, coupled with the data I have collected also helps to further demonstrate the link between EMT, YAP and PRP4K.

The Dellaire laboratory also recently published provocative data indicating that PRP4K, considered previously an exclusively nuclear kinase could be found in the cytoplasm under cellular stress (Corkery, et al., 2018). Additionally, it was found to colocalize with the marker and adaptor of autophagy, p62 in the cytoplasm of stressed cells (Corkery, et al., 2018). While autophagy regulates p62, upregulation of p62 has been implicated in the formation of tumours and resistance to therapy (Islam et al., 2018). Autophagy is an intracellular degradation system that is both pro- and anti-tumourigenic (Yun & Lee, 2018). Because of the presence of cytoplasmic PRP4K, which is the opposite of what is known about most splicing factors, and subsequent PRP4K-p62

colocalization, investigating PRP4K's cytoplasmic functions and its potential role in the autophagy pathway was the primary focus of my study.

Given the observation that under nutrient-deprivation and chloroquine stress PRP4K localized to autophagosomes containing the autophagy adaptor p62 (Corkery et al., 2018), and due to the pro- and anti-tumourigenic aspects of autophagy (Yun & Lee, 2018), I hypothesized that PRP4K's tumour suppressor role might also be linked to cytoplasmic function under cell stress. I chose to further investigate the cellular behaviour and potential role of PRP4K in cell stress induced by conditions that act as inhibitors or activators of autophagy. Since chloroquine induced nucleo-cytoplasmic shuttling of PRP4K and colocalization with the marker of autophagy, p62, I was interested in investigating the shuttling behaviour of PRP4K in response to other autophagy inducers, as well as other forms of cellular stress. Interestingly, both activators of autophagy, such as rapamycin and nutrient deprivation, which either inhibit mTOR or activate AMPK (respectively) and oxidative stress, which is known to activate autophagy through a HIF-dependent manner (Bellot et al., 2009; Efeyan et al., 2015) caused the shuttling phenotype of PRP4K. Each of these stresses resulted in colocalization of PRP4K with p62, Rab7, as well as the most well-known marker of autophagy, LC3 (Figures 3.2.1-3.2.9). This data indicates that stresses that are involved with the autophagy pathway elicit nucleo-cytoplasmic shuttling of PRP4K, which may have a yet to be defined role in autophagy. PRP4K shuttling observed by fluorescence microscopy was further confirmed by subcellular fractionation and Western blot analysis, which demonstrated that PRP4K protein levels are increased by 4-fold in the cytoplasm of chloroquine treated cells (Figure 3.2.3). Additionally, Western blot analysis was preformed to determine if PRP4K levels were decreasing following cellular stress, or if PRP4K was simply shuttling from the

nucleus and into the cytoplasm with no change in protein level. While chloroquine, rapamycin and nutrient deprivation demonstrated relatively consistent protein levels of PRP4K and varying levels of p62 and LC3 depending on the mechanism of autophagy induction or inhibition, oxidative stress demonstrated a rapid decrease of PRP4K following treatment (Figures 3.2.1-3.2.9). This data was intriguing, particularly in the sense that PRP4K is known to be an essential kinase, therefore, substantial cell death would be expected upon loss of PRP4K, which was experienced throughout the time course. However, it was still possible to detect PRP4K protein by immunofluorescence, albeit at a reduced level, by fluorescence microscopy. It was additionally suspected that PRP4K may have precipitated out due to cobalt chloride treatment, giving us a falsely low representation of PRP4K protein expression following oxidative stress. This is a limitation to the interpretation of this study and is a question that is currently under investigation.

Since the immunofluorescence and Western blot observations of PRP4K indicated that PRP4K may play a role in the autophagy pathway (Figures 3.2.1-3.2.9) I then looked at Lamp1, a marker of the lysosome. Given the colocalization of PRP4K with p62 and LC3, which play roles in the recruitment of proteins and the formation and maturation of the autophagosome (Mizushima, 2007; Tanida et al., 2008), we assumed PRP4K may be restricted to autophagic vesicles in the cytoplasm during cell stress. Because chloroquine inhibits fusion of the autophagosome to the lysosome, we expected little to no colocalization between Lamp1 and PRP4K. Rapamycin, oxidative stress and nutrient deprived cells were also not expected to have robust PRP4K-Lamp1 colocalization. Although no colocalization as observed in nutrient deprived or oxidatively stressed cells, as expected, we did observe very minor colocalization between PRP4K and

Lamp1 in chloroquine and rapamycin treated cells (Figure 3.3). This data indicates that PRP4K is not a component of the post-fusion autophagolysosome but may have a more prominent function earlier in the autophagy pathway with p62 and LC3.

Once the shuttling phenotype under various stresses was well characterized and the markers of autophagy under investigation had been analyzed, the next step was determining the mechanism behind the nucleo-cytoplasmic shuttling of PRP4K. The nuclear export protein CRM1 has been shown to allow rapid nuclear exit of various proteins and RNAs and cause nuclear accumulation (Askjaer et al., 1999). Because of this, CRM1 dependent shuttling was investigated as the potential model for PRP4K nucleo-cytoplasmic shuttling. A CRM1 inhibitor, leptomycin B was used to investigate this mechanism. Leptomycin B competitively binds to CRM1, inhibiting the binding of CRM1 to Topo IIα, a process that is required to facilitate nuclear export of various proteins (Figure 3.4.2) (Kudo et al., 1999). When cells were treated with leptomycin B alone, no shuttling behaviour was exhibited, which is consistent with our previous findings of no nuclear to cytoplasmic shuttling when no stress is induced. However, when both chloroquine and leptomycin B were introduced, the nuclear to cytoplasmic shuttling pattern of PRP4K that has been observed in chloroquine treated cells was blocked, and PRP4K was strictly nuclear (Figure 3.4.1). These findings indicate that PRP4K is shuttling in a CRM1-dependent manner. Since proteins that shuttle to and from the nucleus in a CRM1-dependent manner typically contain a nuclear export signal (NES) (Lee et al., 2019), we predict that PRP4K likely also contains a NES.

Following the discovery that the splicing kinase, PRP4K was shuttling in a CRM1 dependent manner, we sought to look at another splicing kinase in attempt to determine if the same shuttling behaviour was exhibited. Due to SRPK1's influential role in

alternative splicing, it was chosen as a second protein kinase to monitor nucleo-cytoplasmic shuttling. We hoped by studying SRPK1, our experiment would tell us whether the shuttling phenotype of PRP4K was specific to PRP4K alone or might also occur among other splicing kinases as well. SRPK1 was previously hypothesized to behave oppositely to PRP4K under stress, shuttling from the cytoplasm to the nucleus based on previous studies looking at the nuclear translocation of SRPK1 in response to activation of EGFR signaling (Zhou et al., 2012). However, our findings demonstrate that SRPK1 shuttles from the nucleus to the cytoplasm during stress, albeit not to the same extent as PRP4K (Figure 3.5). This data indicates that PRP4K's nucleo-cytoplasmic shuttling response to cell stress could reflect a more general response of splicing factors to cell stress, and thus such shuttling could play a role in stress-induced changes in pre-mRNA splicing.

Interestingly, other factors involved in pre-mRNA splicing like the heteronuclear ribonuclear protein A1 (hnRNP A1), also exhibit nucleo-cytoplasmic shuttling in response to cell stress but are found to localize to stress granules (Guil et al., 2006). This prompted my examination of PRP4K's localization relative to stress granules marked by G3BP1 and related processing bodies (P-bodies) marked by DCP1. Stress granules sequester mRNAs and pro-apoptotic proteins in cancer cells, allowing them to recover from stress (Aulas et al., 2020). P-bodies are specifically sites of mRNA decay and form in response to cell stress (Sheth & Parker, 2007). Due to the fact that the treatments were used to stress the cells in various ways, including nutrient deprivation and oxidative stress, it was hypothesized that PRP4K might localize to DCP1 or G3BP1 following stress in a similar fashion to hnRNP A1. Cytoplasmic PRP4K had minor colocalization with G3BP1 in chloroquine treated, rapamycin treated and nutrient

deprived cells, however; this response was not robust (Figure 3.6). However, PRP4K did not colocalize with DCP1 under any stress or in untreated conditions, indicating that PRP4K does not go to p-bodies following cellular stress (Figure 3.7). Taken together, this data indicates that PRP4K is not participating in the part of the stress response involved with p-bodies or stress granules, which furthers the hypothesis that PRP4K may be primarily involved in autophagy.

Since autophagy is also involved in recycling and degradation of cellular organelles such as mitochondria, a process known as mitophagy, I also determined whether PRP4K might be involved in mitophagy. To accomplish this, I looked at the colocalization of PRP4K with Parkin after cellular stress. Parkin is an adaptor protein for mitophagy and is responsible for clearing damaged mitochondria through autophagic degradation (Seirafi et al., 2015). PRP4K strongly colocalized with Parkin following all cellular stresses (Figure 3.8). This phenotype was absent in untreated cells and was strongest in oxidatively stressed cells, with all cytoplasmic PRP4K puncta colocalizing with Parkin (Figure 3.8). This data indicates that PRP4K is involved in mitochondrial clearance, likely through the mitophagy pathway by interacting with Parkin. This opens up an avenue to investigate in the future, in particular coupling Parkin with PINK1 to determine precisely where PRP4K may fit within this complex process, as well as looking at PRP4K colocalization with other markers of mitochondria following stress.

Due to the strong colocalization exhibited between PRP4K and p62 following oxidative stress, this led me to evaluate other p62-associated autophagy pathways specifically linked to oxidative stress. Recently, it has been published that p62 plays a role in the regulation of the KEAP1-NRF2 complex that is a master-regulator of transcriptional programs that mediate survival from oxidative and chemical stress (He et

al., 2020). NRF2 is sequestered in the cytoplasm under normal conditions by interacting with the KEAP1 and CUL3 E3 ligase, which together control NRF2 levels through ubiquitination and proteasome-mediated degradation. Upon oxidative stress, the TRIM16 protein and p62 form a complex with KEAP1 (Jena et al., 2018), triggering the autophagic degradation of KEAP1, which releases NRF2 so that it can translocate to the nucleus (Figure 3.9.1). Given the central role of TRIM16 and p62 in regulating KEAP1 autophagic degradation, and PRP4K's colocalization with p62 under oxidative stress, I first examined if PRP4K and TRIM16 were colocalizing by immunofluorescence microscopy. Following oxidative stress, TRIM16 appeared to form aggregates in the cytoplasm that strongly colocalized with PRP4K (Figure 3.9.2, panel A). I also observed strong colocalization between PRP4K, p62 and TRIM16 (Figure 3.9.2, panel B). Western blot analysis of PRP4K knockdown MCF10A cells also indicated that TRIM16 levels were increased ~2 fold with PRP4K depletion compared to control cells (Figure 3.9.5). This data further indicates that under oxidative stress PRP4K is likely involved in the KEAP1-NRF2 pathway, potentially through the autophagic degradation of KEAP1 mediated by TRIM16.

To further explore the relationship between the NRF2-KEAP1 complex and PRP4K I examined their relative colocalization in HeLa cells in response to $CoCl_2$ induced oxidative stress. Under oxidative stress conditions, cytoplasmic KEAP1 and PRP4K strongly colocalized, as did cytoplasmic KEAP1, p62 and PRP4K (Figure 3.9.3) but PRP4K and NRF2 did not colocalize (Figure 3.9.4). This data indicates that PRP4K is part of the TRIM16-KEAP1-p62 complex at the autophagosome and is not involved in the complex prior to autophagy induction by oxidative stress. To extend these observations I also conducted a Western blot analysis of PRP4K, KEAP1 and TRIM16

levels following oxidative stress. Demonstrated in Figure 3.2.9, it was found that following oxidative stress, PRP4K levels drastically decrease. However, this drastic decrease in PRP4K, coupled with oxidative stress had substantial impacts on KEAP1 and TRIM16 levels. Following oxidative stress, KEAP1 protein levels were abolished (Figure 3.11). It was also found that TRIM16 levels were decreased in oxidatively stressed cells (Figure 3.11).

As a final look into the KEAP1-NRF2 pathway and PRP4K, K48 ubiquitin and PRP4K colocalization was examined. The rationale for looking at K48 ubiquitin stems from the fact that the presence of TRIM16 decreases K48-linked poly-ubiquitination of NRF2 (Jena et al., 2018), and TRIM16 and PRP4K strongly colocalize. Since K48 ubiquitination targets proteins for proteasomal degradation I hypothesized that if PRP4K was being degraded in the cytoplasm in response to oxidative stress, K48-ubiquitin fluorescence signal would colocalize with PRP4K. Additionally, ubiquitin has been shown to act as a signal for autophagy and lysosomal degradation (Komander & Rape, 2012; Randow & Youle, 2014). PRP4K, p62 and K48 Ub colocalized in the cytoplasm of HeLa cells after oxidative stress and did not exhibit any colocalization in unstressed cells (Figure 3.10). This data indicates that PRP4K may be colocalizing with proteins destined for autophagic degradation as a component of the autophagosomes or is itself subject to K48-ubiquitination. Both processes would lead to loss of PRP4K protein expression, however we do not observe colocalization of PRP4K with Lamp1-labelled autophagosomes. Thus, PRP4K may be subject to proteasomal degradation after oxidative stress, which is supported by our preliminary Western blot analysis (Figure 3.2.9).

To summarize, the data that I have collected further demonstrates PRP4K's role in EMT and demonstrates that PRP4K shuttles from the nucleus to the cytoplasm in a

CRM1-dependent manner following treatment with chloroquine or rapamycin, and both nutrient deprivation and oxidative stress; all treatments that induce or inhibit autophagy by various mechanisms. Following treatment, it was determined that cytoplasmic PRP4K strongly colocalizes with markers of autophagy, p62 and LC3, Rab7 (a marker of the late endosome) and the mitochondrial damage marker Parkin following all stresses in HeLa cells. PRP4K was also found to localize with members of the KEAP1-NRF2 complex. While its precise role within the complex is still under investigation, colocalization of PRP4K with TRIM16 and protein aggregates marked by K48 linked ubiquitination could imply a role for PRP4K in the regulation of KEAP1 protein stability via autophagic degradation. Taken together, this data generally implicates PRP4K in the autophagy pathway after cellular stress, with a more specific role in regulation of the KEAP1-NRF2 complex during oxidative stress.

Chapter 5: Conclusions and Future Directions

In this thesis I further investigated the role of PRP4K in EMT and examined a possible role for PRP4K in the cell's response to stress. Additionally, I discovered that PRP4K is involved in the autophagy pathway, and during oxidative stress is likely playing a role in regulating the KEAP1-NRF2 complex via modulation of TRIM16 protein levels and autophagic degradation of KEAP1. Several questions remain in regard to PRP4K's precise role in autophagic degradation of TRIM16-associated aggregates, the autophagy pathway and its behaviour in the stress response. Future research should confirm if PRP4K loss of overexpression affects NRF2 signaling in response to oxidative stress, as well as whether PRP4K kinase activity is essential for its roles in autophagy and/or NRF2 signaling. Finally, given the intriguing results that PRP4K colocalizes with K48-ubiquitin chains, the ubiquitination of PRP4K should also be further evaluated in the context of oxidative stress.

Taken together, the results of this study further demonstrate that PRP4K is a multifunctional kinase and provide some of the first evidence that this kinase has both nuclear and cytoplasmic functions, and in particular a new and as yet to be fully elucidated role in autophagy, oxidative stress and NRF2 regulation. Although PRP4K is generally considered a tumour suppressor, at least one major study suggests under certain conditions PRP4K may be pro-tumourigenic and promote breast cancer and metastasis (Koedoot et al., 2019). Due to the pro and anti-tumourigenic properties of autophagy, it will be important to determine in the future if this new found role of PRP4K in autophagy may in part explain why under some contexts this kinase might also be tumour promoting.

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