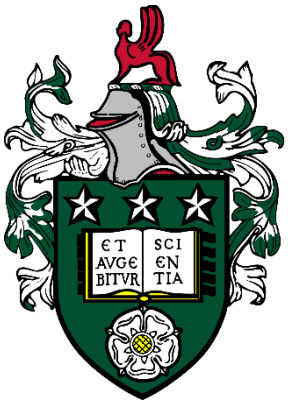

Exploring the intracellular mechanisms associated with JNK signalling in liver cancers

A dissertation submitted by Mr. Nathan Lee in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Molecular Biology from the University of Leeds

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Statement of Originality

Unless otherwise stated in the text, this thesis is the result of my own work

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Abstract

The JNK protein kinases are master regulators of diverse physiological processes of the cell, including apoptosis, survival, differentiation, proliferation, morphogenesis and inflammatory responses. As such, the JNK signalling pathway acts as a central mechanism imperative for maintaining cell homeostasis and relaying key messages to downstream effectors. However, it has become increasingly clear that the persistent activation of JNK proteins can be non-beneficial, aiding the development and progression of cancers. The dichotomy of JNK signalling is stated clearly within the molecular characterisation of liver cancers, where distinct JNK isoforms act to either suppress or activate various cancer-driving mechanisms. With the aid of a wide range of *in vitro* and cell-based experimental techniques, in this study I outline the novel interaction of JNK proteins with the peptidyl-prolyl cis-trans isomerase NIMA-interacting 1, PIN1. Multiple studies have highlighted PIN1 to contribute to the aberrant behaviours of cancer through its unique peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. As the only known isomerase to recognize and act on phosphorylated Serine/Threonine-Proline motifs, PIN1's oncogenic potential is substantiated through the diverse range of key protein group targets that incorporate these binding residues. In this study I detail a mechanistic understanding of JNK's interaction and phosphorylation of PIN1 in cancer, describing the outcome on the stability of PIN1's protein expression. I then describe the effect of this persistent JNK activity on PIN1's biological function within cancer, uncovering novel mechanisms for cancer proliferation and enhanced metabolic functions. Given the widely studied aberrant JNK and PIN1 mechanisms within a variety of tissue-specific cancers to date, these findings advance our understanding of oncogenic JNK signalling pathways and are beneficial for targeting of therapeutical intervention.

Chapter 1 – Introduction

1.1 Overview on cancer

1.1.1.1 Global burden of cancer

The global burden of cancer is reported to have increased to 18.1 million new cases and 9.6 million deaths as of 2018 (*1, 2*). As it stands, the total number of people worldwide who remain alive within 5 years of a cancer diagnosis is approximately 43.8 million. One in five men, and one in six women are reported to develop some variation of cancer within their lifetimes, with one in 8 men and one in 11 women ultimately dying from the disease. The increasing number of new cases recorded is largely attributed to the increasing global population combined with longer life expectancies for the individual. Altercations in social and economic development factors can also contribute to the increased prevalence of specific cancer types in certain regions. This is seen most commonly in rapidly growing economies like Asia, where the development of cancers correlates positively with higher cases of poverty, related infections and lifestyles more typical of an industrialized region (*1, 2*).

Of the reports of cancer for both men and women combined, the global pattern reveals that nearly half of these new cases, as well as over half of all cancer deaths are estimated to occur in Asia. This is somewhat unsurprising, given Asia accounts for a massive 60% of the global population and a significant amount are confined to hyper-city environments with poor social and economic development. Outside of Asia, Europe accounts for 23.4% of global cancer cases and 20.3% of cancer related deaths. Furthermore, the Americas reportedly account for 21.0% of incidences and 14.4% of deaths. In comparison, the proportion of cancer related deaths in Asia and Africa (57.3% and 7.3% respectively) are much higher than the cases of incidences confirmed. This is largely due to these regions having higher proportions of cancer subtypes that are associated with poor

prognosis and elevated mortality rates. This is made worse due to such regions regularly have limited access to correct treatments and timely diagnosis of cancer progression (*1-4*).

Delving into the specific cancer subtypes, it is reported that cancer of the lung, breast and colorectum are the top three most common cancers worldwide by incidence. Collectively these cancers are responsible for one third of all cancer mortalities on a global scale. Approximately 2.1 million people are diagnosed with new cases of either lung or breast cancer as of 2018, with colorectal cancer reporting 1.8 million new cases within the same time period. Lung cancers are also frequently reported incidences and are responsible for large numbers of deaths at approximately 1.8 million attributed to poor clinical prognosis. This is followed by colorectal (881,000 deaths), stomach (783,000 deaths), and liver cancers (782,000 deaths), all of which are also linked to poor clinical outcome(*1-4*).

1.1.1.2 Advances attributed to the genomic era

Our collective efforts to combat cancer have been substantially aided by the sequencing of a broad range of malignant genomes. These efforts, that began with the human genome project in the 1990s (*5, 6*), but now extend to vast genomic research methods have provided extensive insight into the mechanisms that underlie cancer mutations. Much of these have come from advancements in DNA sequencing technologies over the past decade that have resulted in an explosion of cancer related data that can be attributed to this genomic era of research. The combined analyses of molecular data, including DNA copy-number alterations, mRNA and protein expression data have uncovered widespread dysregulations through-out cancers (*7-9*). Such advancements have accelerated the rate at which we are able to comprehend the mechanisms that have emerged to ensure the survival and growth of tumour cells. What we have discovered, is the existence of an elaborate web of networks made up of intricate signalling systems interplaying to create an environment suitable for a tumour cell to thrive and avoid signalling for death. These understandings in this post-genomic era have added significant ammunition towards the way we both recognise and treat human cancer cells in the clinic (*10, 11*).

1.1.1.3 Cancer-driving mechanisms

This is not to say that significant advancements were not made prior to the post-genomic era. In fact, a whole host of critical mechanisms defining both a tumour's genotype and phenotype have been laid out for the world to see, contributing to the arsenal of anti-cancer therapeutics that have been developed. Perhaps the most distinctive of these efforts can be found in the described 'hallmarks of cancer', a pre-genomic pivotal publication summarising the collaborative work to uncover cancer machinery that we can recognise both in clinic and on the bench (*12*). Authors Hanahan and Weinberg, rigorously describe six biological capacities acquired during the multistep development of human tumours. These include sustaining proliferation, resisting cell death, evading growth receptors, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (*12*). Collectively, these hallmarks provide organised principles to tackle the web of networks that comprise these complex neoplastic diseases. Underlying these hallmarks are extensive genomic instabilities to provide tumours with novel mechanisms to grow and survive. Over the past two decades, three new hallmarks of cancer have been recognised largely aided by cross-discipline analysis of cancer genomes (*12-14*). These emerging hallmarks include the reprogramming of energy metabolism, evasion of immune destruction and the overall signalling that contributes to the tumour micro-environment (*12-14*). The individual components that make up each of these hallmarks will be discussed in more depth in later sections.

The accumulation of enormous quantities of molecular data in the post-genomic era has seen the emergence of new branches of science including that of systems biology. These principles are based on the use of integrative genomics that are used to study the molecular events at individual levels, then integrate their effects in a functional or causal framework. This is deemed necessary to combat the complex functional properties of cancer by revealing the existence and principles of molecular interactions that govern fundamental biological mechanisms (*15*). By producing a complete catalogue of the germ line and somatic mutations for each tumour type, the hope is that one can provide an accurate assessment of the functional consequences of each cancer that will inevitably aid in its treatment. However, such analysis is not without its difficulties

and requires a sophisticated level of statistical and mathematical techniques providing opportunities for cross-discipline skills to tackle these dynamic models. Molecular data from proteomic and genomic sources has also aided in our recognition of manipulated post-translational networks of modifications that can occur to proteins influencing the development of cancers. Evidence for the phosphorylation, isomerization, SUMOylation, and ubiquitination used to develop and promote cancers has become extensive, uncovering several layers of signal communication methods that have been hijacked by cancer mechanisms (*16-23*). Many of these post-translational modifications (PTMs) will be discussed in later sections related to their roles in both normal and malignant cell types.

1.2 Liver cancer

1.2.1.1 Subclassifications

To date, primary liver cancer is the second leading cause of death from cancer globally, based on 1-year survival analysis (*24*). Occurring directly in the liver, or spreading by liver metastasis, it claimed the lives of 810,500 patients in 2015 (*25*). Like most cancers, liver cancer is an umbrella term for several distinct classifications of the disease. These include hepatocellular carcinoma (HCC), cholangiocarcinoma and the less common mucinous cystic neoplasm and intraductal papillary biliary neoplasm. HCC accounts for approximately 90% of all primary liver cancers (*24*); initiated in malignant hepatocytes, it is known for its heterogeneity in clinical demographics representing differentiating and aggressive tumour types. cholangiocarcinoma on the other hand, accounts for a smaller 6% of all cases, yet it too is appreciated for its diverse epithelial tumours associated with the epidemiology of the bile duct tissues (*26*). This overall molecular heterogeneity of primary liver cancers causes a massive clinical burden to our societies, with over 854,000 incidences globally in 2015 (*27*). As a result, the five-year survival rate for primary liver cancers is as a low as 31% globally (*28*).

1.2.1.2 Causes

Despite the divergences in histogenic classifications, the root causes of liver cancers are well understood. Viral infections are accountable for a high number

of cases of HCC (*1, 29, 30*) and are also linked with the development of cholangiocarcinoma (*31, 32*). Once infected, 5-10% of individuals become chronic carriers, with around 30% of this population acquiring chronic liver disease and eventually HCC. Specifically, Hepatitis C virus (HCV) or Hepatitis B virus (HBV) are the leading causes of primarily liver cancer progression. In Eastern Asia and sub-Saharan Africa where a higher endemic of HCV and HBV infections is present, it is notable that the majority of HCC cases occur. Individuals infected with HCV or HBV often suffer with viral hepatitis leading to fibrosis, that ultimately result in cirrhosis of the liver. Recent discoveries have also highlighted adeno-associated virus 2 (AAV2) as a novel cause of disease (*33*), particularly for individuals already made susceptible through prior cirrhotic damage. However, other environmental risk factors like excessive alcohol intake and the exposure to aflatoxins can also leave an individual more susceptible to permanent damage of the liver. One of the most notable increases in HCC through environmental risks is obesity. Given curable drugs now exist for HCV infections, the precipitously spreading epidemic's new risk factor is due to risking cases of obesity, type 2 diabetes and non-alcoholic fatty liver disease that are rapidly increasing HCC prevalence in the western world. Given the obesity-associated inflammatory, metabolic and endocrine mediator, these alterations are suspected to contribute to liver tumorigenesis(*1, 34*).

1.2.2 Hepatocellular carcinoma

1.2.2.1 Cell(s) of origin

Hepatocytes constitute 60% to 80% of the total liver mass, with a mixture of other parenchymal (cholangiocytes) as well as non-parenchymal cells (fibroblasts, Kupffer cells, endothelial cells and stellate cells) forming the rest of the liver's anatomy. The localization of a hepatocyte within the overall structure of the liver has a significant effect on its functionality. This is because liver zonation can greatly affect a hepatocytes function without altering its phenotype (*35*). Within the overarching structure of the liver, the cell origin of liver tumours is still of much debate. This is largely due to the heterogeneity of liver cancers resulting in experimental evidence pointing to several emergent sources of the disease (*36*-

38). On one hand, hepatic progenitor cells namely hepatoblasts are declared to be at fault. Subtypes of both HCC and cholangiocarcinoma (iCCAs) can express stem-cell like features (*39-41*) that can be traced back to the common hepatoblasts progenitor suggestive of the origin of disease. This evidence also supports the existence of rare mixed HCC-CCA cells that have a phenotype between hepatocytes and cholangiocytes (*42, 43*). On the other hand, much experimental work has put adult hepatocytes in the spotlight for the admission of liver cancer (*44-46*). Adult hepatocytes have remarkable plasticity owing to the fact that they often dedifferentiate into progenitor cells to restore the hepatocyte population. However, this same property in hepatocytes harbouring genetic alterations, such as the loss of tumour suppressor *TP53*, allows for the expansion of primary liver cancers hosting further oncogenic mutations (*38*). Studies using fate-tracing systems in mouse models have also solidified the role of adult hepatocytes as originators for HCC, with both hepatotoxin-induced and carcinogen-free mouse models implicating hepatocytes, and not hepatoblasts progenitor cells, as the origin of HCC (*44-46*).

1.2.2.2 Development of HCC

Whether originating from progenitor hepatoblasts or adult hepatocytes, the development of HCC is a complex and multi-staged process usually preceded by cirrhosis of the liver. In most cases, HCC from cirrhosis starts with the development of pre-cancerous cirrhotic nodules with low-grade dysplasia termed low-grade dysplastic nodules (LGDNs). From there, the dense nodules subsequentially develop into high-grade dysplastic nodules (HGDNs) harbouring significant genetic alterations in a densely populated oncogenic micro-environment. HGDNs can then transform into the early stages of HCC (classified 0 to A) and progress into more malignant HCC forms (class B and C). The heterogeneity of HCC is such that at any one time, each dysplastic nodule can contain a mean number of 40 functional genomic alterations accumulated in the coding regions (*47, 48*). As a result each tumour in HCC often contains vast mutational diversity with huge alterations that collectively can enhance proliferation, survival, invasion or immune evasion to ensure tumour survival (*49*).

1.2.2.3 Molecular classification of HCC

Clinical and experimental inspection of HCC demonstrate both inter-tumour (tumour to tumour) as well as intra-tumour (within each tumour) heterogeneity. At present, the clinical emphasis is centred on identifying cancers by their molecular subtype, i.e.: inter-tumour identification (*50, 51*). This is undoubtedly an important aspect of diagnosis, and it is through this genomic profiling of patients that we can categorize and personalise treatments. Using the example of chronic myeloid leukaemia (CML), which is defined by the oncogenic fusion of BCR-ABL, resulting in a constitutively active and oncogenic tyrosine kinase. It is only from this inter-tumour identification of BCR-ABL within CML patients that has allowed for targeted treatments resulting in 98% of BCR-ABL patients responding positively. However, despite this, oncogenic mutations arising from genomic, non-genomic and micro-environmental sources within each tumour is cause for strong resistance to treatments in a subset of patients (*50-52*). Such classifications are also relevant for the more accurate molecular identifications of HCC.

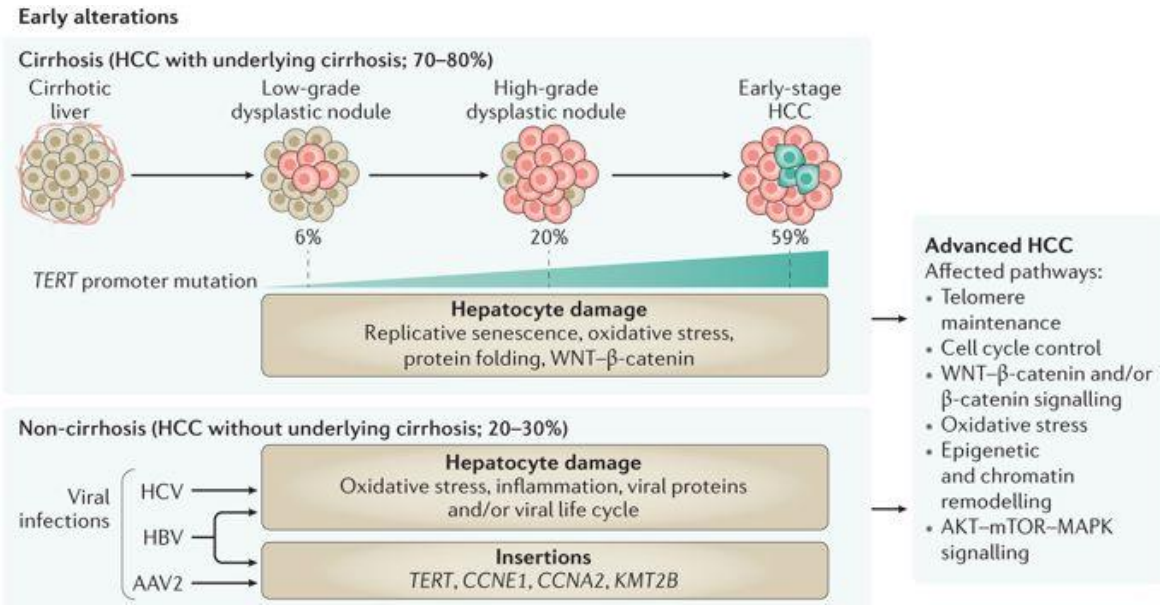


Figure 1.1- Development of hepatocellular carcinoma: Taken from (*24*). Recurrent molecular defects observed in liver carcinogenesis initiated from both cirrhotic and non-cirrhotic sources. Early development of HCC starts with low-grade dysplastic nodules that develop to high-grade dysplastic nodules signifying early stages of disease. Hepatocyte damage from cirrhosis regularly includes replicative senescence, oxidative stress, protein folding and WNT-β-catenin. Telomere reverse transcriptase (TERT) promoter mutations are also commonly seen in early events of HCC initiated from cirrhosis. Non-cirrhotic development of HCC includes viral infections from hepatitis C virus (HCV), hepatitis B virus (HBV), and adeno-associated virus 2 (AAV2) that target oncogenes causing mutagenesis. TERT mutations are also common from viral sources as well as CCNE1, CCNA2, KMT2B insertions. Hepatocyte damage from non-cirrhosis regularly involves oxidative stress, inflammation viral proteins and/ or viral life cycle. Both cirrhotic and non-cirrhotic sources can lead to the development of advanced HCC characterised by altercations in telomere maintenance, cell cycle control, WNT-β-catenin signalling, epigenetic and chromatin remodelling, and AKT-mTOR-MAPK signalling.

Through recent genomic profiling technologies and unsupervised clustering of gene expression patterns, we now identify HCC through 2 major subgroups (*53*). These 2 classes are more broadly referred to as: proliferative and non-proliferative HCC and correlate with pathological and clinical features, as well as etiology and patient outcomes (*24, 53*). The proliferative class of HCC is highly heterogenous, recognizable by its aggressive phenotype in clinic. This subclass often contains enriched proliferative signalling pathways including but not limited to: insulin-like growth factor 1, MTOR; a target of rapamycin, and the aforementioned stem cell features attributed to NOTCH signalling. To add, proliferative HCC types frequently display chromosomal aberrations as well as epigenetic features, including expression patterns of micro-RNAs and DNA methylation patterns.

Non-proliferative HCC subtypes on the other hand, tend to be less aggressive in clinic and consist of highly differentiated tumours. This group retains its hepatocyte-like features and often express an activated canonical Wnt signalling pathway due to mutations in *CTNNB1.* Despite this subclassification of HCC, the extreme heterogeneity of primary liver cancers requires a desperate need to further define individual cases by intra-tumour analysis. One study in primary liver cancer through whole genome sequencing technologies (WGS) observed mutational intratumor heterogeneity in multiple tumour suppressor genes. Another study found diverse genomic alterations in the allelic profiles of frequently mutated genes in HCC biopsies, suggestive that without intra-tumour analysis we may not be capturing the true mutational landscape of each tumour. Only through a more coherent understanding of the link between inter-tumour and intra-tumour heterogeneity in HCC, will we improve patient treatments for the individual and reduce the percentage of recurrence associated with the disease.

1.2.3 Cholangiocarcinoma

1.2.3.1 Cell(s) of origin

Cholangiocarcinomas (CCAs) are subdivided into epithelial tumour classifications based on the anatomical structure of the bile tracts. Primarily CCAs are recognised to be either intrahepatic (iCCA), perihilar (pCCA), or distal (dCCA) based on their anatomical localization from the liver (*54*). Each CC subtype has distinct prognosis, epidemiology and cellular origins. However, despite this, the prognosis of disease remains poor owing to the silent clinical characteristics of CCAs that cause for early detection and diagnosis to prove clinically difficult (*55*). iCCAs can be further divided into large bile duct and peripheral iCCAs; the latter arising from the small intrahepatic bile ducts. Often the characteristics of iCCAs differ from one another based off their subtype origin. The aforementioned large bile duct iCCAs contain peribiliary glands within their walls to which the hepatic stem progenitor cells of this iCCA subtype originate (*56, 57*). They are often shown to have large tubular or papillary growth with taller column-like cell types (*58*). In comparison, peripheral iCCAs lack peribiliary glands, but instead arise from small bile ducts and canals of Hering that result in cuboidal cells with smaller tubular structures and a differentiated subtype characterisation of these tumours (*59*). Overall, iCCAs are also routinely classified based on their tumour phenotypes; with 60-80% know to be mass-forming, 15-35% periductal infiltrating, 8-29% intraductal, and a subset displaying mixed or undefined characteristics (*59-61*).

The best characterization of pCCAs is their proximal separation from iCCAs by the second-order bile ducts, and separation from dCCAs by the insertion of the cystic duct into the extrahepatic biliary tree. pCCAs display exophytic and/ or intraductal growth patterns and are commonly known to infiltrate the periductal regions creating mass forming lesions (*26, 54*). dCCAs on the other hand, are defined to grow along the bile duct between the cystic duct and the ampulla of Vater. They in turn can form two types of precursor lesions including intraductal papillary neoplasms (IPNBs) and biliary intraepithelial neoplasia (BillN) (*26*). Both pCCAs and dCCAs have a similar appearance to iCCAs that derive from the large bile duct, making it difficult to differentiate them in clinic. It is proposed that the

cell of origin(s) for both pCCAs and dCCAs form from mucin-producing cholangiocytes that line the large bile duct and or hepatic progenitor cells (*54*).

1.2.3.2 Molecular cholangiocarcinogenesis

It is widely accepted that cholangiocarcinoma is a progressive and multistep process disease, originating from hepatic stem like progenitors or transformed biliary epithelial cells (*62*). However, the molecular cholangiocarcinogenesis is deeply complex, attributed to a host of risk factors, persistent inflammation of the bile ducts, multicellular origin and tumour heterogeneity, as well as genetic and epigenetic alterations (*26, 63-66*). There is strong evidence to support that the instability of CCAs are initiated by a dysregulation of reparative proliferation in cholangiocytes. This begins a culture of DNA damage, overactivation of more proliferative signalling pathways (including mitogen activated protein kinases (MAPKs), WNT and mTOR signalling), resurgence of mitogenic factors (IL-6 and TGF-b), as well as the overexpression of EGFR and HGF, and silencing of TP53 and p16 genes (*64, 67, 68*). Unsurprisingly, this dramatic change in the cellular environment of cholangiocytes leads to the development of genomic mutations in CCAs as well as the previously mentioned IPNBs and BillNs (*69, 70*). Of note, KRAS mutations are observed in 40% of IPNB lesions and 33% BillNs lesions (*64, 71*) and is now seen as an early molecular event for the development of iCCAs. Many more genetic mutations in CCAs have also been uncovered and range from genes involved in DNA repair, cytokine signalling, cell cycle control, to genomic stability and chromatin remodelling (*62, 64, 72-75*). Interesting we find that there is also a correlation observed between the frequency of genomic alterations and the anatomic localization of the tumours, suggestive again that the localization of progenitor cells within the anatomy can greatly affect their functionality (*76*).

Despite the molecular mess that constitutes primary liver cancers, these molecular and genomic alterations provide some promise in the better identification of the disease through the development of biomarker discovery. For instance, many biliary tract cancer related biomarkers have been suggested, including MAPK, mTOR and PIK/PTEN/AKT factors as well as biomarkers like matric metalloproteinases (MMPs) that can be picked up from the serum and bile. It will be through a combination of both genetic and molecular markers that we

can begin to improve on the identification of CCAs at precancerous and early stages (*62, 77*).

1.3 The hallmarks of cancer

Amongst the complexity that is tumour evolution, it is often hard to conceive of where to start understanding these diseases. It has become clear over the past two decades that cancers are more than the proliferative masses of cells we first naively identified them to be. Instead they constitute a complex network of tumour heterogeneity, often involving multiple cell types that are interlinked in a supportive oncogenic environment, free from normal physiological restraints. The admired perseverance of the scientific community has however, added more and more pieces an ever-complex puzzle, creating insight into the common mechanisms binding cancers together as a whole to dominate and survive. These attributes are widely accepted as established hallmarks of cancer (*12*), adding a degree of certainty to our pursuit when navigating a labyrinth of signalling networks (Figure 1.2).

1.3.1 Sustained proliferative signalling

1.3.1.1 Growth factor signalling

Arguably the most fundamental aspect of cancer cells is their ability to sustain chronic proliferative signalling in the face of adversity. Under normal physiological conditions, several mechanisms are locked in place to ensure the correct regulation of growth and division signals are maintained. This is empirical for the homeostasis of the cell, as both the normal architecture of the cell, and its functions are solely reliant on the timely division of a cell state. For the most part, growth signals are initiated by the binding of growth-factors to cell-surface receptors on either the same cell (autocrine signalling), or proximity cells in the environment (paracrine signalling). Regularly these receptors contain tyrosine kinase domains, that once activated begin a cascade of downstream signalling pathway responses critical for critical responses for cell cycle, proliferation, survival and metabolism of the cell.

Within an oncogenic environment, cancer cells have evolved unique machinery to turn off regulated signalling pathways, allowing for uncontrollable proliferative signalling to take hold. Amongst the mitogen activated pathways in cancer cells, this is understood to regularly occur through the upregulation of excessive growth factor ligands, that simultaneously elicits further upregulation of ligand specific receptors by the cell, resulting in the formation of sustained autocrine proliferative signalling loops (*78*). There is also much evidence of cancer cells ability to also stimulate normal cells in close proximity to the tumour environment, that in return act as paracrine resources of growth factor ligand that can be delivered back to the cancer cells (*79, 80*). Often when there is a shortage of growth factor ligands available in the cell's vicinity, cancer cells have been proven to further upregulate the number of ligand receptors on the surface of the cell (*81, 82*). This causes a hypersensitive signalling response, ensuring maximum signalling capabilities even when resources are scarce.

1.3.1.2 Negative feedback loops

Of equal stature within a normal cell state, is the existence of machinery to turn off proliferative signalling to maintain routine homeostasis. Negative feedback loops are common to signalling pathways, acting as a paralleled and controlled response to immediately turn off activated kinase domains. Defects within these loops act as opportunities for cancer cells to thrive by maintaining unchecked proliferative signalling sources. These loopholes have been found to be common amongst various cancer cell types. A striking example is the loss-of-function mutations found in PTEN phosphatases; a counterpart to PI3Ks that typically would act by degrading its activator PIP_3 products. However, with loss-of-function mutations, PI3K signalling is left unchecked and known to promote tumorigenesis through Atk/PKB transduction (*83-86*).

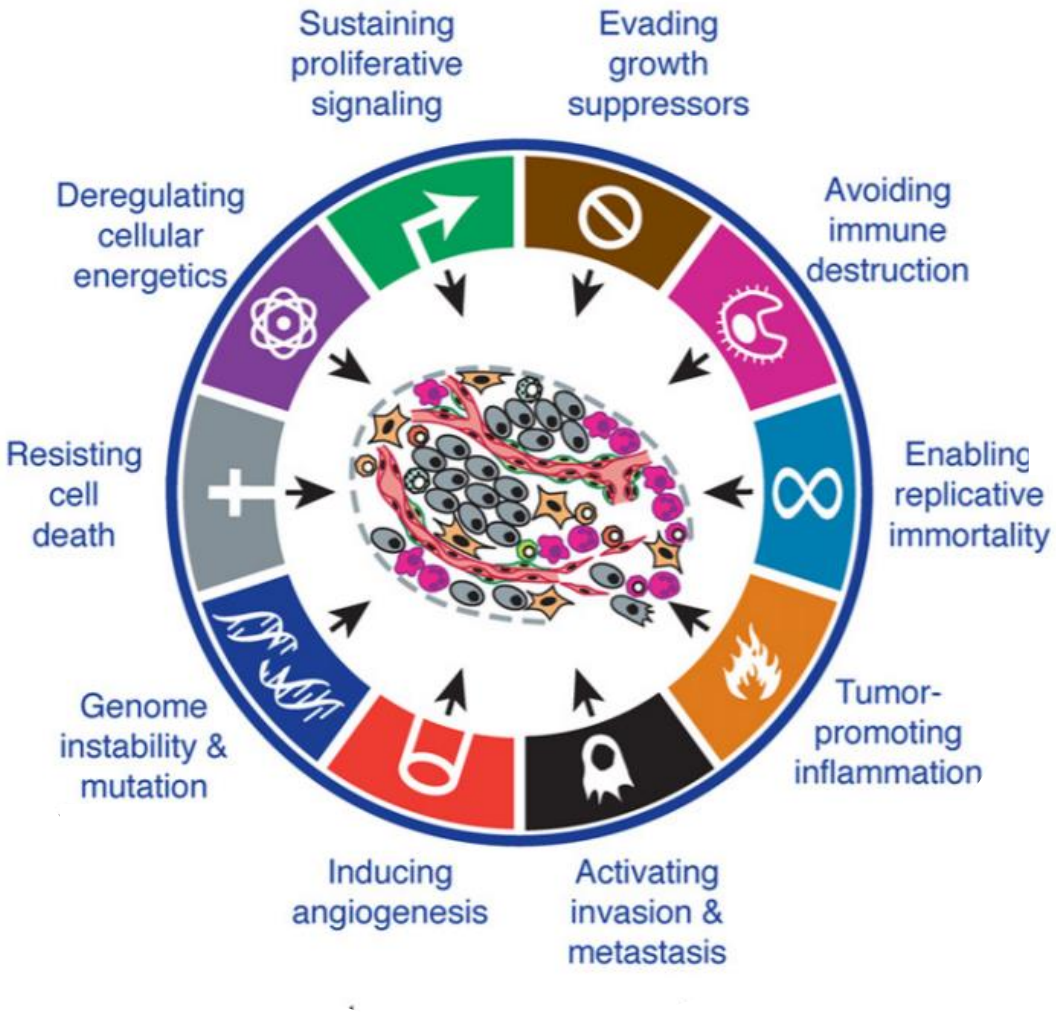


Figure 1.2 - The Hallmarks of Cancer: Taken from (*12, 13*) illustration of the ten recognised hallmarks used to aid tumour growth and survival. These hallmarks are sustaining proliferative signalling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumour-promoting inflammation, activating invasion & metastasis, inducing angiogenesis, genome instability & mutation, resisting cell death, and deregulating cellular energetics.

1.3.1.3 Somatic mutations

Indeed, there are many mechanisms that have evolved allowing cancer cells to harbour oncogenic signalling capacity. More recently through the aid of high-speed DNA throughput technologies we can no appreciate the link between somatic mutations in particular genes to sustained proliferative signalling. Of note, 40% of human melanomas are found to contain activating mutations that alter the structure of the B-Raf protein. This has been linked to a sustained MAPK signalling due to a constitutively active Raf outcome (*87*). Other examples include collective findings of mutations in the catalytic domain of PI3 kinases that consequentially hyperactivate PI3K signalling networks including Atk/PKB signal transduction (*88*).

1.3.1.4 Senescent cells

Despite these mechanisms that allow cancer cells to enhance their survival signalling, the existence of senescent cells challenges our previously conceived 'more is better' thinking. Experimental findings using oncogenes RAS, MYC and RAF have been shown at higher levels to in fact counteract the proliferative signalling response (*89-91*). Instead, cells can become unresponsive, characterised by an enlarged cytoplasm, lack of proliferative cell markers and expression of senescent induced B-galactosidase enzyme. These paradoxical responses reflect the existence of an evolutionary defence mechanism within cells, acting in response to a threshold to protect against sustained signalling that may elicit systemic damage. Unsurprisingly cancer cells have evolved mechanisms to overcome such mechanisms, with evidence of tumours regulating the intensity of these signals to prevent defensive actions, or obliterating these defensive actions all together (*90, 92, 93*).

1.3.2 Evading growth suppressors

1.3.2.1 Tumour suppressors

It has been estimated that 70% of genetic aberrations in solid tumours, represent attempts to evade tumour suppressor genes that fundamentally prohibit cell growth. Dozens of these tumour suppressors have been identified through gain- or loss-of function studies, acting to inhibit the proliferation of damaged/ mutated

cells by arresting cell cycle progression and/ or inducing apoptosis (*92*). The assault on tumour suppressors by cancer cells comes on multiple fronts. Environmental factors such as UV irradiation induce DNA damage, genetic mechanisms cause chromosomal deletions and mutations that ultimately inactivate effectors upstream or downstream of tumour suppressor networks, and finally epigenetic factors can cause DNA methylation and histone modifications. All of these efforts are aimed at overcoming critical gatekeepers of cell cycle progression, that would in turn immensely serve cancer cells through an unopposed and persistent cell proliferation. A common example in solid tumours are mutations that inhibit TP53 functions, that ultimately can lead to the loss of p53 and controlled cell cycle checkpoints. As a result, the cell's ability to arrest and repair DNA errors is no longer possible, leading to an accumulation of genetic mutations and overall instability. Loss of PTEN; the phosphatase responsible for dephosphorylating PIP3 is another common mutation. With an unchecked phosphorylated PIP3, theres is an unopposed activity of PI3K and AKT signalling that can drive tumour growth (*94, 95*).

1.3.2.2 Cell contact inhibition

Other known tumour suppressors act through cell-to-cell contact inhibition mechanisms, that ensure dense populations of normal cells *in vivo* do not excessively proliferate. Of these, the NF2 gene has long been understood to act as a tumour suppressor, with loss-of function studies resulting in an outcome of human neurofibromatosis. The cytoplasmic product of NF2, namely Merlin, acts by coupling E-cadherin adhesion molecules on the cell surface to tyrosine kinase receptors on the transmembrane of the cell. This act not only strengths the adhesivity between cells, but sub sequentially inhibits growth factor signalling by limiting the availability of receptors on the surface to cause mitogenic signals (*96, 97*). LKB1 epithelial polarity proteins work via a similar mechanism to suppressor inappropriate proliferation. When LKB1 is upregulated in organised epithelial structures, Myc-induced transformation is suppressed, and tissue integrity is maintained. However, it's loss results in a destabilised epithelial integrity that in turn allows the cell to become susceptible to Myc-induced transformation (*98-100*).

1.3.3 Resistance to cell death

1.3.3.1 Apoptotic signalling mechanisms

Yet to be discussed, are the programmed cell death mechanisms of apoptosis that are fundamental to the cell's machinery. It has long been established that apoptosis acts as a natural barrier to cancer development, responding to imbalances in the cell's networks including elevated oncogenic signalling and DNA damage associated with hyperproliferation (*90, 91, 101*). The apoptotic response is made possible through a combination of upstream regulators and downstream effector components that interplay to create a proteolytic outcome. Amongst the upstream regulators are those that process extracellular death inducing signals, as well as regulators recognising signals from intracellular origins (*91, 101*). The activation of regulators from either source, result in a cascade or proteolysis, starting with the activation of latent caspases 8 and 9, and ending with the disassembly and consumption of the cell by phagocytic cells or other neighbouring cells. The overall success of apoptosis is then decided by downstream Bcl-2 regulatory proteins that can be both prop-apoptotic, and anti-apoptotic. Bcl-X, Mcl-1 and Bcl-2 proteins are inhibitors of apoptosis, and act by binding and suppressing pro-apoptotic triggering proteins namely; Bax and Bak (*101*). When relieved of this suppression, Bax and Bak disrupt the integrity of the outer mitochondrial membrane resulting in the release of pro-apoptotic proteins including cytochrome c. Cytochrome c once activated results in the cascade of proteolytic enzyme reactions that ultimately result in the disassembly of the cell. Both pro-apoptotic and anti-apoptotic Bcl-2 family members contain BH3 interaction motifs that are coupled to a variety of sensors that signal under cellular abnormality. It is the activation, combination, and interaction of these Bcl-2 family members that ultimately decides the outcome of the apoptotic response (*101*).

1.3.3.2 Abnormalities in apoptotic mechanisms

Unsurprisingly extensive research has highlighted tumour mechanisms that overcome the apoptotic process to enable continued survival. The previously mentioned loss of TP53 tumour suppressor is within this category. Under normal circumstances, TP53 induces apoptosis by upregulating pro-apoptotic BH3

proteins in response to abnormally high levels of DNA breaks and chromosomal abnormalities that induce cell death. In the absence of this TP53 tumour suppressor, this critical damage sensor is eliminated allowing tumour cells to circumvent this mechanism (*102*). Other strategies to circumvent apoptosis include regular upregulation and expression of anti-apoptotic regulators, or simultaneously downregulating pro-apoptotic factors that have the same effect (*91, 102*).

1.3.3.3 Autophagy can produce dormant cancer cells

Another physiological cellular process like apoptosis that can be carried out in response to cellular stress is that of autophagy. Often autophagy is in response to nutrient deficiency of the cell (*103, 104*), and evokes the ability of cells to break down their counterpart sub-units including ribosomes and mitochondria to produce the necessary biosynthesis for the cell's survival. More commonly, low molecular weight metabolites are generated, that support survival of the cell in a nutrient low environment. Similar to apoptosis, this mechanism is controlled by BH3 containing regulators and effectors that decide the autophagy outcome. And in a similar manner, this stress induced response has been shown to be both beneficial for circumventing tumour development, as well as support autophagy's ability to produce dormant cancer cells that will eventually regrow (*103-105*).

1.3.3.4 Necrosis can benefit tumorigenesis

In more recent years the explosive mechanical destruction of cells by necrosis has also been suggested to play a role in tumorigenesis. Unlike apoptosis, that involves the controlled and gradual absorption of the dying cell by neighbouring and phagocytic cells in the vicinity, necrosis was formally seen as an uncontrolled and violent explosion of a cell resulting in pro-inflammatory proteins entering the surrounding tissues. More recently however, there is much evidence to show that necrosis is in fact not as uncontrolled as formally believed, but instead is under genetic control (*106, 107*). This has come from the understanding that cells undergoing necrosis, release proinflammatory proteins that localize innate inflammatory cell to clear up the necrotic debris. However, under tumorigenic conditions where these inflammatory cells can foster angiogenesis, cancer cell proliferation and invasiveness, the localized environment instead receives further

signals for tumorigenesis. Combined with the release of cytokines like IL-1 from necrotic cells that stimulate neighbouring cells to proliferate, necrosis seems to be yet another mechanism for prolonging cancer cells survival (*106-108*).

1.3.4 Enabling replicative immortality

Another well-known hallmark of cancer is the ability of cells to undergo replicative immortality to generate tumours. Under normal physiological conditions, cells are only able to pass through a limited number of cell division and growth cycles before entering stages of senescence or crisis, the latter of which normally results in cell death. On rare occasions, certain cells undergoing crisis will emerge from this stage with the new-found ability to undergo replicative immortality. These select few are thereby known to be immortalized, a transition that is a common trait in cancer cells evolution. Evidence shows that the lifespan of all cells is dependent on telomere lengths; multiple tandem DNA repeats that protect the ends of chromosomes. Accordingly, the length of these telomeres dictates the lifespan of the cell and how many times it may go through division and growth cycles, with each cycle that passes, eroding down the lengths of the chromosome ends. Telomerase, the DNA polymerase that adds telomere repeats to the ends of chromosomes is routinely absence in normal non-immortalized cells (*109*). On the contrary, evidence shows that a vast number of cancer cells express significant quantities of Telomerase, up to approximately 90% increase from normal cells. Given senescence and crisis act as anti-cancer mechanisms by creating barriers to proliferation, increasing the levels of Telomerase results in many cancer cells avoid undergoing these transformations by maintaining sufficient telomere lengths (*109*).

1.3.5 Inducing angiogenesis

In a similar manner to normal tissues, tumour cells require nutrients and oxygen as well as the ability to get rid of metabolic wastes. Indeed, the excessive proliferation and growth of tumours results in a desperate need to fulfil these primary functions which is carried out by the tumour-associated neovasculature, generated through inducing angiogenesis. Within adults, angiogenesis is

transiently turned on under circumstances of wound healing and the female reproductive cycle, used only sparingly under controlled conditions (*12, 110*). However, in contrast, angiogenesis in tumours is always on, allowing for sustained neoplastic growth through the development of continuously formed new blood vessels. It is understood that this angiogenic switch is controlled by both stimulatory and inhibitory regulators, both of which bind to corresponding cell surface receptors on vascular endothelial cells. Stimulatory regulators like the vascular endothelial growth factor A (VEGF-A) result in VEGF signalling that under normal physiological conditions are released sparingly to orchestrate new blood vessel growth. However, it is clear that oncogenic signalling, including Ras and Myc signalling in cancer cells, can also upregulate angiogenic factors that drive enhanced angiogenesis, resulting in the development of vast patterns of neovascularization (*111, 112*).

1.4 Cancer metabolism

The above-mentioned classical hallmarks attributed to cancer cells, do not come without exertion or physiological strain on tumour cells undergoing these dramatic changes. For one, the uncontrolled proliferation associated with tumorigenesis, demands alterations in the cell's metabolic potential to meet an ever-accumulating biomass. This is because typically, any one cancer cell can proliferate from one aberrant cell to more than 10^9 cells; the average number of cells in a tumour of approximately 1cm in diameter (*113*). Therefore, in order to satisfy these enhanced metabolic requirements, cancer cells have developed fascinating and resourceful mechanisms to scavenge nutrients and oxygen from both the tumour itself, and from the tumour microenvironment that surrounds it. These abilities come primarily from cancer-associated metabolic reprogramming, that has been shown to have profound effects to gene expression, cellular differentiation and the overall tumour microenvironment. Indeed many, if not all of the classical hallmarks of cancer mentioned above, either benefit or are a result of the reprogramming of cancer metabolism (*114*). It is fair to say that the metabolic landscape of cancer cells largely dictates which signalling pathways are abhorrently turned on, ultimately allowing cancer cells to proliferation in nutrient deprived conditions and evade death. The importance of better

understanding metabolic changes associated with tumorigenesis has become clear over the past decade, and since then, collectively researchers have made strides to mapping genetic changes to aid therapeutical gain.

1.4.1 Glucose and glutamine

1.4.1.1 Dysregulated Uptake of Glucose and Glutamine

The biosynthetic demands associated with uncontrolled proliferation of cancer cells consequentially results in the cell's need for an increased uptake of nutrients from extracellular sources. The nutrients in question, used to support biosynthesis and survival in mammalian cells are that of glucose and glutamine. These nutrients cannot typically be sourced from within a cancer cell yet are fundamental to the cell's ability to survival and prosper. When taken in by the cell for external sources, glucose and glutamine are catabolised to produce pools of diverse carbon intermediates that feed into numerous pathways providing building blocks for the assembly of macromolecules (Figure 1.3). To add, the simultaneous controlled oxidation of these carbon intermediates allows for cells to capture their reducing power in the forms of NADH and $FADH_2$, that consequentially fuel ATP generation by supplying electrons to the electron transport chain. Glutamine can also contribute a second growth supporting substrate other than carbon, namely, nitrogen. Glutamine is able to provide reduced nitrogen for the de novo biosynthesis of glucose-amine-6-phosphate, purines and pyrimidines; nucleotides that make up the backbone structure of DNA, as well as non-essential amino acids. It has also been linked to the uptake of essential amino acids that are unable to be produced de novo by the cell. This comes from the evidence that glutamine efflux is coupled to the upregulation of LAT1; a neutral amino acid antiporter that can import a broad range of amino acids from extracellular sources including; leucine, isoleucine, valine, methionine, tyrosine, tryptophan and phenylalanine.

1.4.2 The Warburg Effect

Notably the alterations in glucose and glutamine metabolism by cancer cells are not recent findings, in fact the original experiments date back to the late 1920's. The marked increase in glucose consumption by tumours compared to nonproliferating normal tissues was first observed by Otto Warburg nearly a decade ago. This phenomenon coined 'The Warburg Effect' resulted in the assumption that respiration through oxidative phosphorylation (OXPHOS) is impaired in cancer cells. In subsequent years, this statement was regularly debated, and it unfolded that cancer cells do indeed have high rates of glucose metabolism and glycolysis, but OXPHOS is still possible in these cancer cells. This observation has been confirmed in various tumour contexts and now with the aid of PET-based imaging of the uptake of a radioactive fluorine labelled glucose analogs can be used to help diagnosis tumour staging (*115*). The enhanced uptake of glutamine on the other hand, was first noted by Harry Eagle in the 1950's, who noted that the optimal growth of cultured HeLa cells required a 10-100-fold molar excess of glutamine in the cultured medium than other amino acids (*116*). This was then confirmed *in vivo*, showing that the proliferation hepatomas and carcinosarcomas resulted in a depletion of glutamine from the tumour environment (*116-118*). In a similar manner to the G-FDG imaging used for tracing glucose analogs, F-labelled glutamine tracers are also proving to show promise in clinic to further diagnose tumour sub-types (*119, 120*).

1.4.3 Alternative mechanisms of nutrient acquisition

1.4.3.1 Micropinocytosis

Contrary to the dramatic intake of glucose and glutamine by cancer cells, often *in vivo* these nutrients are found to be scare (*121*). This is attributed to the combined increased consumption by tumours themselves, and an often-inadequate tumour vascular supply failing to bring in added nutrients from outside of the tumour micro-environment. As a result, many cancer cells acquire further mutations that allow the cell to drain precious nutrients from alternative and somewhat obscure sources (Figure 1.4). One of these sources results from the activation and

expression of mutant Ras and/ or c-Src alleles, that provide cells with the mechanisms to recover free amino acids from their own lysosomal degradation of extracellular proteins. Unlike the careful intake of low weight nutrients through cell surface transporters under normal cellular conditions, this method involves micropinocytosis; a process by which the extracellular fluid of the cell, led by oncogenic cytoskeleton remodelling, forms large vesicles that trap extracellular content dragging it internally to be fused with the lysosome and subjected to proteasomal degradation (*122*). This oncogenic mechanism has evolved to supply nutrient deficient tumours with a ready supply of free amino acids.

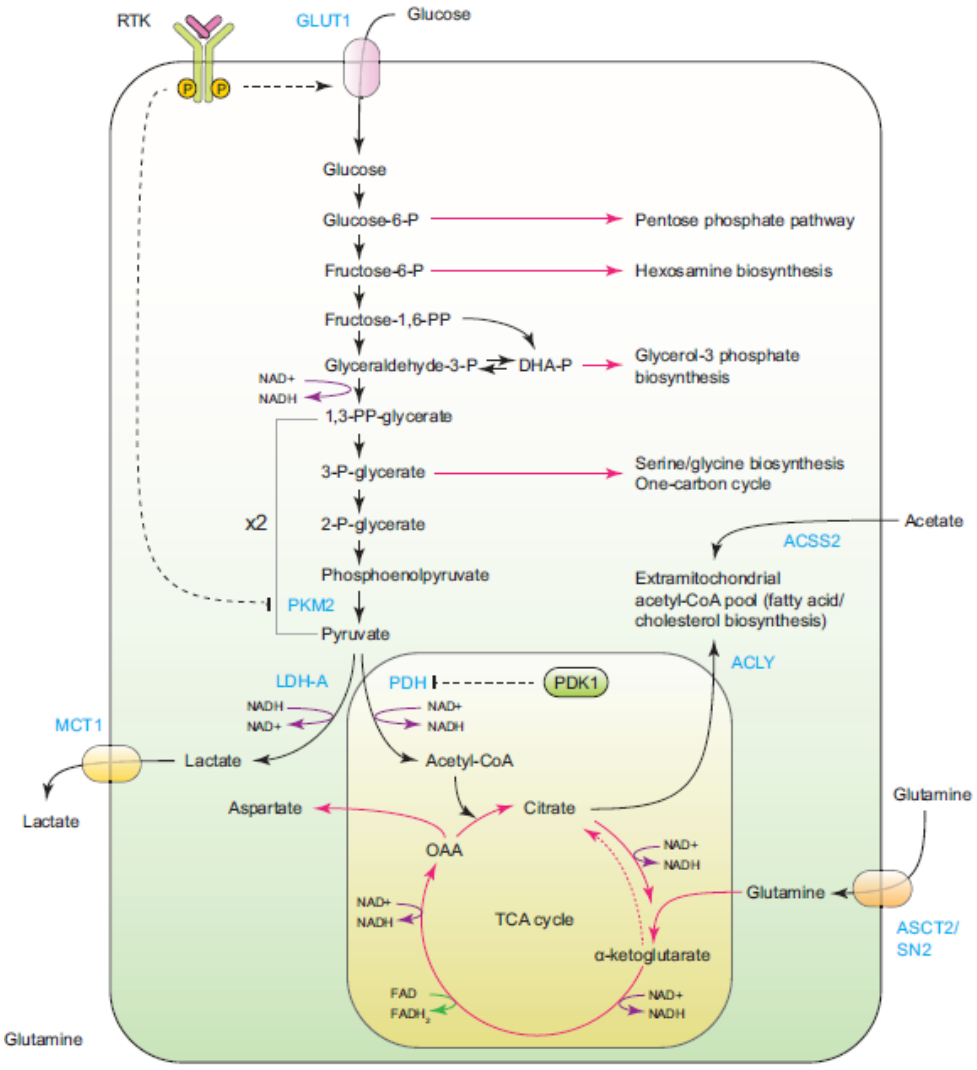


Figure 1.3 - Cellular breakdown of Glucose and Glutamine: Taken from (*13*). Annotation depicts the breakdown of Glucose, Acetate, and Glutamine by the cell to be used in glycolytic and oxidative phosphorylation processes including glycolysis, pentose phosphate pathway, hexosamine biosynthesis, Glycerol-3 phosphate biosynthesis and the serine/ glycine biosynthesis one-carbon cycle. The diverse biosynthetic outputs from carbon metabolism include that by RTK receptor tyrosine kinase, HK2, Hexokinase 2, GLUT1, glucose transporter 1, PKM2, pyruvate kinase M2, ACSS2, acetyl-CoA synthetase 2, LDH-a, lactate dehydrogenase A, PDH, pyruvate dehydrogenase, PDK1, pyruvate dehydrogenase kinase.

1.4.3.2 Entosis

Another alternative mechanism of nutrient acquisition by tumour cells is through a process known as Entosis. Entosis allows free amino acids to be recovered from the consumption and engulfment of entire living cells or apoptotic cell corpses in the proximity to the tumour cell. In a similar mechanism to Micropinocytosis, once digested the material is fused with the lysosome to undergo proteasomal degradation and provide free amino acids in a nutrient deficient environment. Interestingly studies have showed that tumour cells with KRAS mutations are more likely to carry out Entosis then those without the mutation (*123, 124*). This has led researchers to believe that such inter-competitiveness between neighbouring tumours may be another evolved mechanism to develop more aggressive tumour phenotypes.

1.4.3.3 Macroautophagy

In the complete absence of extracellular nutrients, cancer cells have still evolved mechanisms to survive beyond their means. Macroautophagy is a process that cancer cells undergo in the absence of external resources and involves self-catabolism of the tumour's own intracellular macromolecules (*125*). During this process both intracellular macromolecules and whole organelles can be degraded into precious free amino acid building blocks by being broken down in the lysosome of the cell. Given no external sources of nutrients are being used here, Macroautophagy does not result in proliferation of cancer cells, however, it allows for extremely deprived tumours to maintain their viability in culture for weeks at a time (*126*).

1.4.4 The favouring of glycolysis

1.4.4.1 Glycolytic and OXPHOS in normal cells

Under normal physiological conditions, mammalian cells rely primarily on both glycolysis and OXPHOS for their metabolic demands. In fact, these two energies

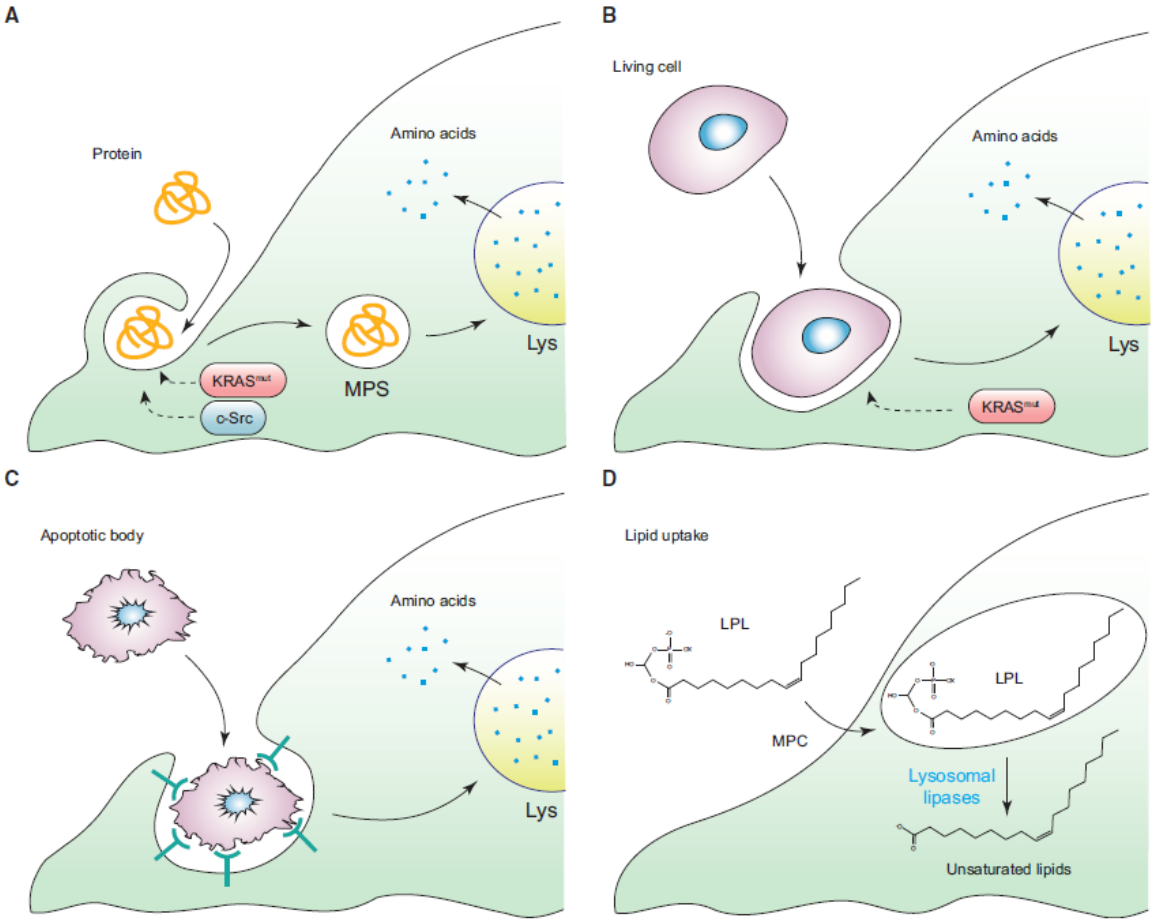


Figure 1.4 - Opportunistic methods of nutrient intake by cancer cells: Taken from (*13*). (A-C) When nutrients are scarce cancer cells can recover amino acids from (A) extracellular proteins by micropinocytosis, (B) entosis of entire living cells or, (C) phagocytosis of dead cells or apoptotic debris from dead neighbouring cells. (D) When oxygen is deficient, de novo desaturation of stearate into oleate is prevented meaning monounsaturated fatty acids such as oleate can be recovered from extracellular lysophospholipids (LPL). MPS, macropinosome; Lys, lysosome; MPC, micropinocytosis

production pathways are tightly coupled, frequently interconverting carbon intermediates to produce the most efficient energy outcome for the cell.

Glycolysis is the more ancient energy production pathway, involving a sequence of ten enzymatic reactions that converts glucose into pyruvate whilst simultaneously producing ATP. Working in parallel to glycolysis is the Pentose Phosphate Pathway (PPP), producing NADPH alongside glycolysis through the breakdown of carbon intermediates. Occurring in the cytoplasm, glycolysis can produce ATP under anaerobic conditions, albeit only two molecules. Once the carbon intermediates are broken down into pyruvate by glycolysis, under aerobic conditions, pyruvate can enter the mitochondria to be oxidised to acetyl CoA that combines with oxaloacetate to start the tricarboxylic acid (TCA) cycle. It is here that 36 ATPs can be produced, and as a result, under normal conditions OXPHOS is responsible for supplying 70% of energy for the cell (*127*). However, under anaerobic conditions, OXPHOS is no longer functional, and pyruvate is instead converted into lactate and excreted into the extracellular space through monocarboxylate transporters (MCTs).

1.4.4.2 Redefining the Warburg Effect

The Warburg Effect was originally founded on the assumption that cancer cells had irreversibly damaged their mitochondrial function, and were therefore unable to undergo OXPHOS, selecting for glycolysis instead. However, as previously mentioned, later findings challenged this misconception, indicating that many tumours can still undergo OXPHOS and that contradictory to this, removing mitochondrial DNA actually reduces the tumorigenic potential of cancer cells (*128, 129*). Therefore, it is apparent that cancer cells can undergo both glycolysis and OXPHOS, and rather this information points to the existence of a regulated metabolic state in cancer cells that are opting to choose glycolytic avenues over OXPHOS. At first this information was confusing, most researchers including Otto Warburg assumed that the focal point for glucose metabolism by the cell was to maximise the generation of ATP. However, a deeper look at the requirements of proliferative cells shows that surprisingly the act of proliferation requires a relatively low threshold of ATP consumption and much higher requirements for precursor molecules and reducing equivalents in the form of NADPH. We find that the process of glycolysis alone is sufficient to carry out these needs, and in

contrast, the mitochondrial activity of the TCA cycle only acts to negative regulate of glucose metabolism. It is for these reasons that often we find cancer cells low on OXPHOS activity and strictly carrying out glycolysis, converting pyruvate into lactate to be excreted into the extracellular space. Doing so prevents pyruvate from entering the mitochondria which in turn prevents the accumulation cytosolic NADH and mitochondrial ATP production, leaving glycolytic metabolism free from feedback repression (*13*).

1.4.5 Selection of key glycolytic enzyme upregulation

1.4.5.1 Hexokinases

The fundamental shift in our understanding of tumour glucose metabolism has spurred increased efforts in exploring tumorigenic mechanisms manipulating glycolysis and OXPHOS pathways. One overarching principle that has emerged in tumour metabolism, is through the regulation of key glycolytic enzymes involves in the catabolic process of glucose. Glycolysis is a ten-enzymatic step process, involving both reversible and irreversible reactions; the latter being referred to as committed steps. These committed steps provide opportunities for cancer cells to maximise the metabolic output from glycolysis without being compromised through any reversible reaction processes (*130*). The first, and arguably most important of these committed steps is catalysed by hexokinases (HK). There are four well described isoforms of HKs in mammalian cells (HK1-4), each of which phosphorylate glucose into glucose-6-phosphate (G6P) (*131-133*). This step has unequivocal importance, as it simultaneously traps glucose in the cell, preventing it from being exported by glucose transporters, and produces G6P that acts as the convergence point for not only glycolysis, but also the PPP, the hexosamine pathway and glycogen synthesis.

Unsurprisingly cancer cells have evolved oncogenic mechanisms to facilitate this first committed step in glucose metabolism. This occurs by increasing glucose uptake and inducing high levels of the hexokinase 2 isoform (HK2), in addition to HK1 which is already expressed in normal cells (*134, 135*). Both HK1-2 have been shown to bind to the outer mitochondrial membrane and voltage dependent anion channel (VDAC) whilst simultaneously using ATP derived from

mitochondria to carry out the phosphorylation of glucose. This allows for the coupling of OXPHOS with glycolysis which we have already shown to be useful for tumorigenesis (*127*). Furthermore, despite HK1-3 being allosterically regulated by their own catalytic products of G6P, within an oncogenic environment it is unlikely that high levels of G6P will ever accumulate, as G6P will quickly be catabolised to carry out metabolic processes important for the cell. Collectively this information is suggestive as to why HK1-2 are regularly upregulated by cancer mechanics (*136, 137*).

1.4.5.2 PFK1 and PKM2

The second committed step in glycolysis involves the catabolism of F6P into fructose-1-6-bisphosphate (F1,6BP). This rate limiting step is carried out by the allosterically regulated Phosphofrunctokinase-1 (PFK11), to which its inhibition or activation will alter the flux of glucose through glycolysis. Both PFK1 and HK1-2 act to increase the flux of glucose in glycolysis whilst simultaneously consuming ATP in the process. The third and final committed step however, requires pyruvate kinases that generates ATP alongside the production of pyruvate; the final step in glycolysis. This rate limiting step is attenuated in cancer cells, achieved in most part through the use of a low-affinity pyruvate kinase M2 (PKM2) to catalyse the reaction (*138*). This is critical in the regulation of glycolysis, as unlike PKM1 that constitutes an active tetramer, the isoform of PKM2 activity is regulated by various metabolites (*139*). As a result, PKM2 activity is frequently bent to the will of cancer cells to adapt to a variety of conditions that require different levels of metabolites present. This is strongly supported by the findings that PKM2 expression varies significantly from cancer to cancer. Often, it's expression is completely absent such as in the case of breast cancer and colon cancer samples, but other times it is pivotal to the tumour development, including the development of leukaemia (*138*). The low-activity of PKM2 also benefits cancer cells by creating a backlog of carbon intermediates. This causes a diversion of metabolites into the previously mentioned pathways, including PPP and serine biosynthesis, both of which use carbon intermediates to fuel cancer cell growth and proliferation.

1.4.5.3 LDH

Despite the low-activity PKM2 creating a backlog of carbon intermediates, its glycolytic product, pyruvate, is rapidly converted into lactate and exported from the intracellular space. This conversion is carried out by lactate dehydrogenases (LDH), of which isoforms LDHA and LDHB are highly expressed. The former has a high affinity for pyruvate, responsible for its conversion into lactate, whereas the latter has a higher affinity for lactate and converts the back reaction. Cancer cells predominately favour the expression of LDHA (*140, 141*), and shuttle lactate out of the cell via monocarboxylate transporters (MCTs) to ensure the backwards reaction by LDHB is significantly reduced. There are several reasons for this behaviour, firstly, too much lactate within the cell results in a highly acidic intracellular environment that is toxic for the cell's homeostasis. Secondly, lactate acts to inhibit PFK1 activity, sub sequentially inhibiting the second committed step in glycolysis (*140, 141*).

1.4.6 Mechanisms of reprogramming to enhance cancer progression

1.4.6.1 Oncogenic signalling to promote glycolysis

Despite the absence of any concurrent mechanisms for the reprogramming of glucose metabolism amongst all cancers, there are many evolved mechanisms that hijack the committed steps of glycolysis, as well as the rate of glucose uptake itself, to fulfil a tumour's anabolic needs. Both oncogenes and tumour suppressors are at fault here and have multiple roles in manipulating glucose metabolism of the cell, some of which overlap with roles mediated by hypoxia and the transcription factor hypoxia inducible factor 1 (HIF1) to accelerate cellular metabolism. For one, the transcription factor HIF1 increases HK2 and GLUT1 expression to respectively enhance glucose phosphorylation and glucose uptake by the cancer cell. GLUT1, as well as other glucose transporters, are also victims of further upregulation and translocation to the plasma membrane by KRAS, BRAF and activated AKT oncogenic signalling (*36, 142-144*). Furthermore, HK2 expression is markedly increased by oncogenic MYC signalling, and once phosphorylated by AKT, increases its association for the mitochondria which in turn enhances its intracellular activity further (*145, 146*). HIF1 and AKT are also

responsible for the allosteric activating of PFK1. HIF1 does this by increasing the expression of PFKFB3, which generates F2,6BP, the allosteric activator of PFK1. AKT on the other hand, activates PFK1 by phosphorylation and activation of PFKFB2, yet another example of oncogenic stimulus working in harmony for the overall benefit of the tumour's condition. HIF1 is also responsible for increasing the expression of LDHA and MCT4, allowing for further conversion of pyruvate into lactate to be excreted from the intracellular space.

1.4.6.2 Oncogenic metabolic signalling in hepatocellular carcinoma (HCC)

Perhaps the most well-established mechanisms for glucose metabolism come from the disease state of hepatocellular carcinoma (HCC). The reason being that under normal tissue conditions, the core function of hepatocytes is to regulate glucose levels around the liver. In a resting physiological state, hepatocytes express GLUT2 transporters in the plasma membrane to cater to the fact that glucose is both consumed and exported by liver cells and thus in constant reversible flux through GLUT2 channels. To add, glucose is phosphorylated into G6P by HK4, known for its low affinity for glucose as well as its allosteric regulation by glucose itself. Intriguingly, hepatocytes are also able to undergo gluconeogenesis, a process that runs opposite to glycolysis and catalysed by enzymes that override the committed reactions in glycolysis. However once hepatocytes have transformed and developed into HCC, a dramatic change can be seen in the way the liver prioritises its metabolic functions. For instance, we find that within the disease state, GLUT1 is now predominately favoured over GLUT2, enhancing the influx of glucose over its exportation to other cells. HK4 expression is found to be significantly suppressed, favouring instead the tumour benefiting, high affinity HK2 of which we have already discussed (*147*). Thirdly, there is a characteristic isoform switch of aldolase; responsible for the reversible conversion of aldol and fructose 1,6, bisphosphate into triose phosphates and dihydroxyacetone phosphate (DHAP). In normal hepatocytes Aldolase B is the major isoform, providing enzymatic activity for both glycolysis and gluconeogenesis reactions. But in HCC, Aldolase B is suppressed, and instead replaced by higher levels of Aldolase A (*148, 149*). The latter is more efficient at the conversion to triose phosphates and DHAP and is better suited to the one-way glycolytic flux that is favoured of HCC.

Overall gluconeogenic enzymes are largely suppressed in HCC, favouring instead enzymes that increase glycolytic flux. To this end, normal hepatocytes express pyruvate kinase isoform, PKL that helps interchange PEP and pyruvate. However, in HCC the low-activity PKM2 is preferentially expressed that is beneficial to growth and proliferation of cancer cells. We find as well, that LDHA expression is enhanced, increasing the flux of pyruvate to lactate that is otherwise reduced under normal conditions (*150-152*).

1.4.7 JNK signalling contributes to the Warburg Effect

In the urgency to better understand mechanisms associated with the altered glucose metabolism of tumour cells, there has been subsequent effort to uncover key signalling pathways responsible for feeding these metabolic shifts. As we have discussed, the Warburg Effect; the genetic reprogramming of tumour cells to selectively favour glycolysis, is associated with the activation and inhibition of many known oncogenes and tumour suppressors. To this end, one oncogenic signalling family that has emerged and is associated with cellular metabolic functions, is that of the c-Jun N terminal Kinase (JNK) pathway. The JNK pathway, which will be discussed in detail in later sections, accounts for three proteins (JNK1, JNK2, JNK3) that are encoded by three separate genes, respectively, *JNK1 (mapk8). JNK2 (mapk9), JNK3 (mapk10)*. Under normal physiological conditions, the ubiquitously expressed JNK1 and JNK2 proteins are found in most tissues, whereas JNK3 proteins are restricted to certain domains (*153*). All JNK proteins are involved in diverse cellular signalling processes, ranging from proliferation, differentiation, survival as well as apoptosis. Furthermore, it has become apparent that the manipulation of the JNK signalling pathway in the context of cancer is well documented, with oncogenic roles for specific JNK isoforms emerging after decades of scientific debate (*154*).

1.4.7.1 Linking cancer metabolism with apoptosis: JNK signalling

Previous publications from our laboratory have highlighted the oncogenic roles of JNK signalling and its contributions to the Warburg Effect (*155*). One novel mechanism outlined in these studies, portrays the maintenance of a low-enzymatic activity of PKM2, a well-known contributor resulting in an enhanced

glycolytic function of tumour cells. PKM2 is a frequent target favoured by cancer cells, due to the protein's ability to be tightly regulated by external metabolites, such as the binding of its substrates; PEP and ADP, as well as post-translationally through mechanisms such as phosphorylation (*156, 157*). In these studies, low-enzymatic PKM2 activity was established through altercations in the pro-apoptotic JNK1 signalling, caused by subsequent upregulation and overexpression of a novel anti-apoptotic protein poly (ADP-ribose) polymerase (PARP)14 in HCC. PARP14, is a pro-survival protein that is shown to promote the Warburg effect in HCC and sustain the survival of hepatoma cells both *in vitro* and *in vivo*. Its elevated expression is regularly found in human HCC cell lines as well as primary tumours, and is associated with a worsened clinical prognosis and the onset of HCC (*154*).

PARP14 expression has been shown to suppress the apoptotic functions of JNK1 signalling in cancer tissues (*154*). In these studies, this same mechanism results in the inhibition and modulation of PKM2 activity, allowing for the enhanced aerobic glycolysis in cancer cells. This was uncovered through loss of function experiments, highlighting an enhanced PKM2 activity in PARP14-depleted cancer cells. This enhanced activity was reversed by knocking down of JNK1, which simultaneously resulted in the reversal of the impaired aerobic glycolytic phenotype and apoptosis. Collectively these findings position JNK1 as a direct mediator of the tumorigenic metabolic function (*154, 155, 158*).

1.5 The c-Jun N-terminal Kinase (JNK) Pathway

1.5.1 Normal physiological roles of JNK signalling

The c-Jun N-terminal Kinase (JNK) signalling pathway is a well-studied cellular protein kinase family. At their core, each individual protein kinase making up this pathway, acts by catalysing the transfer of terminal phosphate groups of ATPs, to specific amino acid sequences on their substrate target proteins. This simple act is responsible for the modification of substrate proteins to undergo further protein interactions, subcellular localizations, activations, as well as protein degradations. It is truly a universal mechanism used to carry out critical and

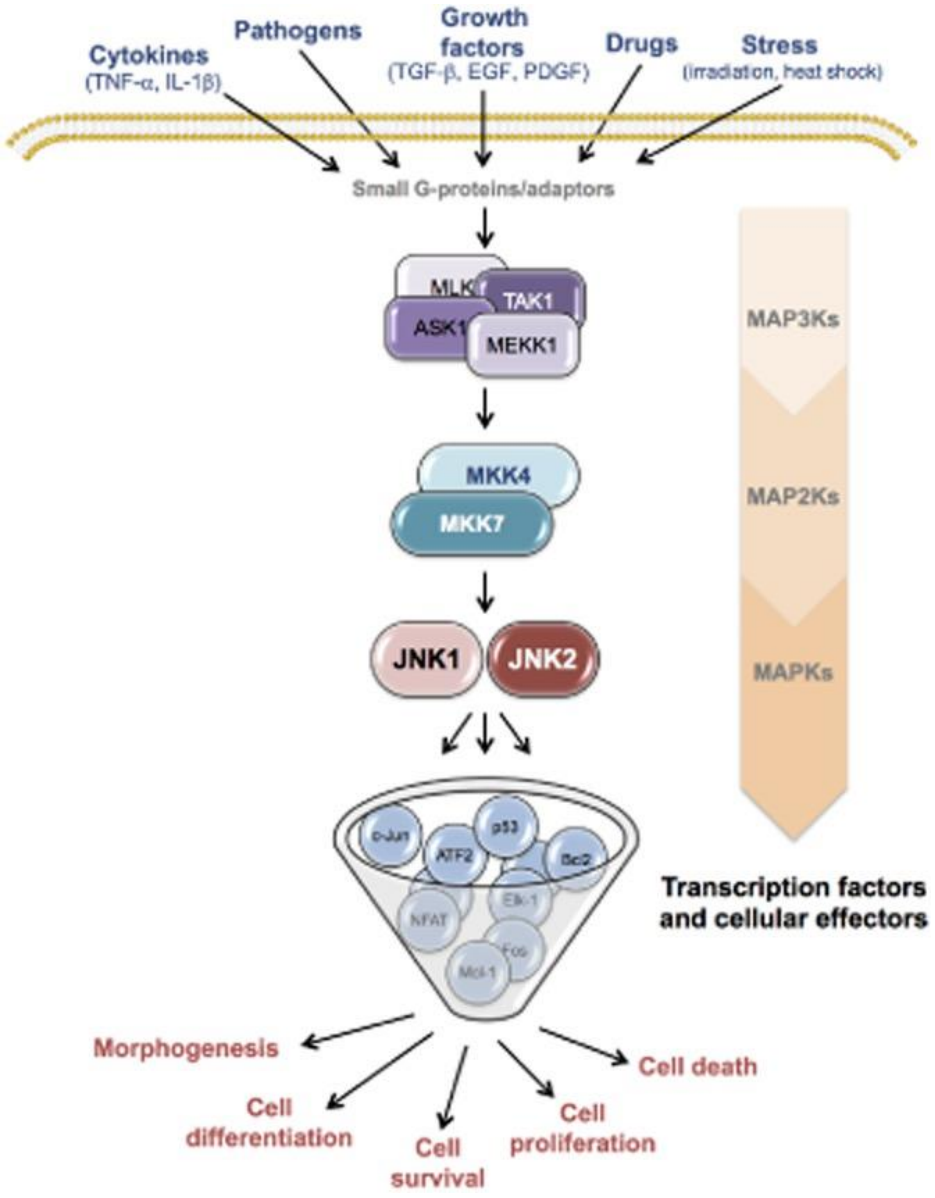


Figure 1.5 - Schematic representation of the JNK pathway: Taken from (*159*). Annotation represents MAPK signalling pathway for JNK proteins under normal physiological conditions. Upon activation by extracellular signalling including cytokines, pathogens, growth factors, drugs and stress, a phosphorylation cascade is activated whereby the MAP3Ks activate the MAP2Ks, in turn activating the JNK isoforms (JNK1 and JNK2). The JNK isoforms are responsible for diverse cellular outcomes including morphogenesis, cell differentiation, cell survival, cell proliferation and cell death.

diverse cellular functions. JNK proteins fall under the umbrella of Mitogen-activated protein kinase (MAPKs); a serine-threonine family of kinases that play important regulatory roles within the cell.

1.5.1.1 A phosphorylation cascade of activation

As shown in Figure 1.5, JNK signalling can be initiated by a range of diverse extracellular stimuli including: cytokines (TNF-a, IL-1b), pathogens, growth factors (TGF-b, EGF, PDGF), stress (irradiation, and heat shock) as well as being a frequent target for therapeutical intermission. Once activated, a MAP3K activates a MAP2K, that in turn activates a MAPK, in a process referred to as a phosphorylation cascade of activation. More specifically, MKK4 and MKK7 are activated via dual phosphorylation by MAP3Ks, such as the members of MEKK family, the mixed-lineage kinase family, the apoptosis signal-regulating kinase family, TAK1 and TPL2. MKK4 and MKK7 are the two MAPK2 protein kinases that in turn, directly phosphorylate the aforementioned JNK isoforms; JNK1-3. They do so by phosphorylating JNKs on threonine 183 (Thr^{183}) and tyrosine 185 (Tyr^{185}) residues, that are present in a conserved tripeptide motif (Thr-Pro-Tyr) within their activation loop (*160*). The kinase activity of JNKs can also be substantially regulated by their interactions with scaffolding proteins, as well as dual-specificity phosphatases and NF-kB transcription factors (*161-163*).Once activated by the upstream MAP2Ks, JNKs phosphorylate and in turn activate a range of downstream cellular and nuclear proteins including the transcription factor activator protein-1 (AP-1) – formed by dimerization of the Jun protein with the Fos proteins, activating transcription factor 2 (ATF-2), c-Myc, p53, Elk1, NFAT and cell death regulators of the Bcl-2 family in the mitochondria. In line with this, JNK signalling is attributed to diverse cellular outcomes such as proliferation, transformation, differentiation, cell survival, as well as cell death.

1.5.2 JNK signalling in cancer

1.5.2.1 Oncogenic roles of JNK

The diverse outcome of JNK signalling mechanisms makes its roles particularly interesting in the context of cancer (Figure 1.6). Earlier findings surrounding JNK signalling in cancers found strong evidence of several oncogenes such as RAS, c-fos, Met and Bcr-ABL behaving by JNK-dependent mechanisms. Collectively, this data implicated JNK's involvement in cellular transformation; a process pivotal in the development of tumours. This was later supported by the discovery of the oncogenic role of c-Jun; the downstream substrate of JNK that is required for cellular transformation when induced by the oncogene Ras (*164-167*). Mouse models of intestinal cancer have further supported the oncogenic function of c-Jun, adding the need for the substrate's phosphorylation by JNK to carry out these tumorigenic needs. This study found that muted phosphorylated forms of c-Jun (namely JunAA/JunAA/Apc^{min}) could not result in cellular transformation, and these mice sub sequentially developed smaller and fewer polyps (*168*). Whats more, the presence of hyperactivated JNK is strongly associated with multiple cancer lines and tissue samples, with further evidence of JNK depletion in certain tissues acting to suppress tumorigenesis (*169, 170*). Taken as a whole, there is significant evidence depicting JNK signalling in the development of tumours.

1.5.2.2 Tumour suppressive roles of JNK

However, despite this there is a substantial body of evidence favouring JNK signalling in tumour suppressive roles. For instance, one study describes loss of JNK to play a key role in the early stage development of breast cancer by promoting mammary gland neoplasia (*171*). Whats more, JNK-1 null mice in a DMBA/TPA-induced skin cancer model were shown to develop greater numbers of papilloma's compared with wild-type mice (*157*), indicating JNK1 to negatively regulate tumorigenesis. JNK-1 null mice are also highly susceptible to tumour development after inoculations with both melanoma and lymphoma cell lines (*172*), and can spontaneously develop intestinal cancers (*173*). Overall, loss of JNK studies support a tumour suppressive role of the pathway, and have been shown to result in genomic instability that is associated with cancer progression.

The genetic changes that develop in the absence of JNK, contribute substantially to tumour development and reflect the function of JNK to promote genome maintenance in response to stress. Examples of this include JNK-mediated phosphorylation of SIRT6 that simulates double stranded DNA break repair, as well as JNK mediated phosphorylation of DGCR8 that induces transcription coupled nucleotide excision repair. This loss of JNK signalling in this case, would ultimately result in defects to DNA repair, resulting in increased genomic instability (*171*).

1.5.2.3 Emergence of distinct roles for the JNK isoforms.

It is therefore apparent that there are both tumour supportive, and tumour suppressive roles for JNK signalling in cancer. These discrepancies caused much debate among the scientific community for decades, deliberating how it is that one signalling pathway can result in completely opposing outcomes. The wealth of research that followed, described specific roles for the JNK isoforms; predominately JNK1 and JNK2, that are expressed ubiquitously in numerous tissues. It became clear that in order to study JNK signalling in cancer, we must approach the topic in an isoform specific, as well as tissue specific functionality. What is true of the mechanisms of one JNK isoform does not necessarily equate to the same response in a different tissue context. This approach is especially important in the development of cancer therapeutics aimed at JNK inhibition, and there is continued efforts in designing JNK inhibitors that can selectively target specific isoforms (*174*).

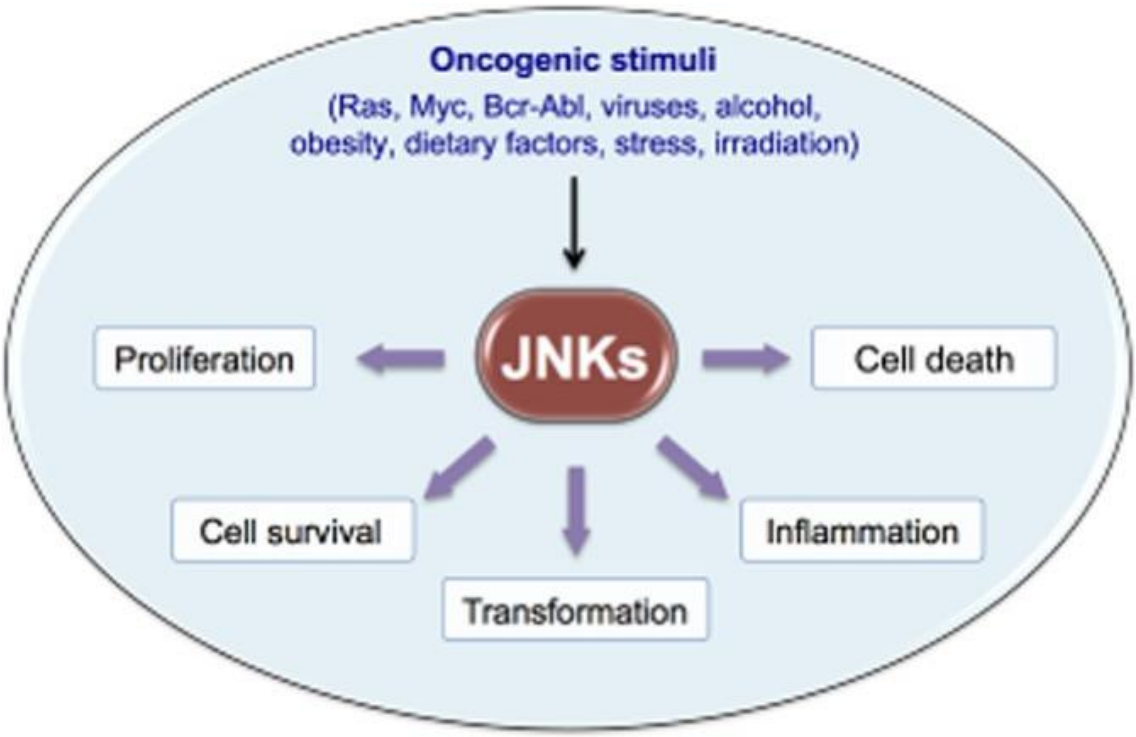


Figure 1.6 - JNK signalling in Cancer: Taken from (*159*). Annotation depicts the key cellular outcomes involved in JNK signalling in cancer. JNK activation in response to extracellular and intracellular signalling mechanisms including Ras, Myc, Bcr-Abl, viruses, alcohol, obesity, dietary factors, stress, and irradiation promote oncogenic outcomes affecting proliferation, cell survival, transformation, inflammation and cell death in cancer.

A fantastic example portraying the diversity of JNK isoform functionality, can be found in Multiple Myeloma (MM). MM is characterised by tumours containing terminally differentiated B-cells, that accumulate clonal and long-lived plasma cells in the bone marrow and extramedullary sites. A screening of MM cells lines and samples compared with normal plasma cells highlighted a high expression of JNK2 isoforms in MM conditions, suggestive of a pathogenic role for JNK2 in this disease. Subsequent silencing of JNK2 expression resulted in an enhancement of MM apoptotic cell death, whereas ablation of JNK1 did not alter the proliferative rate of MM cells. To add, JNK2 silencing was also associated with an increased JNK1 activity, and the suppression of JNK1 activity in already

JNK-2 depleted MM cells, restored the regular growth rate of these malignant cell types (*175-177*). Collectively, these findings state in MM that JNK2 signalling induces survival signals whereas JNK1 signals induce apoptosis. In the oncogenic environment of MM, JNK2 signalling has evolved mechanisms to suppress pro-apoptotic JNK1 signals benefiting tumour growth and survival. In this context, we can appreciate the extreme diversity in JNK isoform functions, with one isoform signalling for cell death, whilst the other is simultaneously directing cell survival. Interestingly, these opposing roles are not seen in normal MEF cellular states with the ablation of the JNK2 gene resulting in an increased expression of c-Jun and cellular proliferation (*178, 179*). This suggests that the competitive nature of cancer cells can attenuate these oncogenic functions of both isoforms to benefit cancer progression.

1.5.3 JNK signalling in liver cancers

1.5.3.1 JNK mechanisms of cell death

Adhering to the tissue specific functionality of the JNK isoforms, JNK signalling mechanisms in liver cancers contrast to those already discussed in MM. Here, we see conclusive examples of JNK mechanisms at play, in both the initiation and progression of disease. As we have already discussed, a preliminary stage of HCC development often begins with cirrhosis of the liver. To add, HCC also regularly develops from severe liver fibrosis, both of which involve numerous rounds of hepatocyte death, inflammatory responses and compensatory liver

regeneration. All of these factors have been shown to involve the over-activation of JNK mechanisms in injured hepatocytes (*162, 180, 181*), pertinent to JNK's involvement in establishing liver cancer. Several mechanisms have been uncovered linking JNK signalling to liver cancer development. The first, implicates JNK signalling in the development of HCC by inducing hepatocyte cell death which in turn promotes compensatory proliferation (*181*). For example, diethyl nitrosamine (DEN)-induced HCC mice studies shows the deletion of IkB kinase B (IKKB) in hepatocytes to induce activation of JNKs. The subsequent JNK activation results in signalling for cell death and the triggering of compensatory proliferation of surviving hepatocytes that greatly enhances the development of HCC (*162, 182*). This cellular response is reversed when JNK1 genes are silenced that sub sequentially restore IKKb expression in hepatocytes. This JNK mechanism involving first cell death is further supported by evidence that JNK1 deficient mice are much less susceptible to DEN-induced HCC development. This is accompanied by a decreased expression of cell cycle regulators including cyclin D and VEGF that suggest JNK1 has tumour promoting roles in HCC (*183*). Whats more, JNK1 has been implicated in the upregulation of p53's modulator of apoptosis, namely PUMA; a pro-apoptotic BH3-only protein during fatty acid induced hepatocyte apoptosis (*184, 185*).

1.5.3.2 Other JNK roles in liver cancer

There is also strong evidence to support JNK's roles in the development of HCC by regulating hepatocyte proliferation directly. Again, using the DEN-induced HCC mouse model, JNK1 was shown to decrease the expression of the proliferation inhibitor p21 through transcriptional expression of c-Myc (*186*). Silencing of JNK1 in DEN-induced HCC models results in a reduced proliferation of tumours, with the genetic inactivation of p21 in the same mice is shown to restore hepatocyte proliferation. To add to this, in more than 50% of HCC samples for patients compared with normal tissues, JNK1 is found to be hyperactivated (*169, 187*). This hyperactivation correlates with an increased tumour size as well as an elevated expression of cell cycle and proliferative markers (*187*). Despite this, there is also several sources highlighting JNK's tumour suppressive roles in HCC. For example, one genetically engineered mouse model contains JNK1 deletions in hepatocytes and JNK2 deletions in the

entire organism. What followed was observations of an enhanced development of HCC in these knock out mouse models compared to wild-type controls. The increased HCC development in the knockout mice was shown to stem from an enhanced death rate of hepatocytes (with JNK1 deletions), with subsequent compensatory proliferation, as well as an increased expression of c-Myc and c-Jun. This study demonstrates JNK's ability to be tumour suppressive in hepatocytes, whereas it is known that in non-parenchymal cells of the liver, JNK activity is reported to promote HCC development (*169*). Collectively, we can appreciate that the tissue-specific and isoform specific functions of JNK remain true in liver cancer, and there needs to be continued efforts to detail JNK mechanisms in specific tissue contexts.

1.5.4 JNK signalling in cholangiocarcinoma

1.5.4.1 Triggering of cholangiocellular tumorigenesis

With regards to JNK signalling in cholangiocarcinoma, molecular mechanisms are still being uncovered. Hyperactivated JNK signalling have been observed in a numerous cancer types including intrahepatic cholangiocarcinoma (ICCA) (*188*). Close examination of cholangiocellular tumours revealed JNK signalling to induce a biliary proliferation program in hepatocytes or liver bipotential cells (*189*), sub sequentially implicating JNK signalling in the pathogenesis of human cholangiocellular tumours. This was shown to be possible, due to mitochondrial defects in hepatocytes that trigger ROS accumulation and the recruitment of Tnf-producing Kupffer cells. Collectively, this response creates a favourable niche that allows biliary proliferation to occur via JNK signalling mechanisms (*190*).

Other studies have highlighted further roles for JNK in the promotion of ICCA progression through its interactions with Breast cancer type 1 susceptibility protein-associated protein-1 (BAP1). BAP1 has been reported to act as a tumour suppressor in a wide spectrum of tumour types. Clinically, low BAP1 expression is positively correlated with aggressive tumour characteristics including large tumour size, presence of lymphatic metastasis and advanced tumour node

metastasis staging. In ICCA, we find that BAP1 functions as a tumour suppressor by inhibiting JNK signalling mechanisms, detailing a role for abhorrent activated JNK in the development and progression of ICCA (*159, 191*).

1.5.4.2 Abnormal accumulation of bile acids

Finally, some studies have implicated bile acids as an initiation to malignant JNK signalling in CCA. Bile acids are routinely produced by hepatocytes from cholesterol and transported to the small intestines via the bile duct. This process is well known to assist nutrient absorption, allowing the gastrointestinal system to digest and metabolize critical nutrients. However, under malignant conditions, such as during the development of CCA, risk factors include the abnormal accumulation of bile acids due to obstructions and cholestasis. This has opened up a new line of enquiry on the mechanisms behind bile acid functions, highlighting their role in the active regulation of the biliary endothelium. In fact, we find that the bile-acid component deoxycholic acid is responsible for activating EGFR and inducing COX-2 expression. This in turn activates p42/44, p38 MAPK, as well as JNK signalling pathways (*192*). This has been shown to promote cellular proliferation and convey resistance to Fas-induced apoptosis in CCA(*193*). Further understandings of the mechanisms by which bile acids contribute to CCA are needed.

1.6 The Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PIN1)

1.6.1 The PIN1 isomerase

1.6.1.1 pSer/Thr-Pro residues

Central to the JNK signalling pathway is the common mechanism of proline (Pro) directed Ser/Thr phosphorylation (pSer/Thr-Pro). All MAPKs including JNKs, ERKs and p38s, are at the core, operated by a sequence of Pro-directed kinases. In fact, global phosphorylation studies has identified that over one-quarter of all

phosphorylation sites involve pSer/Thr- Pro residues (*194-196*) and account for families of protein kinases such as cyclin-dependent kinases (CDKs), MAPKs, as well as numerous oncogenes and tumour suppressor mechanisms in cancer (*19, 20, 196*). Of particular interest in regard to pSer/Thr-Pro directed proteins, is the ability of proline to adopt both *cis* and *trans* confirmations. The interconversion between spatial proline confirmations can occur spontaneously yet slowly in nature, and sub sequentially confer a complete change in the overall proteins' configuration (*21, 197-199*). This has huge ramifications to signalling process mechanisms, as the *cis-trans* configuration of proteins alters their ability to interact with both upstream and downstream substrate proteins. As a result, the spatial confirmation of a proline directed peptide bond can dictate which pools of proteins it is able to interact with, thereby altering the overall outcome of the cell's signalling response (*20, 197-201*).

1.6.1.2 The unique isomerase PIN1

Although these *cis-trans* conformational switches can occur spontaneously, yet slow in nature, the process of proline interconversion is dramatically enhanced when catalysed by peptidyl-prolyl isomerases (PPIases). The PPIase superfamily includes cyclophilins, FK506-binding proteins (FKBPs), and parvulins (*200*). Thanks to the wide range of protein kinase families containing proline directed peptide bonds, it is unsurprising that PPIase activity is commonly involved in the structural transformation, folding, subcellular location, stability, activation and interaction of multiple proteins (*20, 21, 202*). Despite this, the unique pSer/Thr-Pro structure of a subset of protein kinases further renders the peptide bonds inaccessible to the majority of PPIases, as most PPIases do not have the thermodynamic energy to overcome this proline-directed peptide sequence (*201*). The exception is that of the peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PIN1) from the paruvulin subfamily of PPIases, that consequentially has critical roles in the functions of pSer/Thr-Pro kinases, both under normal physiological conditions as well as in cancer (*20, 21, 200-202*) (Figure 1.7).

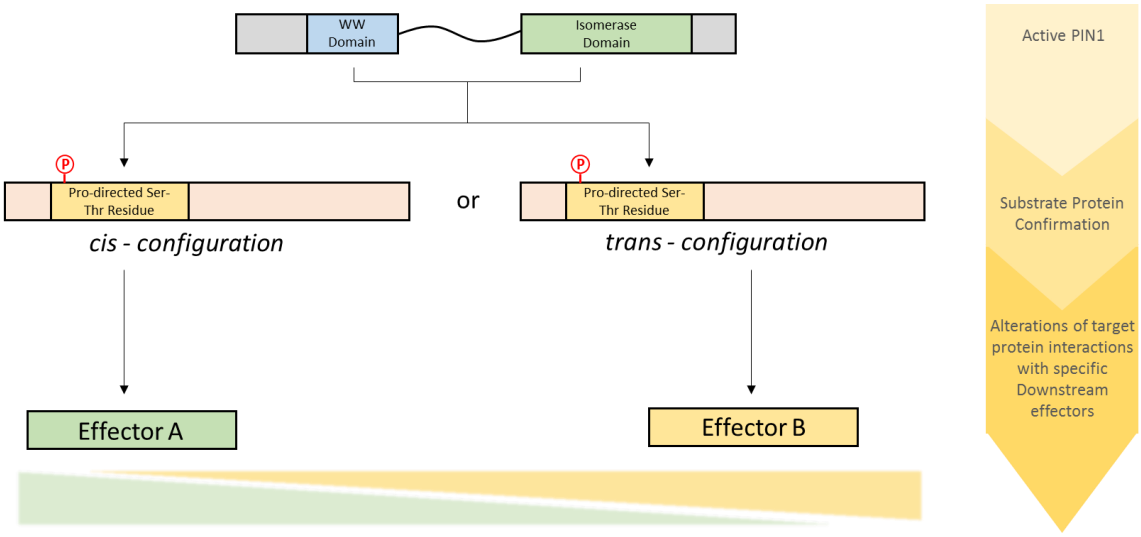


Figure 1.7 - PIN1 isomerase activity dictates downstream effector interactions: Annotation shown above depicts the mechanism of action by which Pin1 targets pro-directed Ser/Thr residue protein effectors. Targeting of Pin1 results in cis-trans confirmation of protein effector configuration, in turn affecting their ability to interact with further downstream molecules. Pin1's MoA and isomerase activity makes it a unique bottleneck target for therapeutical benefits.

PIN1 is a small (18 kDa) enzyme made up of a WW domain and a catalytic domain with a flexible linker. Several lines of research show the WW domain is responsible for targeting PIN1 close to its substrates, that sub sequentially allows for the PPIase domain to come into close proximity and carry out *cis-trans* isomerisation of the substrate protein. Both these domains have been shown to be critical for the function of PIN1, both *in vitro* and *in vivo (21, 198, 200, 201, 203-207)* and have provided much insight into the mechanisms of PIN1 when regulating its substrates. The unique isomerization of PIN1 is of particular interest regarding the phosphorylation-based signalling mechanisms. This is because PIN1's PPIase activity is accountable for the confirmation of both Pro-directed kinases and phosphatases; both of which play antagonistic roles in the regulation of phosphorylation (*23, 138, 161, 208, 209*). Given both parties are functional only in specific confirmations, PIN1 has the unique ability of deciding the phosphorylation outcome for many protein substrates altering the overall cellular fate (*21, 210*). One example is the critical functions of PIN1 for the phosphorylation of JNK1 proteins; the previously mentioned protein kinase responsible for diverse cellular outcomes. Phosphorylation at Thr^{183} in the Thr-Pro motif of the JNK1 phosphorylation loop is a rate limiting step in the activation of JNK1. One study found PIN1 to directly target and promote JNK1 activity by catalysing the prolyl isomerization of the phospho-Thr-Pro motif in JNK1 from a *trans* to *cis* confirmation. This was supported by results indicating PIN1 knockdown cells to be defective in JNK activation and resistant to oxidative stress (*211*).

1.6.2 Dysregulation of PIN1 cell cycle functions in cancer

The unique isomerase activity of PIN1 is characteristically displayed in its roles regarding the regulating of cell cycle functions (Figure 1.8). Despite its first being identified as a mitotic regulator, PIN1 has since been shown to regulate the function of a range of phosphorylated proteins in other phases of the cell's cycle (*199*). Primarily, the ability of a cell to undergo proliferation is tightly regulated within the resting G0 phase. Genes involved in cell cycle regulation including cyclin A, cyclin E, cyclin-dependent kinase (CDK) 2 and Cdc25 are transcriptionally activated by the E2F family of proteins early in the G1 stage in

order to progress through the G1 checkpoint and into S stage of division. Routinely, the retinoblastoma protein (Rb) binds to E2F proteins inhibiting the release of E2F proteins into the nucleus and the sub sequent transcription of cell cycle regulators (*92, 199, 212*). A major regulatory event for the cell to proceed in the G1 checkpoint stage is the activation of CDK by cyclin D1. This results in the activation of CDK4/6 and following phosphorylation and dissociation of Rb from E2F proteins promoting cell cycle progression (*213*).

1.6.2.1 G0-G1 stage

Given cyclin D1 is a major regulator of the G1 checkpoint, it is no surprise that its dissociation is regularly attributed to uncontrolled cell proliferation and tumour formation. Interestingly however, the dysregulation of cyclin D1 correlates with the overexpression of PIN1 in cancers (*198, 199*), and cyclin D1 expression has been shown to be controlled by PIN1 at both transcriptional and translational checkpoints. At a transcriptional level, PIN1 is shown to bind to the phosphorylated Thr246- Pro motif of B-catenin that protects B-catenin from protein degradation. This stabilization of B-catenin consequentially enhances cyclin D1 expression (*21, 198, 199*), contributing to the dysregulation of cell proliferation in cancer. The enhanced expression of cyclin D1 is then acted upon post-translationally by another PIN1 mechanism. This time, PIN1 binds to the

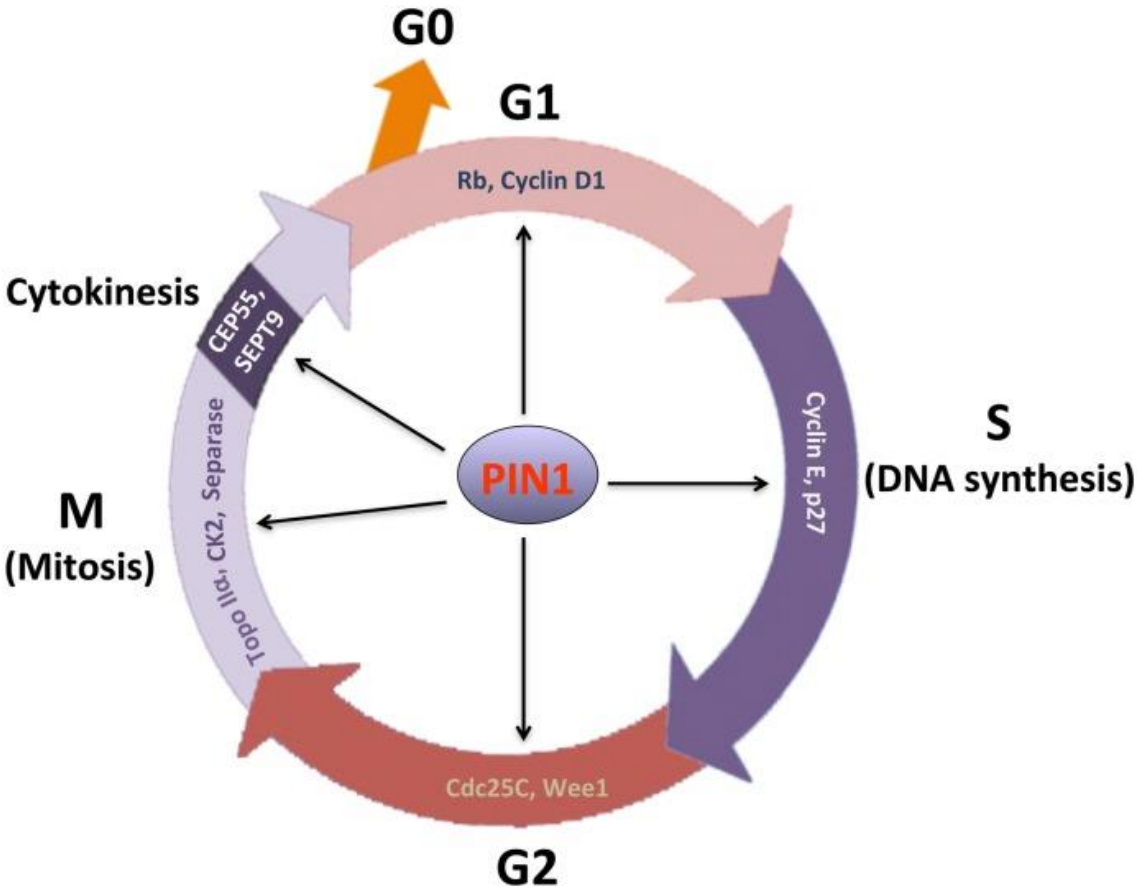


Figure 1.8 - Essential role of PIN1 in cell cycle regulation: adapted from (*199*). Annotation describing the essential role of PIN1 in regulation of the cell cycle progression. PIN1 regulates the function of various cell cycle proteins through phosphorylation-dependent prolyl isomerization. To progress the G1 stage, PIN1 activated cyclin D1-CDK4/6 by inactivation of retinoblastoma protein (Rb) allowing for the accumulation of cyclin D1. To promote the G1-S phase of cell cycle transition, PIN1 increases the stability of the Cyclin E-CDK2 inhibitor, p27 thus increasing Cyclin E activity. The G2-M stage of the cell cycle is enhanced by PIN1 activity on Cdc25c, that consequentially affects Cyclin B-CDK1 activity. In addition, PIN1 can regulate chromosome condensation and segregation by interacting with topoisomerase (Topo) IIα, casein kinase (CK) and separase. Finally, during cytokinesis, PIN1 alters the activities of the centrosomal proteins (CEP55) and septin 9 (SEPT9) the mediate midbody abscission.

phosphorylated Thr286-Pro residue of Cyclin D1, increasing its stability and nuclear accumulation resulting in the same outcome. More recent work has also shown PIN1 to interact directly with phosphorylated Rb, increasing its binding to cyclin D1-CDK4/6 and parallel disassociation from E2F proteins that normally act to inhibit cell cycle progression (*213*). Collectively these findings highlight PIN1 mechanisms that induce the release of E2F proteins by Rb that in turn promote cell cycle progression past the G1 checkpoint.

1.6.2.2 G1-S stage

PIN1 has further roles in cell cycle regulation through actively promoting the proportion of S phase cells undergoing proliferating. Following on from an enhanced CDK4/6 activation by cyclin D upregulation, another round of Rb phosphorylation is carried out by cyclin E-CDK2 complex formation that promotes cells to enter the S phase (*21, 198, 199*)..The expression of cyclin E, and sub sequent binding to CDK2 is regulated by an inhibitor p27 that prevents premature entry into the S phase. Interestingly the evolved mechanism of PIN1 involves the paradoxical stabilization of cyclin E-CDK2 inhibitor, p27. This mechanism is still however, beneficial to cell cycle progression, as studies have correlated the stabilization of the p27 inhibitor with its increasing impairment of its CDK2 inhibitory activity. As a result, cyclin E-CDK2 activity is increased, promoting cell proliferation in the S stage (*21, 198, 199*).

1.6.2.3 G2-M stage

Much of PIN1's regulation within the G2-M phase of transition appears to involve altercations of the cyclin B-CDK1 complex; a critical step in the initiation of mitosis. In order for mitosis to be carried out, a progressive activation of CDK1 is required through first the binding of cyclin B, then the sub sequent dephosphorylation of Thr13 and Tyr15 residues on the CDK1 structure (*199*). Cdc25C phosphatase is responsible for regulating the dephosphorylation of the CDK1 residues which in turn is controlled by PLK1 that acts by phosphorylating Cdc25c thus increasing its nuclear translocation and dephosphorylation of CDK1. Interestingly PIN1 has emerged as another regulator of Cdc25c, with evidence of both its activation and inhibition of Cdc25c activity (*199*). Studies have highlighted

PIN1's ability to both activate and inhibit cyclin B-CDK1 formation through its opposing effects on Cdc25c activity. Given that the effects of PIN1 activity on Cdc25c are frequently altered in the presence of other kinases / phosphatase, it is speculated that PIN1 may play a supportive role to ensure maximal activation of the Cyclin B-CDK1 complex and the ultimate transition into mitosis events (*199*). Other PIN1 mechanisms in G2-M stage are more straightforward including PIN1's inhibition of protein kinase Wee1 function. Wee1, under normal circumstances induces the phosphorylation of CDK1 Thr14 and Tyr15 residues, resulting in the inhibition of the cyclin B-CDK1 complex. However, in an oncogenic environment PIN1 is found to bind Wee1 and impair its functions, thus allowing for an enhanced cyclin B-CDK1 complex formation (*199*).

1.6.3 Sustaining proliferative signals

PIN1 also has distinctive roles in sustaining proliferative signals in cancer. As we have discussed, under normal circumstances cellular proliferation is tightly regulated by both intracellular and extracellular signalling mechanisms (*12, 13*). Despite this, one of the hallmarks of cancer entails the ability of tumours to encompass methods to overcome tightly regulated systems and prolong signal inductions that they require. Here, we find many examples incriminating PIN1 to overcome such regulated proliferative signalling pathway to benefit tumour cell progression. Often this dysregulation is attributed to the altercations in PIN1's activity due to enhanced expression and/ or PTMs on PIN1 that enhance its activity (Figure 1.9). One distinctive role of PIN1 is through its effects on the Estrogen receptor α (ERα) that is frequently found upregulated in breast cancer (*214*). ERα is shown to promote proliferation of breast cancer cells by regulating the expression of Estrogen response element (ERE)-containing genes. Concurrently ERα expression has been subject to therapeutical targeting in clinic and its presence is now associated with a positive patient outcome (*215*). PIN1 enhances ERα expression by increasing its transcriptional activity, ERE binding affinity, as well as inhibiting E6AP induced degradation of ERα in breast cancer (*206, 216*). These proliferative mechanisms are supported by the fact that both PIN1 and ERα expression is substantially reduced during by garlic extract diallyl trisulfide treatment of breast cancer cells (*217*).

Additionally, oncogenic PIN1 expression is involved in the proliferation of glioblastoma, endometrial carcinoma, acute myeloid leukaemia (AML), and hepatocellular carcinoma (HCC) (*218*). This is predominately through its activation of the nuclear factor (NF)-kb pathway (*203, 205*). Studies shows that cytokine stimulation induces the binding of PIN1 to the (NF)-kb subunits of RelA, enhancing its nuclear localization and stability. PIN1 was further shown to associate with other subunits of the (NF)-kb pathway, even in the absence of activating stimuli, to modulate their transcriptional and oncogenic activities. Through interactions with c-Rel proteins PIN1 is found to markedly increased the transformation of primary lymphocytes, as well as increase cell transformation by interaction with the potent viral (NF)-kb oncoprotein, v-Rel. Collectively these results show that PIN1 is a critical regulator of Rel/ (NF)-kb transformational activity (*205*).

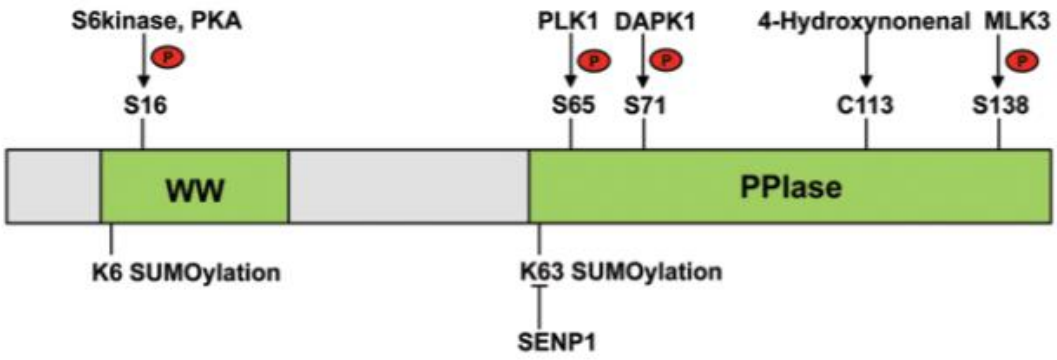


Figure 1.9 - Known PTMs affecting PIN1 regulation in cancer: adapted from (*198*). Schematic representation of PIN1 protein structure including the known post-translational modifications that regulate PIN1 activity and cellular localization in cancer. (P) denote sites of phosphorylation. The PIN1 protein structure is 18 KDa in size with two major protein domains of a WW domain and a PPIase domain.

1.6.4 Downregulation of tumour suppressors

We have already discussed the oncogenic role of PIN1 in downregulating the Rb tumour suppressor in the cell cycle phases (*198, 199*). It is apparent however, that the targeting of tumour suppressors is a common PIN1 mechanism to enable tumour functionality (Figure 1.10). Of note, is the critical tumour suppressor of promyelocytic leukaemia protein (PML) that is expressed in numerous tissue types (*219*). Routinely PML is found to be mutated in cancer, allowing for enhanced survival and proliferative mechanisms to go unchecked. PML has several important roles in the regulation of cellular processes including apoptosis, viral infection, DNA damage repair, cell cycle regulation, and senescence (*220*). Observations has shown PIN1 to associate with PML, targeting it for degradation in a phosphorylation dependent manner. This was found to be dependent on an ERK2 mechanism that inhibits PIN1's binding and subsequent degradation of PML. Consistent with these findings, the inhibition of ERK2 resulted in an enhanced PIN1 function to degrade the tumour suppressor of PML causing an increased survival and proliferation of these cancer subtypes (*220*)

PIN1 has additional methods of regulation on the previously mentioned ERα in breast cancer by controlling its tumour suppressor, Runt-related transcription factor 3 (RUNX3) (*221*). The expression of RUNX3 is inversely correlated with the expression of ERα in breast cancer cell lines. This portrays RUNX3 tumour suppressive function on ERα by reducing the stability of ERα expression to inhibit ERα-dependent transactivation and Estrogen-dependent proliferation. However, RUNX3 has an increased ubiquitin dependent degradation in the presence of PIN1 expression, that occurs through PIN1's deactivation of the E3 ligase FBXW7 that consequentially results its dimerization thus promoting its self-ubiquitination. In the absence of FBXW7, RUNX3 expression quickly dissipates, reducing ERα expression and promoting tumour proliferation.

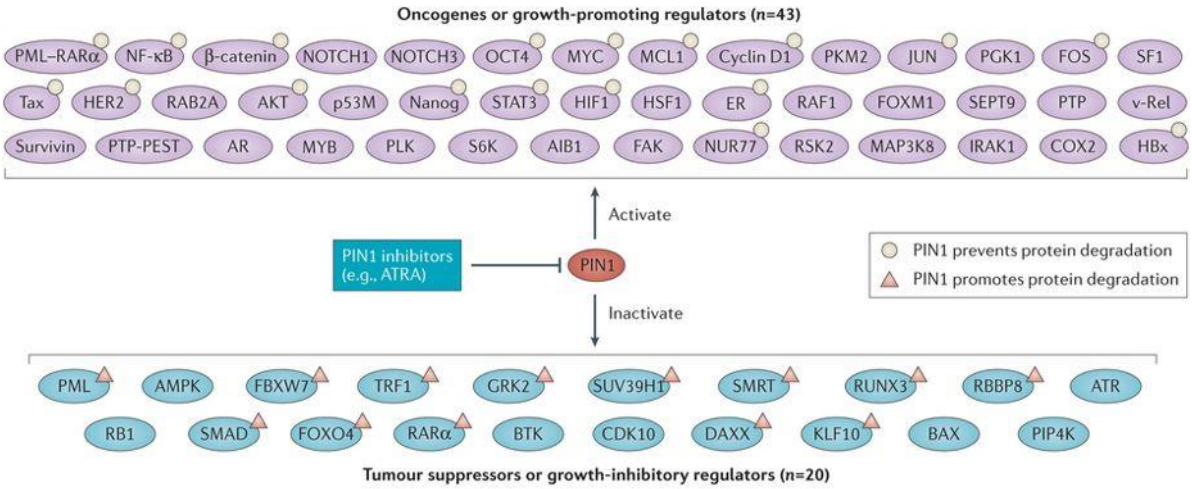


Figure 1.10 - PIN1 targets multiple oncogenes and tumour suppressors: adapted from (*200*). Annotation depicts the regulation of various oncogenes and tumour suppressors through the *cis* to *trans* isomerization of PIN1. PIN1 activity on these substrates can dictate a substrate's phosphorylation status, cellular localization, protein or DNA interactions, as well as substrate stability. PIN1 is detailed to activate over 40 oncogenic substrates and at least 20 tumour suppressor substrates, many of which are shown to have well-established roles in cancer stem cells (CSCs). Current PIN1 inhibitors available in clinic include the gold standard ATRA that blocks PIN1 activity *in vitro* and *in vivo*

1.6.5 Resistance to cell death

Several well-known mechanisms of PIN1 in cancer focus on its ability to modulate pro-apoptotic signals. These evolved mechanisms to evade programmed cell death are a characteristic hallmark of numerous cancers, and has been discussed at length in previous sections. It is clear that the oncogenic functions of PIN1 act as powerful barriers to block pro-apoptotic signalling and can occur through PIN1's ability to both downregulate pro-apoptotic factors and simultaneously upregulate anti-apoptotic factors. To address the former, PIN1 has several mechanisms aimed at inhibiting pro-apoptotic factors. The mitochondrial proteins of BAX and BAK are known to induce apoptosis by enhancing the release of cytochrome *c.* In human eosinophils, PIN1 works to inhibit BAX-induced apoptosis by preventing its mitochondrial translocation (*222*). What's more, PIN1 is also shown to block the Fas-FADD apoptotic pathway through its dephosphorylation of FADD. Under normal circumstances, Fas signalling increases the nuclear translocation of FADD by phosphorylating the protein on it Ser194 residue and simultaneously inhibiting PIN1's phosphorylation of the Ser16 residue. However, exo-genetic expression of PIN1 maintains the cytoplasmic location of FADD, sub sequentially blocking the Fas-FADD pathway. Other mechanisms involve PIN1's ability to upregulate anti-apoptosis factors, including the B-cell lymphoma 2 (BCL-2) family proteins that inhibit apoptosis by directly inactivating BAX and BAK (*223*). Research highlights PIN1's role to enhance the stabilization and cell death resistance of BCL-2 that sub sequentially promoting myeloid cell leukemia-1.

Some anti-apoptotic mechanisms of PIN1 have evolved to retain within specific tissue subtypes, in part attributed to the diverse tumour heterogeneity we can observe between cancers. In hepatocellular carcinoma (HCC), PIN1 is highly expressed and has been shown to contribute to hepatocarcinogenesis (*224*). This mechanism involves the inhibition of apoptosis by direct suppression of caspase-3 and caspase-9 activity. What's more, down-regulation of surviving in PIN1 overexpressed cells attenuates the anti-apoptotic effect induced by PIN1 suggestive that the suppression of caspase activity is mediated through a PIN1-survivin interaction in HCC (*224*).

1.6.6 Evasion of immune destruction

1.6.6.1 Manipulation of TLR responses

Over the past decade there has been considerable progress in the understanding of how cancers evade destructive immunity within the body. So much so that the evasion of immune destruction is now widely appreciated as an emerging hallmark required for the development of all cancer types (*14*). Unsurprisingly, research has highlighted roles for PIN1 in the immune evasion by cancer cells, using these evolved mechanisms to allow tumours to thrive in inflamed micro-environments and suppress immune reactivity. This is largely attributed to PIN1's influence on the Toll-like receptors (TLRs); cell surface and cytoplasmic pathogen-associated molecular patterns (PAMPs) that act as first responders to infection by initiating an innate reaction (*225*). In plasmacytoid dendritic cells, TLR7/TLR9 are frequently activated in response to PAMPs to induce an innate response. Their activation results in the autophosphorylation of interleukin (IL)-1 receptor associated kinase-1 (IRAK1) within the TLR complex, which in turns allows for the secretion of type 1 interferon (IFN-αβ) by activating the transcription factor IFN-regulatory factor 7 (IRF7) (*21, 199, 205, 218*). However, when PIN1 expression is elevated in diseased conditions, the activated TLR complex results in PIN1-mediated isomerization of IRAK1 causing it to dissociate from the TLR complex (*20, 21, 198, 199*). This PIN1 mechanism plays a critical role in the development of systemic lupus erythematosus, and therefore offers a promising strategy for therapeutical treatment (*226*). Other PIN1 mechanisms involve TLR3/TLR4 complex formations and act to reduce IFN-β production. In poly(I)poly(C) and RIG-1 stimulated immune cells, IFN-β is induced in response to viral threat by the recruitment of IRF3 to the TLR3/TLR4 complex. The optimal production of type 1 IFNs are crucial for maintaining immune homeostasis and to eliminate invading viruses. Here, we find PIN1 reduces the transcription activity and promote ubiquitin dependent degradation of IRF3 which sub sequentially reduces IFN-β production by these immune cells (*227*). These results elucidate a novel mechanism of PIN1 to control innate antiviral responses (*228*).

1.6.6.2 PIN1 mechanisms for immune escape

PIN1 is also known to participate in the active escape of tumours from circulating immune cells seeking their destruction. One method by which this occurs, is through the heightened production of Indoleamine-pyrrole 2,3-dioxygenase (IDO) by dendritic cells (DCs) circulating the body. IDO production has been identified as a feedback mechanism to exhaust local tryptophan; a unique amino acid required for T cell proliferation on which its depletion largely limits T lymphocyte functions (*229*). Given IDO is produced by the simultaneous activation of NOTCH and PI3K pathways in cytotoxic T lymphocyte associated protein 4 (CTLA-4) stimulated dendritic cells, a role has emerged for PIN1 to enhance the production of IDO providing protection for cancer cells from T lymphocyte interrogation. Research shows PIN1 increases the enzyme activity of casein kinase II to abolish the PTEN-mediated suppression of PI3K whilst simultaneously sustaining NOTCH signalling in cancer (*197, 230*). Collectively these results indicate the use of PIN1 activity to provide protection to cancer cells from T lymphocyte attack.

Another action of PIN1 to evade immune responses, is through the elevated production of the transforming growth factor-β (TGF-β). Encoded by 33 genes, TGF-β is known to play critical roles in immunoregulation; inhibiting T cell proliferation, cytokine production, and the induction of FOX3P regulatory T cells (iTregs) into circulation (*231, 232*). PIN1 is clearly shown to increase TGF-β production by stabilising it at both mRNA and protein levels (*204, 233*). Consequentially, the increase levels of TGF-β by PIN1, are shown to enhance TGF-β induced invasion and migration of cancers, making them more difficult to be interacted with and destroyed by the appropriate immune cell mechanisms (*234*).

1.6.7 Tumour-promoting inflammation

The ability of a tumour to influence chronic inflammation is correlated to cancer initiation and progression (*108, 235*). As we have discussed in previous sections, one mechanism to initiate inflammation is through the necrotic destruction of cells. Previously thought of as an uncontrolled response to cell death, we now appreciate necrosis as another mechanism to promote tumour formations. By

releasing necrotic cell content into the tumour micro-environment, an inflammatory response is initiated involving the recruitment of innate inflammatory cells into the local space (*108, 235*). These inflammatory cells include the recruitment of neutrophils; innate granulocytes found abundantly in the body. Oncogenic PIN1 is found to enhance NADPH oxidase activity within neutrophils to produces reactive oxygen species (ROS) that cause inflammation, and act to destroy invading microbes in the vicinity. This mechanism occurs through the translocation of p47phox from the cytosol to the cell membrane, that sub sequentially increases NADPH oxidase activity thus enhancing the inflammation response in the tumour micro-environment. The relationship between inflammation and cancer progression is not new (*108*), but instead is well understood to promote cancer through the oncogenic production of cytokines, chemokines, upregulation of transcription factors and further recruitment of inflammatory cells (*235*). Indeed, the attributions of PIN1 to inflammation are also widely known(*236*) and the vast tumour-promoting networks within cancer will likely overlap PIN1, inflammation and the progression of cancer in many contexts.

1.6.8 Metabolic reprogramming of cancer

Regarding the previously discussed metabolic reprogramming of cancer, here too we find PIN1 to be a crucial regulator for this oncogenic response. The Warburg Effect; known for the favouring of the conserved glycolytic function even in the presence of oxygen, causes a metabolic shift in tumour cells. This shift is frequently seen in the enhanced landscape of glycolytic enzyme functions required to meet the demands for the tumour's glycolytic needs. Amidst this change, the PKM2 isoform is regularly found to be upregulated in cancer. PKM2 is the rate limiting glycolytic enzyme that catalyses the irreversible transphosphorylation between phosphoenolpyruvate (PEP) and adenosine diphosphate to produce pyruvate and ATP. The activity of PKM2 in cancer is notoriously low, resulting in a backlog of carbon intermediates and reduced rate of pyruvate production by this pyruvate kinase isoform. This is extremely favourable for cancer cells, as the high conversion of pyruvate initiates an OXPHOS response that negatively impacts the proliferation of the tumours (*13,*

150). Furthermore, the backlog of carbon intermediates are shuttled into various metabolic pathways further fuelling the tumour's needs (*13, 115*). Intriguingly, PIN1 is found to increase the nuclear translocalization of PKM2 in cancer. This was demonstrated to occur through the EGFR-activated ERK2 pathway that resulted in the phosphorylation of PKM2 at Ser 37 by ERK2. As a result, phosphorylated Ser 37 PKM2 recruits PIN1 for isomerisation in turn promoting the binding of the nuclear importin α to PKM2. PKM2 is then sub sequentially translocated to the nucleus to act as a co-activator of β-catenin, inducing oncogenic c-Myc expression that leads to the upregulation of GLUT1, LDHA, and a positive feedback loop to enhance PKM2 expression (*237*). Collectively, these results indicate a role for PIN1 in enhancing PKM2 nuclear activity to promote the Warburg Effect.

1.7 Post-Translational crosstalk of proteins

We have to some degree, deduced the extensive incrimination of two major protein pathways involved in the development and oncogenic potential of cancers, namely JNK and PIN1 signalling pathways. Despite this, we have merely scratched the surface of the signal transduction machinery that are regularly deceived and hook-winked into promoting the survival of malignant cells. In reality, the protein network of any one cell, more closely resembles that of the inner workings of a beehive; thousands of independent bodies, cross-linking and each contributing to the same goal, the overall survival of the hive. This protein network within cells, is more commonly referred to as the protein interactome, consisting of protein-protein interactions (PPIs) as well as epigenetic modifications that form the backbone of signal transduction pathways responsible for the diverse physiological outcomes of the cell at any time. Over the past decade, advancements in computational biology have provided scope into the extensive protein interactomes that exist, both within normal and cancerous cell types. Indeed, these advances have resulted in the emergence of network medicines that offer a platform to therapeutically explore key molecular mechanisms in cancer. This network insight highlights hubs within the protein

networks that are representative of proteins and/ or genes that provide conserved and essential roles in oncogenic signal transduction. Network analysis collectively allows us to prioritise cancer related genes to target in clinic. Network analysis has also uncovered large bodies of post-translational modifications (PTMs) between proteins; evolved mechanisms for proteins to crosstalk with one another and influence their outcomes within the cell. Already we have highlighted substantial cross-talk for even the JNK kinase, and PIN1 isomerase mechanisms alone, providing insight into how extensive PTMs are being used in both normal and cancer conditions (*238*). In this section, we will address some of these PTMs that occur between proteins in more depth, including how they can be influenced to promote tumour progressions.

1.7.1 Mechanisms of phosphorylation

1.7.1.1 Overview

Of the many PTMs that are in effect, phosphorylation is by far the most widely used in signal transduction. Carried out by protein kinases, phosphorylation affects every basic cellular process including, growth, survival, metabolism, division, proliferation, differentiation, transport, immunity, muscle contraction, as well as learning and memory (*22, 239*). It is no wonder then, that the overarching family of protein kinases that carry out these roles make up 2% of the entire genome within eukaryotes (*22, 240, 241*). They act by catalysing the transfer of terminal phosphate groups from ATP to specific amino acids in the protein, usually Ser, Thr and Tyr residues (Figure 1.11). The result of this PTM often involves further altercations in the substrate protein's structure, allowing for additions mechanisms such as protein interactions, kinase activations, cellular translocations and targeting of downstream substrates to occur (*194*). Phosphorylation is also a frequent indicator used by other proteins to initiate additional PTMs, whether that involves the negative regulation of phosphorylation by counterpart phosphatases, or further modification by sumoylation, acetylation, AMPylation, isomerization, or ubiquitination (*194, 242*). Therefore, the small action of adding a terminal phosphate group to a substrate protein, frequently

results in the accumulation of PTMs to the same substrate target, each of which may confer a different message implicating the substrate protein in various signal transductions (*19*).

Given the cell's wide spread use of phosphorylation as a universal language to communicate amongst the diverse protein networks, it is fascinating that any one protein kinase can still be entirely selective of just a few phosphorylation targets, within a sea of roughly 700,000 potential phosphorylation sites that they may encounter (*23*). Even within the same substrate protein, any one protein kinase can differentiate between multiple phosphorylation sites, adding an addition layer of specificity to this PTM. It is clear therefore, that several methods have evolved in the cross-talk of proteins to ensure that phosphorylation can be used as precise signal to carry out specific cellular functions.

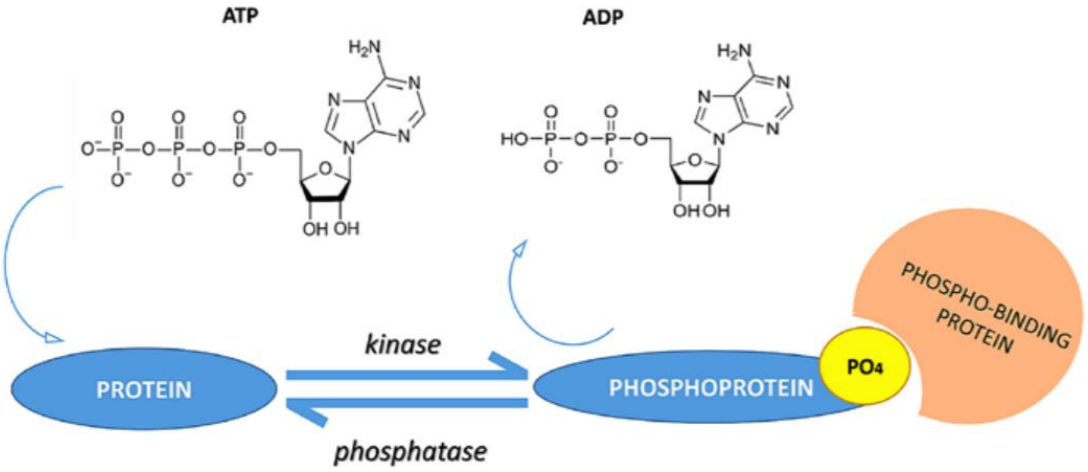


Figure 1.11 - The role of phosphorylation in protein signalling networks: adapted from (*194*). Schematic representation of the mechanism of protein regulation that contributes to protein signalling networks in the cell. Phosphorylation regulation includes the action of kinases acting on phospho-binding sites on substrates. These actions are frequently reversed by phosphatases present in the cell. The act of phosphorylation uses ATP, adding a phosphate group to the substrate protein and producing a phosphoprotein and ADP. This action can result in activation of the phospho-protein form allowing for the binding mechanics to other proteins in its new configuration.

1.7.1.2 Specificity of kinases

Amongst the diverse family of protein kinases, roughly 80% constitute Ser/Thr specific kinases, with the remaining made up by Tyr-specific kinases (*22, 239, 240*). The characteristic amino acids of both groups creates an immediate layer of substrate specificity for the different protein kinases; dictating which substrate protein groups they may interact with downstream. This is predominately caused by the depth of the catalytic clefts; the active region of the protein kinase that will confer phosphorylation to the substrate targets. Ser/Thr specific kinases notoriously have much more shallow clefts than Tyr specific kinases, limiting the ability of one group of kinases to phosphorylate substrate targets from the other group with few exceptions (*243*). This kinase specificity is then further enhanced by the amino acid signature found at the N terminal and C terminal sequences that encompass the phosphorylation site. Where the active site of the kinase interacts on the substrate, situated at either side are four amino acids the confer additional barriers to binding. Therefore, active protein kinases must not only have complementary binding sites, but must also align the neighbouring sequences to the binding site successfully in order to overcome the energy needed to undergo phosphorylation (*244*). This is also the reason why free amino acids provide poor substrates for protein kinases, simultaneously preventing the activity of protein kinases outside of their jurisdiction (*245, 246*).

1.7.1.3 Docking sites

Another layer of substrate specificity comes between interaction domains on the kinase with the docking motifs present on the substrate (*23, 247*). Often the interaction domains are optimally spaced from the kinase domain on the same protein to allow for easier kinase activity once the interaction domain has bound to the substrate. Docking interactions have been identified in several protein groups, including the MAPKs such as on the JNK proteins. Typically, JNK substrates contain docking domains (D domains) between 50-100 residues away from the phosphorylation site on the substrate protein. D domains increase the efficiency of phosphorylation by JNK proteins, as binding to the D domain increases the affinity of the kinase domain of JNK for its phosphorylation site (*248*). Other MAPK substrates also have these conserved sites, including transcription factor -2 (ATF2), the transcription factor ELK1, and myocyte

enhanced factor 2A and 2C (MEF2A and MEF2C) (*212, 247, 249-251*). It should be noted however, that these docking interactions are not limited to the MAPKs or indeed phosphorylation mechanisms. For example, the aforementioned isomerase PIN1 is also known to have an interaction domain, distal to the PPIase domain of the protein. In this case it is a WW domain that plays critical roles in binding PIN1 substrates to enhance its isomerase activity (*207, 252*). It is therefore clear that docking sites are ubiquitously used to enhance protein-protein interactions within the cell. Interestingly, there are also several examples within the cell's protein network of conditional docking sites. This involves the recruitment of kinases to substrates on the condition that have previously been phosphorylated at specific sites, that in turn influences the ability of the same, or other kinases to further phosphorylate the substrate protein. This mechanism has been shown to prevent the substrate kinase activity, until the substrate has incurred prior phosphorylation. This mechanism is shown to sequester kinase activities of the substrate protein, ensuring they remain in an inactive state until the protein has received a degree of PTMs (*23*).

1.7.1.4 Scaffolds

Aside from the direct interaction of protein kinases with their substrates, another evolved mechanism is through the use of intermediary adaptor or scaffold protein complexes. Scaffolds act as mediators for the interactions of two or more proteins that are recruited to the complex, allowing for the alignment of kinases with substrates that previously would not be available due to an incompatible, or hidden binding sites (*253*). By facilitating the binding of protein kinases in proximity to their substrate proteins, often scaffolds will allow for kinases to adopt unique configurations that allow temporary access to substrates also bound to the scaffold complex (*253-255*). Indeed, we find that scaffolds can both be passive, as mentioned above, as well as actively modify protein kinases to alter their functionality. For example, within yeasts, the Ste5 scaffold allosterically activates the autophosphorylation of a Tyr in the activation look of MAPK Fus3. After autophosphorylation of the Tyr site, MAPK Fus3 becomes activated and downregulated the signalling output from the mating pathway. Thus, scaffolds are not necessarily just passive structures to bring together kinases with their substrates, but can also serve to activate or inactivate the kinases themselves

consequentially blurring the lines between what constitutes a scaffold compared with a protein substrate (*254*).

1.7.2 Phosphorylation used to promote cancer

It is abundantly clear that phosphorylation is a key PTM utilized by large families of protein kinases to operate seamlessly within the cell. It therefore comes as no surprise, that phosphorylation is also a key contributor to the development of cancer. In part, this has been shown to occur through mutational gain or loss of phosphorylation mechanism that respectively over activate or inhibit signal transduction critical for cellular processes (*256*). Often this involves genetic altercations resulting in amino acid substitutions that directly interrupt the stability and function of proteins integral to the signal transduction pathway (*208, 209*). However, more commonly, it is mutations within ubiquitously expressed kinases and phosphatases that cause prolonged damage to the homeostasis of a healthy cell. The resulting altered phosphorylation of kinases and phosphatases frequently disrupts the delicate balance of activation or inactivation of cancer-associated proteins (*257*). What follows are examples of major protein kinase groups frequently targeted to promote oncogenesis.

1.7.2.1 Mitogen-Activated Protein Kinases (MAPKs)

A familiar protein kinase group targeted to promote oncogenesis, is that of the MAPKs, including the JNK signalling pathways. As we have discussed, MAPKs relay signalling messages from the cell surface to the nucleus by a cascade of phosphorylation mechanisms. As a result, this makes them prime targets to relay oncogenic signals to promote tumorigenesis. Indeed, the high activation of JNKs kinase activity are seen in multiple cancers, and correlate positively with disease progression and poor clinical outcomes (*155, 171, 173*). In fact, it has long been known that this hyperactivation of JNK is responsible for the activation of several oncogenes in cancer tissues, including Ras, c-fos, Met and Bcr-Abl (*160, 164, 165*). Furthermore, the hyperactivation of JNK was first recognised to contribute to the transformational properties of Ras, through its prototypical downstream target of c-Jun (*258*). Collectively, the JNK signalling pathway provides evidence

of cancer mechanisms enhancing phosphorylation signal transductions for the tumour's own gain.

Interestingly, a critical step for the activity of JNK is regulated primarily by the previously mentioned PIN1 isomerase activity (*124*). PIN1 is shown to directly target and promote JNK1 activity by modulating the phosphorylated JNK1 form. It is proposed that partially active JNK becomes fully active after conformational changes induced by PIN1 activity. Through initial phosphorylation of the Thr183 in the JNK phosphorylation loop, the protein kinase is made partially active resulting in the partial opening of a docking groove nearby to the phosphorylation loop of JNK. This structural change allows for the recruitment and activity of PIN1, that causes JNK to undergo a conformational change involving the flipping out of the phosphorylation loop and complete exposure of the nearby docking domain creating an active JNK isoform with the ability to phosphorylate its targets (*211*). This model is supported by the correlation between high PIN1 expression and simultaneously high JNK expression in a range of cancers (*259*).

1.7.3 Mechanisms of ubiquitination

Just as phosphorylation is a widely used PTM for most protein activation, it is rare for any cellular protein, at some point in its life to not encounter ubiquitination. In fact, we now recognise over 1000 proteins to be actively involved in the process of ubiquitination; a dynamic system that dictates protein outcomes as well as numerous cellular functions (*260*). Initiated by the binding of a small 76 amino acid ubiquitin to the substrate protein, this process was originally described for its methods of labelling proteins to undergo destruction via the proteasome. However, in subsequent years, many novel roles involving ubiquitination have emerged, attributed largely to the addition PTMs that can occur to a ubiquitin molecule, once it binds to the substrate protein. These additional PTMs add a layer complexity, to an already dynamic system, that has justified its referral as the ubiquitin code borrowing nomenclature from the histone code; used to describe the complex interplay of histone modifications (*261*). In the sections that follow, we will focus on the foundations to which the ubiquitin code is built, as well as highlight roles of ubiquitin in protein trafficking, protein degradation, and its manipulation to promote cancer (*17, 262, 263*).

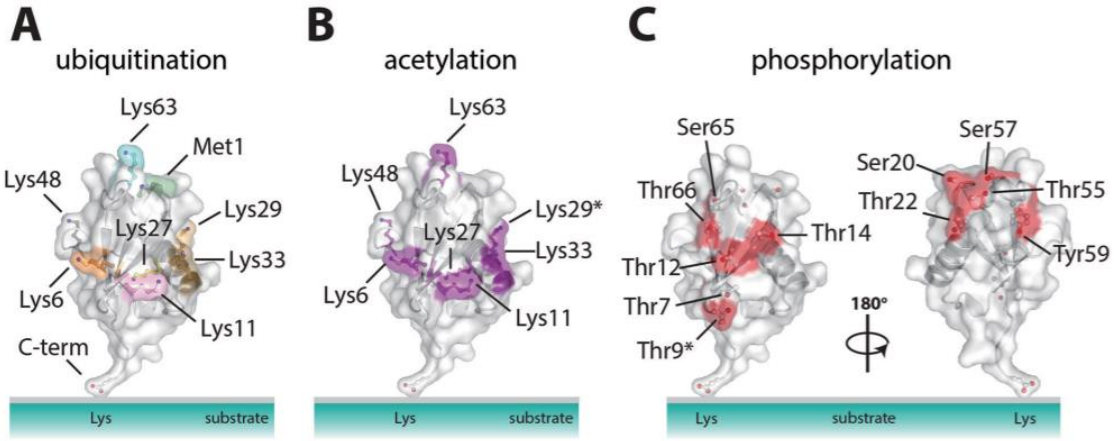


Figure 1.12 - Modification sites on the ubiquitin molecule: Taken from (*262*). Annotation depicts the modification sites on ubiquitin molecules. Ubiquitin is shown as a semi-transparent surface where post-translation modifications are shown of (A) ubiquitination of lysine residues including Lys63, Lys29, Lys33, Lys11, Lys27, Lys6, Lys48 and Met1. (B) Acetylation of lysine residues Lys63, Lys29, Lys33, Lys11, Lys27, Lys6, Lys48. (C) Phosphorylation of Ser, Thr and Tyr residues including Ser65, Ser57, Ser20, Thr66, Thr12, Thr7, Thr9, Thr14, Thr22, Thr55, and Tyr59.

1.7.3.1 The ubiquitin code

The initial attachment of a ubiquitin molecule to a substrate protein involves three enzymatic steps. The first step activates the ubiquitin molecule through the formation of a thioester bond with the ubiquitin activating enzyme, E1. Once activated, the second step involves the delivery of ubiquitin to the E2 ubiquitin conjugating enzyme by E1. Then, the conjugation is completed by the E3 ligase that catalyses the transfer of ubiquitin from E2 to a lysine residue in the substrate protein. This process results in the mono-ubiquitination of substrate proteins; bound by a straight or modified ubiquitin molecule at any respective lysine residue; the latter of which represents any modifications of ubiquitin through phosphorylation and acetylation. Given the numerous lysine residues present on any one protein target, often protein substrates can be mono-ubiquitinated multiple times at various sites throughout the tertiary protein structure (Figure 1.12). Therefore, already the act of mono-ubiquitination has its own layer of complexity, and behaves by dynamic tagging of substrate proteins in turn dictating protein regulation (*264, 265*). This tagging of ubiquitin has been shown to influence protein degradation, protein complex formation as well as intracellular localization and trafficking (*17, 262*).

1.7.3.2 Chains and branches

Alternatively, the modification of the N terminus of any one of the seven lysines present on the substrate attached ubiquitin, can lead to the formation of polymeric ubiquitin chains. This involves the stacking of ubiquitin molecules to the substrate linked ubiquitin aided by E3 ligase machinery. These chains can constitute anything from short chains, containing no more than two ubiquitin molecules, to long complex chains encompassing up to ten moieties (*17*) (Figure 1.13). Often these ubiquitin chains are homogenous where the same residue is modified during the elongation process. Indeed, chains commencing from Met1, Lys11, Lys48 or Lys63 residues on the substrate linked ubiquitin characteristically produce these chain types and their formation is an essential requirement to their function. Other chains have mixed topologies, due to the linking of ubiquitin moieties from different residues within the chain which again affect the overall ubiquitin function (*266*). There is also extensive evidence for the formation of

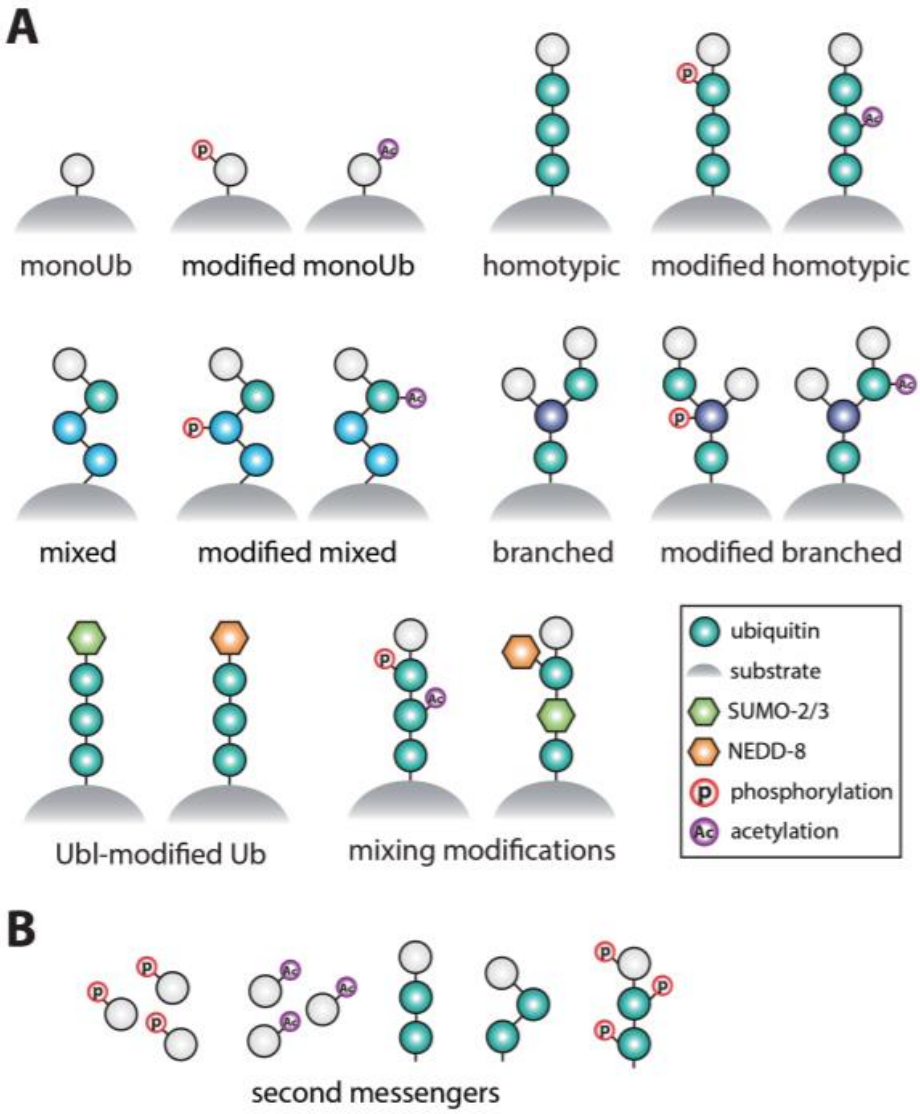


Figure 1.13 - Ubiquitin chain variations add complexity to the ubiquitin code: Taken from(*262*). Annotation depicts the variety of chain and branch combinations that can occur from a single binding of a ubiquitin molecule to a substrate lysine. (A) Representation of possible mono and poly-ubiquitin chains that can form from binding a lysine on a substrate protein. (B) Representation of unanchored ubiquitin molecule and chains present within the cell that with or without further modifications can act as second messengers affecting protein regulation.

branched ubiquitin chains, where any one of the ubiquitin moieties within the chain can be ubiquitinated at multiple lysines resulting in fork like structures. The

ubiquitin chain topologies, whether it be homogenous or heterogenous, affect the confirmations of the overall chain. Ubiquitin chains can either adopt compact confirmations, where the adjacent moieties interact with one another, or open confirmations, when no interfaces are present accept the linkage site. The type of confirmation have been understood to largely dictate the overall binding and recognition mechanics of the ubiquitin chains. For example, the homogenous Lys48 chain structure adopts a compact confirmation, allowing for the ubiquitin moieties to interact via their Ile44 patches that are exposed to their substrates (*266-268*). On the other hand, Met1 and Lys63 linked ubiquitin chains are known to adopt open confirmations with a resulting structure that allows high conformational freedom where binding partners can exploit the distance and flexibility between chain moieties (*269*).

1.7.3.3 Specific ubiquitin chain-linked functions

Intriguingly, the topology and lysine specificity of a substrate-attached ubiquitin chain is becoming increasingly linked to specific cellular functionality (*19, 260, 266, 267*). Such functionality can be remarkably specific if we consider the confirmation, length, branch formation, lysine specificity and overall topology that can individualise a ubiquitin chain signal. The most studied of these chain types is the formation polyubiquitin chains from the Lys48 residue. It has long been known that Lys48 chains of specific lengths, function as a marker for proteolytic targeting of the linked substrate protein for destruction via the 26s proteasome (*270, 271*). Indeed, it was through these discoveries that the entire ubiquitin system was first believed to be dedicated solely to the destruction of proteins. Since then, Lys48 has been appreciated to also regulate the activity of some transcription factors including Met4; responsible for the activation of genes in the methionine biosynthetic pathway (*19, 262, 272, 273*). In this example, the depletion of methionine within the cell results in the rapid polyubiquitination of Met4. However, although Met4 is conjugated to Lys48 chain formations, the transcription factor is shown to escape degradation via the proteasome.

Structural analysis shows this is made possible through the Met4 transcription factor containing its own ubiquitin binding domain that sub sequentially interacts with its own ubiquitin chains to restrict the chain length to below the threshold needed for targeting to proteasomal degradation, thus prolonging its half-life and activity within the cell (*226, 274, 275*).

In contrast to Lys48 chains, Lys63 chains were first recognised to carry out non-proteolytic functions in damage repair, cellular signalling, intracellular trafficking and ribosomal biogenesis (*16, 18, 276*). For example, Lys63 polyubiquitination is important for the protein kinase activation of interleukin-1 (IL-1) and toll-like receptor (TLR) pathways. These experiments identify TRAF6, a RING domain protein important for IKK activation by IL-1 and TLR pathways, to act as a Ub E3 to mediate IKK activation. Together with the E2 activity, TRAF6 catalyses the polyubiquitination of Lys63 target proteins including, TRAF6 itself (*277*). Lys63 also plays key roles in protein kinase activation in multiple pathways resulting in NF-kB activation (*273*). These protein kinase pathways include those of the TNF receptor, T cell receptor, NOD-like receptor , and RIG-1 like receptor pathways, as well as DNA damage and viral proteins (*278*).

The extensive research into the mechanisms behind ubiquitin signalling is not limited to Lys48 and Lys63. Since their discoveries, intense work has helped uncover detailed mechanisms behind the other lysine specific ubiquitin chain formations including the Met-1, Lys6, Lys11 and emerging Lys27- linked ubiquitin chains (*262*). In doing so, it becomes clear that the ubiquitin code is well established in diverse cellular functions. Respectively, Met-1 linked chains are key positive regulators of NF-kB signalling as well as involved in inflammation and immunity (*56, 279-282*). Lys-6 linked chains are associated with UV genotoxic stress and regulators of DNA damage responses (*283-285*). Lys11-linked chains on the other hand, are used as additional proteasomal degradation signals in cell cycle regulation (*286, 287*), with an emerging role for Lys27- linked chains in protein recruitment mechanism (*272, 288*), however more work is needed to establish the latter.

1.7.3.4 Unanchored ubiquitin

Finally, in addition to the functions of substrate-linked ubiquitin chains, the presence of unanchored ubiquitin moieties and ubiquitin chains that exist independently within the cell's matrix can perform second messenger-like functions. For instance, monoubiquitin is a prominent component of cell lysates and can regularly undergo modifications like phosphorylation and acetylation. Such modified ubiquitin moieties have been shown to act as new signalling molecules within the cell. One such example is that of free Ser65-phosphorlyated ubiquitin, that is reported to activate kinase signalling (*289, 290*). The same holds true for unanchored polyubiquitin chains, with evidence of their direct activation of TAK1 and IKK that are necessary signals for protein kinase regulation (*291*). Alternative roles for unanchored polyubiquitin chains includes their regulation of NF-kB signalling mechanisms. A distinct example of this is shown when cells are stimulated with IL-1b that lead to the activation of the previously mentioned TRAF6 E3 ligase. TRAF6 catalyses the synthesis of unanchored Lys63 polyubiquitin chains that result in TAK1 activation, in turn phosphorylating IKKb and causing NF-Kb activation (*263*). There are also examples of unanchored polyubiquitin chains aiding the entry of viruses into a host's cell. The influenza virus is found to enter a host cell's aggresome system; an area of high misfolded protein concentration within the cell, by carrying unanchored polyubiquitin chains. This strategy allows for viruses to 'uncoat' and replicate after their escape from the endosome during entry into the host cell (*292, 293*).

1.7.4 Hijacking of the ubiquitination system in cancer

The integrate workings of the ubiquitination signalling system sees it engaged in critical and diverse cellular functions. Consequentially, the dysregulation of components for such an elaborate network has huge consequences to human disease. Altercations in the ubiquitin code are now emerging and play distinct roles in the initiation and progression of various tumour formations. It is clear that through the hijacking of the elaborate ubiquitin network, cancer has evolved alternative strategies to further aid the survival and growth of malignant cells.

1.7.4.1 Blocking ubiquitin binding in cancer

Often such strategies involve altercations in the abilities of ubiquitin to tag proteins for destruction to the 26s proteasome. This role in protein destruction dictates the half-lives of most major protein families operating in the cell and ensures that active proteins are well regulated to avoid 'always on' proteins from causing harm to the cell's homeostasis (*294, 295*). Given the abundance of ubiquitin components including ubiquitin moieties, unanchored ubiquitin chains, and ubiquitin ligases present in the cell's make up, the ubiquitin system has evolved to readily tag active proteins for destruction, as well as other distinct cellular functions as soon as the protein confirmation is activated to reveal a ubiquitin binding domain (UBD). Often, protein-protein interaction can ultimately sequester UBDs of a protein, sub sequentially preventing ubiquitin ligases from initiating a substrate-linked ubiquitin chain formation to catalyse a response from the ubiquitin code. In breast cancer, cathepsin S (CTSS), a cysteine protease, is known to contribute to tumour growth, angiogenesis and metastasis (*295*). This is in part through active CTSS's ability to induce proteolytic degradation of BRCA1; a tumour suppressor responsible for double strand DNA break repair activity. One study found that the depletion of CTSS through RNAi as well as mutant CTSS expression enhanced the protein stability of BRCA1 by inhibiting its ubiquitination, thus restoring BRCA1 tumour suppressive functions within the cell (*295*). Other examples are found in prostate cancer involving the androgen receptor (AR) shown to promote tumour cell growth through ligand-dependent transcription and control of target gene expression (*294*). Canopy FGF signalling regulator 2 (CNPY2) expression controls AR protein levels within prostate cancer. This is found to occur through CNPY2 decreasing the ubiquitination activity of the E3 ligase specific for the AR, namely, myosin regulatory light chain interacting protein (MYLIP). Collectively, these studies provide examples of tumour's benefiting their own survival by enhancing protein-protein stability by preventing ubiquitin substrate binding for proteasomal degradation (*294, 295*).

1.7.4.2 Oncogenic targeting of the ubiquitin ligase system

Alternative strategies adopted by cancers to promote numerous cellular processes include the manipulation of ubiquitin ligases (E3s). As we have discussed, E3s are responsible for recognising, interacting with and ultimately

ubiquitinating protein substrates. They do so, in both a temporal and spatial manner, largely dictated by the ubiquitin chain topologies that determine which E3 ligase the substrate-linked chain can interact with. In turn, this specific interaction dictates the fate of the substrates, tagging them for destruction, subcellular localization, protein recruitment, or other distinct cellular functions (*16, 17, 262, 263, 265, 272, 273, 278*). Consequentially, this makes E3 ligases useful oncogenic targets to promote the needs of a tumour. Only through better understanding these E3 functions can we aid the development of anti-cancer therapeutics. One example of oncogenic manipulation of E3 ligases is in the regulation of MAPK signalling in cancer. As we have discussed, the MAPK pathway, the overarching family of the JNK pathways, play critical decision making roles in the proliferation, differentiation, survival and death of a cell (*157, 159, 160, 162, 170, 181, 183, 185, 187, 248*). They are also notoriously hyperactivated in cancer tissues and associated with poor clinical outcomes and disease progression. The oncogenic RAS, a regular instigator of tumorigenic MAPK signalling, is found to regularly evade ubiquitination signals allowing for its sustained expression in cancer (*296*). Under normal physiological conditions, RAS signalling is targeted by the E3 'NEDD4' limiting its activity. This occurs as part of a negative feedback loop, whereby RAS induces transcriptional upregulation of NEDD4, which in turn binds RAS to limit its activity (*296*). However, the same cannot be said for the oncogenic RAS, which is able to escape regulation by the NEDD4 E3 and sustain MAPK signalling. Given that NEDD4 upregulation also increases PTEN degradation, the oncogenic RAS is able to further enhances its activity without regulation leading to RAS-driven tumour altercations. Collectively, tumours can clearly benefit from the targeting of ubiquitin components, to sustain malignant protein activity within the cell (*296, 297*).

1.7.4.3 Deubiquitylating enzymes in cancer development

Unsurprisingly, the reversal of E3 ligase activity in cancer is also subject to manipulation. Deubiquitylation enzymes (DUBs) recognize ubiquitylated proteins and remove their ubiquitin tags providing a homeostatic balance to the ubiquitin machinery in unmutated cell types. Most human DUBs are cysteine proteases, like the previously mentioned CTSS, and include sub-families that act as direct

antagonists of both oncogenic and tumour-suppressive E3 ligases. The subfamily of ubiquitin-specific proteases (Usp) includes the DUB ‘USP28’ that is regularly overexpressed in colon and breast tumours. USP28 is required for the stability of MYC in human tumour cells. It does so, by interacting with FBW7alpha, a ubiquitin ligase and counteracting its actions. Thus, overexpression of USP28 enhances the stability of MYC in the nucleus shown to be essential for tumour cell proliferation (*298*). DUBs are also critical in the regulation of the NF-kB pathway. In this case, the tumour suppressor gene CYCLD, giving rise to the DUB CYLD, is frequently mutated in skin cancer (*299*). the DUB CYLD contains a C terminal ubiquitin hydrolase domain that targets the removal of Lys63 linked chains from several mediators of the cytokine-induced NF-kB pathway. However, thanks to the mutated tumour suppressor gene, CYLD expression is often reduced in skin cancer, as well as kidney, liver, and uterine cervix cancers suggestive of tumour suppressive roles for CYLD in a range of cancers. This was found to occur due to CYLD blocking of BCL3, a transcriptional co-activator that associates with NF-kB components in the nucleus to downregulate cell proliferation. In the absence of CYLD, BCL3 is left unchecked, resulting in sustained cell proliferation that benefits the tumour’s outcome (*299*).

Chapter 2 – Hypothesis and Aims

The disparity of JNK signalling mechanisms in cancer over the years has been a cause for much controversial debate. Despite high phospho-active JNK expression being regularly linked with numerous cancer subtypes, the overall outcome of JNK signalling within the cell has remained for a long time, elusive (*159, 165, 166, 173, 185, 212, 248, 249, 251, 306-308*). Strong evidence for JNK's roles in relaying both survival and death signals to the nucleus have been frequently uncovered, which begs the question: how can one signal pathway elicit completely opposing outcomes?

Not until the focus was on the functional variations between separate JNK isoforms (JNK1, JNK2), did such contradictory findings start to make sense. It became clear that specific JNK isoforms could often play antagonistic roles when relaying signal messages, messages that sub sequentially dictated the outcome of the overall cellular decision. More so, the function of these isoforms was often inconsistent among different tissues contexts, resulting in a generic appreciation that all JNK signalling mechanisms must be treated in both, tissue-specific and isoform-specific manners.

Previous findings from our laboratory had identified a novel downstream substrate of JNK, namely PIN1. PIN1 was shown to be regulated post-translationally by active JNK2 isoform signals in the context of Multiple Myeloma. Given the extensive literature surrounding PIN1's upregulation and contributions to a plethora of cancer tissues, we aimed to assess whether a similar interaction was occurring between active JNK and PIN1 in liver cancers. Furthermore, in response to the frequent dysregulation of PIN1 in the wider context of cancer, we also aimed to detail an *in vitro* mechanistic interaction between JNK and PIN1. This last aim was done in the hope that our findings on PIN1 may have significant impact to our colleagues working in other areas of cancer research.

Chapter 3 – Material and Methods

3.1 - Gene expression profiling

Gene expression profiling studies were derived from publicly available datasets (GSE2658 and GSE9782) (*300-303*) to examine PIN1 expression levels of 528 patients diagnosed with MM and enrolled in phase 2 and phase 3 clinical trials with bortezomib. PIN1 expression levels of these patients were compared with normal bone marrow samples or monoclonal gammopathy of unknown significance. Dunn's post hoc tests were performed between data sets as a non-parametric pairwise multiple comparison method to understand significant difference (*P<0.05 and ***P<0.001). Data was visualised as Kaplan-Meier overall survival (OS) and Progression free survival (PFS) comparing high levels of PIN1 expression in patients (above the median) with low levels of PIN1 expression in patients (below the median).

3.2 - Cell line cultures

Human embryonic kidney lines (HEK293T) were cultured in Dulbecco's modified Eagle Medium (DMEM 1X) high glucose (41965-039), supplemented with: 10% FBS heat inactivated (cat no.10500065 Invitrogen), 1% L-Glutamine (200mM 100X cat no. 25030-123 Invitrogen) and 1% PES solution (cat no. 15140-130 Invitrogen) referred to in text as complete medium. CCLP1 cell lines were cultured in complete DMEM media mix: DMEM (1X) 41965-039 + MIX of 10% FBS, 1% Glutamine, 1% PES; (L-Glutamine 200mM (100X) (cat no. 25030-123, Invitrogen), Penicillin-Streptomycin (PES) Solution (cat no. 15140-130, Invitrogen), Fetal Bovine Serum (FBS) heat inactivated S. America (cat no. 10500064, Invitrogen, Lot no: 07Q0116k)

3.3 Protein synthesis inhibition treatments

HEK293T or CCLP1 cell lines were counted at 4 x 10^5 cells per 1 well of a 6 well plate and resuspended in fresh 2ml of the appropriate medium: HEK29T: (DMEM

1X) high glucose (41965-039), supplemented with: 10% FBS heat inactivated (cat no.10500065 Invitrogen), 1% L-Glutamine (200mM 100X cat no. 25030-123 Invitrogen) and 1% PES solution (cat no. 15140-130 Invitrogen) or CCLP1: complete DMEM media mix: DMEM (1X) 41965-039 + MIX of 10% FBS, 1% Glutamine, 1% PES; (L-Glutamine 200mM (100X) (cat no. 25030-123, Invitrogen), Penicillin-Streptomycin (PES) Solution (cat no. 15140-130, Invitrogen), Fetal Bovine Serum (FBS) heat inactivated S. America (cat no. 10500064, Invitrogen, Lot no: 07Q0116k). 6 well plates were then incubated at 37c o/N, medium was removed during the morning of the following day and replaced with 2ml fresh medium containing CHX treatment at 150 ug/ul (Sigma C7698-5G, stock 5000ug/ul. Cells were incubated at 37c with CHX for 0,3,6,9,12, 24-hour periods then subsequentially lysed in the appropriate buffers for further experimental analysis (CoIP or MLB – see section 2.15).

3.4 JNK activity inhibition treatments

HEK293T or CCLP1 cell lines were counted at 6 x 10^5 cells per 1 well of a 6 well plate and resuspended in fresh 2ml of the appropriate medium: HEK29T: (DMEM 1X) high glucose (41965-039), supplemented with: 10% FBS heat inactivated (cat no.10500065 Invitrogen), 1% L-Glutamine (200mM 100X cat no. 25030-123 Invitrogen) and 1% PES solution (cat no. 15140-130 Invitrogen) or CCLP1: complete DMEM media mix: DMEM (1X) 41965-039 + MIX of 10% FBS, 1% Glutamine, 1% PES; (L-Glutamine 200mM (100X) (cat no. 25030-123, Invitrogen), Penicillin-Streptomycin (PES) Solution (cat no. 15140-130, Invitrogen), Fetal Bovine Serum (FBS) heat inactivated S. America (cat no. 10500064, Invitrogen, Lot no: 07Q0116k). 6 well plates were then incubated at 37c o/N, medium was removed during the morning of the following day and replaced with 2ml of fresh medium containing 5uM of JNK inhibitor (SP600125, CAS no. 129-56-6). Cells were incubated at 37c with SP600125 vs DMSO controls for 0, 12, 24, 48, 72-hour periods then subsequentially lysed in the appropriate buffers for further experimental analysis (CoIP or MLB – see section 2.15).

3.5 Proteasomal inhibition treatments

HEK293T or CCLP1 cell lines were counted and seeded at 2 x 10^6 cells per 10cm plate or 4 x 10^5 in 60mm plates respectively with fresh 2ml of the appropriate medium: HEK29T: (DMEM 1X) high glucose (41965-039), supplemented with: 10% FBS heat inactivated (cat no.10500065 Invitrogen), 1% L-Glutamine (200mM 100X cat no. 25030-123 Invitrogen) and 1% PES solution (cat no. 15140-130 Invitrogen) or CCLP1: complete DMEM media mix: DMEM (1X) 41965-039 + MIX of 10% FBS, 1% Glutamine, 1% PES; (L-Glutamine 200mM (100X) (cat no. 25030-123, Invitrogen), Penicillin-Streptomycin (PES) Solution (cat no. 15140-130, Invitrogen), Fetal Bovine Serum (FBS) heat inactivated S. America (cat no. 10500064, Invitrogen, Lot no: 07Q0116k). Cells were then incubated at 37c o/N, medium was removed during the morning of the following day and replaced with 10ml of fresh medium (HEK293T) or 2ml of fresh medium (CCLP1) containing 20uM of MG132 (Sigma M8699). Cells were incubated at 37c with MG132 vs DMSO controls for 0 to 6-hour periods then subsequentially lysed in ubiquitination buffers for further experimental analysis (CoIP or MLB – see section 2.16). Prior to Co-immunoprecipitation experiments, cell lysates treated with MG132 for ubiquitination analysis.

3.6 - Transient transfections

HEK293T cells are counted and plated at 2 x 10^6 cells per 10cm plate with 5ml of (DMEM 1X) high glucose (41965-039), supplemented with: 10% FBS heat inactivated (cat no.10500065 Invitrogen), 1% L-Glutamine (200mM 100X cat no. 25030-123 Invitrogen) and 1% PES solution (cat no. 15140-130 Invitrogen) during the early afternoon of day one. The following evening (~30 hrs post seed), Day 2, each plate is transfected with a calcium phosphate mix of 30ug DNA, 375ul H_2O, 125ul $CaCl_2$, and 500ul of 2XBBS. Mix is vortexed during preparation and then subsequentially dropped onto each plate in random distribution whilst moving the plate back and forth (total of 1ml mix per plate). Morning of Day 3 (14-16h post-transfection), medium is removed and replaced with 5ml of fresh medium without PBS washing. Day 4 cells are collected, or drug treatment

protocols are carried out (see section 2.3-6). For drug treatments on cells (CHX or JNK inhibitors), HEK293 or CCLP1 cells were grown and seeded in 10 x 60mm plates at 400'000 cells per plate. Concentrations of each drug were made up to the recommended stock concentration by the supplier. We then carried out serial dilutions and examined the toxicity (by cell death) as well as the experimental outcome on the cell population to optimise the drug concentration for further experiments.

3.7 – Lentiviral infections

Lentiviral production: HEK293T cells were plated at 1.5 x 10^6 cells per 10 cm plate at D0 in 5ml of HEK293T medium: (DMEM 1X) high glucose (41965-039), supplemented with: 10% FBS heat inactivated (cat no.10500065 Invitrogen), 1% L-Glutamine (200mM 100X cat no. 25030-123 Invitrogen) and 1% PES solution (cat no. 15140-130 Invitrogen). 20 x 10 cm plates were used for each lentiviral batch production process. At D1 in the late afternoon, cells were transfected using the calcium phosphate method, see section 2.6 for detail. Per plate calcium phosphate mix contained: 375ul dH_2O, 10ug of PwPI vector of interest, 5ug of pMD2G (co-plasmid), 5ug of R8.91 (co-plasmid), 125ul of $CaCl_2$, and 500ul of BBS [2X]. On D2 following transfection, medium was changed for 10ml of fresh medium (described above). Virus-containing medium is collected on D3 in 250ml centrifuge tubes and spun at 15000rpm for 5' at 4°C. 10ml of HEK293T medium is replaced to each plate which are returned to the incubator o/N at 37c. Virus containing medium is filtered using 0.22um GV PVDF membrane 150ml with pump under sterile conditions then stored at -80°C. D4 is a repeat of D3 to collect a second round of virus containing medium. D4 includes lysing of transfected and non-transfected plates to be analysed by FACs analysis for virus concentration sampling. Virus containing medium from Days 3-4 are ultra-centrifuged at 23'000 rpm for 1.5 hours at 4°C following which the medium is removed. New medium is added to the virus pellets and resuspended then stored in -80c for use in further experiments. Lentiviral infections: CCLP1 cells were seeded at 1 x 10^5 in 1 well of 6 well plate on D1 in 2ml of CCLP1 complete medium: DMEM (1X) 41965-039 + MIX of 10% FBS, 1% Glutamine, 1% PES; (L-Glutamine 200mM (100X) (cat no. 25030-123, Invitrogen), Penicillin-Streptomycin (PES) Solution (cat no.

15140-130, Invitrogen), Fetal Bovine Serum (FBS) heat inactivated S. America (cat no. 10500064, Invitrogen, Lot no: 07Q0116k). On D2 all cells were treated with 3ug/ul of Polybrene (Sigma TR-1003) for 15' at 37°C followed by the addition of selected shRNA (see lentiviral production for method) at 10ul. For all cells medium was removed on D3 and replaced with 2ml of fresh medium following which cell growth was observed for the next 2-4 days before further experimentation. Incorporation of lentiviruses into the host cells was quantified by GFP expression using microscopy. Batch lentivirus production was carried out for JNK and PIN1 protein silencing within HEK293 and CCLP1 cell lines. The sequences for both protein targets are outlined in the table below:

shRNA Target	Species	Sequence
PIN1	*Human*	5'-CGGGAGAGGAGGACTTTGA-3'
JNK1/2	*Human*	5'-GATCAGTGGAATAAAGTTA-3'

3.8 - Co-Immunoprecipitation and Phos-tag™

HEK293T cell lysates were resuspended in co-IP buffer containing 40mM Tris-Cl pH7.5, 1% NP-40, 150mM NaCl, 5mM EGTA pH8, 1mM DTT, 2 tablet cocktail inhibitors in 50ml Roche (118735800DI) EDTA free, 10μM chymostatin 0.002mg/ml leupeptin, 0.002 mg/ml antipain, 1mM PMSF, 20mM NaF, and 1mM Na_3VO_4. Following these lysates in co-IP buffer were incubated with 30ul of a 1:1 slurry of protein A/G plus-agarose (Santa Cruz Biotechnology) in the presence of a specific antibody for at least 4h at 4°C. The beads were washed four times with 1ml of lysis buffer and then subjected to SDS-polyacrylamide gel electrophoresis for analysis of existing protein complexes by probing with antibodies specific for proteins potentially bound in complex. For lysates undergoing Phos-tag™ procedures to determine phosphorylated forms, cells were resuspended in modified lysis buffer (MLB): 0.5% NP-40, 100mM NaCl (5M), 50mM Tris-Cl pH 7.5 (1M), 1 tablet of cocktail inhibitor Roche (118735800DI) EDTA free, 50mM NaF, 1mM Na_3VO_4, 30mM Na pyrophosphate, 0.5mM PMSF, 0.002 mg/ml leupeptin, 0.002mg/ml antipain. Lysates in MLB were analysed by SDS-

polyacrylamide gel electrophoresis containing Mn^{2+} with composition based on the Laemmli method.

3.9 - Western blotting and antibodies

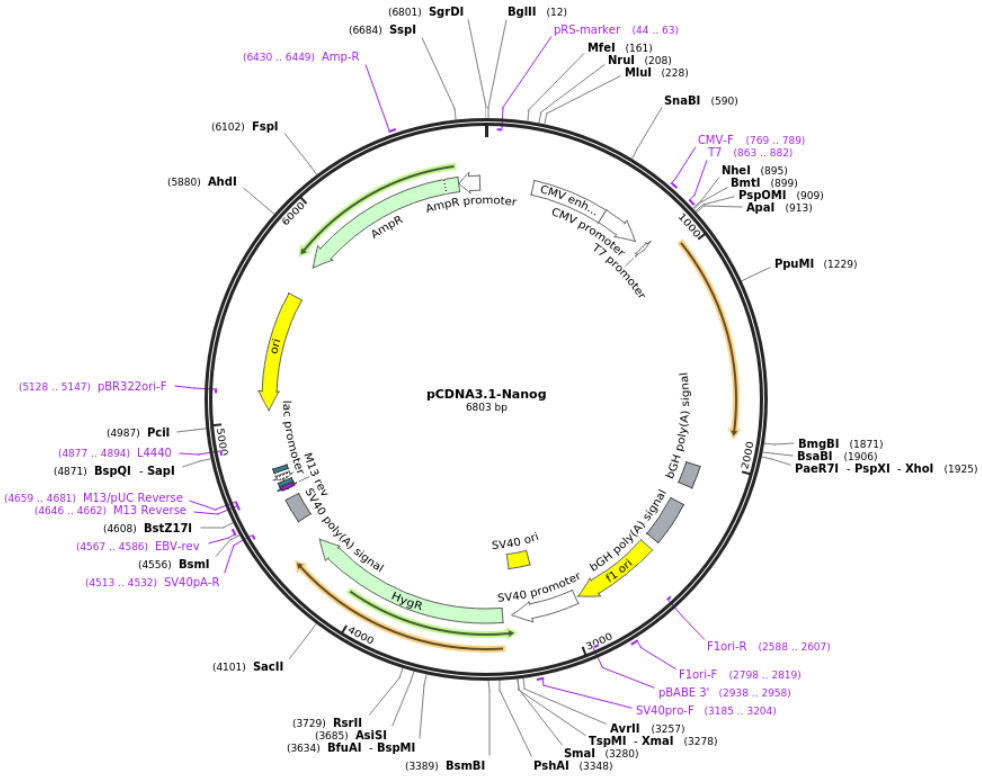
HEK293T cell extracts were resuspended in appropriate buffers (Co-IP or MLB) then protein concentrations were determined using a standard colorimetric assay (Bio-Rad). 10/15µg of protein were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis adjusting the percentage of polyacrylamide depending on expected band size (10% JNK and phospho-active JNK (46-55 kDa), 15% for PIN1 (~18 kDa)), then transferred onto nitrocellulose membrane (Amersham™ Protran™ Premium 0.45uM NC). Immuno-detection procedures were carried out using chemiluminescent methods (Amersham™ ECL reactants) to enhance visualising of secondary HRP-conjugated antibodies; goat anti-mouse IgG (sc-2031 Santa Cruz Biotechnology), and ECL anti-rabbit GE healthcare (cat no. NA9340V) bound to respective primary antibodies: anti-HA-probe (Y-11) (Santa Cruz Biotechnology, anti-FLAG M2 Sigma Aldrich F3165, anti-PIN1 (H-123 sc-15340 Santa Cruz) and phospho-active JNK (cs#9251 Cell Signalling). For all experiments where densitometry analysis was performed through Western Blot bands, results were normalised to control bands (Tubulin). For all Western Blots, control antibodies were included for comparison.

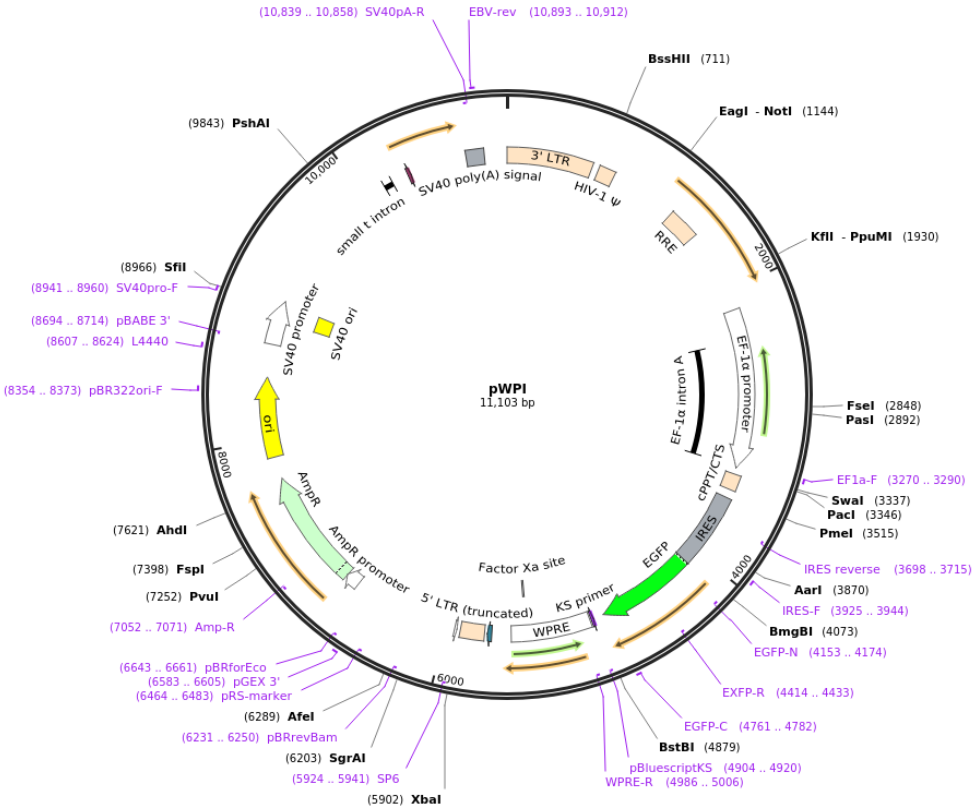
3.10 - Kinase assays

In vitro JNK1 activity on GST-PIN1 Kinase assay was performed using GST alone as control. The in vitro phosphorylation was performed in the presence of [γ-^{32}P] -ATP (Perkin-Elmer) for 40 min at 30°C. Reactions were stopped by the addition of SDS sample buffer, boiled and the phosphorylated proteins were resolved by 10% SDS-polyacrylamide gel electrophorese. The gel was dried and subjected to radiography. Further detail of experiment should be directed towards the supervisor: Salvatore Papa

3.11 – Cloning and mutagenesis

Commercially available plasmid vectors: pcDNA3.1-FLAG-C/ HA-C, pWPI-GFP bicistronic GFP, pGex-2T and pET-28a were previously acquired and digested appropriately to be inclusive of desired complementary DNA (cDNA) between chosen restriction sites. Expression plasmid of constitutive-active JNK1 (JNK^{CA}; LZRS-FLAG-MKK7-JNK1a1) was previously a gift from J. Zhang (*304*) and sub-cloned between BamHI and XhoI sites in pcDNA3.1-FLAG and HA-C expressing vectors. pcDNA-FLAG-PIN1/ HA-PIN1, pWPI-FLAG-PIN1, pET28-PIN1 and pGEX-PIN1 were obtained by sub cloning of the full length human cDNA of PIN1 (NM_006221) insert between restriction sites BamHI/EcoRI, PmeI, BamHI/XhoI and EcoRI/BamHI of expression vectors respectively. All vector maps are shown below. PCDNA3.1 was selected as the cloning backbone when amplifying the quantity of DNA to be stored or used in transient transfection of HEK293 cells. PWPI vectors were selected for infections into cancer cell lines and GFP expression with pET28 vectors allowing for expression of recombinant proteins through bacteria transduction. pcDNA-JNK1 and pcDNA-JNK2 were previously obtained as described elsewhere (*305*) and sub-cloned into pWPI expression vector between PmeI restriction sites. cDNA inserts were ligated into expression vectors with T4 DNA ligase (ThermoFisher).

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EBV-rev (10,893 .. 10,912)
BssHII (711)
EagI - NotI (1144)
KflI - PpuMI (1930)
FseI (2848)
PasI (2892)
EF1a-F (3270 .. 3290)
SwaI (3337)
PacI (3346)
PmeI (3515)
IRES reverse (3698 .. 3715)
AarI (3870)
IRES-F (3925 .. 3944)
BmgBI (4073)
EGFP-N (4153 .. 4174)
EXFP-R (4414 .. 4433)
EGFP-C (4761 .. 4782)
BstBI (4879)
pBluescriptKS (4904 .. 4920)
WPRE-R (4986 .. 5006)
XbaI (5902)
SP6 (5924 .. 5941)
SgrAI (6203)
AfeI (6289)
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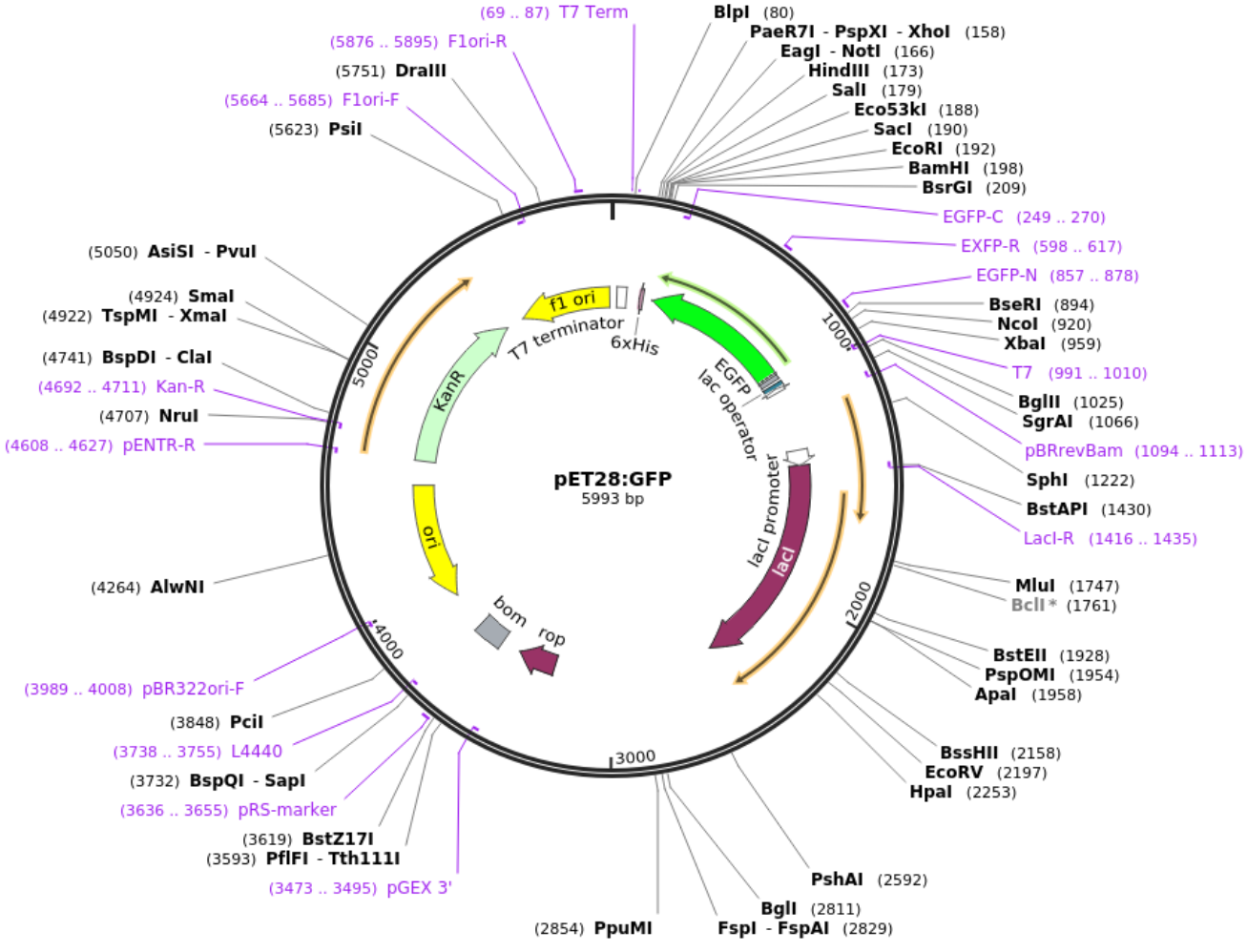
pBRforEco (6643 .. 6661)
pGEX 3' (6583 .. 6605)
pRS-marker (6464 .. 6483)
Amp-R (7052 .. 7071)
PvuI (7252)
FspI (7398)
AhdI (7621)
pBR322ori-F (8354 .. 8373)
L4440 (8607 .. 8624)
pBABE 3' (8694 .. 8714)
SV40pro-F (8941 .. 8960)
SfiI (8966)
PshAI (9843)
3' LTR
HIV-1 ψ
RRE
EF-1α promoter
EF-1α intron A
cPPT/CTS
IRES
EGFP
KS primer
WPRE
Factor Xa site
5' LTR (truncated)
AmpR promoter
AmpR
ori
SV40 promoter
SV40 ori
SV40 poly(A) signal
small t intron
2000
4000
6000
8000
10,000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(69 .. 87) T7 Term
(5876 .. 5895) F1ori-R
(5751) DraIII
(5664 .. 5685) F1ori-F
(5623) PsiI
BlpI (80)
PaeR7I - PspXI - XhoI (158)
EagI - NotI (166)
HindIII (173)
SalI (179)
Eco53kI (188)
SacI (190)
EcoRI (192)
BamHI (198)
BsrGI (209)
EGFP-C (249 .. 270)
EXFP-R (598 .. 617)
EGFP-N (857 .. 878)
BseRI (894)
NcoI (920)
XbaI (959)
T7 (991 .. 1010)
BglII (1025)
SgrAI (1066)
pBRrevBam (1094 .. 1113)
SphI (1222)
BstAPI (1430)
LacI-R (1416 .. 1435)
MluI (1747)
BclI* (1761)
BstEII (1928)
PspOMI (1954)
ApaI (1958)
BssHII (2158)
EcoRV (2197)
HpaI (2253)
PshAI (2592)
BglI (2811)
FspI - FspAI (2829)
(2854) PpuMI
(3473 .. 3495) pGEX 3'
(3593) PflFI - Tth111I
(3619) BstZ17I
(3636 .. 3655) pRS-marker
(3732) BspQI - SapI
(3738 .. 3755) L4440
(3848) PciI
(3989 .. 4008) pBR322ori-F
(4264) AlwNI
ori
rop
bom
lacI
lacI promoter
lac operator
EGFP
6xHis
T7 terminator
f1 ori
KanR
(4607) NruI
(4692 .. 4711) Kan-R
(4741) BspDI - ClaI
(4922) TspMI - XmaI
(4924) SmaI
(5050) AsiSI - PvuI
1000
2000
3000
4000
5000

3.12 - DNA purification and Gel electrophoresis

XL-1 Blue competent cells (Agilent Technologies) (#200130), deficient in endonuclease (endA) and recombination (recA), were transformed by heat shock with ligated plasmid and DNA was purified using Invitrogen mini-prep DNA kits (#K210011). Correct ligations of plasmids containing desired cDNA inserts were checked by restriction enzyme digest combinations: ThermoFisher Fast Digest: XhoI, NotI, PmeI (mssI), PstI, BamHI, EcoRI, Kpn21, BglII, EcoRV, XbaI and BioLabs; XbaI (R0145S) following manufactures instructions to evaluate expected band sizes on 0.6-1% agarose gels with the aid of loading dyes; Xylene and Bromophenol blue (ThermoFisher 6X Gel loading dye cat no. R0611) running at approx. 4000bp and 400bp respectively. Samples of correctly ligated purified DNA from Invitrogen mini prep procedures were then transformed into Invitrogen DH5-alpha strains and grown overnight in LB broth at 37°C 225rpm. The following day, Invitrogen Maxi Prep protocols were followed according to manufacturer's instructions to increase the concentration and volume of purified DNA.

3.13 - Recombinant protein expression and dialysis exchange

His-tagged protein purification was carried out by first transforming of previously discussed pET-28 vectors with desired human full-length PIN1 cDNA into ThermoFisher one shot BL-21(DE3) Chemically competent E.coli responsive to IPTG. Bacteria colonies were grown in LB broth and allowed to reach optimum density (OD=0.375) before administration of IPTG and expression of His-tagged recombinant proteins at 25°C for at least 14hrs. Cell lysates containing recombinant proteins were chemically and physically degraded to lyse cells and resuspended in lysis buffer (50mM NaH_2PO_4 [Sigma Aldrich S8282], 300mM NaCl [Sigma Aldrich S7653], 10mM $MgCl_2$ [Sigma Aldrich M8266], glycerol [Sigma Aldrich G5516], 5mM Imidazole [Sigma Aldrich I5513]) then loaded onto Qiagen Ni-NTA Spin Columns, washed (50mM NaH_2PO_4, 300mM NaCl [Sigma Aldrich S7653], 10mM $MgCl_2$ [Sigma Aldrich M8266], glycerol, 20mM Imidazole [Sigma Aldrich I5513]) and eluted (50mM NaH_2PO_4 [Sigma Aldrich S8282], 300mM NaCl [Sigma Aldrich S7653], 10mM $MgCl_2$ [Sigma Aldrich M8266],

glycerol [Sigma Aldrich G5516], 500mM Imidazole) following manufacture guidelines. Recombinant protein concentration were determined using a standard colorimetric assay (Bio-Rad) and expression checked by SDS-page and commaisse blue staining. After correct expression was determined, purified proteins were loaded into ThermoFisher SnakeSkin™ Dialysis tubing and suspended in Dialysis buffer (0.1M sodium phosphate) at +4°C for 24hrs. For GST protein purification of expression vector pGex-2T-PIN1, selected plasmid were transformed into BL-21 lines and colonies were grown to optimal density (OD between 0.5 -1.0) using Jenway 6305 spectrometer before IPTG administration. Cells were chemically and physically degraded and clear lysates were incubated with a 50% slurry of Glutathione-agarose beads (Sigma cat no. G4510) for at least 2 hrs at +4°C repeating incubation with fresh beads twice more before eluting recombinant protein from beads.

3.14 Buffers

Modified lysis buffer (MLB): of stock 0.5% NP-40 [100%] [Sigma Aldrich 127087-87-0], 100mM NaCl [5M] [Sigma Aldrich S7653], 50 mM Tris-Cl pH 7.5 [1M], 1 cOmplete EDTA-FREE cocktail inhibitor [Roche COEDTAF-RO] per 50ml. Added Fresh each time: 50 mM NAF [1M] [Sigma Aldrich S7920], 1 mM Na_3VO_4 [200 mM] [Sigma Aldrich S6508], 30 mM Napyrophosphate [MW 446.06] [Sigma Aldrich P8010], 0.5 mM PMSF [Sigma Aldrich P7626], 10 mM chymostatin [10 mM] [Sigma Aldrich C7268], 0.002 mg/ml leupeptin [1 mg/ml] [Sigma Aldrich L2884]., 0.002 mg/ml antipain [1mg/ml] [Sigma Aldrich 10791].

Co-IP Buffer: of stock 40 mM Tris-Cl pH 7.5 [1M], 1% NP-40 [100%] [Sigma Aldrich 127087-87-0],, 150 mM NaCl [5M] [Sigma Aldrich S7653], 5 mM EGTA pH 8 [0.2M], 1 mM DTT [1M], 2 cOmplete EDTA-FREE cocktail inhibitor [Roche COEDTAF-RO] per 50ml. Added Fresh each time: 10 uM chymostatin [10mM], 0.002 mg/ml leupeptin [1mg/ml] [Sigma Aldrich L2884], 0.002 mg/ml antipain [1mg/ml] [Sigma Aldrich 10791], 1 mM PMSF [1M], 20 mM NaF [1M] [Sigma Aldrich S7920], 1 mM Na_3VO_4 [200 mM] [Sigma Aldrich S6508].

Averil's Buffer: of stock 20 mM HEPES pH 8.0 [1M] [Sigma Aldrich H3375], 350 mM NaCl [5M] [Sigma Aldrich S7653], 20%

3.15 Glucose consumption

Glucose consumption of CCLP1 cells lines treated with lentiviral transfections of shNS and shPIN1, as well as shPIN1 + reconstituted shRES-PIN1-WT, shPIN1 + reconstituted shRES-PIN1(S115A), and shPIN1 + pWPI control [10ul lentiviral + 60ul of shRNA] were compared. All cell conditions were carried out in both experimental and biological triplicates (total n=9). Each cell condition were seeded at D0 at densities of 1 $x10^4$ in 6 well tissue culture sterile plates with a volume of 2ml of complete DMEM media mix: DMEM (1X) 41965-039 + MIX of 10% FBS, 1% Glutamine, 1% PES; (L-Glutamine 200mM (100X) [cat no. 25030-123, Invitrogen], Penicillin-Streptomycin (PES) Solution [cat no. 15140-130, Invitrogen], Fetal Bovine Serum (FBS) heat inactivated S. America [cat no. 10500064, Invitrogen, Lot no: 07Q0116k]. At D1-D3 after infection, cells were reseeded at 80'000 cells per 6 well plate in 1ml of DMEM 41965-039 + MIX. 8 hours post reseed, medium was changed to a fresh 1ml of the same DMEM mix. Following this, after 72 hours from the change of medium, 100ul samples of the medium were collected, spun at 3500rpm for 3 minutes, then frozen at -80c. All cell conditions were simultaneously collected using trypsin and cells from repeat experiments of the same condition were pulled together and lysed in equal buffer of MLB between all conditions, snap frozen at -80°C, then collected the supernatants. Supernatants for each condition were then ran on a Bradford assay to quantify protein concentrations used for equalising results of the glucose assay kit (GAGO-20) Sigma. Standard curves were prepared using complete DMEM (41965-039 + MIX) shown in the table 1.

Table 1 – Standard curves for glucose assay

Standard	Glucose (nmol)	Ul medium / ul standard	H_20 (ul)
S1	25 nmol	10 ul medium	390 ul
S2	20 nmol	240 ul S1	60 ul
S3	15 nmol	150 ul S2	50 ul
S4	10 nmol	133.2 ul S3	66.8 ul
S5	5 nmol	100 ul S4	100 ul
S6	2.5 nmol	100 ul S5	100 ul
S7	1.25 nmol	100 ul S6	100 ul
Blank	0 nmol	0	100 ul

1ul of each supernatant condition was used per well in a 96 well plate of the glucose assay kit (GAGO-02). 96 well plates were left at 37°C for 30 minutes covered by foil, then 80ul of 12N H_2SO_4 was added to each well, shaken for 10 minutes and the optimum density (OD) was acquired

Chapter 4 Results: Active JNK interacts with PIN1 and phosphorylates residue S115

4.1 Phospho-active JNK is highly expressed in liver cancer

In support of the findings that both phospho-active JNK and PIN1 are often highly expressed in a multitude of cancer tissues, we sought to identify the expression of both proteins in liver cancer cell lines. In light of the wider and more pressing need to differentiate liver cancers by molecular classification of their subtypes, we set out to determine the expression of these proteins across divergent liver cancer classifications. Hepatocellular carcinoma (Hep3B) and cholangiocarcinoma (CCLP1, SG231, HuCCT-1) cell lines were thereby cultured and lysed to quantify the endogenous expression of both proteins in question. Amongst the four liver cancer lines assessed, PIN1 expression showed large variability; depicting a higher PIN1 protein expression in Hep3B and CCLP1 lines than SG231 and HuCCT-1 counterparts (Figure 4.1). This finding exemplified the often-drastic variability within diseased livers and the need for better molecular classification to address this. On the other hand, the expression of phospho-JNK was noticeably elevated in all cancerous tissue samples suggestive of an intrinsic need for high phospho-JNK in all liver tumour tissues assessed. Given prior data confirming an interaction between active JNK and PIN1 in MM, we moved forward to assess whether similar interactions would occur between JNK and PIN1 in liver cancers.

4.2 Silencing of JNK reduces PIN1 protein expression in liver cancer

Phospho-active JNK is often found to have both dysregulated activity and/or expression in cancers, leading to alterations to downstream substrate functions

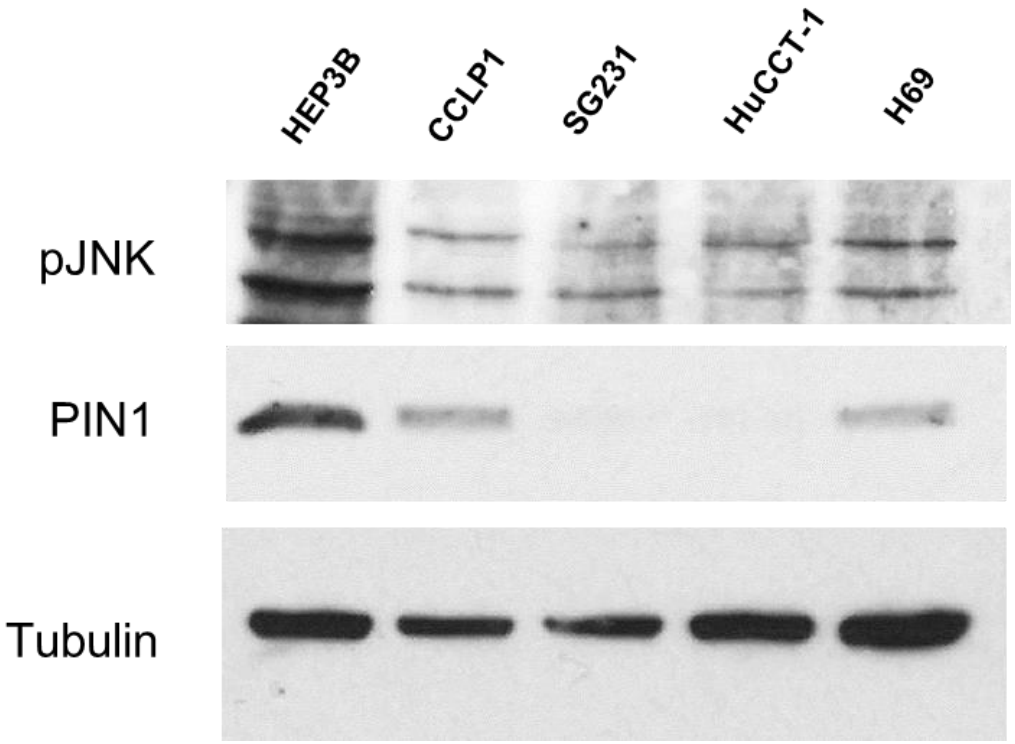


Figure 4.1: Endogenous expression of p-JNK and PIN1 in liver and bile duct cancers.: hepatocellular carcinoma (Hep3B), cholangiocarcinoma (CCLP1. SG231, and HuCCT-1), and non-tumour (H69) cell lysates were subjected to western blot analysis using antibodies against; phospho-JNK, Total-JNK (TOT-JNK), and PIN1 to determine expression in all cell lines. Each cell line was lysed in the same MLB buffer and loaded at equal concentrations (ug/ul) into the western blot. Antibodies against tubulin were used as loading controls. Experiment has been conducted in triplicate and all antibodies were probed onto the same gels.

critical for the cellular outcome. Given the high phospho-active JNK expression found in liver cancers (Figure 4.1), we sought to understand how silencing of phospho-active JNK signals may alter PIN1's protein expression in HEP3B and CCLP1 liver cancer subsets. These cell lines were selected due to their enhanced expression of PIN1 comparable to SG231 and HuCCT-1 lines. We therefore knocked down *JNK1/2* in Hep3B and CCLP1 cell lines using lentiviral infections to stably transduce *shJNK1/2* into the human cell lines. As the introduction of lentivirals causes subsequent stress and frequent upsets in the homeostasis of the cell, a non-specific shRNA (*shNS*) was used as a control to account for these side effects. All cell lines were then cultured for five days post infection to allow for a stable integration of the knock down. This knockdown was then confirmed by western blot, probing for JNK expression (Figure 4.2)

Both CCLP1 and Hep3B cells showed a decrease in PIN1 protein expression following treatment with *shJNK1/2*. This outcome was not reciprocated in *shNS* control conditions (Figure 4.2). These results supported our preliminary findings in MM (not shown) and was suggestive of a novel mechanism in liver cancer cells between JNK and PIN1 proteins. Given our prior knowledge that JNK does not regulate *PIN1* at a transcriptional level (results not shown), we hypothesised that a post-translational interaction between JNK and PIN1 may be occurring in tumorigenic liver tissues. We therefore directed our next experiments to answer this question.

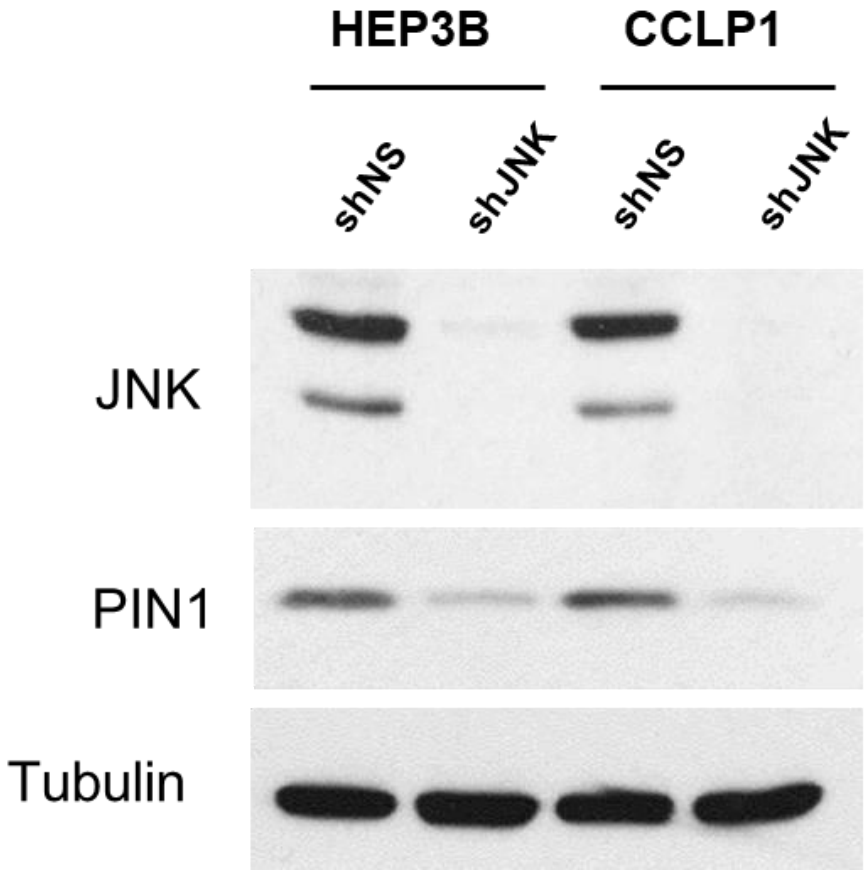


Figure 4.2: Silencing of JNK reduces PIN1 protein expression in Liver cancer: Selected hepatocellular carcinoma and cholangiocarcinoma cell lines; Hep3B and CCLP1 respectively, were subjected to lentiviral *shJNK1/2* or *shNS knock* downs. Five days following lentiviral infections, both cell lines treated and untreated with shRNA were lysed in the same buffer and a BSA test was used to quantify the lysate concentrations (ug/ul). All lysates were then loaded at equal concentrations onto a western blot and probed with antibodies against PIN1 and JNK expression. Antibodies for tubulin expression were used for controlled loading. Experiment has been conducted in triplicate.

4.3 Constitutively Active JNK interacts with PIN1

To elucidate an endogenous interaction between phospho-active JNK and PIN1 in liver cancers, we chose to lyse cholangiocarcinoma cell lines (CCLP1) in the appropriate buffers and carry out an immunoprecipitation (IP); pulling down with active-JNK antibodies and probing by western blot to detect subsequent PIN1 interactions. We opted to take forward the CCLP1 cell line for the majority of our experiments over Hep3B. Despite both cell lines potentially fitting the hypothesis of a post-translational interaction (Figure 4.2); less was researched of the cholangiocarcinoma and limitations in project time would prevent the repetition of our experiments in too many cell lines. Despite our best efforts, we struggled technically to uncover an endogenous interaction between active-JNK and PIN1 (Results not shown). This was a consequence of the unavailability of appropriate active-JNK and PIN1 antibodies that could successfully IP the endogenous proteins in question.

Therefore, to confirm the interaction, we opted to conduct over-expression studies using tagged proteins (HA, FLAG tags) to confirm the mechanistic interaction between phospho-active JNK and PIN1 exclusive of cancer tissues. Using transient transductions in HEK293 cells, we co-expressed a FLAG tagged constitutively active JNK (FLAG-MKK7-JNK1) and HA tagged PIN1 (HA-PIN1). All cells were then lysed, quantified and immunoprecipitation was carried out using FLAG-conjugated beads to pull down constitutively active JNK with HA antibodies used for the detection of PIN1 expression. Our findings confirmed a mechanistic interaction between constitutively active JNK1 and PIN1 (Figure 4.3).

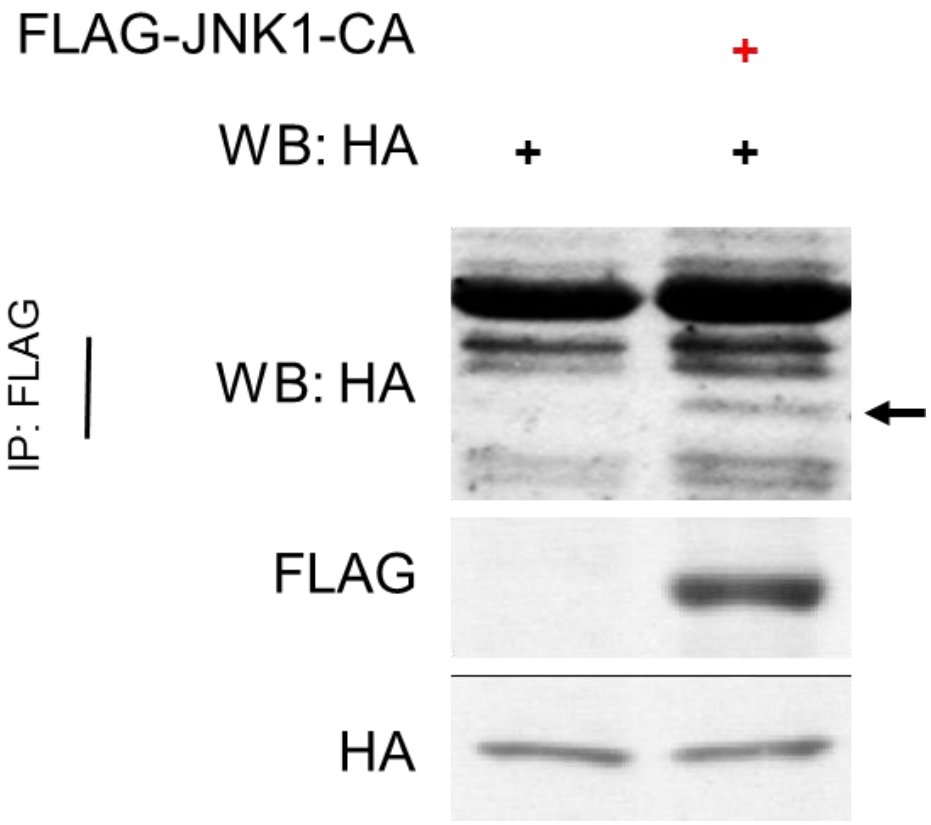


Figure 4.3: Constitutively active JNK interacts with Pin1 / Overexpression in HEK293: HEK293 cells were transiently transfected with a combination of FLAG-JNK1-CA and HA-PIN1 plasmids and left for 48hrs to express the exogenous proteins. All HEK293 cells were then lysates in the same buffers and a BSA test was carried out to quantify lysate concentrations (ug/ul). Immunoprecipitation was then performed on both lysates using equal volumes of FLAG-M2 conjugated beads to subsequently pull-down FLAG tagged proteins. Post washing and boiling of the beads for loading into a WB analysis, antibodies were used to probe for HA expression by western blots. Total expression of FLAG and HA are shown from original lysates as controls and black arrow indicates the detection of HA-PIN1 in the pull-down. Experiments are replicated in triplicate.

4.4 PIN1 interacts with both active JNK isoforms

Due to the history of JNK isoforms (JNK1 and JNK2) carrying out divergent and often opposing effects in both the normal physiological state of cells, we agreed to extend the previous over-expression studies to explore interactions of PIN1 with both JNK isoforms. The initial data in MM had reported a post-translational effect on PIN1 attributed solely to the JNK2 isoform. We therefore deemed it important to understand whether under normal physiological conditions PIN1 could mechanistically bind to both isoforms of JNK. In a similar manner to the last experimental design, we over-expressed HA-tagged PIN1 in HEK293 cells but now with the considerations of both FLAG-tagged constitutive active JNK1 and JNK2 isoforms. The results showed that PIN1 did in fact interact with both JNK isoforms when over-expressed in HEK293 cells, showing no preference for any particular isoform (Figure 4.4). Collectively with the preliminary findings in MM, this data suggests the oncogenic environment of cancer tissues can result in specific JNK isoforms preferentially binding to PIN1 in a tissue-specific manner. This is not surprising given the disparity of JNK signalling mechanisms in cancer that are already widely appreciated. What remained unclear at this stage, was the specificity surrounding the mechanism by which phospho-active JNK interacts with PIN1. In both liver cancer and over-expression conditions the interaction was evident, however given the cellular environment for both experiments, it remained unknown whether this interaction was direct or through the aid of other proteins present in the cell's environment creating scaffold formations to aid binding.

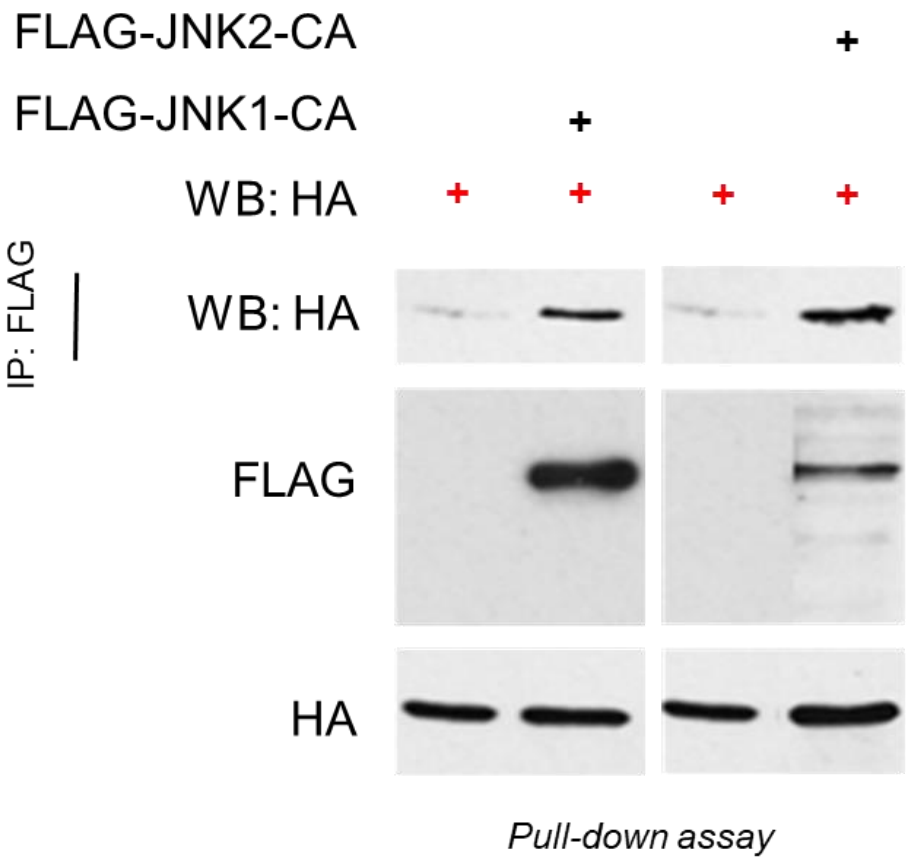


Figure 4.4: Pin1 interacts with both constitutively active forms of JNK by overexpression in HEK293 cells: HEK293 cells were transiently transfected with a combination of FLAG-JNK1-CA, FLAG-JNK2-CA and HA-PIN1 plasmids and left for 48hrs to express the exogenous proteins. All HEK293 cells were then lysates in the same buffers and a BSA test was carried out to quantify lysate concentrations (ug/ul). Immunoprecipitation was then performed on both lysates using equal volumes of FLAG-M2 conjugated beads to subsequently pull-down FLAG tagged proteins. Post washing and boiling of the beads for loading into a WB analysis, antibodies were used to probe for HA expression by western blots. Total expression of FLAG and HA are shown from original lysates as controls, with the top gel displaying the outcome of binding between HA-PIN1 and both FLAG-JNK isoforms. Experiments are replicated in triplicate.

4.5 Recombinant protein production method for HIS-PIN1

To elucidate an answer to this question, an *in vitro* interaction experiment using recombinant proteins was required. Doing so ordered for us to design and purify a recombinant PIN1 protein to be used in a direct recombinant pull down experiment with a previously gifted recombinant active JNK protein (see references). We therefore designed a histidine (HIS)-tagged PIN1 plasmid supported in a vector (pET-28) containing the lac operon (Figure 4.5). The plasmid was transfected into *E. coli* strains that were grown to an optimum density for an IPTG induction (synthetic lactose mimic) allowing for the translation of recombinant PIN1 proteins in the bacterial cells. After expression of the recombinant proteins for 24 hours at 25c, the proteins were then lysed from the bacterial cells and purified by chemical and sonication methods. Following this, all proteins underwent an exchange of buffers to produce stable recombinant proteins free of bacterial contaminants, suitable for an *in vitro* interaction experiment. All recombinant proteins generated were shown to be of accurate kDa and significant concentration through western blot analysis.

4.6 Phospho-Active JNK interacts with PIN1 *in vitro*

With both recombinant proteins now in our arsenal, to query the mechanism of interaction between Active-JNK and PIN1, we incubated both active recombinant JNK with His-tagged PIN1 proteins under *in vitro* conditions. Following incubation of both proteins, we pulled down the recombinant JNK using total JNK antibodies combined with A/G beads and probed for the presence of PIN1 by western blot analysis. The findings concluded that active JNK interacted directly with PIN1 *in vitro,* supportive of a protein-protein confirmation that does not require the presence of any other subsidiary cellular-residing proteins to occur (Figure 4.6).

4.7 Inactive JNK does not interacts with PIN1 *in vitro*

Thus far we had spent our efforts understanding the mechanistic interaction surrounding active JNK with PIN1 through overexpression and *in vitro* techniques. Given the extensive literature surrounding the dysregulation of active-JNK within cancers, these results strongly suggest that PIN1 may be a target of active-JNK in liver cancers. However, prior to interrogation in liver cancer cell lines, we decided that it would be useful to understand if this interact was an outcome of JNK's active structural state. We were therefore curious to understand if the described interaction between phospho-active JNK and PIN1 also occurred in the absence of JNK activity. To find out, we produced a recombinant GST tagged inactive JNK protein (GST-JNK1) and immunoprecipitated by GST in the presence or absence of His-PIN1. After incubation of GST-JNK with His-PIN1 *in* vitro, using GST as a control for non-specific binding sites, we noted that PIN1 did not interact with the inactive form of JNK *in vitro* (Figure 4.7). These results indicated that the interaction between both proteins is exclusive to the activity of JNK however these results do not rule out the potential interaction of inactive JNK with PIN1 through subsidiary proteins in the cell.

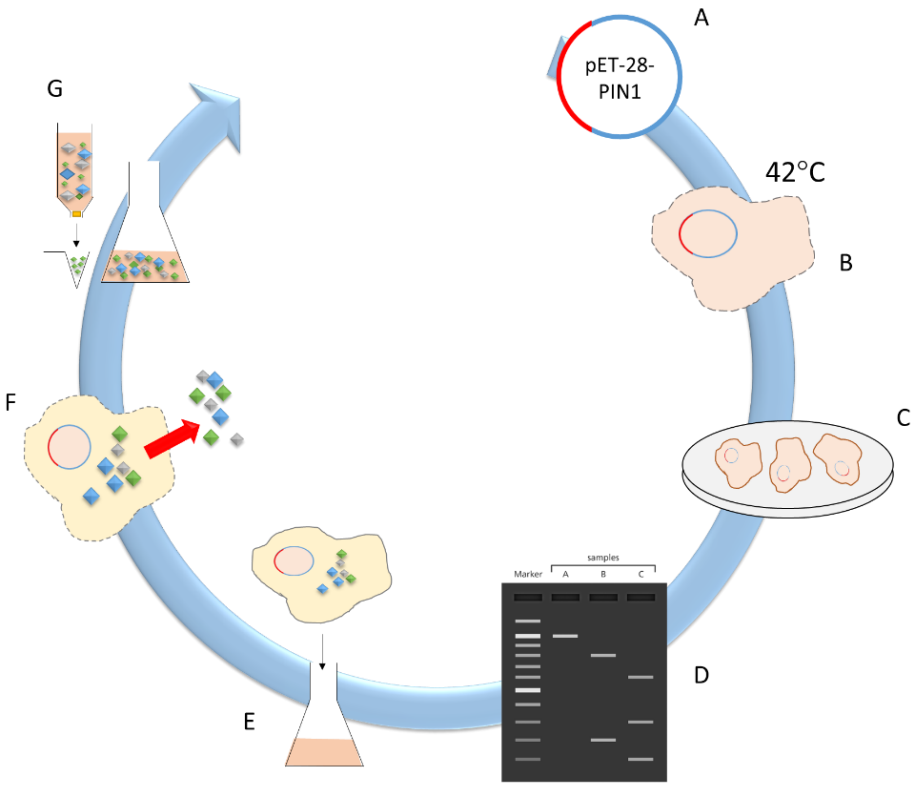


Figure 4.5: Annotation depicting the Process for purification of His-tagged Pin1 Recombinant Proteins (a) PIN1 cDNA is isolated from previous vectors through specified digestion with restriction enzymes. Results of the digest are then run on an agarose gel to extract and elute the purified PIN1 cDNA before using in a ligation reaction with pET28 vectors **(b)** Ligated plasmids are transformed into XL-1 blue E.coli cells through heat shock at 42°C to increase volumes of plasmid DNA **(c)-** Colonies were grown overnight at 37c and 10 colonies were picked for mini-prep testing. **(d)-** Selected Mini preps were checked for the correct ligation of DNA by gel electrophoresis. **(e)** Correctly ligated PIN1 DNA in the pET28 vector are then transformed into BL-21 lines containing inducible Lac operon. **(f)-** At the optimum growth for the bacterial cells after inoculation of the overnight culture into 1L of bacterial medium ITPG (lactose mimic) is administered to induce expression of recombinant proteins within the bacterial culture. **(g)-** After overnight expression at 25c, cell culture are lysed, and column purification techniques then allowed for the separation of recombinant protein (green diamonds) from other bacterial proteins in the supernatant. Recombinant proteins finally underwent buffer exchange ($P0_4$-) and were tested for concentration and correct kDa by western blot analysis.

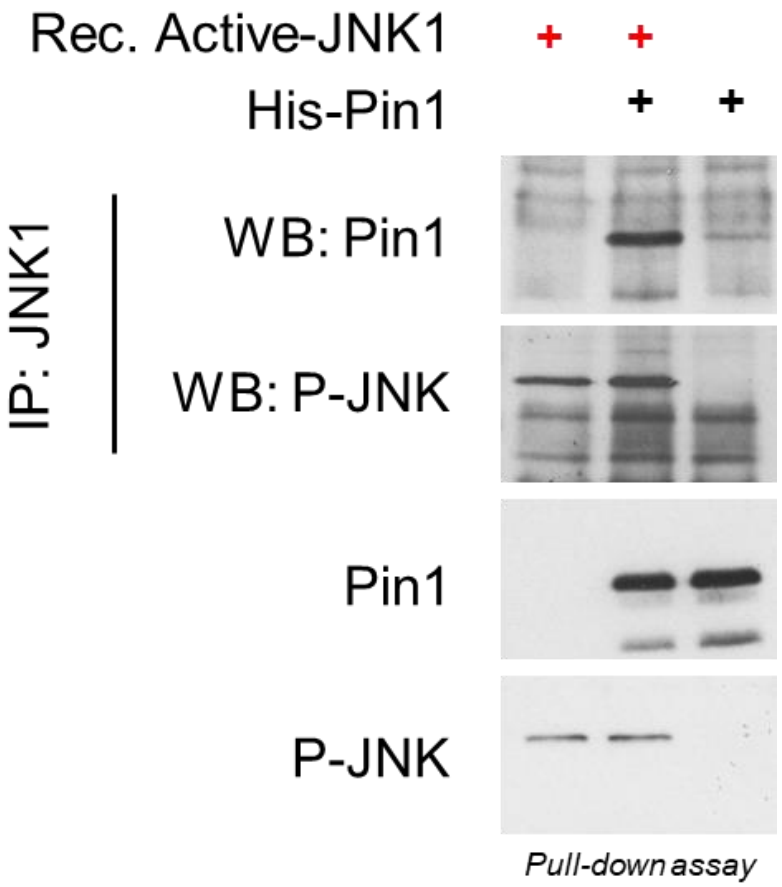


Figure 4.6: Recombinant Proteins phospho-active JNK and His-PIN1 interact *in vitro*: Recombinant active JNK1 (14-327 Sigma-Aldrich; JNK1α1/SAPK1c) was incubated with His-tagged Pin1 recombinant protein in appropriate buffers at equal loading concentrations (see methods). Pull-down of recombinant active JNK by JNK antibodies allowed for detection of PIN1 binding (middle lane) after probing with antibodies to detect Pin1 through western blot analysis. (Bottom) Western Blots for Pin1 and Phospho-JNK show a controlled concentration of both recombinant proteins loaded. Results are representative of three experimental repeats.

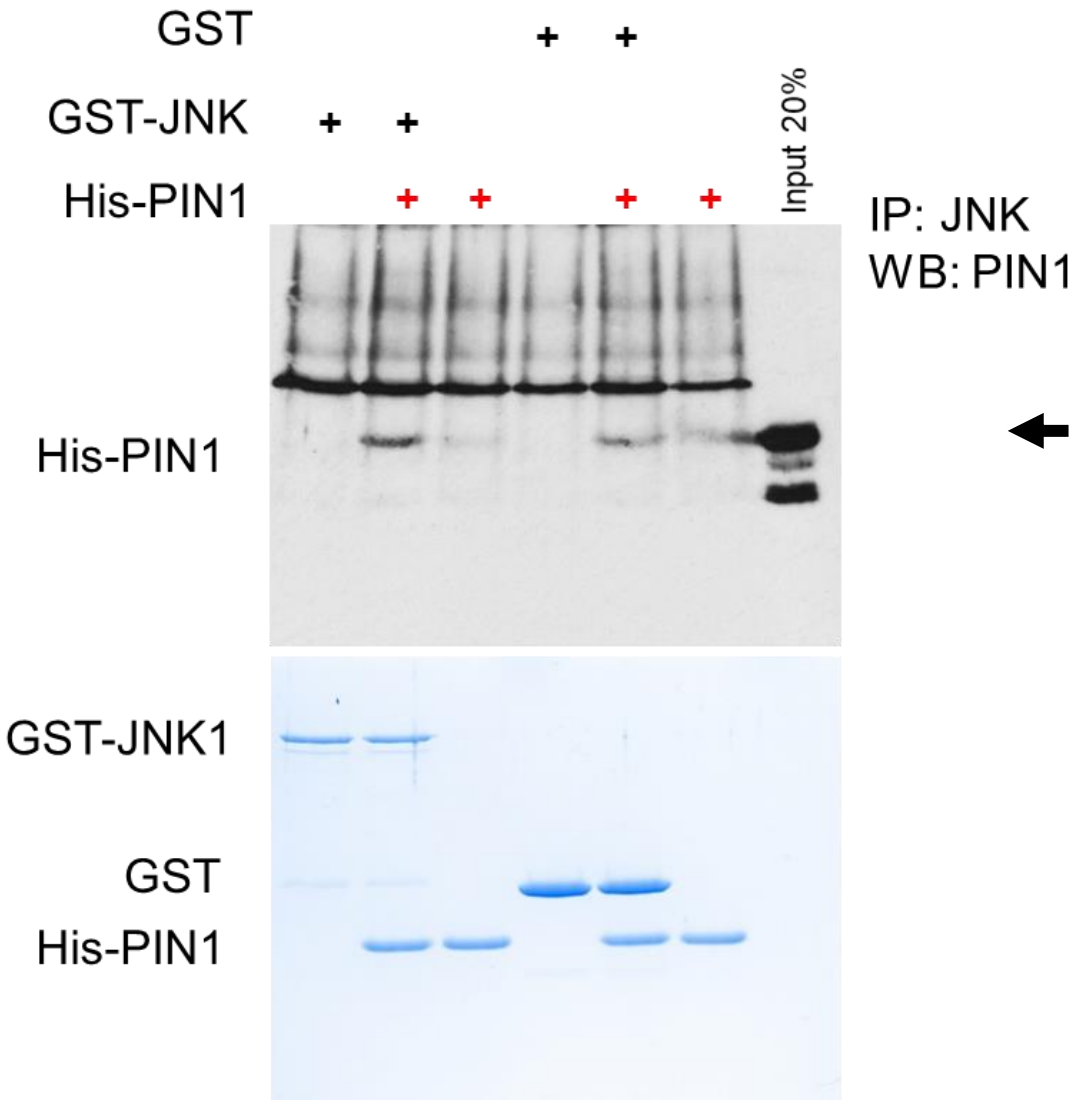


Figure 4.7: Total JNK does not interacts with PIN1 *in vitro*: (Top) Immunoprecipitation of recombinant protein GST-JNK post incubation with His-tagged PIN1 recombinant proteins in appropriate buffers and at equal concentrations loaded. GST recombinant proteins were used as an immunoprecipitation control in the same buffers. Post immunoprecipitation using Total JNK antibodies, western blot analysis was used to detect PIN1 presence through western blot analysis. Coomassie blue gel (bottom) indicates equal loading controls for all recombinant proteins, and western blot includes positive control of His-PIN1 input (20% total load). Experiment is representative of three experiments.

4.8 Phospho-active JNK phosphorylates PIN1 *in vitro*

Our results now confirmed a direct conformational interaction between active JNK and PIN1. Consequentially this data led us to query how such an interaction may affect the role of PIN1 post-translationally in the cell. Given the notorious kinase activity of active JNK proteins, we first explored the possibility that JNK was not only interacting with PIN1 but was also resulting in its phosphorylation. With that being said, using the previously mentioned recombinant proteins of active JNK and His-PIN1 (Figure 4.5), we performed a kinase assay to measure the potential for active JNK to catalyse the transfer of phosphate groups onto the PIN1 substrate. A commaisse gel was present to indicate equal loading of active JNK and His-PIN1 proteins. We discovered that the kinase assay of JNK was shown to phosphorylate PIN1 *in vitro* (Figure 4.8), a result that was not seen in control experiments using active JNK or PIN1 proteins alone.

4.9 Both active JNK isoforms phosphorylates PIN1 *in vitro*

Shortly after confirming the capability of active JNK to phosphorylate PIN1 *in vitro*, it occurred to us that our previous experimental design had not been mindful of questioning this phosphorylation by both JNK isoforms. The last experiment in question was also lacking a positive control to confirm the kinase activity of active JNK recombinant proteins. In light of this, more recombinant proteins were designed including both JNK1 and JNK2 isoforms as well as a GST-tagged c-JUN substrate; a known substrate to be phosphorylated by active JNK. When carrying out the newly designed kinase assay, the findings showed clearly that PIN1 could be phosphorylated by both JNK isoforms *in vitro*, suggestive of a mechanistic affinity of both isoforms for PIN1 in this context (Figure 4.9). The same outcome was not found for active JNK's activity on GST control proteins alone.

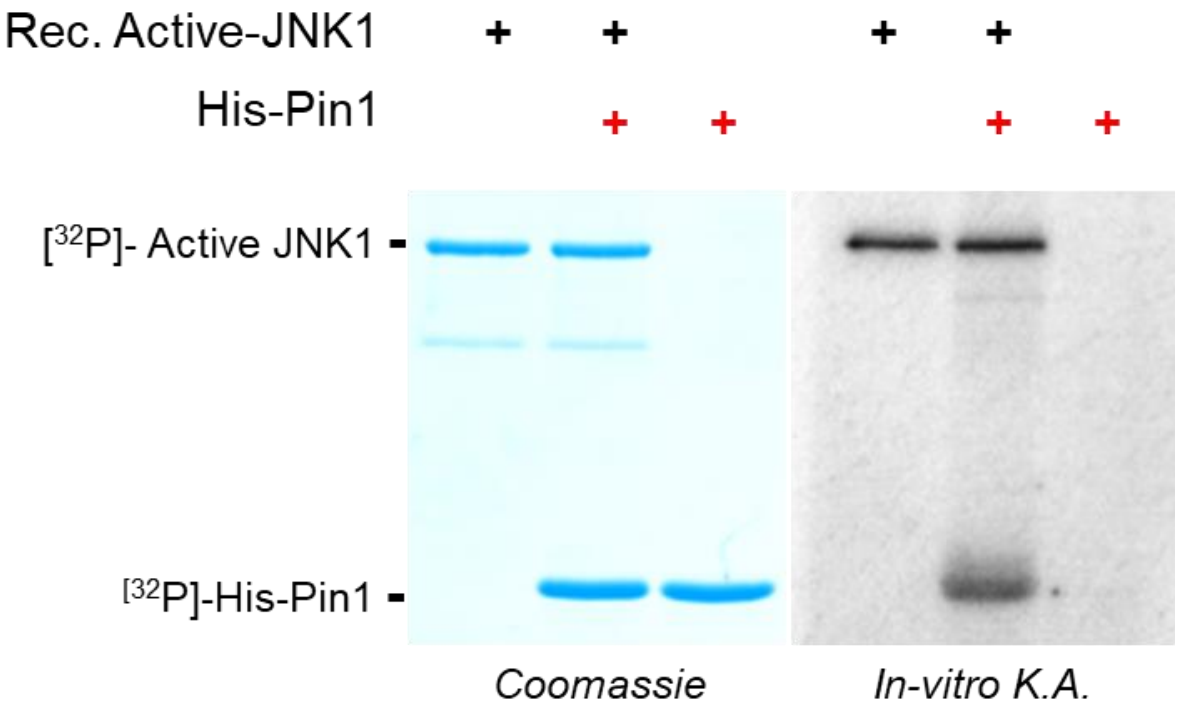


Figure 4.8: Recombinant active-JNK phosphorylates PIN1 *in vitro:* Kinase activity of phospho-active JNK shows phosphorylation of His-tagged PIN1 through incorporation of radioactive labelled phosphate groups in the presence of active-JNK only. Rec.Active JNK1 was incubated in varied combinations with His-PIN1 *in* vitro prior to subjection to kinase assay techniques. (Right) Coomassie Blue shows equal loading of both recombinant active JNK1 And His-PIN1. (Left) No activity can be observed in control groups of Active-JNK alone and His-Pin1 alone on when in combination of both active JNK1 and His-PIN1 (Middle lane). Results show a repeat of at least three individual experiments. Kinase assay technique was performed by Salvatore Papa due to limited authorisation to conduct kinase work. All recombinant protein work was carried out by me to supplement this work.

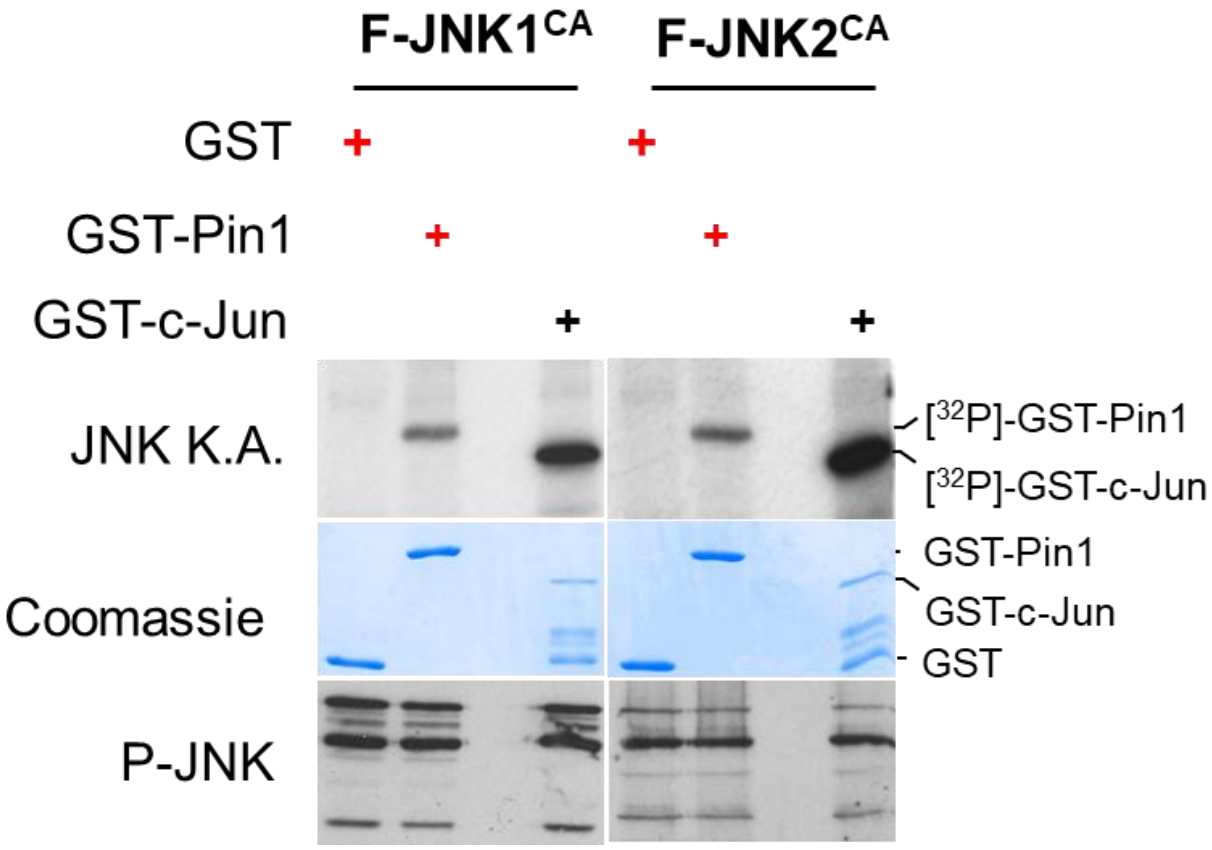


Figure 4.9: Both isoforms of phospho-active JNK phosphorylates PIN1 *in vitro:* Both Phospho-active JNK1 and JNK2 from HEK293 cell extracts phosphorylate GST-tagged PIN1 recombinant proteins. Recombinant proteins of GST, GST-PIN1, GST-c-Jun, FLAG-JNK1-CA, and FLAG-JNK2-CA were incubated in various combinations prior to subjection to kinase assay experiments. The activity of both JNK isoforms are confirmed through the incorporation of radio-actively tagged ATP to known downstream substrate c-JUN (GST-c-JUN). GST alone is used as a control showing no incorporated activity. Coomassie blue staining depicts controlled protein expression. Western Blots for Phospho-JNK expression in cell extracts used as controls. Results show a repeat of three individual experiments. Kinase assay technique was performed by Salvatore Papa due to limited authorisation to conduct kinase work. All recombinant protein work was carried out by me to supplement this work.

4.10 Phos-Tag™ method for recognizing phosphorylated protein forms

Still absorbed with questions surrounding the phosphorylation of PIN1 by active JNK, we were curious if we could further uncover specifics of this interaction, both from a mechanistic viewpoint as well as specifically in liver cancer cells. To address the former, we adopted a Phos-Tag™ method for quantifying phosphorylated forms of proteins through western blot analysis (Figure 3.10). In brief, the integration of positive metal ions (Mn^{2+} and Zn^{2+}) into a western blot matrix and sub sequential running of phosphorylated proteins through the matrix, allowed for the slowed migration (shift) of proteins containing PO_4^- groups due to their interaction with the positive ions embedded in the gel matrix. As a result, phosphorylated forms of the protein are shifted respective of their degree of phosphorylation.

4.11 Constitutive-active JNK phosphorylates PIN1 at one major site

We set out to apply this technique to an over-expression experiment, analysing the effects of FLAG-tagged active JNK vs inactive JNK on the phosphorylation of HA-tagged PIN1. We found that only co-expression of the active form of JNK with PIN1 resulted in the presence of a shifted band, indicative of a phosphorylated PIN1 form (Figure 4.11). This phosphorylated band was confirmed by its degradation in the presence of a calf intestine phosphatase (CIP); an enzyme that actively targets phosphorylated proteins and breaks them down. What's more, the fact that only one phosphorylated band of PIN1 was present with active JNK, suggested the phosphorylation was occurring at one major residue in PIN1's protein structure. With these results in mind, we went on question the phosphorylation of PIN1 by active JNK in cholangiocarcinoma.

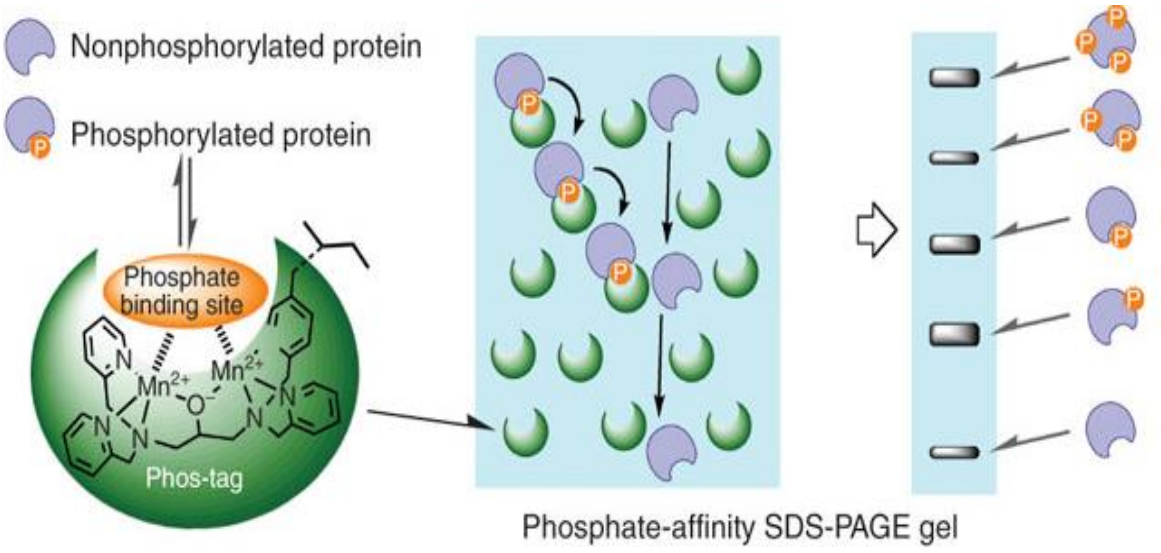


Figure 4.10: Annotation of the methods of Phos-Tag to bind phosphorylated forms of protein: Annotation depicting the use of a phosphate binding molecule (Phos-Tag^{TM}) containing Mn^{2+}or Zn^{2+} molecules embedded into SDS- gels (blue background) to capture phosphorylated proteins and reduce the speed of motility. Consequential addition of +1 charge by binding of Phos-Tag^{TM} molecules results in a shifted band indicating phosphorylated forms. Proteins with higher levels of phosphorylation can also be detected and visualised by increased shifts of bands on SDS-page

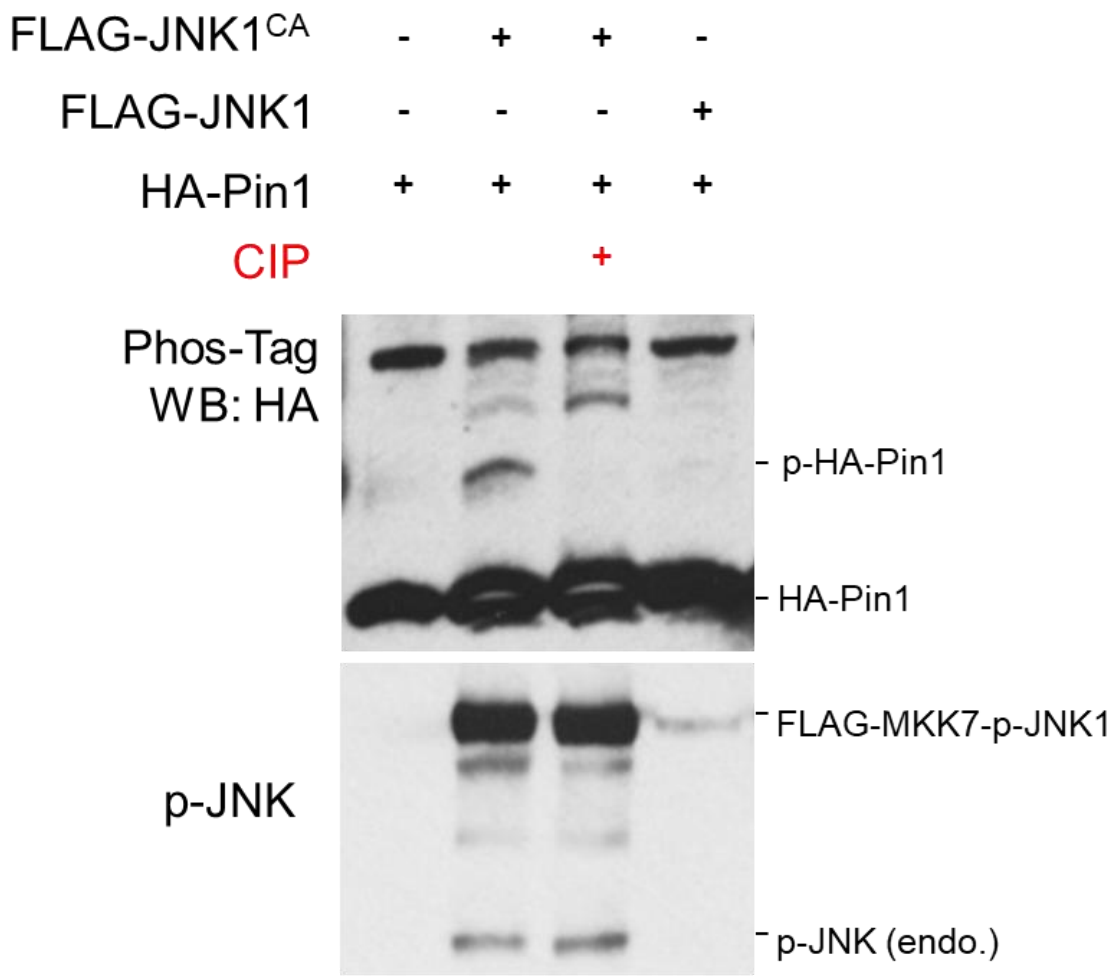


Figure 4.11: Constitutive-active JNK phosphorylates Pin1 by overexpression: HEK293 cells were transiently transfected with a combination of FLAG-JNK1-CA, FLAG-JNK1, HA-Pin1 plasmids and incubated for 48hrs before all cells were lysed in the appropriate buffers. Calf intestine phosphate (CIP) was added as a control to assess phosphorylation bands vs a controlled lane. Phos-Tag™ Western Blot using antibodies against HA indicates Pin1 is phosphorylated in the presence of constitutively active JNK. Phosphorylation is depleted upon addition of calf intestine phosphate (CIP) and no phosphorylation is observed in the presence of the non-active form of JNK or with HA-Pin expression alone. Antibodies probing against p-JNK depict controlled loading expression**.** Experiment shown is representative of three repeats.

4.12 Phospho-active JNK's phosphorylation of PIN1 occurs in cholangiocarcinoma

Still absorbed with questions surrounding the phosphorylation of PIN1 by active JNK, we turned our attentions to whether the mechanistic protein-protein interaction would also result in PIN1 phosphorylation within the context of cholangiocarcinoma. To this end an elegantly designed Phos-Tag™ approach was used in CCLP1 cell lines treated with or without SP600125 and/or shRNA specific for a JNK1/2 knockdown. In this manner, we could explore whether both the activity and expression of active JNK resulted in the endogenous phosphorylation of PIN1. Our findings indicated that when either phospho-JNK was inhibited, or its expression was silenced, there was a reduction in the endogenous phosphorylation of PIN1 (Figure 4.12). These results confirmed that both the activity and expression of JNK affect the phosphorylation of endogenous PIN1 in cholangiocarcinoma.

4.13 Mass spectrometry identified active JNK to phosphorylate PIN1 at Serine 115.

Now eager to uncover the specific site of PIN1 phosphorylation by active JNK, we decided to collaborate in order to analyse the outcome of active JNK's phosphorylation of PIN1 by mass spectrometry. Doing so required for us to send both recombinant proteins in various combinations and patiently wait for the return of the data, data that would hopefully elucidate a specific site of PIN1 phosphorylation. Upon receiving the mass spectrometry analysis, we were pointed towards a specific site of PIN1 phosphorylation by active JNK, markedly a serine at position 115 (S115) of the PIN1 amino acid sequence (Figure not shown). Further analysis of the PIN1 protein sequence revealed that immediately adjacent to the S115 was another serine positioned at 114 (S114). Initially this finding gave rise to some

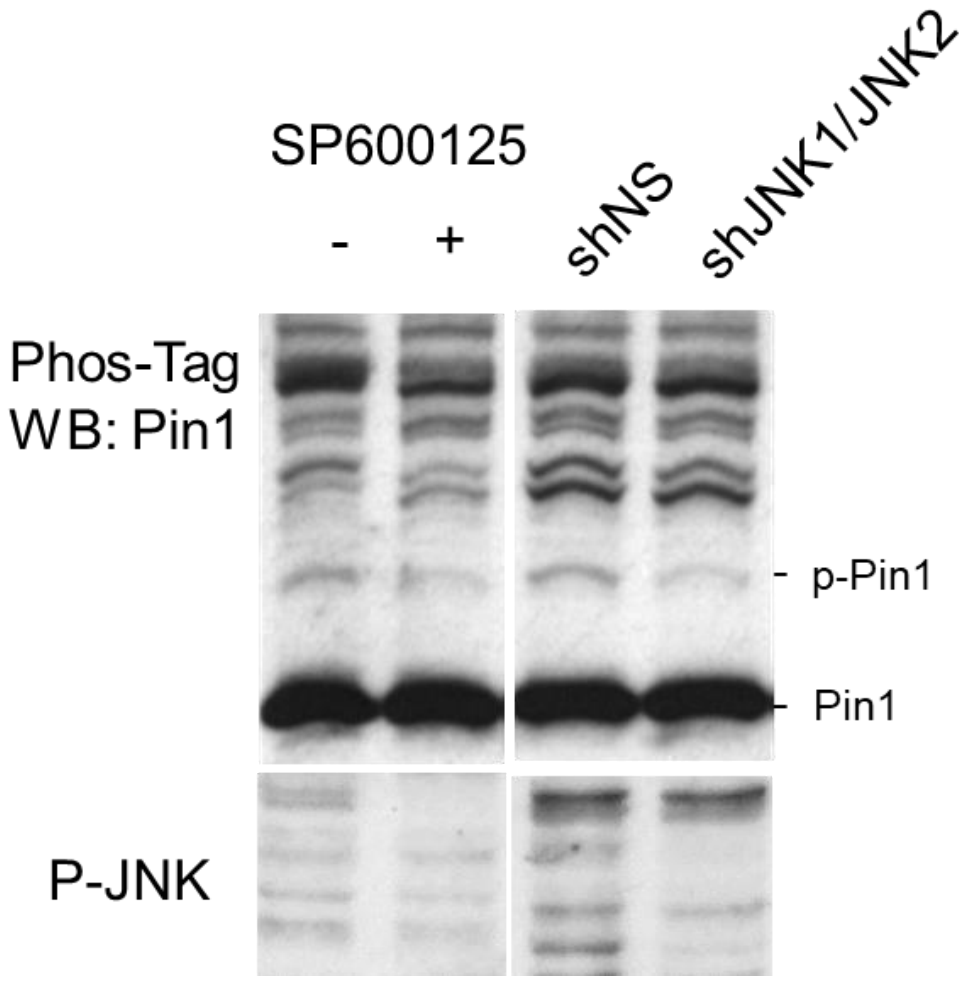


Figure 4.12: Phospho-Active JNK phosphorylates Pin1 in CCLP1 cell lines: CCLP1 cells were treated in the presence/ absence of JNK inhibitor (SP600125) (Left) and shJNK1/2 vs shNS knock down (Right). All cells were then lysed in the appropriate buffers and a BSA test was performed for concentration. All cells were loaded at equal concentrations in a Phos-Tag™ Western blot gel. Antibodies were then used to probe to detect Pin1 expression revealing that the phosphorylated Pin1 band is reduced in the presence of both SP600125 and shJNK compared with no treatment and shNS respectively. Western blots against p-JNK depict controlled loading expression.

concerns; hypotheticals that the spatial closeness of the two serines may have resulted in an error when identifying the phosphorylation site.

To ensure that this was not the case we opted to carry out mutagenesis, cloning individual mutant PIN1 plasmids that had replaced the serine at either 115 or 114 for an alanine; sub sequentially an amino acid structurally unable to be phosphorylated. These mutants were hereon named S115A and S114A. In addition, an S138A was also created to account for a wider literature reading that had attributed the phosphorylation of PIN1 at serine 138 to an upstream regulator of JNK.

4.14 Constitutive-active JNK phosphorylates PIN1 at Serine 115

Returning to the technique of Phos-Tag™, we were now able to simulate identical conditions for the phosphorylation of the wild-type PIN1 by active JNK. However, this time, we also extended the experiment to question active JNK's ability to phosphorylate point mutant PIN1 S114A, S115A and S138A. The results of the experiments described a maintained phosphorylated form of PIN1 in the presence of active JNK for each of the WT, S114A and S138A expressed PIN1 proteins. However, for S115A conditions, no phosphorylated bands were observed, indicative that the S115 position was indeed the site of PIN1 phosphorylation by active JNK (Figure 4.14).

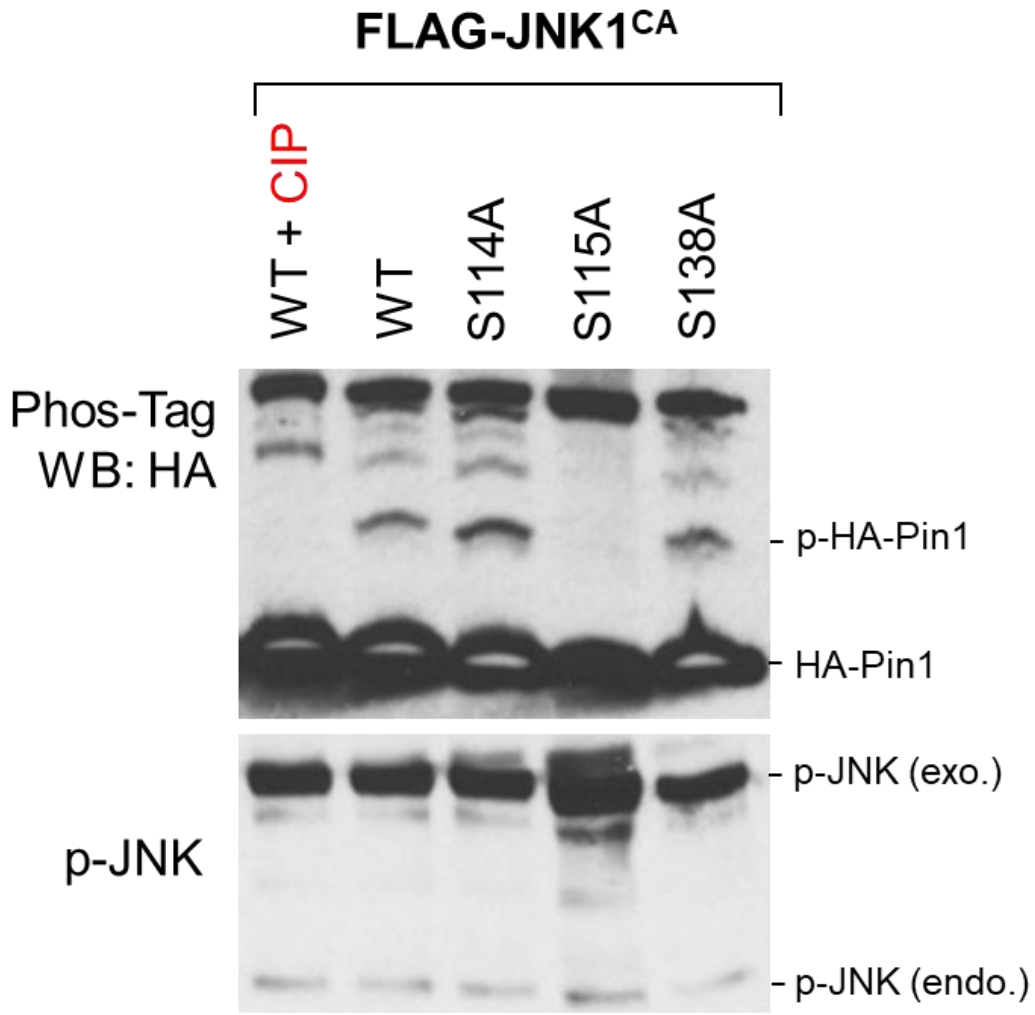


Figure 4.14: Constitutive-active JNK phosphorylates Pin1 at Serine 115 / Overexpression in HEK293: HEK293 cells were transiently transfected with a combination of FLAG-JNK1-CA, HA-Pin1-WT, HA-Pin1-S114A, HA-Pin1-S115A, HA-Pin1-S138A plasmids and incubated for 48hrs before all cells were lysed in the appropriate buffers. Calf intestine phosphate (CIP) was added as a control to assess phosphorylation bands vs a controlled lane. Figure shows Phos-Tag™ Western Blot using antibodies against HA indicating that Pin1 cannot be phosphorylated by FLAG-tagged constitutive active JNK (JNK1-CA) when Pin1 Serine (S) is mutated to an Alanine (A) at position 115 (S115A). JNK1-CA directed Pin1 phosphorylation bands are present for expression of Pin1 wild-type (WT) and mutants S114A and S138A. Furthermore, phosphorylated Pin1 WT band is depleted upon addition of calf intestine phosphate (CIP). Western blots against p-JNK depict controlled loading expression. Results shown are representative of three repeats.

4.15 Constitutive-active JNK phosphorylates PIN1 at serine position 115 in vitro

In an attempt to confirm these results, we created mutant recombinant proteins of S114A, S115A and S138A to accompany the existing WT PIN1 protein. Following this, we subjected all WT and mutant PIN1 recombinant proteins to the kinase activity of active JNK to understand the site of phosphorylation through *in vitro* techniques. The results showed that incorporation of radio-actively labelled phosphate groups to the S115A PIN1 mutant was significantly decreased in the presence of active JNK, a trait not seen in all other mutant and WT conditions (Figure 4.15). This experiment confirmed our findings and concluded the first chapter of results describing active JNK to interact with and sub sequentially phosphorylate PIN1 at serine position 115. We now turned our focus to examine the importance of this mechanistic interaction on PIN1 and what the biological outcome of this interaction has in cancer.

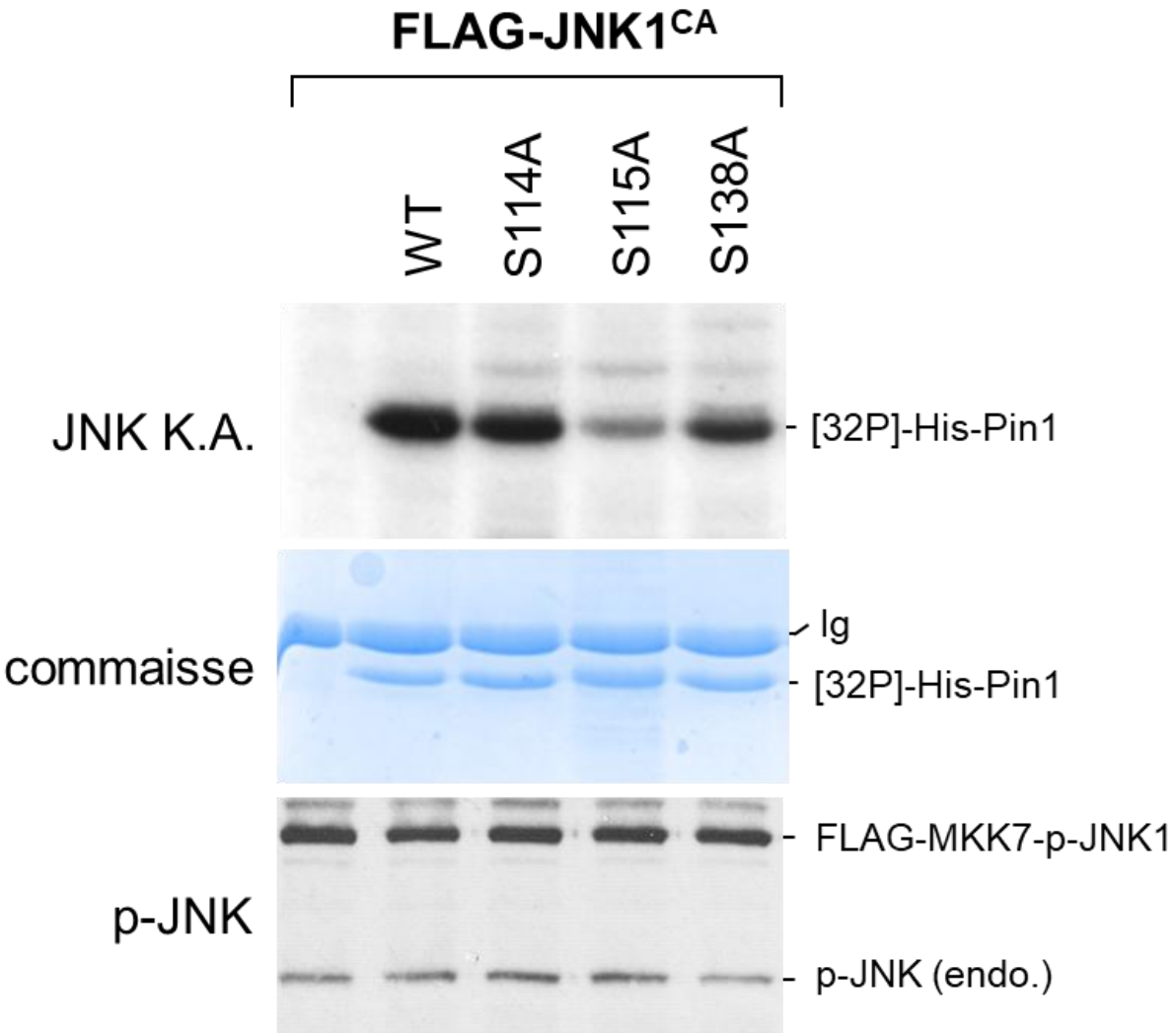


Figure 4.15: Constitutive-active JNK phosphorylates Pin1: Kinase assay of FLAG-tagged constitutive-active JNK expression from HEK293 cell extract on Pin1 point mutated Serine (S) to Alanine (A) mutants at residues indicated by mass spectrometry analysis; S114/S115/S138. Constitutively active JNK is shown to maintain phosphorylation capability of His-tagged recombinant protein wildtype (WT) and mutants S114A and S138A however phosphorylation capability is significantly decreased on S115A. Figure includes western blots using antibodies against p-JNK shown to depict controlled loading expression. Coomassie Blue of His-Pin1 WT and point mutants are shown to depict equal presence of protein expression loading. Kinase assay technique was performed by Salvatore Papa due to limited authorisation to conduct kinase work. All recombinant protein work was carried out by me to supplement this work.

Chapter 5 Results: Active JNK's stabilises PIN1 by phosphorylation at S115 important for proliferative functions in cancer

With strong evidence now supporting a post-translational interaction between active JNK and PIN1 at site S115, we could now turn our attention to explore the importance of this novel mechanism and its functions in cancer. Several post-translational modifications on PIN1 have previously been established. These include known sites for phosphorylation, sumoylation, ubiquitination and oxidation that are shown to regulate the stability, substrate-binding ability, PPIase activity and subcellular localization of PIN1. These post-translational modifications of PIN1 are frequently aberrant in cancer and contribute to its frequent high expression and over activate state. In fact, the over-activation of PIN1 has been linked with the upregulation of more than 50 oncogenes and proliferation-promoting factors, whilst simultaneously inhibiting more than 20 tumour suppressors and proliferation restraining factors (*21, 198, 199*).

Under a normal physiological state, cell proliferation is strictly regulated by both intracellular and extracellular pathway signals, however cancer cells use novel means to adapt allowing for mechanisms of sustained proliferation, a known hallmark of cancer. It has become evident that one of these mechanisms is through the over-expression of PIN1, leading us to question how our findings regarding the novel post-translational interaction between active JNK and PIN1 would affect the overall proliferation of cancer cells.

5.1 Recovery of Pin1 in JNK1/2 silenced cells only partial restores proliferative function

To explore this question, we decided to knock down *JNK1/2* expression in CCLP1 cell lines, assessing the effect on the proliferation of cancer cells reconstituted with exogenous PIN1 vs *shJNK1/2* treated cells alone. This was done in order to isolate the function of PIN1 in cancer cells absence of JNK expression, by tracking alterations in cell growth for each condition following 12 days post infection. Our first observation was a complete loss of cell proliferation by cells

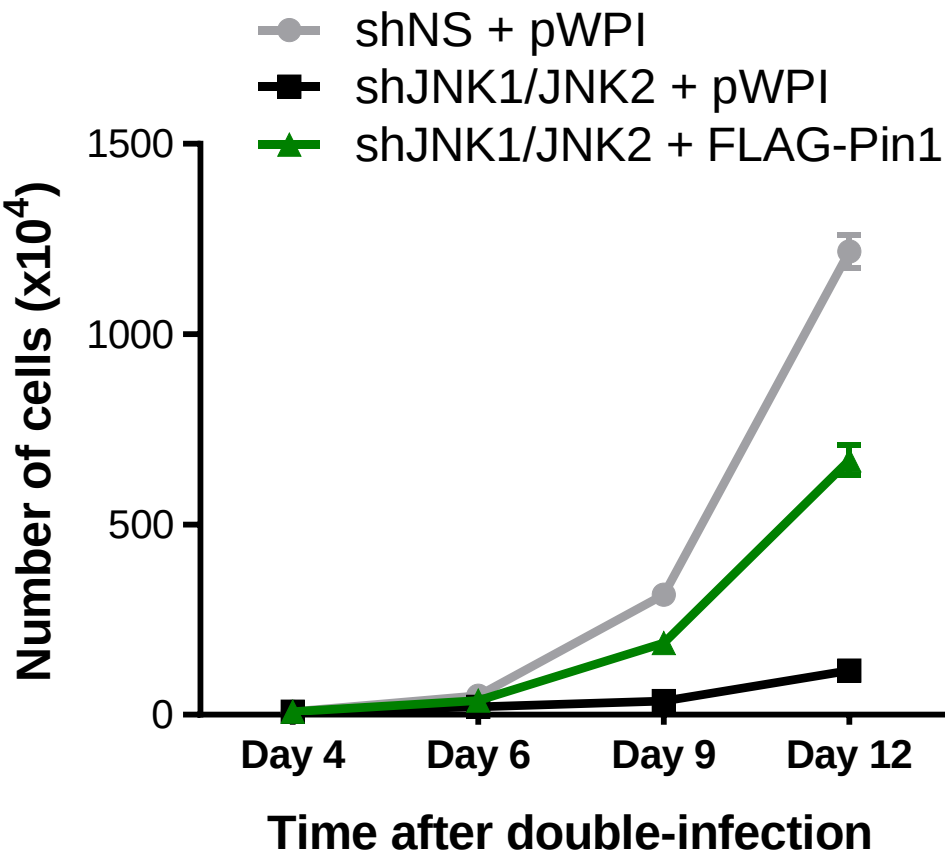


Figure 4.1: Reconstitution of PIN1 in JNK1/2 silenced cells only partial restores proliferative function in cancer: (Analyses performed by Dr Pui Mann Choy, Institute of Hepatology London). XY scatter plot describes number of cells (x10^4) (CCLP1) counted in each condition after 4 days post lentiviral infection with either: shJNK/1/shJNK2 + reconstituted FLAG-PIN1 (Green line), shJNK1/shJNK2 + PwPI (control) (Black line), or shNS + PwPI (control) (Grey line). Cells were split and counted for 12 days following infection. Error Bars are indicative of mean values between three counts for each condition at each time point. Experiment is a representative of over three repeats.

treated with *shJNK1/2* (black line) compared with the *shNS* treated control cells (grey line) (Figure 5.1). This loss of function supported the fundamental need for JNK expression in maintaining cell proliferation. Next, to evaluate how the loss of JNK expression affected PIN1's role in proliferation, we compared *shJNK1/2* + control cells with *shJNK1/2* cells reconstituted with PIN1 (green line). To our interest, we found that cells expressing exogenous PIN1 in the absence of JNK could only partially restore the proliferative function of cells with endogenous levels of both JNK and PIN1 present (grey line). These results pointed to an intrinsic need for both JNK and PIN1 proteins to be present to restore full proliferative function of cancer cells. Given our results from chapter 1 detailing PIN1 as a downstream substrate of JNK, we hypothesised that our described post-translational interaction of active JNK with PIN1 may in turn affect the expression and/ or activity of PIN1 in cancer. If this was deemed true, in the absence of JNK, PIN1 would indeed be unable to work at full effect, explaining why PIN1 alone would be unable to restore full proliferative function of the cell.

5.2 Constitutive-active JNK stabilises PIN1 protein expression

We therefore chose to first examine changes in PIN1 protein expression in the presence and absence of active JNK. This was achieved using a transient transfection method to over-express both proteins in a cellular environment followed by cycloheximide (CHX) treatment to block further protein synthesis occurring. CHX was used for a 0-3-6-9 hrs time period, lysing cells at each point to assess alterations in PIN1 expression that was either co-expressed with active JNK or alone over the stated time course. After carrying out the experiment several times, we soon discovered that the time at which we chose to block protein synthesis after the transient transfection (i.e. 0hrs CHX treatment) was imperative for the successful examination of the post-translational interaction between both proteins. Leaving too long of a period after expression of the plasmids, resulted in the accumulation of high concentrations of both proteins (HA-PIN1 and FLAG-MKK7-JNK1). This in turn made it difficult to interpret the results. In essence, finding the optimal time for CHX treatment

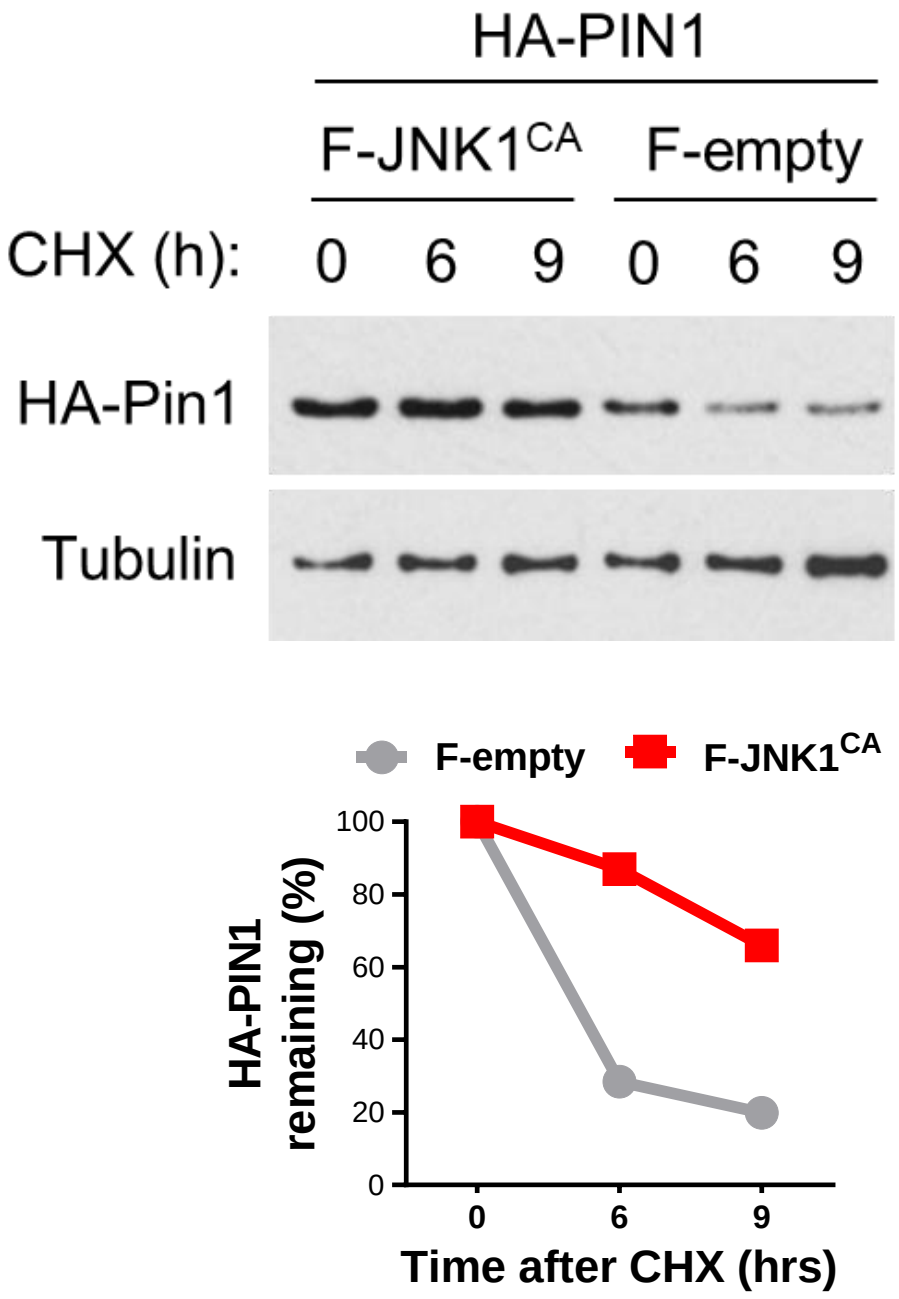


Figure 5.2: Constitutive-active JNK stabilises Pin1 protein expression: (Top) Western Blot analysis showing time course of 0-3-6hrs post protein synthesis inhibition (CHX) indicates Pin1 expression is stabilised in the presence of FLAG-constitutive active JNK compared to HA-Pin1 expression alone (control). HA-Pin1 expression was detected using antibodies against HA and tubulin expression for control of loading. (Bottom) XY scatter plot indicates decrease of Pin1 protein expression over time treated with CHX. Blue line indicates Pin1 expression in presence of active JNK compared to grey line indicative of Pin1 expression alone.

would prevent any newly formed PIN1 from obscuring our understandings of the post-translational effect of active JNK on the protein expression of PIN1. Following the optimization of drug treatment, we found that when both active JNK and PIN1 were expressed together, the protein expression of PIN1 over 9 hours post CHX treatment remained stable (Figure 5.2). On the other hand, in the absence of active JNK, PIN1 protein expression slowly degraded within the same time frame. These results indicated that the post translational interaction with JNK is important for PIN1 protein stability in the cell.

5.3 Presence of Phospho-active JNK stabilises PIN1 protein expression in CCLP1 cell lines

Leading on for this experiment, we carried out a similar method in CCLP1 cell lines in order to elucidate if JNK's effect on PIN1 stability was consistent in a cancer environment. We therefore knocked down *JNK1/2* expression and waited 5 days to ensure the knockdown took effect. Following this, we seeded equal numbers of *shJNK* and control *shNS* cells and treated them both with CHX for 0-12-24 hours analysing PIN1 protein expression by western blot at each time point. Again, we found that in the absence of JNK, PIN1 protein expression was unstable (red line) compared with its expression in the presence of JNK (red line) (Figure 5.3). Under these conditions, optimization of the CHX treatment lead to a time-course of 0-12-24 hours of treatment visualised by plotting the data in a line graph format. This extended time for alterations in PIN1 expression in the absence of JNK was unsurprising given the oncogenic environment of the experiment. Indeed, we have already pointed out that PIN1 is post-translationally modified by other known proteins likely to be over-expressed in the cancer environment leading to a longer stability of PIN1 than in the transient transfection conditions.

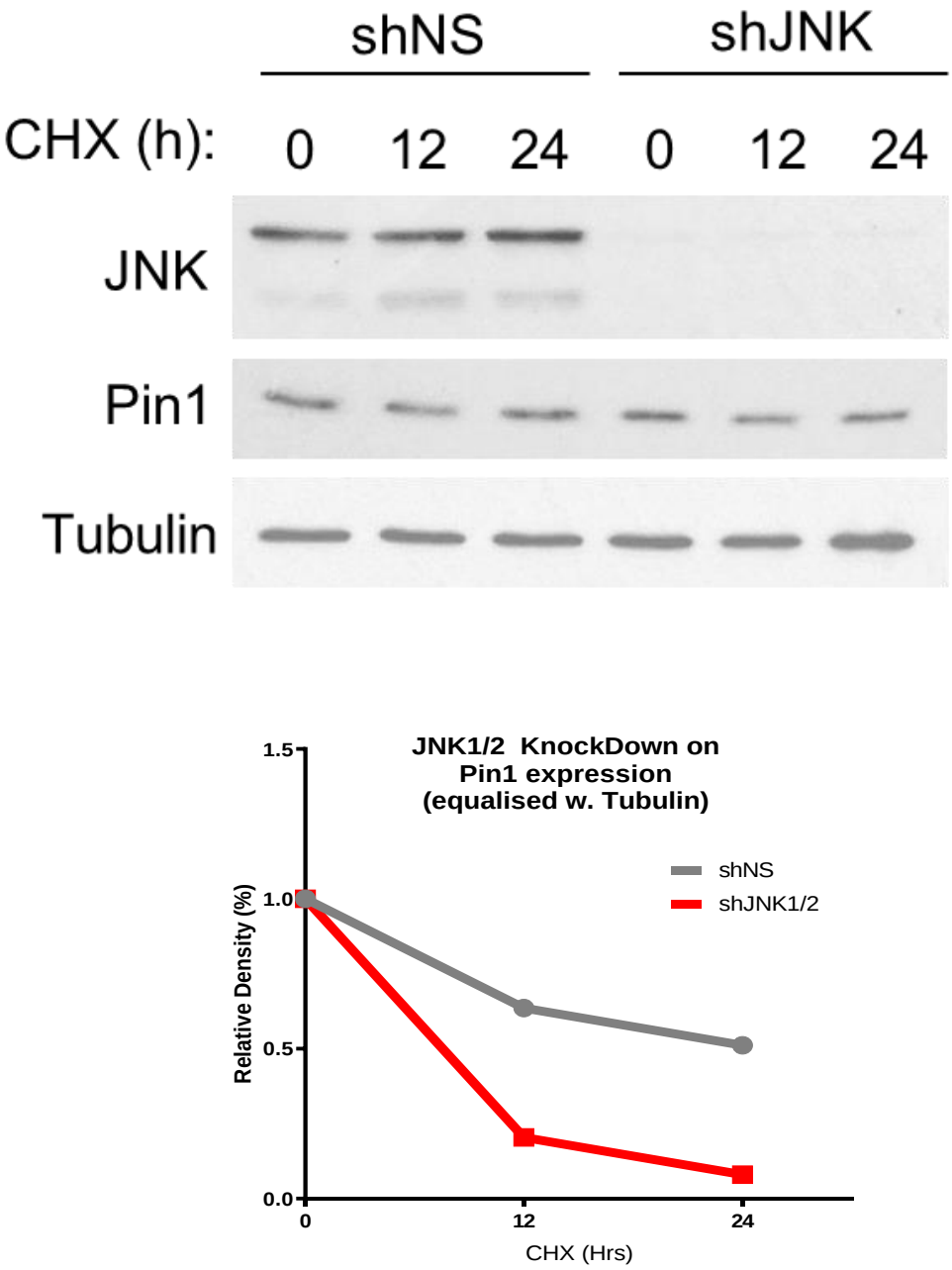
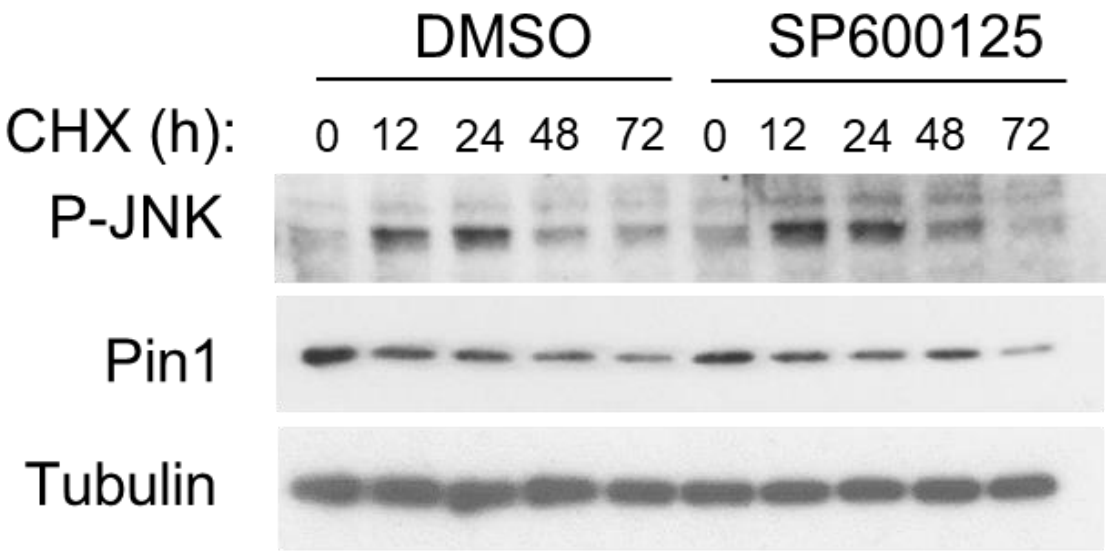


Figure 5.3: Presence of Phospho-active JNK stabilises Pin1 protein stability in CCLP1 cell lines: (Top) Western Blot analysis using antibodies to detect endogenous Pin1 expression in CCLP1 cell lines shows Pin1 protein is stabilised in the presence of phospho-active JNK (shNS control) post treatment with CHX for 0-12-24 hours compared with expression when JNK is knocked down in cell lines (shJNK). Antibodies against JNK and tubulin are included for controlled expression. (Bottom) Scatter XY plot showing enhanced reduction of pin1 expression in the absence of JNK (right line) compared with control treatment (black line).



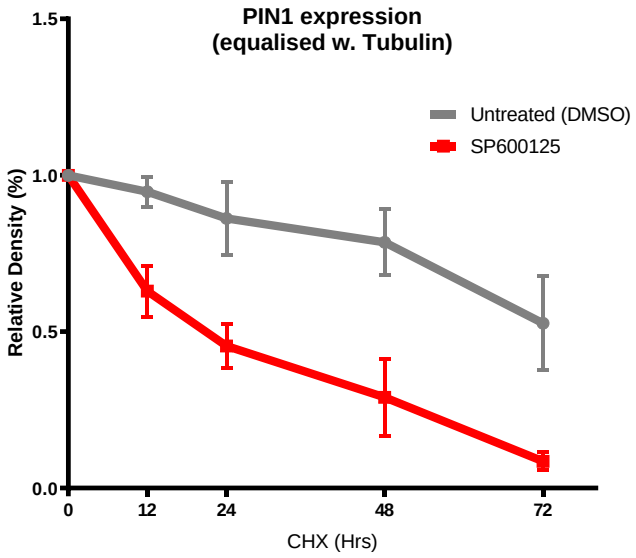


Figure 5.4: Phospho-active JNK inhibition reduces Pin1 protein stability in CCLP1 cell lines: (Top) Western Blot analysis using antibodies to detect endogenous Pin1 expression in CCLP1 cell lines shows Pin1 protein is destabilised in the presence of JNK specific inhibitor (SP600125) during treatment with CHX for 0-12-24 hours compared with Pin1 protein stability under DMSO control conditions. Antibodies against tubulin are included for controlled expression. (Bottom) Scatter XY plot showing enhanced reduction of pin1 expression during JNK activity inhibition (red line) compared with control treatment (black line). Error bars indicate SEM calculated from two experimental replicates.

5.4 Phospho-JNK inhibition reduces PIN1 stability in CCLP1 cell lines

Next, we aimed to assess how the activity of JNK affected PIN1 protein stability in a cancer environment. We had already outlined its requirement for its interaction with PIN1, and were curious to understand if this extended to the stability of PIN1 expression. To this end, we seeded equal numbers of CCLP1 cells and treated them with either a JNK inhibitor (SP600125) or control (DMSO) to inhibit the kinase potential of JNK. Following this, CHX treatment was carried out for 0-12-24-48-72 hours, lysing cells at each time point to assess changes in PIN1 expression. After equalising to tubulin, CCLP1's treated with SP600125 (red line) showed a reduced PIN1 expression over 72 hours whereas control cells remained more stable (black line) (Figure 5.4). These results indicated that JNK activity was necessary for our observed PIN1 protein stability in cancer cells.

5.5 Reconstitution of shRNA resistant PIN1 restores proliferative function in PIN1 silenced cells

Given we had now established that PIN1 protein expression is unstable in the absence of active JNK, we returned to our previous experimental findings surrounding proliferative functions in cancer (Figure 5.1) In this previous experiment we had detailed that both JNK and PIN1 protein expression are needed for the full proliferative function of cancer cells. Following this, in later experiments (Figures 5.2-4.4), we described that the presence of active JNK was necessary for stable PIN1 protein expression. Collectively, these results suggest that the maintained stability of PIN1 in the presence of JNK expression may restore full proliferative functions in cancer and provides evidence as to why in the absence of JNK, PIN1 expression could not fully restore cell proliferation (Figure 5.1). Therefore, to answer this question, we set out to uncover whether the reconstitution of shRNA-Resistant PIN1 (*shRES-PIN1*) would restore full proliferative functions in CCLP1 cell lines treated with *shPIN1* knockdown. To do so, we carried out lentiviral infections of *shPIN1* combined with either reconstituted exogenous *shRES-PIN1* (green line), or an empty vector control (Pwpi) (Black line). After 5 days to ensure the knockdown had took full effect, we reseeded both

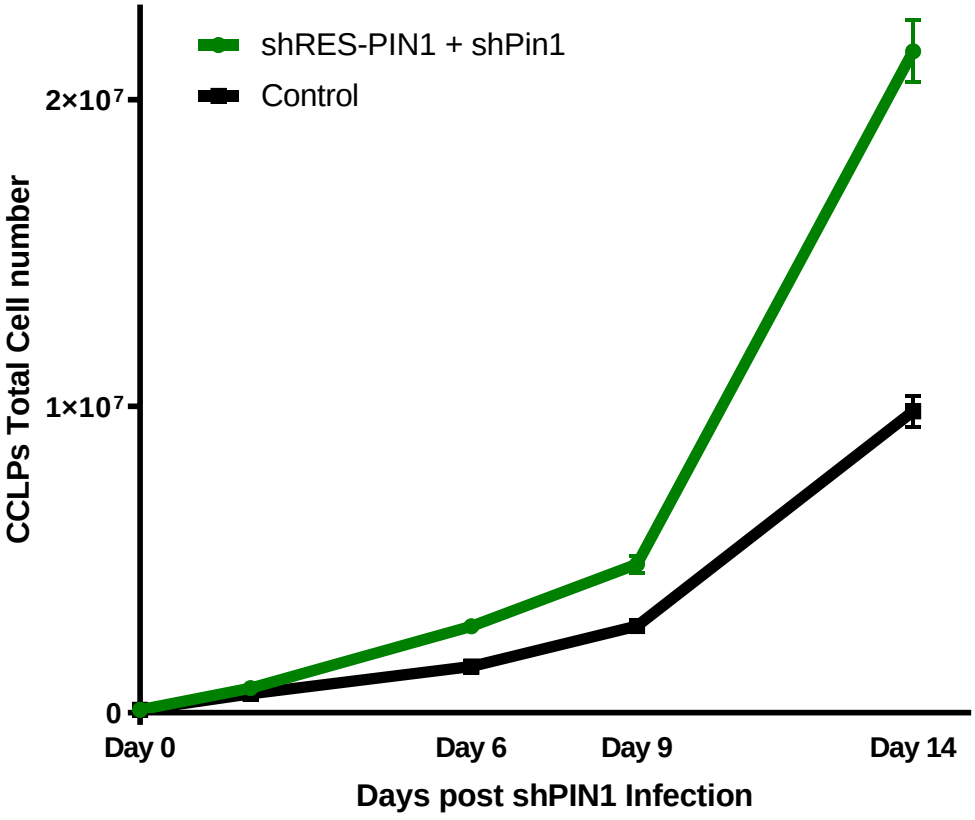


Figure 5.5: Reconstitution of shRNA-resistant PIN1 restores proliferative function in shPIN1 cancer cells: XY scatter plot describes number of cells (x10^4) (CCLP1) counted in both conditions after 4 days post lentiviral infection with either: shPIN1 + reconstituted shRES-PIN1 (Green line), or shPIN1 + PwPI (control) (black line). Cells were split and counted for 14 days following infection. Error Bars are indicative of mean values between three counts for each condition at each time point. Experiment is a representative of over three repeats.

conditions at equal numbers of cells and followed the growth of cell numbers over 14 days (Figure 5.5). This knockdown viability of *shPIN1* was later confirmed through western blot analysis in subsequent experiments (Figure 5.6). Figure 5.5 confirmed that silencing of PIN1 reduced the overall cell proliferation of cancer cells. However, upon reconstitution of exogenous *shRES-PIN1*, full proliferative function of the cancer cells were restored. These results indicated that the endogenous expression of active-JNK in CCLP1 cell lines stabilises PIN1 expression to maintain proliferative function.

5.6 Reconstitution of shRNA resistant PIN1 restores cell cycle function in shPIN1 cancer cells

In light of active-JNKs stability of PIN1 proliferative function in cancer cell lines, we sought to understand whether the downstream proliferative mechanism of PIN1 could be further uncovered. PIN1 is well established as a master regulator of cell cycle substrates, both in normal tissues and in cancers (*198, 199*). We therefore set out to question the expression of these downstream cell cycle substrate targets, including cyclin D and cyclin A expression, and how they may be affected by the constitutive activation of JNK expression in these cells. To this end, following the 14-day lentiviral infections of *shPIN1* with either control or exogenous *shRES-PIN1*, as well as further conditions of shNS with either control or exogenous shRES-PIN1, we collected all cell conditions and lysed in the appropriate buffers. Next, we analysed all cell lysate conditions by western blot analysis using antibodies to probe for Cyclin D1, Cyclin A, PIN1 and Tubulin. We were able to confirm both the effective knockdown of PIN1 by *shRNA* as well as strong expression of the exogenous *shRES-PIN1* in cancer cells. Furthermore, the reduced proliferative function seen by control (shPIN1 + control) conditions in Figure 5.5 (black line) were reflective of a reduced cell cycle expression of Cyclin D1 and Cyclin A (Second from left) in Figure 5.6. Cell cycle expression for both PIN1 substrates were then recovered by the reconstitution of exogenous shRES-PIN1 (right). These results show clearly that the slowed proliferative function of PIN1 observed is caused by a reduced ability of PIN1 to maintain expression of downstream cell cycle targets.

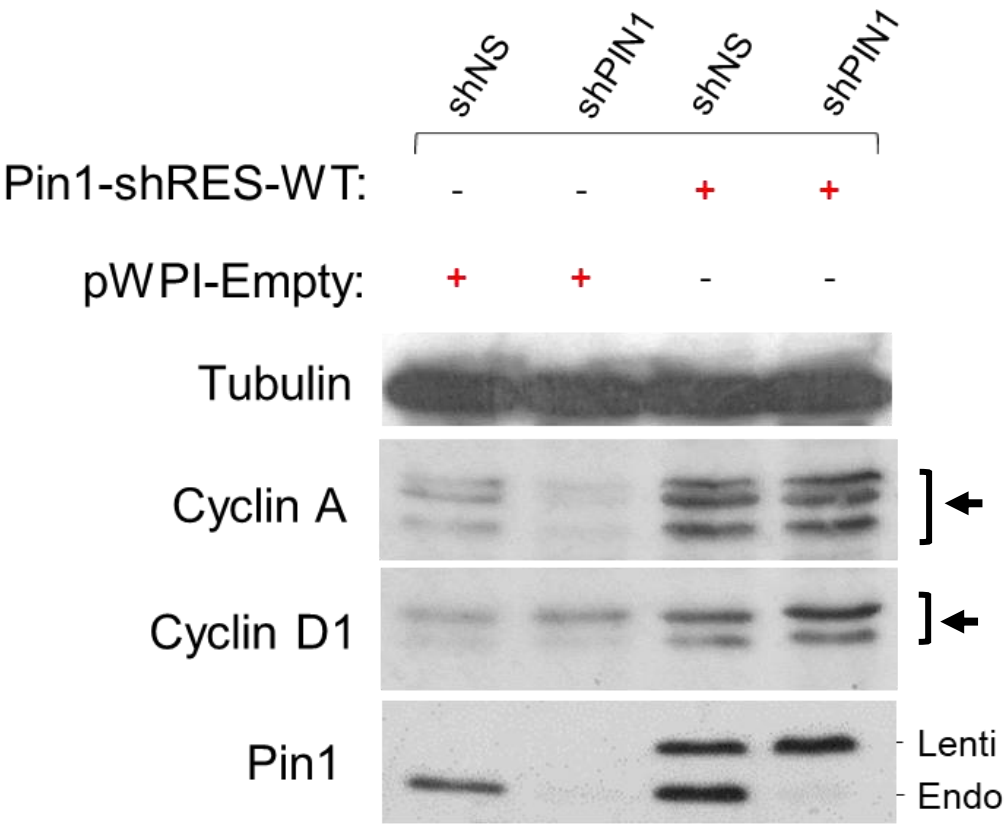


Figure 5.6: Reconstitution of shRNA-resistant PIN1 restores cell cycle function in shPIN1 cancer cells: Western blot analysis of CCLP1 cell lysates at day 14 post lentiviral infection with either (left to right): shNS + pWPI (control), shPIN1 + pWPI (control), shNS + reconstituted PIN1-shRES-WT, or shPIN1 + PIN1-shRES-WT. All cell conditions were lysed and loaded into western blots at equal concentrations. Western blots used antibodies to detect PIN1, Cyclin D1, Cyclin A expression. Lentiviral protein expression is denoted as (Lenti), with endogenous protein expression denoted as (Endo). Antibodies against tubulin were used as controls. Experiment is a representative of two.

5.7 Constitutive-active JNK stabilises PIN1 protein expression by phosphorylation at Serine 115

We were now concrete in the knowledge that the post-translational interaction between active JNK and PIN1 stabilises PIN1 protein expression in cancer. Given our findings from chapter 1 regarding active JNK's phosphorylation of PIN1 at serine 115, we sought to determine whether this phosphorylation at S115 was an important factor for the stability of PIN1 expression. To this end, we returned to the mutant plasmids namely HA-PIN1 (WT), HA-PIN1 (S114A), HA-PIN1 (S115A), HA-PIN1 (S138A), but this time over-expressing them in combination with active JNK using a transient transfection method. CHX was treated for a 0-3-6-9-hour time period, lysing cells at each stage and analysing PIN1 protein expression by western blot. We found that for each the WT, S114A and S138A in combinations with active JNK, PIN1 protein expression remained stable over 9 hours of CHX treatment (Figure 5.7). However, with a mutation at S115 preventing active JNK from phosphorylating PIN1 at this site (S115A), the stability of PIN1 was dramatically reduced. These results were evidence that the phosphorylation of PIN1 at S115 by active JNK resulted in the stability of PIN1 expression in cells.

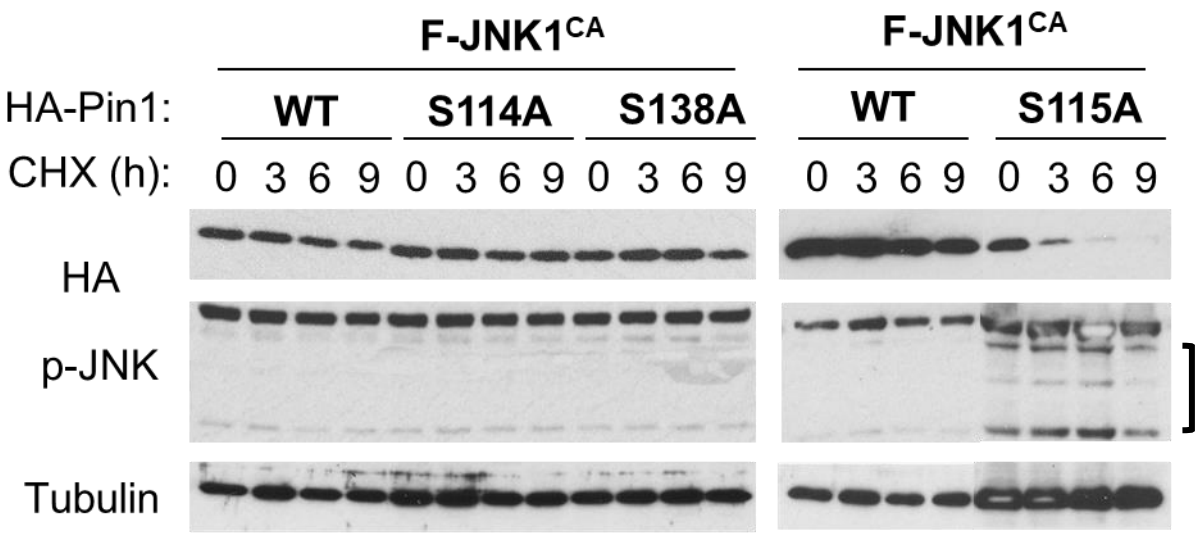


Figure 5.7: Constitutive-active JNK stabilises Pin1 protein expression by phosphorylation at Serine 115: Western Blot analysis using antibodies against HA to detect protein stability of WT/ S114A / S138A/ Pin1 expression in the presence of FLAG-tagged constitutive-active JNK overexpressed in HEK293 cell extracts. WB shows FLAG-tagged constitutive-active JNK stabilises WT / S114A / S138A Pin1 protein expression over 0-3-6-9hrs of CHX protein synthesis inhibition treatment however S115A Pin1 mutant is highly unstable [concentration of S115A loaded 3x of other cell extracts] (Right). An additional WT control was included at 3x concentration of other cell extracts (Left) to account for the instability of S115A mutant. WB using antibodies against p-JNK and tubulin are used as controls of loading. Results shown are a repeat of three individual experiments

5.8 Phospho-mimic PIN1 S115E cannot maintain Pin1 stability alone

In an attempt to support of this finding, we set out to understand if the constitutive phosphorylation of PIN1 at serine 115 in the absence of active JNK would maintain PIN1 protein stability over time. We therefore designed a phospho-mimic mutant of PIN1, namely S115E by replacing the serine at position 115 with a glutamic acid (E). The rational for this amino acid substitution is that glutamic acids resemble a similar structure to a phosphorylated serine residue, and therefore allowed us to design an 'always on' phosphorylated site at position 115 of PIN1. Following this we transiently transfected the S115E mutant PIN1 and compared its expression against WT PIN1 in the absence of active JNK expression. We found that over 0-3-6-9 hours of CHX treatment, S115E in the absence of active JNK behaved in a similar manner to WT-PIN1. Both WT and S115E PIN1 proteins were shown to reduce in expression over the given time (Figure 5.8).

These results may either point to an unsuccessful phosphorylation mimic by the S115E, or that phosphorylation of S115 in the absence of a conformational interaction with active JNK cannot stabilise PIN1 expression in the same manner. The latter may readily explain why our findings have shown the interaction and phosphorylation of PIN1 with active JNK to be dependent on one another.

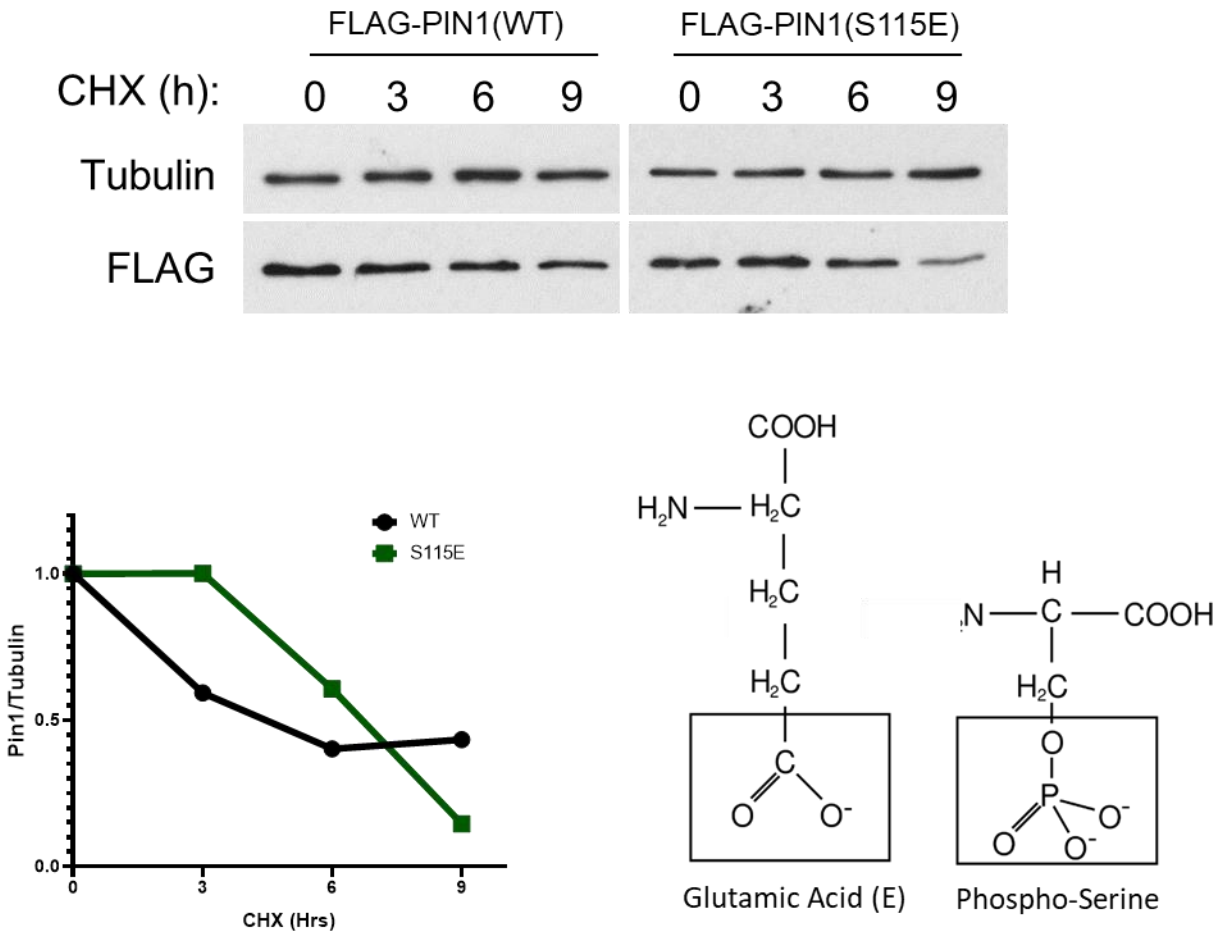


Figure 5.8: Phospho-mimic PIN1 S115E cannot maintain PIN1 stability alone: (A): Western blot analysis of HEK293 cell lysates transfected with either FLAG-PIN1 or FLAG-PIN1(S115E) treated with 0-3-6-9 hours of cycloheximide (CHX) stimulation (25ug/ul). WB use antibodies to detect FLAG expression, with tubulin used as loading control. Each cell condition was lysed in the same buffer and loaded at equal concentrations into the western blot **(B):** XY scatter plot indicates PIN1's protein concentration (relative density) across 0-3-6-9 hours of CHX for both WT (black line) and S115E phospho-mimic (green line) stability equalised to tubulin loading. **(C):** Depiction of the similarities in chemical structures of glutamic acid (E) (Left) and Phospho-serine (Right). Structural representation of the ability for Glutamic Acid structure to mimic a phospho-serine residue **(A-B):** Representative of over three experimental repeats.

5.9 Phosphorylation of PIN1 at S115 is critical for restoring proliferative function in cancer cells

Following the findings that PIN1 in the absence of JNK could only partial restore the proliferative function to cancer cells, we had hypothesised that a post-translational interaction of JNK on PIN1 may be necessary for the full restoration of cellular proliferation. Indeed, within this chapter alone, we have confirmed this hypothesis; detailing the unstable expression of PIN1 in the absence of JNK expression or activity. Furthermore, we have also pinpointed active JNK's phosphorylation of PIN1 at S115 as the mechanism for PIN1's stability and isomerase activity in cancer cells. Collectively these findings describe PIN1 as a downstream effector of JNK in the cell, and give cause to why PIN1 in the absence of JNK signals can only partial recover the proliferative function of cancer cells. With this in mind, we set out to prove that the phosphorylation of PIN1 at S115 by active JNK was critical to restore full proliferation in cancer cell lines.

To this end, we knocked down endogenous levels of *PIN1 (shPIN1)* in CCLP1 cell lines regularly expressing phospho-active JNK. Following this, we used lentiviral infections to exogenously express either *shPIN1* resistant WT-PIN1 (shRES-PIN1-WT), or *shPIN1* resistant PIN1 (S115A) (shRES-PIN1-S115A) in cells now absent of endogenous PIN1. We then followed the proliferation of CCLP1s containing exogenous PIN1-WT, vs exogenous PIN1 (S115A) over 14 days post infection to determine how the phosphorylation at S115 by active JNK affected the proliferative function of PIN1 (Figure 5.9).

We found that *shPIN1* CCLP1 cells reconstituted with exogenous PIN1-WT were able to rescue full cell proliferation (green line) compared with *shPIN1* control cells (black line) that showed a significantly reduced proliferation in the absence of PIN1. To our interest however, *shPIN1* CCLP1 cells reconstituted with exogenous PIN1 containing the mutation S115A (grey line) were unable to rescue cell proliferation, showing similar cell proliferation as cells absent of all PIN1 expression.

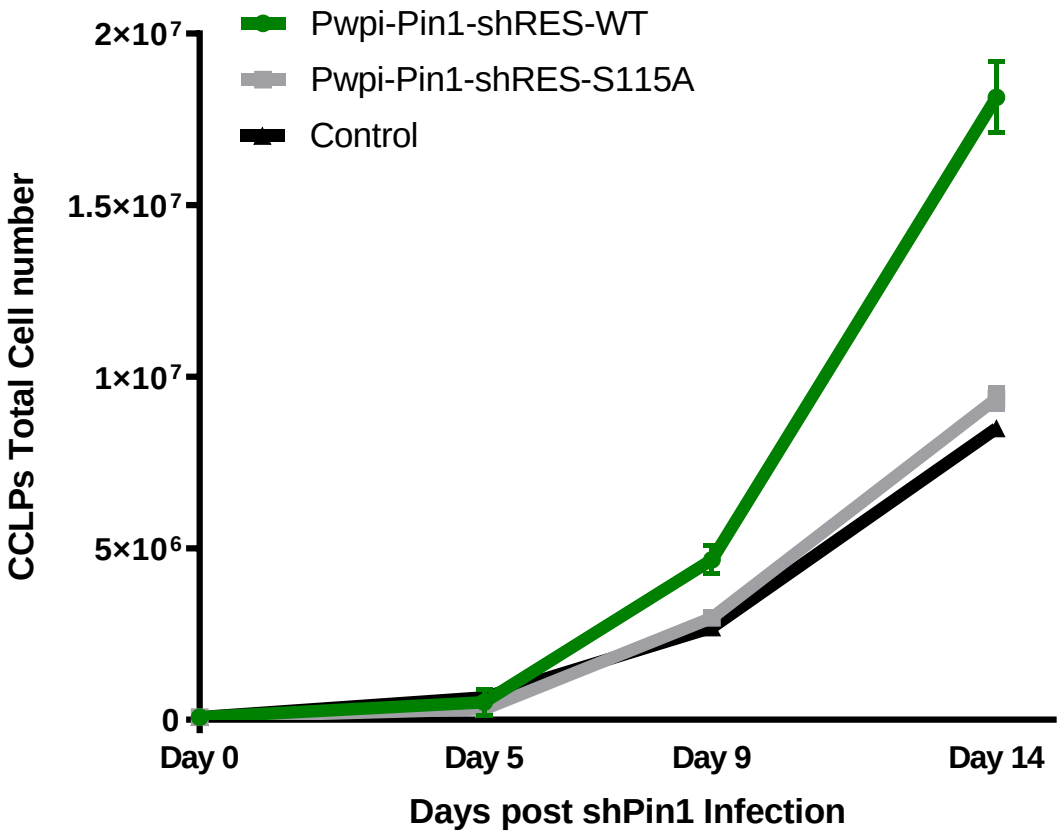


Figure 5.9: Phosphorylation of PIN1 at S115 is critical for restoring proliferative function in cancer cells: XY scatter plot describes number of cells (x10^4) (CCLP1) counted in both conditions after 4 days post lentiviral infection with either: shPIN1 + reconstituted *shRES-PIN1* (Green line), *shPIN1* + reconstituted *shRES-PIN1(S115A)* (grey line), or *shPIN1* + *PwPI* (control) (black line). Cells were split and counted for 14 days following infection. Error Bars are indicative of mean values between three counts for each condition at each time point. Experiment is a representative of over three repeats.

5.10 Phosphorylation of PIN1 at S115 restores cell cycle function in cancer cells

These results were striking, confirming clearly that the phosphorylation of PIN1 at S115 by active JNK is critical for the full proliferative capability of cancer cells. To explore how this defect in proliferation may be occurring at a biochemical level, we lysed cells of each condition at day 14 of the experiment and resuspended them in the appropriate buffers. Following this, we probed for cyclin (A, and D1) expression in each condition; known cell proliferative targets of PIN1. For CCLP1 cells containing endogenous (shNS) and exogenous (shRES-PIN1-WT) PIN1 expression, cyclin A and D levels were shown have high expression. However, for CCLP1 cells lacking endogenous PIN1 and /or reconstituted with shRES-PIN1-S115A we saw a reduced cyclin expression. This data showed that the phosphorylation of PIN1 at S115 by active JNK results in further downstream signalling, critical for the proliferative function of cancer cells (Figure 5.10).

5.11 Phosphorylation at PIN1 S115 enhances overall isomerase activity

Now that we had established the phosphorylation at S115 to be critical for PIN1 stability, we questioned how this post-translational modification affected the activity of PIN1. As discussed already, the over-activation of PIN1 is linked to the upregulation of numerous oncogenes and down-regulation of tumour suppressors. For these reasons the identification of post-translational modifications critical to PIN1's activity is of much interest to elucidate its role in cancer. Given our findings surrounding the instability of PIN1 in the absence of JNK's phosphorylation at S115, we believed that the reduction in PIN1 expression may be accompanied by a reduction in PIN1's isomerase activity. However, at present, no method allowed for direct analysis of PIN1 isomerase activity within cancer cells, and therefore we opted to explore the activity through an *in vitro* method. This technique measured the isomerase activity of PIN1 through its conformational change of a green fluorescent substrate. As a result, the green

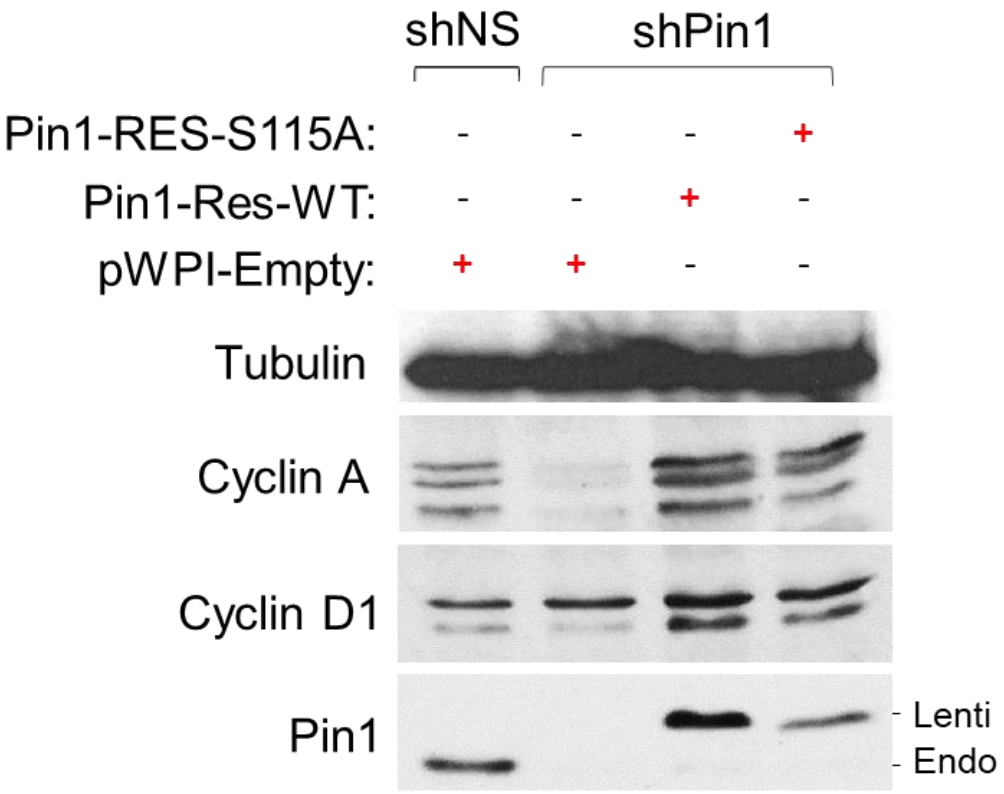


Figure 5.10: Phosphorylation of PIN1 at S115 restores cell cycle function in cancer cells: Western blot analysis of CCLP1 cell lysates at day 14 post lentiviral infection with either (left to right): shNS + Pwpi control, shPIN1 + Pwpi (control), shPIN1 + reconstituted PIN1-shRES-WT, or shPIN1 + PIN1-shRES-S115A. All cell conditions were lysed in the same buffer and loaded at equal concentrations into the western blot. Expression of lentiviral proteins is denoted as (Lenti) with expression of endogenous proteins denoted as (Endo). Western blots used antibodies to detect PIN1, Cyclin D1, Cyclin A expression. Antibodies against tubulin were used as controls. Experiment is a representative of two.

fluorescence substrates released specific wavelengths of light that can then be read as a measure of PIN1's activity *in vitro.*

In order to determine the effect of S115 phosphorylation on PIN1's isomerase activity, we were gifted a phosphorylated S115 PIN1 (pS115 PIN1) recombinant protein from a collaborator at the University of Leeds (Bayliss Lab). We then matched the concentration and buffer solution of pS115 with our own WT-PIN1 recombinant protein and assessed the ability of each recombinant protein to elect conformational changes in the green fluorescent substrates over time. Over the course of four hours we noted an initial increase in WT-PIN1's isomerase activity, followed by a plateau, then steady reduction in its isomerase activity (Figure 5.11). As for pS115 PIN1 however, we saw a steady and continuous increase in its isomerase activity over the same time period. These results indicated that pS115 PIN1 had an enhanced and prolonged isomerase activity compared with non-phosphorylated PIN1 *in vitro.* We concluded that active JNK's phosphorylation of PIN1 at S115 was also crucial for PIN1 constitutive activity.

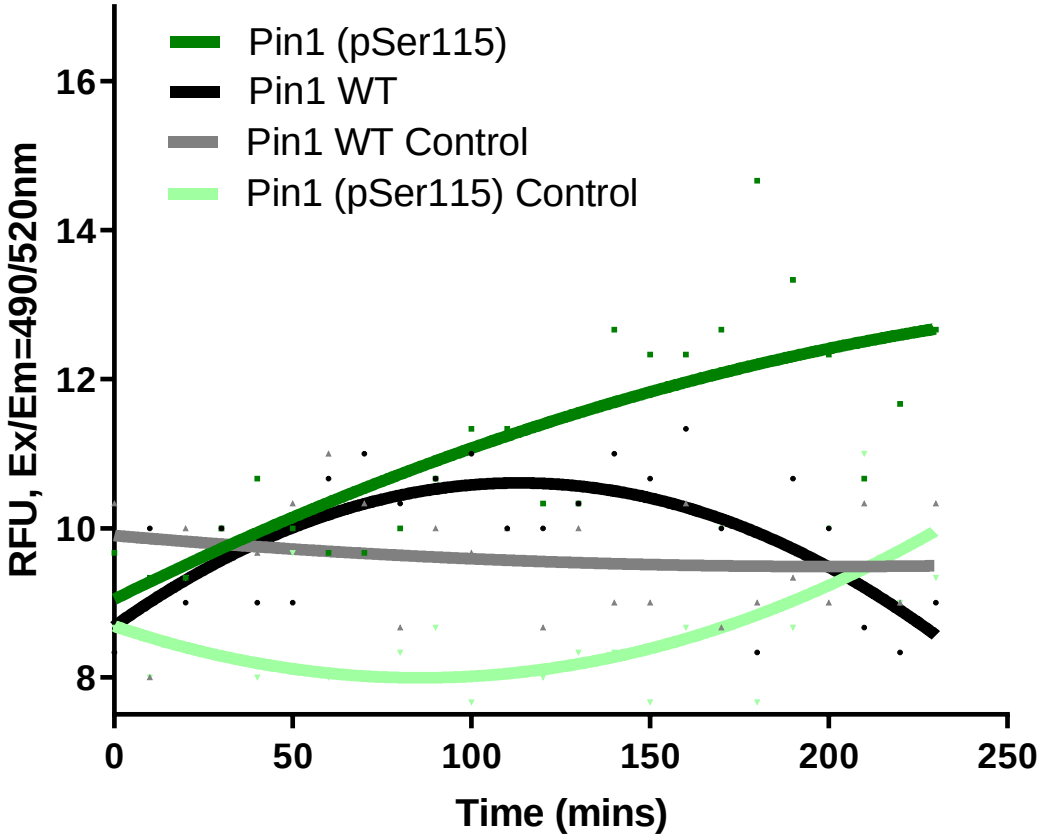


Figure 5.11: Phosphorylation of Pin1 residue S115 enhances overall isomerase activity in vitro: XY scatter plot shows in vitro isomerase activity of recombinant proteins of phosphorylated S115 Pin1 recombinant protein vs Pin1 WT recombinant protein. Results show a consistent increase in RFU over 250 minutes for phosphorylated S115 Pin1 (Dark Green line) however Pin1 WT (Black line) isomerase activity is reduced after 110 minutes. All recombinant proteins were loaded at equal concentrations to the plate assay and treated under the same conditions. Results indicate phosphorylation of Pin1 at S115 prolongs the isomerase activity of Pin1 in vitro. Pin1 isomerase activity was measured using a green florescent protein substrate protein with negative controlled conditions of the plate assay shown for both the phosphorylated S115 Pin1 (Light green line) and WT-Pin1 conditions (Grey line). All recombinant proteins used in the assay were of equal concentration with a fixed concentration of green florescent protein substrate and other buffers used. Results shown are repeated in triplicate.

5.12 Phospho-JNK expression and inhibition protects against PIN1 protein degradation via the proteasome

We had now identified that active JNK's stability of PIN1 expression was critical for its isomerase activity and proliferative function in cancer cells. The next logical step was to explore the mechanism of PIN1 degradation in the absence of its interaction with active JNK. Several possibilities lay before us, cellular protein degradation can be attributed to mechanisms involving lysosome degradation, proteasome degradation, or exiting of the cell through endosomal vesicles. Given that roughly 80% of cellular degradation occurs via 26s proteasomes within the cell's machinery, we opted to first explore this route of protein degradation. To this end, we knocked down *JNK1/2* in CCLP1 cells then waited five days for the infection to take effect. Following this we reseeded cells in all conditions at equal numbers and treated with a proteasome inhibitor (MG132) or control (DMSO). After 6 hours of treatment at 10uM we harvested cells and probed for PIN1 expression by western blot. We found that PIN1 expression in *shJNK1/2* treated cells (black) was significantly reduced compared with control *shNS* cells (grey) (Figure 5.12). However, after *shJNK1/2* cells were also treated with MG132, we saw a significant accumulation in PIN1 expression (green). These results indicated that in the absence of JNK signalling, PIN1 was being degraded by the cell's 26s proteasome machinery.

To pinpoint the need for JNK's activity in preventing PIN1 from being degraded via the proteasome, we also treated CCLP1 cells with the JNK inhibitor SP600125. Following this, cells were then treated with MG132 or control (DMSO) to block the proteasome function. Again, we found that when CCLP1 cells were treated with MG132 alone, PIN1 expression was significantly reduced compared with control cells. On the other hand, in combination with MG132 treatment for 6 hours, we saw an accumulation of PIN1 expression return. These results pointed to the need for phospho-active JNK signalling for the prevention of PIN1 degradation via the proteasome.

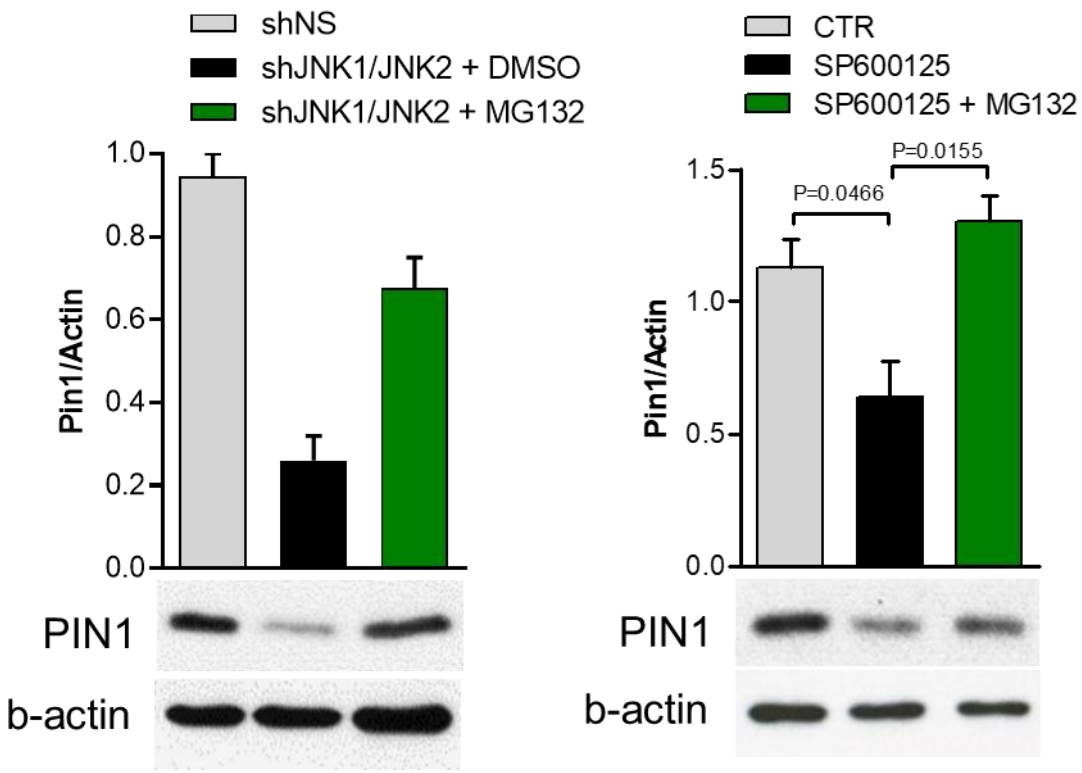


Figure 5.12: Phospho-Active JNK expression and activity protects against Pin1 protein degradation via the proteasome in cancer: (Analyses performed by Dr Pui Mann Choy, Institute of Hepatology London). Western Blot analysis using antibodies to detect against endogenous Pin1 expression in CCLP1 cell lines in the presence / absence of JNK expression (Left) and JNK inhibition (Right). All conditions are treated or untreated with 10uM proteasome inhibitor MG132. Antibodies for b-actin are used as loading controls. Bar graphs (Left and Right) indicate Pin1 relative density equalised to B-actin. Error bars are representative of SEM, with T-tests performed between data sets: P=0.0466, P=0.0155.

5.13 Active JNK phosphorylation of PIN1 at S115 reduces degradation via the proteasome

Given our previous findings that active JNK stabilises PIN1 by phosphorylation at S115, we sought to identify whether this mechanism was preventing the degradation of PIN1 via the proteasome. Therefore, following on from the last experiments, WT-PIN1 and PIN1 mutant (S115A) were transiently transfected in the presence of active JNK using HEK293 cells. After protein expression was established, cells of equal numbers containing either WT-PIN1 or PIN1 (S115A) both in the presence of active JNK were treated with MG132 or control DMSO for a 6-hour time period. All cell conditions were then lysed and PIN1 expression was analysed by western blot. For WT-PIN1 in the presence of active JNK we noted no significant difference in expression between MG132 treated cells and control cells. These results confirmed that WT-PIN1 is stabilised by active JNK and not degraded via the proteasome. On the other hand, PIN1 (S115A) expression in the presence of active JNK was significantly reduced for control conditions (DMSO), supportive of the fact that in the absence of S115 phosphorylation PIN1 is unstable (Figure 5.13). When the same conditions were treated with MG132, we saw the return of PIN1 (S115A) expression, indicative that phosphorylation at S115 protects PIN1 from degradation via the 26s proteasome.

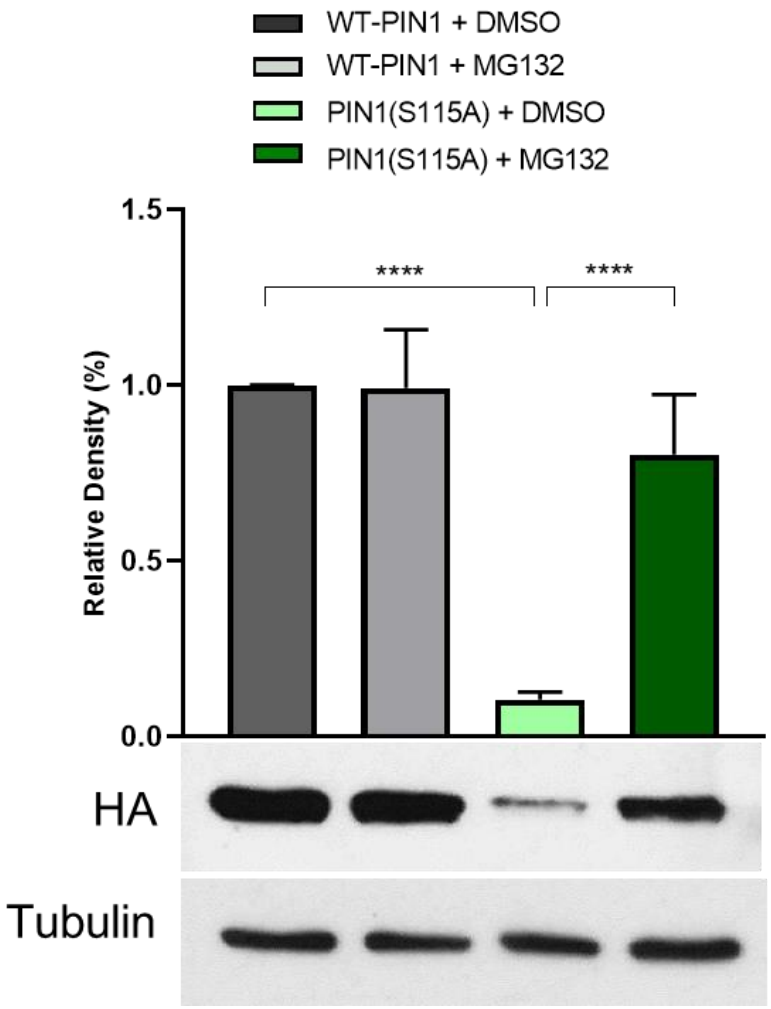


Figure 5.13: Active JNK's phosphorylation of PIN1 at S115 reduces degradation via the proteasome: Western Blot analysis of HEK293 containing FLAG-MKK7-JNK1 co-transfected with HA-WT-PIN1 and HA-PIN1(S115A) in the presence and absence of 20uM MG132 stimulation (proteasome inhibitor). Antibodies against FLAG, and HA are used to show JNK and PIN1 expression respectively, with tubulin used as control for loading. Column graph quantifies the relative density of HA-WT-PIN1 and HA-PIN1(S115A) expression treated with and without MG132 equalised to Tubulin load. One-way ANOVA analysis compares the reduction of HA-PIN1(S115A+ DMSO with all other conditions (<0.0001). Error bars are representative of SEM, with T-tests performed between data sets. Experiment is a repeat of two results.

5.14 Optimization of technique to identify PIN1 ubiquitination

Conclusive data now pointed to active JNK's phosphorylation at S115 as a critical factor in protecting PIN1 from degradation via the proteasome. Consequentially this area of our research had brought us to explore the ubiquitin code; a complex network of post-translational modifications involving ubiquitin enzymes that target proteins for degradation via the proteasome machinery of the cell. We hypothesised that in the absence of active JNK, PIN1 would be targeted for ubiquitination, and thus focused on uncovering this mechanism through techniques in the lab.

5.14.1 Data suggestive of poly-ubiquitinated PIN1 in the absence of active JNK expression

At first attempt, using transient transfections in HEK293 cells, we co-expressed HA-PIN1 and Myc-Ubiquitin in various combinations with FLAG-active JNK or FLAG-JNK1, either untreated (UT) or treated with MG132. It was essential that we included an exogenous Myc-tagged Ubiquitin in the cohort to differentiate from the endogenous ubiquitinated proteins in the cell when probing by western blot. When analysing total expression of HA-PIN1 by western blot, initially we noted the presence of smeared bands of high molecular weight (55-250 kDa) when cells were treated with MG132. Given our findings, we believed that the accumulated PIN1 smears under MG132 treatment depicted ubiquitinated forms of PIN1 that were unable to be degraded via the proteasome when blocked. These findings were concurrent with the poly-ubiquitination of other proteins founds in literature that presented as a smear thanks to the binding of numerous ubiquitin molecules (8kda) adding an increased range of molecular weight to the protein.

What's more, the intensity of the PIN1 smears were supportive of our findings that active JNK protected PIN1 from ubiquitination. Lower intensity PIN1 smears were present when co-expressed with active JNK whereas higher intensity PIN1 smears were observed in the absence of JNK expression. These findings were indicative of the accumulation of unstable PIN1 in the absence of JNK and when the cell's proteasome is blocked. However, despite these findings, we later noted

that we were missing the control for Myc-Ubiquitin expression alone, and therefore, set up new transfections to repeat results that would be inclusive of this factor. Upon repeat of these western blots using new cell extract, we discovered that part of the PIN1 smear; bands residing between 55-70 kDa, were in fact the product of a non-specific HA band present across all extracts. With this in mind we moved forward under the assumption that PIN1 was poly-ubiquitinated in the absence of active JNK, however now producing PIN1 smears between 70-250 kDa.

5.14.2 Technical difficulties with the immunoprecipitation of poly-ubiquitinated PIN1

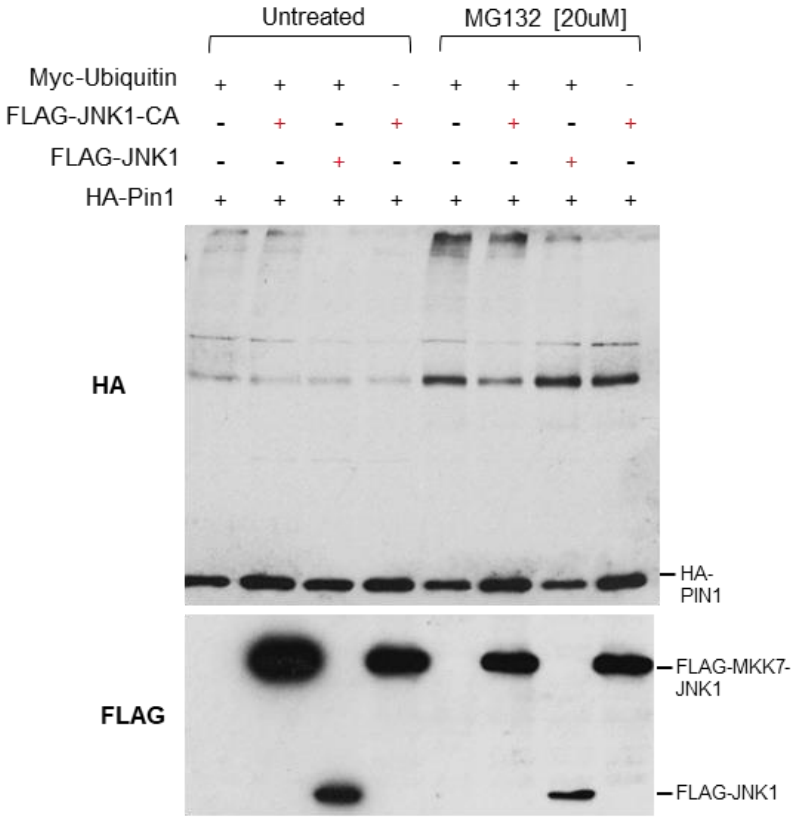
In order to pinpoint the specific ubiquitination of PIN1, we needed to perform immunoprecipitations (IP); pulling down PIN1 and probing for ubiquitinated PIN1 forms attached to the total protein expression. Thus far our western blot results from Figure 4.14 had given us an indication of where to expect ubiquitinated forms of PIN1, however alone these results would not suffice. Therefore, we pulled down PIN1 using a combination of PIN1 antibodies and AG beads, probing for ubiquitin expression. These findings were inconclusive, depicting a steady background from the beads in all conditions.

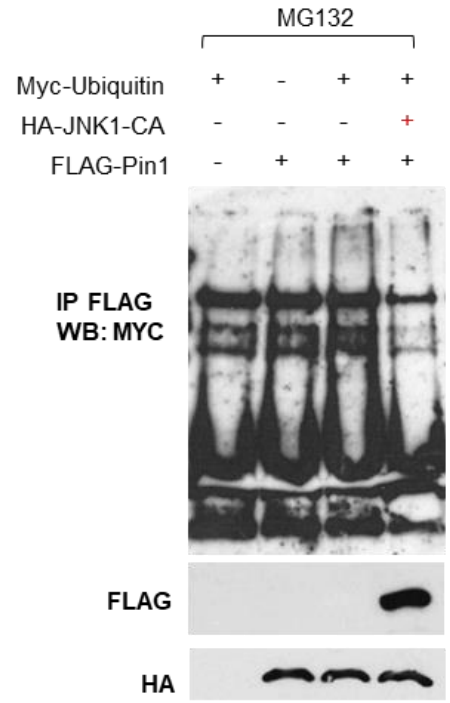
Upon reflection we realised this combination of antibodies / beads would also pull down the endogenous levels of the proteins. Therefore, we then pulled down PIN1 using the HA tag by aid of HA-conjugated beads and probed for Myc expression. Here we noted that there was a reduced smear when PIN1 was in combination with active JNK expression, a result consistent with past findings. However overall, we were disappointed at the amount of background still present using these conditions. Across the next months we attempted to uncover the poly-ubiquitination of PIN1 through various immunoprecipitation techniques; cloning our plasmids with different tags and altering the antibody-bead combinations. As shown in Figure 4.14 we found there to be background problems for many of the IP combinations making the results difficult to interpret, however we persisted due to a consistent trend of a reduced smear of PIN1 in the presence of active JNK compared with PIN1 alone.

5.14.3 GST-TUBES technique to target poly-ubiquitinated proteins

With the aim of reducing antibody-bead background, we purchased a GST-tagged tubes plasmid and produced recombinant proteins of GST-TUBEs. TUBEs are high affinity ubiquitin traps that bind to poly-ubiquitin chains and allow for successful pull downs using the GST-tag combined with Glutathione reduced beads. Given the difficulties with producing a reliable IP thanks to various backgrounds from beads and antibodies, this approach held potential to stop these issues from arising. We therefore set up another IP, however this time including GST-TUBEs bound to beads to pull down PIN1 and look for ubiquitinated PIN1 forms using GST antibodies. To our interest we noted the presence of a novel band present when FLAG-PIN1 with HA-Ub was co-expressed together. This band was also seen in combination with active JNK albeit much fainter. What was surprising was the size of the band, appearing at the much lower molecular weight of ~30kda, roughly only 8kda more than the size of non-ubiquitinated PIN1.

These last findings suggested that only one ubiquitin chain was attached to PIN1 in the absence of JNK, pointing towards the mono-ubiquitination of PIN1 instead of the previously assumed poly-ubiquitination. Indeed, we began to uncover other literature on PIN1 that also pointed towards its mono-ubiquitination, and collectively these results may have explained why we were unable to identify the poly-ubiquitin forms through various IP techniques whilst simultaneously missing mono-ubiquitinated forms by running western blot gels aimed at analysing a higher kDa range. Despite the GST-TUBEs technique providing a reduced background from the IP, we were still unable to get a consistent result, often seeing no mono-ubiquitinated bands present in either condition. On reflection these results were unsurprising given that GST-TUBEs are designed to bind most effectively to poly-ubiquitinated proteins; with the ability to attach to over 4 tandem repeats of a poly-ubiquitin chain.





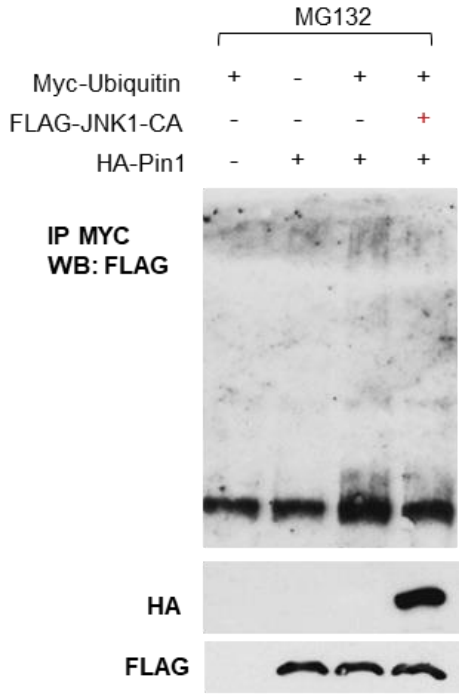


Figure 5.14: Optimization of technique to identify PIN1 ubiquitination: (A): Western blot analysis of HEK293 cell lysates transfected with combinations of HA-PIN1, FLAG-JNK1. FLAG-MKK7-JNK1 and/or Myc-Ubiquitin. Each transfection condition is both treated and untreated with MG132 [20uM] for 4 hours to monitor effect of proteasome blocking. Antibodies against HA are used to detect PIN1 expression, with FLAG detection of JNK and JNK-CA used as controls. **(B):** Immunoprecipitation of HEK293 cell lysates transfected with combinations of HA-PIN1, FLAG-JNK1-CA, and Myc-Ubiquitin all treated with MG132 under same conditions as (A). Results show immunoprecipitation of FLAG and WB using antibodies to detect MYC. Antibodies for HA and FLAG expression are used as controls. **(C):** Immunoprecipitation of HEK293 cell lysates shown in (B). Results show reverse immunoprecipitation of MYC and WB using antibodies to detect FLAG expression. Antibodies for HA and FLAG expression are used as controls. **(A-C):** Representative of over three experimental repeats.

5.15 Active JNK protects PIN1 from mono-ubiquitination

In an attempt to further address the problematic issues surrounding the excessive background from immunoprecipitation methods so far, we adopted a new approach to identify the role of JNK in the ubiquitination of PIN1. Through previous experimental techniques, we had noted that both HIS-conjugated beads and FLAG-conjugated beads (M2-FLAG) provided the least background during immunoprecipitations. We therefore opted to use a combination of both these antibody-beads as an attempt to reduce the background that were obscuring our results thus far. This required further cloning for a His-tagged ubiquitin construct to be overexpressed in HEK293 cell lines with existing HA-MKK7-JNK1 and FLAG-PIN1 constructs. This method confirmed a reduced background in preliminary immunoprecipitation experiments with these constructs. Whats more, further literature readings surround ubiquitin methods suggested a quenching technique involving the treatment of cell lysates with detergents and boiling prior to the incubation with conjugated beads. This was deemed appropriate to remove excess unbound and secondary messenger ubiquitin present in the cell lysates that may obscure the results of the immunoprecipitation. Finally, from the results outlined in Figure 5.14, we had evidence to suggest PIN1 was undergoing mono-ubiquitination over poly-ubiquitination mechanisms of degradation. To account for this, we made up western blot gels of alternative acrylamide concentrations in order to best visualise any ubiquitination occurring at lower kDa protein sizes. We therefore overexpressed all three constructs in HEK293 cell lines in various conditions and treated all conditions with MG132 10uM at the change of medium, 12 hours after the transfection of the plasmids. Cells were harvested and lysed after 6 hours of MG132 treatment, then were quenched prior to incubation with His-conjugated beads. We then conducted western blot analysis of the IPs using antibodies against FLAG, HA. In the absence of active JNK expression, PIN1 was shown to be mono-ubiquitinated, indicated by a band 8kda higher than the normal PIN1 protein kDa. Furthermore, this band was not apparent in the presence of active JNK, confirming that active-JNK protects PIN1 from mono-ubiquitination (Figure 5.15).

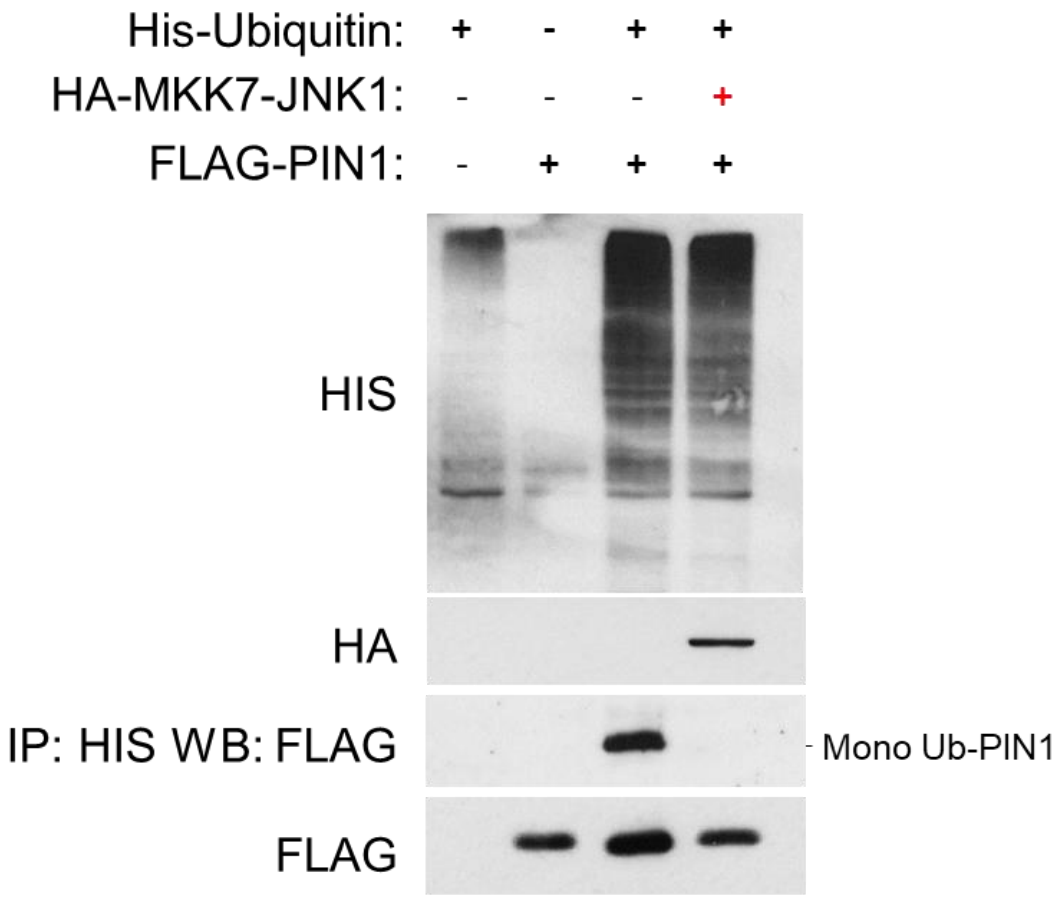


Figure 5.15: Active JNK protects PIN1 from mono-ubiquitination via the proteasome: (Analyses performed by Dr Alessio Lepore, University of Leeds). Immunoprecipitation of His-Ubiquitin in the presence and absence of HA-MKK7-JNK1 and FLAG-PIN1. Western blot analysis uses antibodies to detect PIN1 ubiquitination in the presence and absence of HA-MKK7-JNK1. Antibodies against FLAG, HA and Histidine are used as controls for total gel loading. All conditions are treated with 10uM MG132 (proteasome inhibitor). Result shown is a repeat of more than three experiments.

5.16 Active JNK protects PIN1 from mono-ubiquitination by phosphorylation at S115

Following on from Figure 5.15 and our prior evidence surrounding the phosphorylation of PIN1 by active JNK at S115 residue, we sought to understand if the protection of PIN1 from mono-ubiquitination by active-JNK was the outcome of phosphorylation at PIN1's S115 residue. Using the same constructs from Figure 4.15, with the addition of a FLAG-PIN1(S115A) construct, we carried out a similar experimental procedure for the immunoprecipitation of WT-FLAG-PIN1 vs FLAG-PIN1(S115A) in the presence of HA-MKK7-JNK1 and His-Ubiquitin. This involved the over-expression of all constructs in HEK293 cell lines followed by treatment of 10uM of MG132 for 6 hours the following day. Cells were again lysed and subjected to quenching prior to incubation with His-conjugated beads. Western blot analysis was then carried out to detect FLAG, HA expression as well as His expression on a standard western blot to quantify the ubiquitin background.

With our previous experiments pointing towards PIN1 being preferentially mono-ubiquitinated by active JNK, we continued to run western blot gels to accommodate for a lower kDa range to observe a similar event. In the presence of active JNK we confirmed that no mono-ubiquitination occurs on WT-FLAG-PIN1. Contrary to this, when PIN1 was mutated at S115A residue even in the presence of active-JNK, we saw mono-ubiquitination of PIN1 (Figure 5.16). These results confirm that the identified post-translational phosphorylation at S115 of the PIN1 structure by active-JNK is critical for protecting PIN1 from ubiquitination via the 26s proteasome.

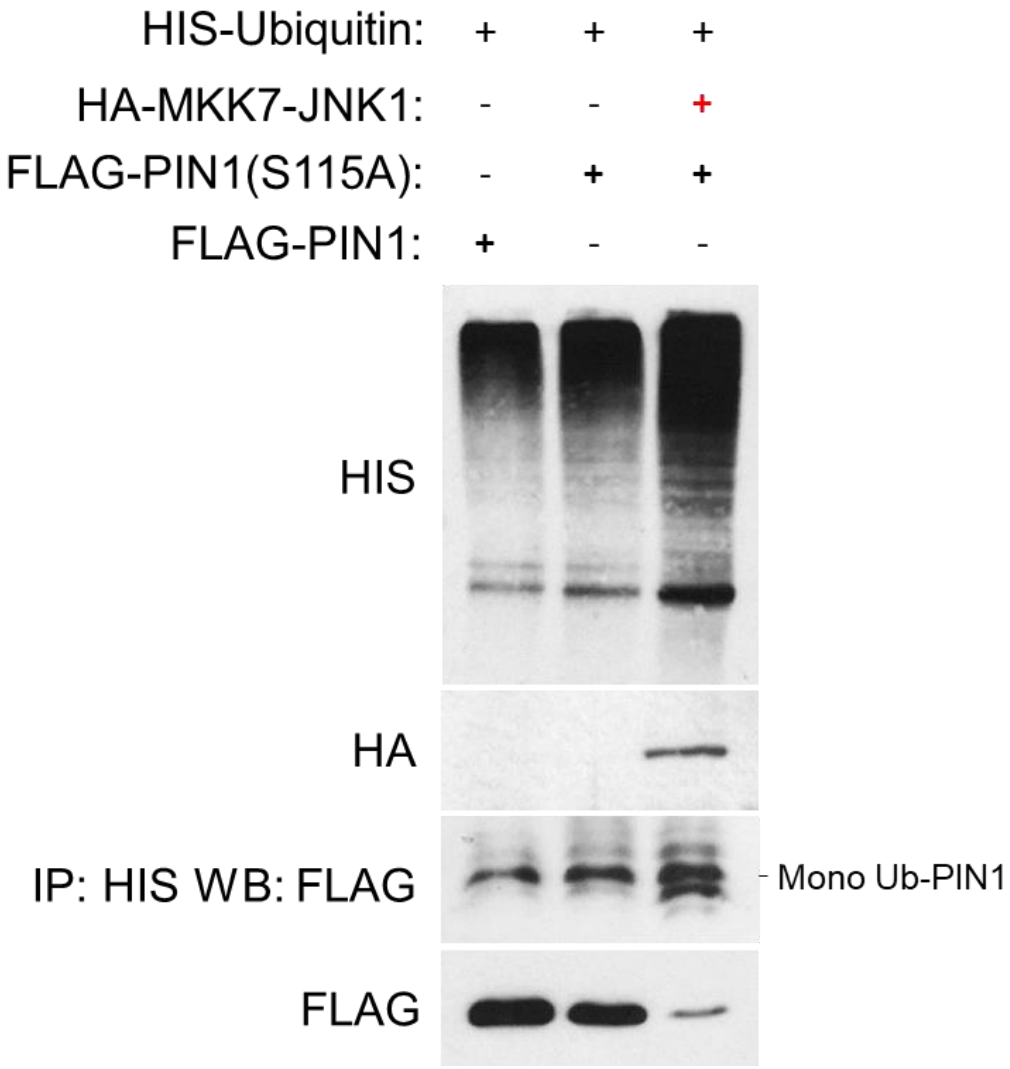


Figure 5.16: Phosphorylation of PIN1 S115A by Active JNK protects from mono-ubiquitination: (Analyses performed by Dr Alessio Lepore, University of Leeds). Immunoprecipitation of His-Ubiquitin in the presence of FLAG-PIN1 or FLAG-PIN1(S115A) with and without HA-MKK7-JNK1. Western blot analysis uses FLAG antibodies to detect PIN1 ubiquitination. Antibodies against FLAG, HA and Histidine are used as controls for total gel loading. All conditions are treated with 10uM MG132 (proteasome inhibitor). Result shown is a repeat of two experiments

Chapter 6 Results: Phosphorylation of PIN1 S115 by active JNK is important for glycolytic function in cancer

In the past two chapters we have detailed a novel JNK mechanism on PIN1; describing how the biological outcome of this mechanism is beneficial for the proliferation of cancer cells. Due to the plethora of Pro-Ser/Thr protein targets of PIN1 combined with its abhorrent over-activation in cancer, our described novel mechanism outlining active JNK's ability to stabilise and alter PIN1 activity in cancer will likely have other impactful implications.

One well-established role of PIN1 in cancer is in the reprogramming of cellular metabolism. Under normal physiological conditions, the metabolic pattern of glucose in mammalian cells is largely dependent on an oxygen environment. Mitochondrial oxidative phosphorylation (OXPHOS) is the preferred pathway; efficiently breaking down glucose and generating high levels of ATP for the cell's needs under aerobic conditions. However due to the cell's high demand for energy, most mammalian cells also undergo glycolysis; an anaerobic and more ancient evolutionary pathway for energy production. As a result, under normal conditions most cells are seen to couple both metabolic pathways to meet the cell's energy requirements. Despite the clear advantages for cellular metabolism by oxidative phosphorylation, the major aerobic metabolic pattern in cancer cells remains selective of glycolysis. This phenomenon is referred to as the Warburg Effect; a metabolic adaptation that benefits cancer progression through rapid glucose consumption combined with a large supply of glycolytic intermediates.

In the mix of this complex metabolic pattern, PIN1 has surfaced as a crucial regulator of the Warburg Effect. PIN1 is shown to enhance the nuclear translocation of PKM2 resulting in an enhanced expression of GLUT1; controlling the level of glucose influx into the cell, as well as LDHA; responsible for inter-conversion of pyruvate and L-lactate. With this research in mind, we set out to determine the effect of our novel JNK mechanism on PIN1 and its role in cancer cell metabolism.

6.1 Knockdown of PIN1 in liver cancer causes reduced glycolytic enzyme expression

Given the wealth of research detailing PIN1 as a critical regulator of the Warburg Effect, we sought to understand the outcome of PIN1 expression on key glycolytic enzymes in liver cancer cells. The Warburg Effect benefits the survival of cancer cells through rapid ATP production and a backlog of glycolytic intermediates that are shuttled into divergent metabolic pathways. High volumes of glycolytic intermediates produced by cancer cells taking in glucose and glutamine, result in rapid and unchecked biosynthesis beneficial for the cell's extended needs.

This dynamic shift in the cell's metabolic programming does not go unnoticed and is often seen through the upregulation or enhanced expression of glycolytic enzymes that are working in overdrive to maintain cellular homeostasis. For these reasons, we opted to knock down PIN1 expression in CCLP1 cell lines and compare the effect on glycolytic enzyme expression with control shNS conditions. Following treatment with lentiviral shPIN1 or shNS, we waited five days to ensure the knock down took full effect before lysing all cell conditions and screening for expression of HK2, GLUT1, PDK1, PKM2, and LDH-A; known glycolytic enzymes involved in the Warburg effect. The results showed that in the absence of PIN1 expression, there was a significant reduction in HK2 and PDK1; two glycolytic kinases central to the glucose metabolism processes of the cell (Figure 6.1). These results are suggestive of a functional need for PIN1 in maintaining glycolytic enzyme expression central for cancer cell metabolism. We therefore decided to quantify how PIN1 affected the glucose expression of the cell in our next experiments.

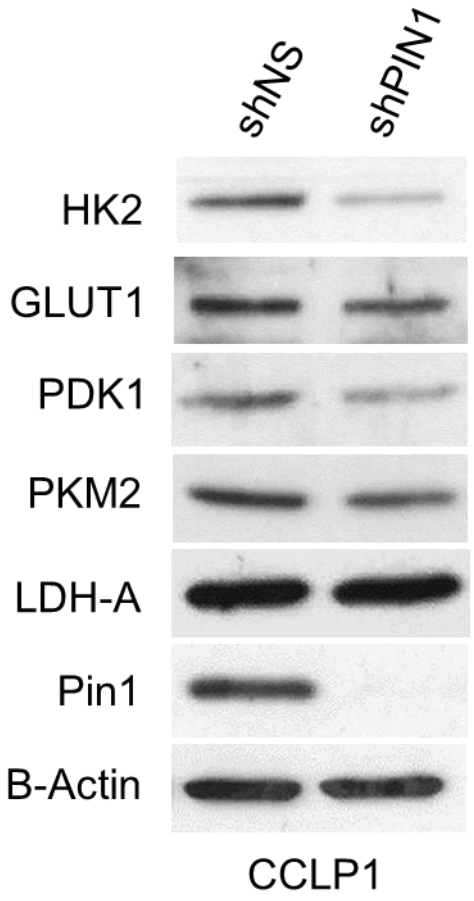


Figure 6.1: Knockdown of PIN1 in liver cancer caused reduced HK2 expression: Western blot analysis of CCLP1 cell lysates 4 days post lentiviral infection with shNS (control)(left) or shPIN1 (right). All cells 4 days post infection were lysed in the same buffer and loaded at equal concentrations onto the western blot. Antibodies are used for the detection of glycolytic enzymes: HK2, GLUT1, PDK1, PKM2 and LDH-A. Antibodies for PIN1 and B-Actin are used as knockdown and loading controls respectively. Results are representative of two experimental repeats.

6.2 Silencing of PIN1 in CCLP1 cells reduces glucose consumption

The upregulation of the rate limiting Hexokinase 2 (HK2) in cancer cells containing endogenous levels of PIN1 is suggestive of support for increased catalysis of glucose into G6P; the first obligatory and rate-limiting step in the metabolism of glucose by the cell. To assess whether the presence of PIN1 was resulting in an increased glucose consumption by cancer cells, we set out to measure alterations in glucose intake by cells containing endogenous PIN1 (shNS) vs those knocked down with PIN1 (shPIN1) in CCLP1 cells. To do so, in a similar method to past experiments we used lentiviral methods to establish shRNA into CCLP1 cells and waited 5 days to ensure a stable knockdown of PIN1 was successful. Following this, we reseeded equal volumes of cells treated with either shNS or shPIN1 and replaced cells with fresh medium at time point 0 hours. After 72 hours in the incubator we collected samples of the medium for cells of each condition and quantified the glucose intake of cells through spectrophotometry. We found that CCLP1 cells in the absence of PIN1 expression consumed less glucose over 72 hours than cancer cells with endogenous PIN1 levels (Figure 6.2). These results supported previous experiments suggestive of a reduced glucose metabolism of cancer cells in the absence of PIN1 expression.

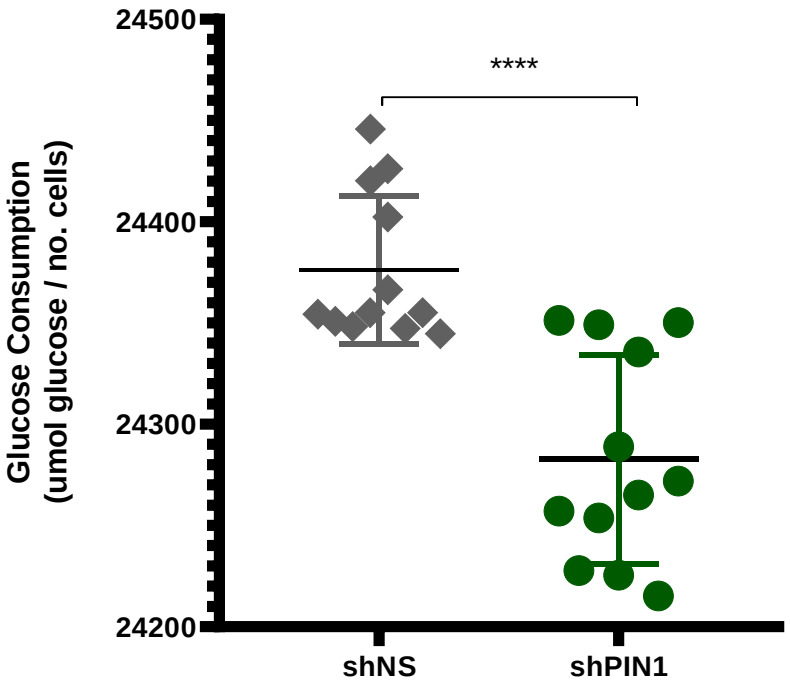


Figure 6.2: Knockdown of PIN1 reduces rate of glucose consumption in cancer: Glucose consumption analysis of CCLP1 cell lysates 7 days post lentiviral infection with either shNS (control)(grey), or shPIN1 (green). Aliquots of cell medium were taken for each condition, 72 hours post equalising of infected cells by both seed density ($x10^4$) and medium volume (ul). Glucose consumption was calculated by the concentration of glucose oxidised by all cells in each condition. For both shNS and shPIN1 cells, 4 experimental and 3 biological repeats were carried out (n=12). Unpaired T test were performed between data sets (<0.0001).

6.3 Phosphorylation of PIN1 at S115 is critical for glycolytic enzyme expression

Following the findings that silencing of PIN1 reduced HK2 expression in cancer cells, we sought to understand the role of active-JNK's phosphorylation of PIN1 on HK2 expression. We therefore silenced shPIN1 in the same manner shown in Figure 5.1, but this time reconstituted CCLP1 cells with shRNA resistant WT-PIN1-shRES (green), or mutant PIN1(S115A)-shRES (light green). To appreciate the normal endogenous PIN1 expression of the cell line, we also included a control condition for the lentiviral infection namely; Empty + shNS (black). All cell conditions were then lysed, and western blot analysis was used to quantify HK2 expression. We found a significantly reduced expression in HK2 expression for cancer cells reconstituted with the mutant PIN1(S115A)-shRES (light green), compared with WT-PIN1-shRES (dark green) or control conditions (Figure 6.3). These results stated that JNK's phosphorylation of PIN1 at S115 is necessary to maintain normal HK2 expression in cancer cells.

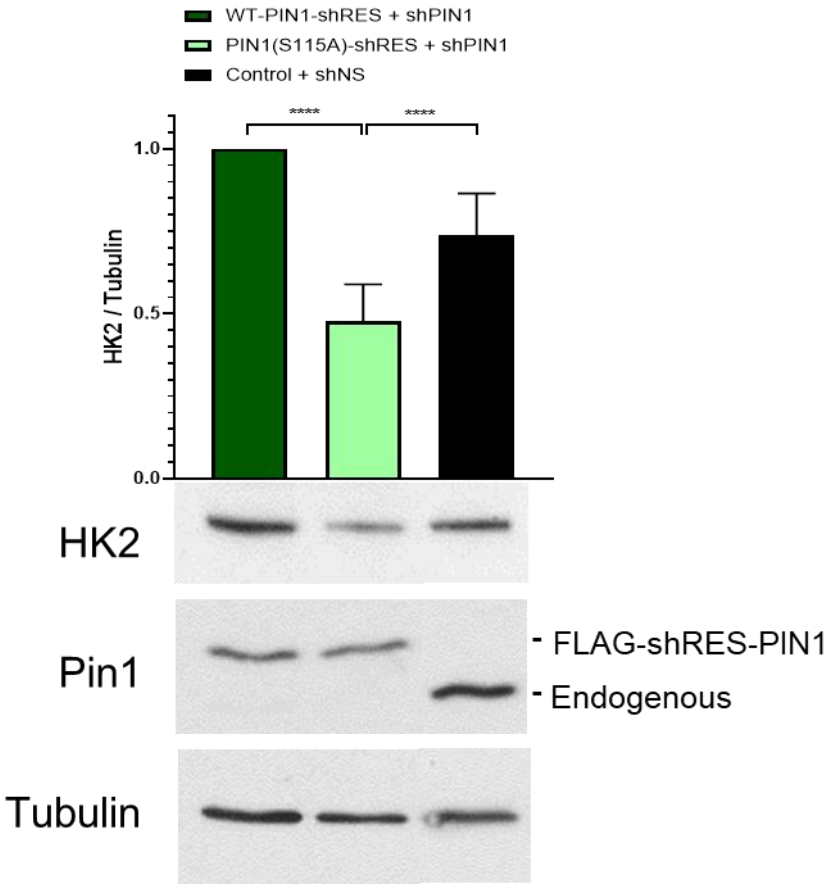


Figure 6.3: Phosphorylation of PIN1 at S115 is critical for enhanced HK2 expression in cancer: Western blot analysis of CCLP1 cell lysates 4 days post lentiviral infection with either: shPIN1 + reconstituted shRES-PIN1 (Dark Green), shPIN1 + reconstituted shRES-PIN1(S115A) (Light Green), or shNS + PwPI (control) (Black). Antibodies are used for the detection of HK2, with Tubulin and PIN1 used as controls for loading and lentiviral success respectively. Column graph displays the relative density of HK2 expression equalised to tubulin expression. One-way ANOVA statistical tests are carried out using shPIN1 + reconstituted shRES-PIN1(S115A) (Light Green) as comparison <0.0001. Experiment is an example of two repeats.

6.4 Reconstitution of phosphorylated S115 PIN1 enhances the glucose consumption of cancer cells

To further elucidate the role of JNK's phosphorylation of PIN1 on cancer cell metabolism, we opted to quantify the glucose consumption of the cell conditions described in Figure 6.3. To this end we established lentiviral infections of either; shPIN1 + reconstituted shRES-PIN1 (dark green), shPIN1 + reconstituted shRES-PIN1(S115A) (light green), or shPIN1 + control (grey). We then waited 5 days to ensure the knockdown and reconstitution of exogenous PIN1 took full effect. After this, again we reseeded all cell conditions to equal numbers and equal medium volume. Following 72 hours of incubation we took samples of medium from each condition and analysed the glucose consumption. We saw a marked increase in glucose consumption for cancer cells reconstituted with exogenous WT-PIN1 (Dark Green), compared with control cells (Figure 6.4). Interestingly cancer cells reconstituted with the mutated S115A PIN1 showed no significant increase in glucose consumption compared to control cell conditions. These findings highlight the phosphorylation of S115 PIN1 as a requirement for enhanced glucose consumption by cancer cells.

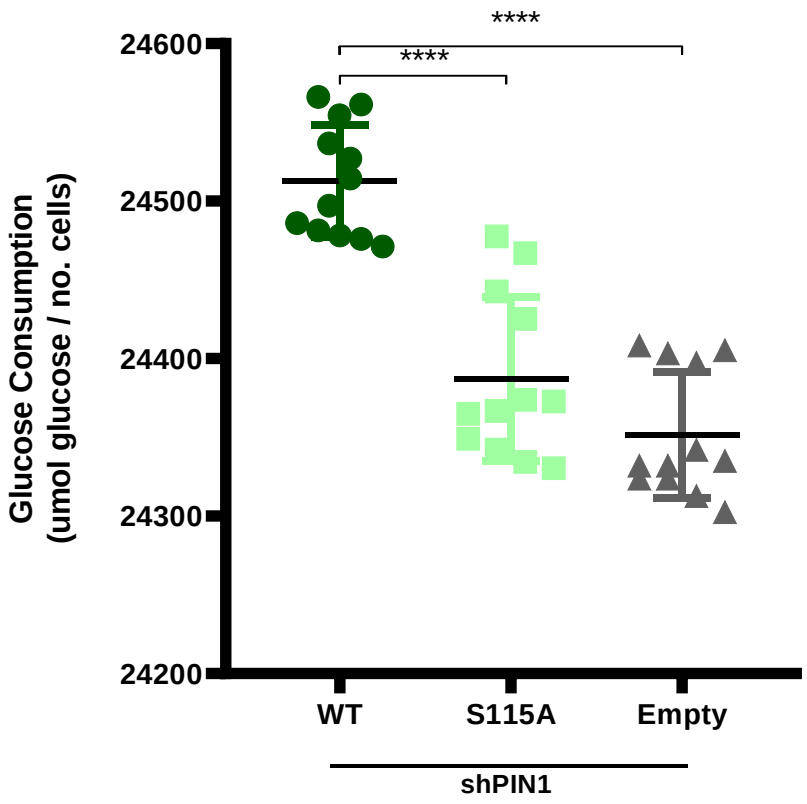


Figure 6.4: Phosphorylation of PIN1 at S115 is critical for the recovery of glucose consumption in cancer cells: Glucose consumption analysis of CCLP1 cell lysates 7 days post lentiviral infection with either: shPIN1 + reconstituted shRES-PIN1 (Dark Green), shPIN1 + reconstituted shRES-PIN1(S115A) (Light Green), or shPIN1 + PwPI (control) (Grey). Aliquots of cell medium were taken for each condition, 72 hours post equalising of infected cells by both seed density (x10^4) and medium volume (ul). Glucose consumption was calculated by the concentration of glucose oxidised by all cells in each condition. For all conditions, 4 experimental and 3 biological repeats were carried out (n=12). Unpaired T test were performed between data sets (<0.0001).

6.5 Simplified representation depicting the glycolytic pathway in liver tumours

At this stage of the project we were presented with an opportunity to further explore the roles of glycolytic enzymes in the progression of liver cancer. In our supplemental readings surrounding the study of PIN1's metabolic function of liver cancer cells, we discovered that the wider biochemical events underlying the progression of cirrhosis to hepatocellular carcinoma (HCC) were largely not well understood. With liver cancer representing the second most common cause of cancer-related death, there is real immediacy in the need for better molecular characterization of primary liver cancers like HCC and cholangiocarcinoma.

We therefore opted to undertake a parallel study; examining the gene expression of glycolytic enzymes upregulated in precancerous cirrhotic livers and their associated risk in the development of HCC. To this end we began by exploring the ten enzymatic reactions within the glycolytic pathway through which glucose is converted into pyruvate (Figure 6.5). The functional diversity of glycolytic enzymes is further increased through the presence of multiple isoforms able to catalyse each glycolytic reaction. For these reasons, we focused our efforts on evaluating the mRNA expression from glycolytic enzymes predominately expressed in the liver (Figure 6.5). The results of this study shown here in Figure's 3.5 to 3.12 have been published in Frontier's Cell Biology Press titled 'High Expression of Glycolytic Genes in Cirrhosis correlates with the Risk of Developing Liver Cancer (31 October 2018).'

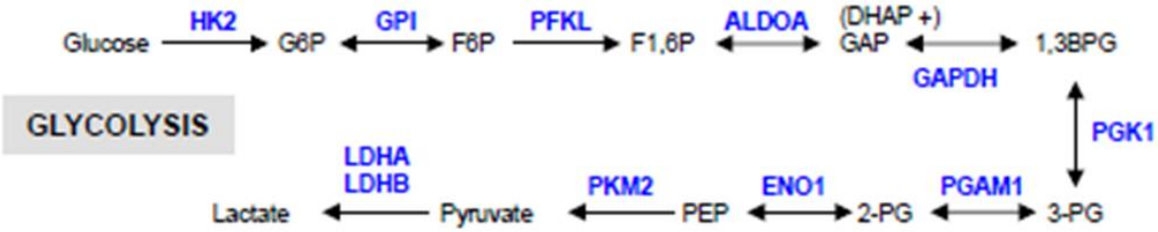


Figure 6.5: A simplified representation depicting the glycolytic pathway in liver tumours: Abbreviations of the enzymes are as follows: hexokinase 2 (HK2), glucose-6-phosphate isomerase (GPI), phosphofructokinase liver isoform (PFKL), aldolase A (ALDOA), glyceraldehyde 3 phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1), phosphoglycerate mutase 1 (PGAM), enolase 1 (ENO1), and pyruvate kinase M2 (PKM2), lactate dehydrogenase (LDH). Abbreviations of the metabolites are as follow: glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose 1,6-biphosphate (F1,6BP), glyceraldehyde 3-phosphate (GAP), and dihydroxyacetone phosphate (DHAP), 1,3-biphosphoglycerate (1,3BPG), glycerol-3-phosphate (3-PG), glycerol-2-phosphate (2-PG), phosphoenolpyruvate (PEP).

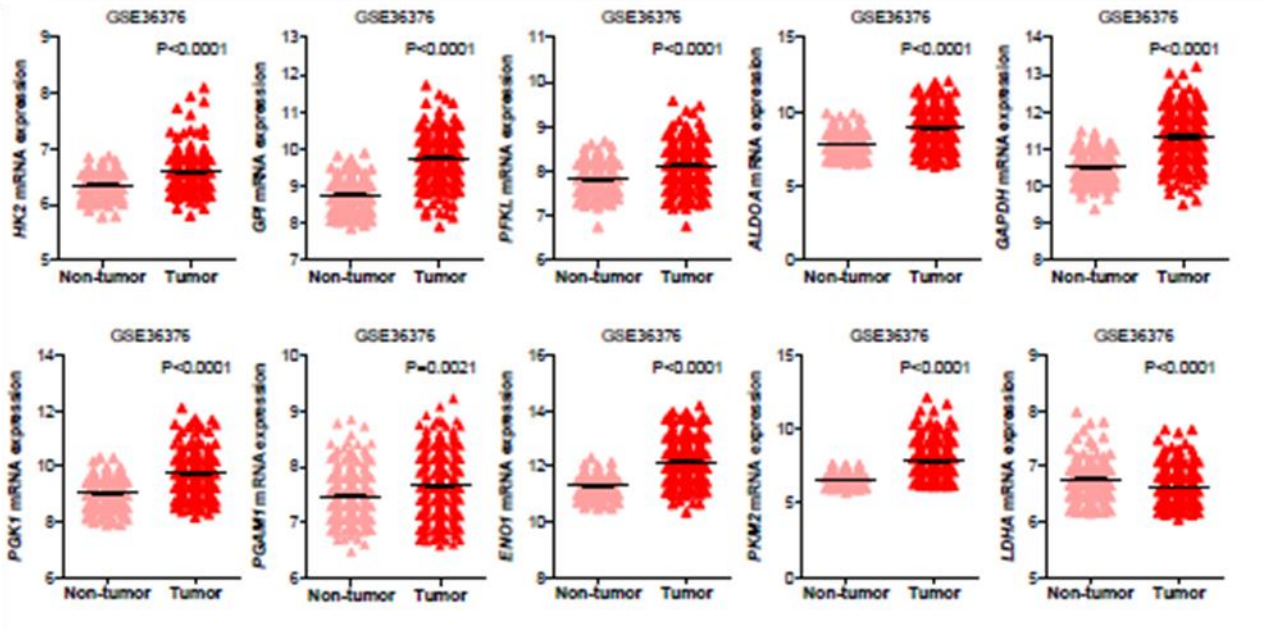


Figure 6.6: Glycolytic enzyme transcript levels in HCC and non-tumour patients: Scatterplots showing the transcript levels of different glycolytic enzymes in the clinical data set GSE36376 consisting of HCC (*n* = 240) and adjacent non-tumour (*n* = 193) liver tissue (Lim et al., 2013). The horizontal lines indicate mean ± SEM *P*-values were calculated by non-parametric Mann–Whitney tests.

6.6 Glycolytic enzyme transcript levels in non-tumour and HCC patients

To start, we chose to assess the variability in glycolytic enzyme levels between HCC and non-tumour conditions. As shown in Figure 6.6, the expression for the majority of glycolytic transcripts, including the rate limiting HK2 (Hexokinase 2), ALODA (Aldolase, fructose, bisphosphate A), PFKL (6-phosphofructokinase, liver type), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), and PKM2 (Pyruvate kinase M2), was significantly increased in HCC livers compared to non-tumour conditions ($P<0.0001$). The exception was PGAM1; although still shown to be significantly higher in tumour cells compared with non-tumour ($P=0.0021$) it was not elevated to the same degree as the other enzymes analysed.

Interestingly however, the analysis of mRNA for LDHA (Lactate dehydrogenase A) provided the opposite; describing a decreased LDHA expression from non-tumour to HCC samples. Given the role of LDHA in the conversion of pyruvate into lactate away from the mitochondria, a reduced LDHA expression would indicate the favouring of glycolytic-derived pyruvate being shuttled into mitochondrial oxidative pathways. As a result, we may see a consequential increase in metabolic processes involving the mitochondrial TCA and oxidative phosphorylation pathways. To support these results, we conducted a similar analysis from ‘The Cancer Genome Atlas’ (TCGA) dataset consisting of a cohort of 371 primary HCC tumours and 50 normal liver samples (Figure 6.7). In this dataset we also observed a significantly higher expression of all glycolytic enzymes in primary cancer samples compared with normal livers; with the exception of LDHA that again was seen to decrease in HCC (Figure 6.7).

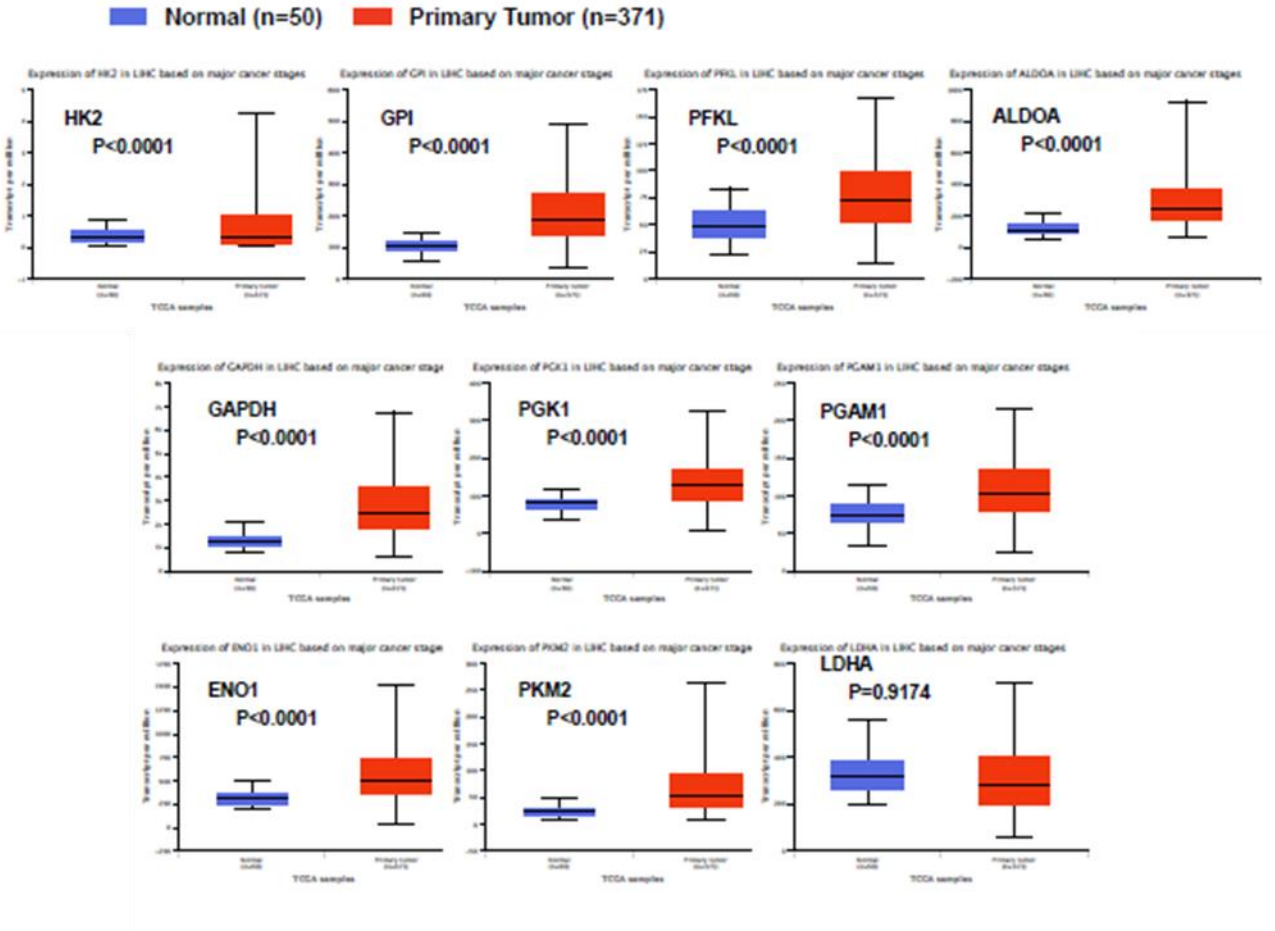


Figure 6.7: Differential glycolytic gene expression in normal liver tissues vs primary tumour tissues: Boxplots showing differential gene expression of glycolytic enzymes among normal liver tissues (*n* = 50) vs. primary tumour tissues (*n* = 371) (TGA-LIHC samples) analysed using the UALCAN bioinformatic tool of genomic database (Ally et al., 2017; Chandrashekar et al., 2017). Values are expressed as transcript per million. For each box plot, the whiskers represent the 2.5–97.5th percentile range of values, the lower and up boundaries denote the 25th and the 75th percentile of each data set, respectively, and the horizontal line represents the median value for each group. *P*-values were calculated by *t*-test.

6.7 Expression of genes in the Pentose phosphate pathway (PPP)

Running in parallel to the glycolytic pathway is the ancient metabolic Pentose Phosphate pathway (PPP); responsible for the generation of NADPH, pentose and ribose 5-phosphate. During the oxidative phase of the PPP, energy from the conversion of glucose-6-phosphate to Ribose-5-phosphate from the glycolytic pathway is used to reduce two molecules of NADP+ to NADPH. This is an obligatory requirement for the synthesis of nucleotides in the non-oxidative state of the PPP (Figure 6.8 (A)). Given the significant increases in glycolytic enzymes found in HCC compared to non-tumour samples, we opted to explore the mRNA expression of enzymes involved in the PPP. As a result, we found that in HCC compared to normal livers, transcript levels for PPP enzymes including G6PD (Glucose-6-phosphate dehydrogenase), PGLS (6-phosphogluconolactonase) and PGD (6-phosphogluconate dehydrogenase) were significantly elevated (Figure 6.8 (B-C)). Combined with the analysis for the increased demand in glycolysis, these data are telling of a need for sustained ATP and cellular building block production required by abnormal hepatocyte proliferation.

6.8 Gene expression analysis of the oxidative mitochondrial metabolism of HCC

Next, we chose to explore the mRNA expression for enzymes involved in mitochondrial oxidative metabolism. Once in the mitochondria, pyruvate is converted into acetyl-CoA by the pyruvate dehydrogenase complex (PDH). Acetyl-CoA in turn then enters into the TCA cycle to generate NADH and FADH2, transferring their electrons to the electron transport chain to generate ATP through oxidative phosphorylation. Upon assessment of PDHA1 expression; that sub sequentially encodes the E1 alpha 1 subunit of the PDH complex, we found it to be significantly elevated in HCC across two datasets compared to normal livers and non-tumour tissues (Figure 6.9(A)). However, analysis of SDHB (Succinate dehydrogenase); responsible for the conversion of fumarate in the TCA was significantly reduced in non-tumour samples. This data is in line with observations that low SDHB expression in HCC benefits the Warburg Effect.

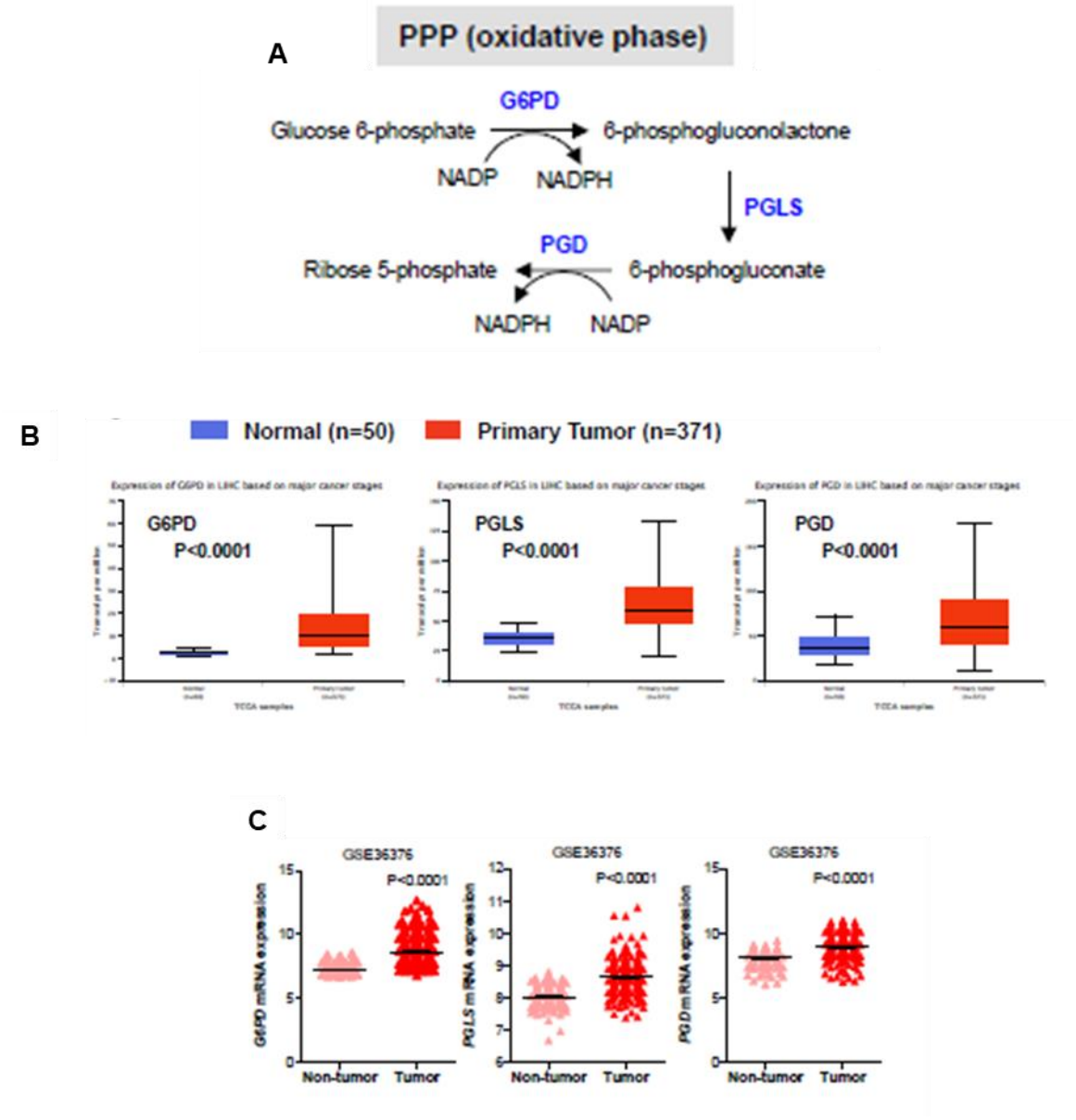


FIGURE 6.8: Expression of genes in pentose phosphate pathway (PPP): (A) Diagram of the oxidative phase of the PPP. Abbreviations of the enzymes are as follows: glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconolactonase (PGLS), 6-phosphogluconate dehydrogenase (PGD). Activation of the two dehydrogenase enzymes, G6PD – the rate-limiting enzyme and PGD, results in the production of NADPH, H+ ions, and ribose 5-phosphate. **(B,C)** Gene expression analyses showing enhanced expression of G6PD, PGLS, and PGD in primary HCC tumour samples compared to either adjacent non-tumour samples in GSE36376 data set **(B)** or normal liver tissues in TGA-LIHC data set **(C)**, respectively. *P*-values were calculated by nonparametric Mann–Whitney tests in **(A)** or by *t*-test **(B)**.

However, expression of IDH2 (Isocitrate dehydrogenases 2) that converts isocitrate to alpha-ketoglutarate in the TCA was less clear; with one data set showing elevated expression in HCC contrasted by the other data set showing the opposite outcome.

Utilizing the same gene expression analysis, we turned our attention to enzymes involved in oxidative phosphorylation through the formation of the electron transport chain complexes namely; NDUFA5 (complex I), UQCRB (complex III), and COXA5 (complex IV). With the exception of COXA5, NDUFA5 and UQCRB were either constant or significantly reduced in HCC compared to non-tumour samples (Figure 3.9(B)). However, all three enzymes were shown to be elevated compared with normal liver samples (Figure 6.9 (B)). Collectively this data shows both glycolytic and oxidative metabolism are elevated in HCC compared to normal livers, while the expression of oxidative enzymes is either comparable or reduced against non-cancerous livers. This data is in keeping with the understanding that some cancer genotypes display a need for both glycolytic and oxidative metabolism.

6.9 Glycolytic Gene Expression is associated with poor patient prognosis

To understand the importance of key regulators of aerobic glycolysis and oxidative phosphorylation in HCC compared to normal livers, we decided to evaluate their prognostic value in patients. We therefore used an online tool ‘GEPIA’ to plot patient survival curves (Kaplan-Meier methodology) based on the glycolytic metabolic expression in HCC. HCC patients were then divided into two groups according to the median value of each gene transcript of HK2, PKM2, ALDOA, and LDHA. We showed that patients in the high glycolytic expression group had a reduced overall survival rate (Figure 6.10 (A)). Other glycolytic enzyme expression including that of PFKL showed an inverse trend associated with overall patient survival, however these data were not significant (P=0.420).

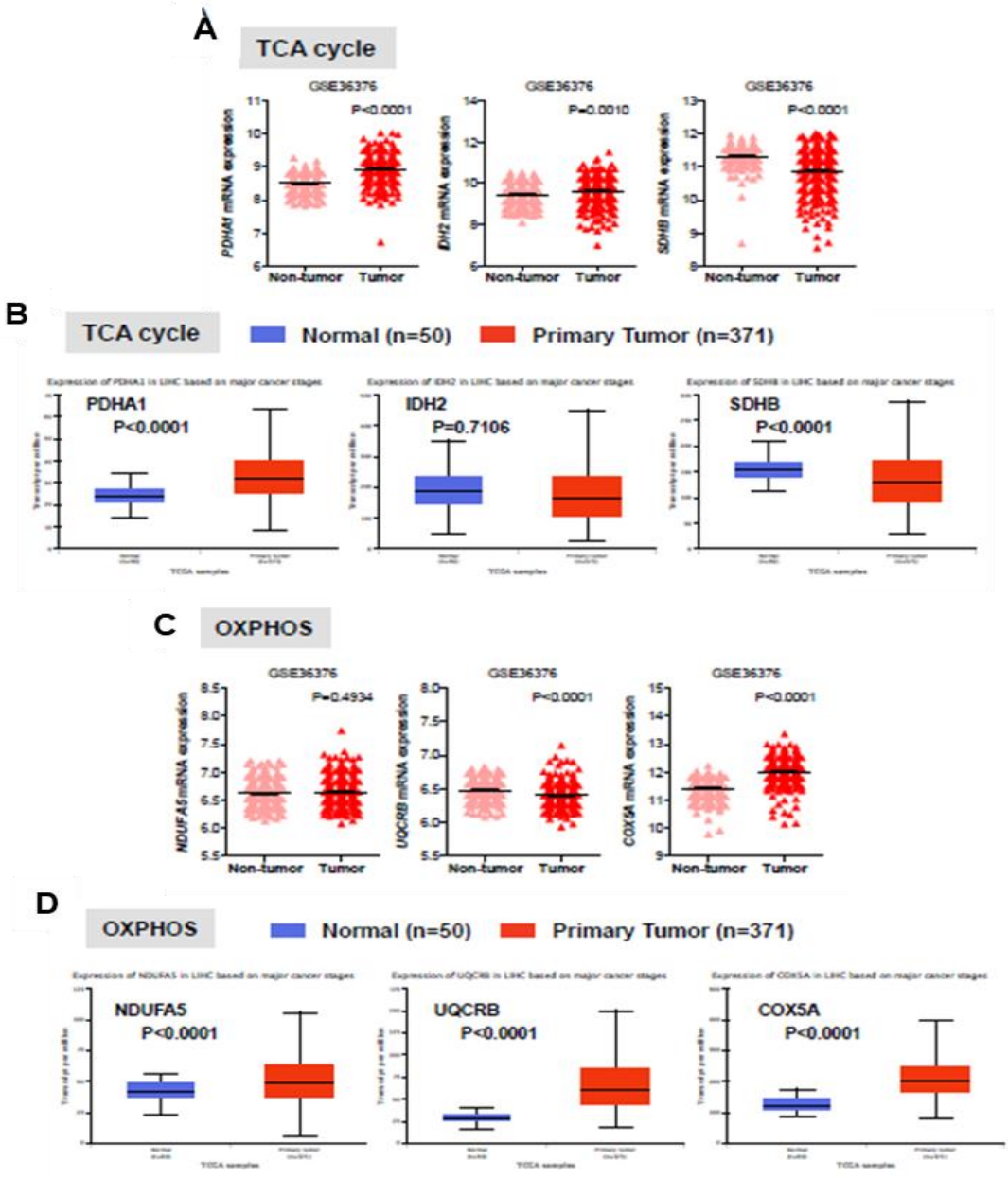


FIGURE 6.9: Gene expression analysis of the oxidative mitochondrial metabolism in HCC. (A,C) Scatterplots showing the transcript levels of representative TCA **(A)** and OXPHOS **(C)** enzymes in the clinical data set GSE36376 consisting of HCC (*n* = 240) and adjacent non-tumour (*n* = 193) liver tissue (Lim et al., 2013). The horizontal lines indicate mean ± SEM *P*-values were calculated by nonparametric Mann–Whitney tests. **(B,D)**Boxplots showing differential gene expression of TCA **(B)** and OXPHOS **(D)** among normal liver tissues (*n* = 50) vs. primary tumour tissues (*n* = 371) (TGA-LIHC samples) analysed using the UALCAN bioinformatic tool of genomic database (Ally et al., 2017; Chandrashekar et al., 2017). Values are expressed as transcript per million. For each box plot, the whiskers represent the 2.5–97.5th percentile range of values, the lower and up boundaries denote the 25th and the 75th percentile of each data set, respectively, and the horizontal line represents the median value for each group. *P*-values were calculated by *t*-test.

Examination of genes involved in PPP (Figure 6.10 (B)) also showed the overall survival rate of patients to be significantly lower in those expressing high G6PD. Given G6PD is the rate limiting enzyme of the PPP, this has important clinical implications. However, upon analysis of metabolic genes involved in the mitochondrial metabolism we found them to have no prognostic value (Figure 6.10 (C-D)). Neither genes utilized in the TCA or oxidative phosphorylation correlated with a reduced overall patient survival. Collectively this data highlights clinical implications for HCC patients with high glycolytic metabolism over high mitochondrial metabolism.

6.10 Gene expression of Glycolysis and PPP enzymes in cirrhotic and HCC livers

Thus far our analysis had been conducted between cohorts of HCC and normal liver patient samples. However, liver cirrhosis represents the majority of precancerous state of HCC and therefore may be a unique model to investigate markers for the early detection of HCC *in vivo.* To better examine the metabolic alterations in early liver carcinogenesis, we opted to explore the glycolytic and mitochondrial metabolism expression in three independent clinical data sets. Together these data sets represent 94 cirrhotic livers and 34 healthy livers. Upon examination of mRNA expression of the same glycolytic enzymes looked at previously in HCC, we found a statistically significant difference in HK2, ALDOA and PKM2 expression between cirrhotic and normal livers in all three data sets

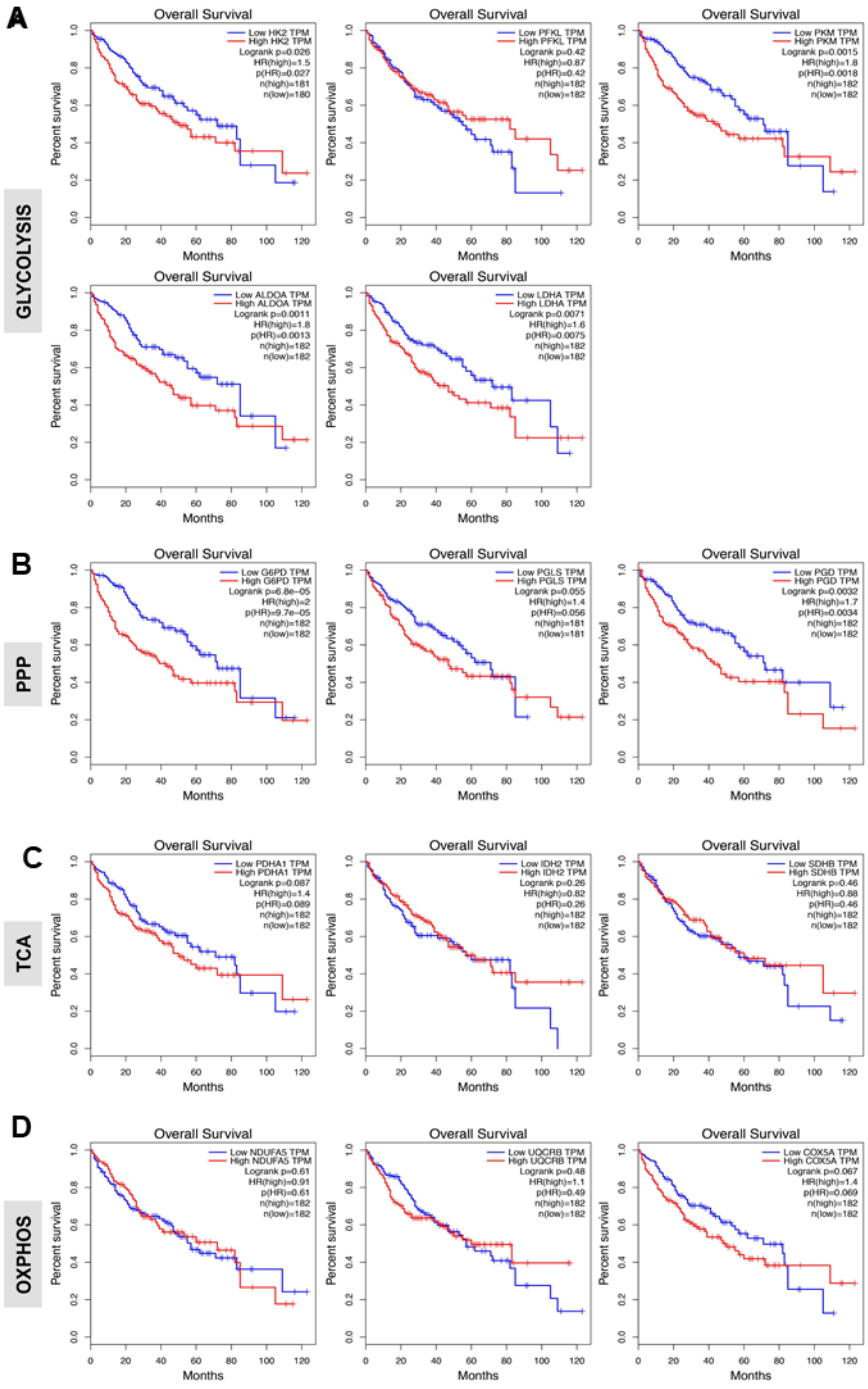


FIGURE 6.10: Glycolytic gene expression is associated with poor patient prognosis. (A,B) High expression of genes associated with glycolysis and oxidative phase of PPP significantly correlates with poor overall patients' survival. Shown are the Kaplan-Meier overall survival curves of HCC patients according to the designated gene expression levels above or below the median value based on TGA-LIHC data set and analysed with the GEPIA bioinformatic tool of genomic database (Ally et al., 2017; Tang et al., 2017). **(C,D)** Kaplan-Meier overall survival curves of HCC patients in TGA-LIHC data set showing homogenous prognostic significance of TCA and OXPHOS genes.

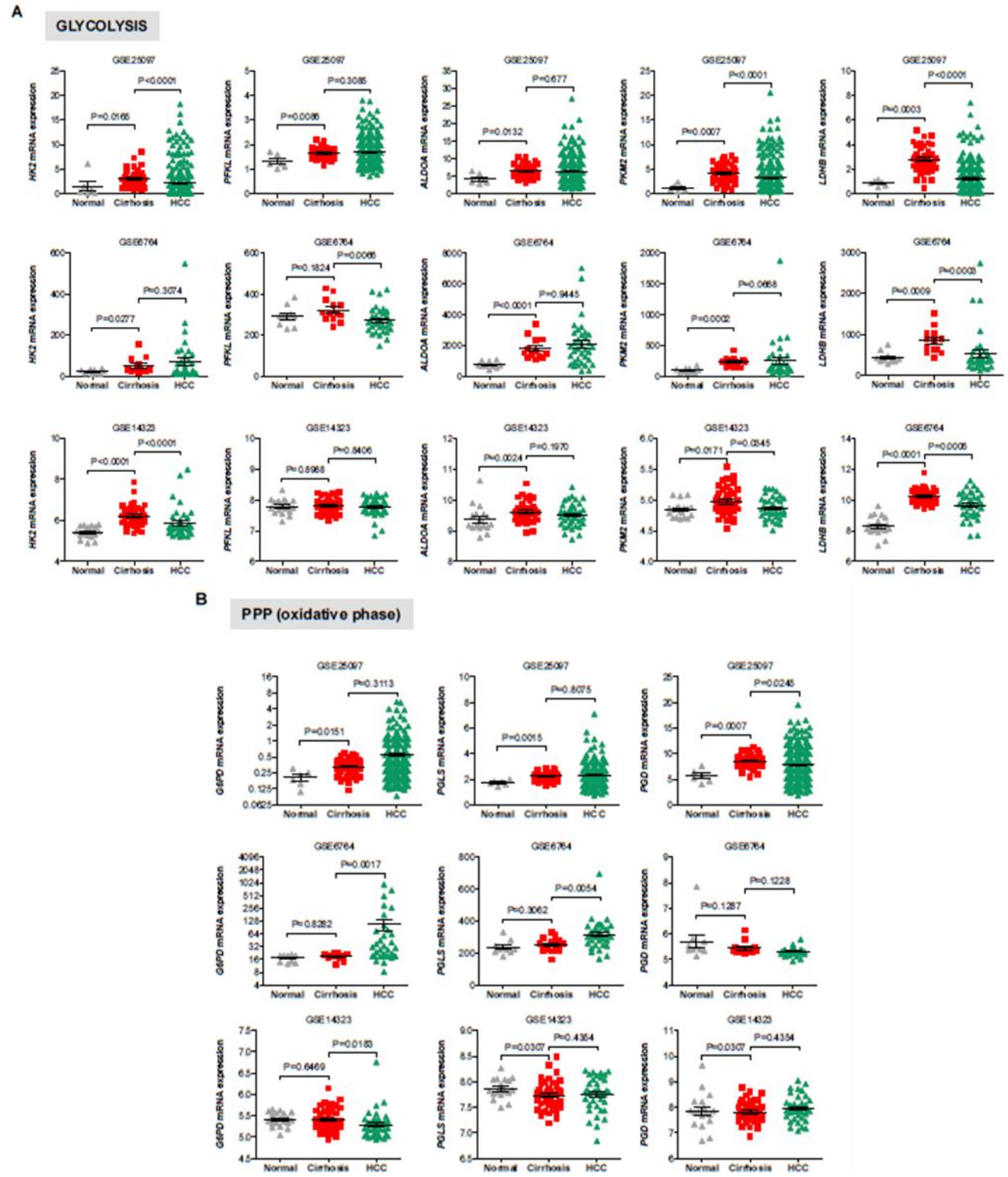


Figure 6.11: Gene expression of glycolysis and PPP enzymes in cirrhotic and HCC livers. (A) Scatterplots showing the increased expression of transcript levels of representative glycolytic enzymes in cirrhotic livers compared to HCC livers and normal healthy liver tissues in three independent clinical data sets GSE25097, GSE6764, and GSE14323 (Wurmbach et al., 2007; Sung et al., 2012; Levy et al., 2016). **(B)** Scatterplots showing differential gene expression of genes related to oxidative PPP among cirrhotic livers compared to HCC livers and normal healthy liver tissues in three independent clinical data sets GSE25097, GSE6764, and GSE14323 (Wurmbach et al., 2007; Sung et al., 2012; Levy et al., 2016). The horizontal lines indicate mean ± SEM *P*-values were calculated by non-parametric Mann–Whitney tests.

analysed. Given LDHA was not included throughout all datasets, we instead analysed LDHB expression and found it also increased in cirrhotic livers compared to healthy individuals. On the other hand, in data set GSE25097, PFKL expression was significantly higher in cirrhotic livers compared to healthy individuals yet remained constant between both groups in the other data sets.

Collectively we find this data to be in line with our findings in HCC; cirrhotic expression of glycolytic HK2, ALDOA and PKM2 are elevated similarly to HCC compared with healthy individuals and associated with poor overall patient survival. On the other hand, despite the findings of high PFKL expression in one cirrhotic data set, elevated PFKL expression is not associated with a prognostic value. Overall this data is suggestive of a cirrhotic gene expression signature similar to that of HCC.

6.11 Gene expression of TCA and OXPHOS enzymes in cirrhotic livers

Next, we calculated the gene expression of enzymes in the oxidative phase of PPP, as well as TCA and OXPHOS. For the former we found that in one data set (GSE25097) all three PPP genes analysed including the rate limiting G6PD were significantly increased in cirrhotic livers compared to healthy individuals. Despite this in the two other data sets no changes was observed except PGLS that was notably reduced in cirrhotic tissues. Interestingly we observed no significant increase of glycolytic and PPP gene expression in HCC compared with cirrhosis, suggestive that the reprogramming of glucose metabolism may occur at the pre-cancerous stages. As for the TCA enzymes, expression of PFHA1, IDH2 and SHDB was constant in all data sets between cirrhotic and normal livers. Likewise. OXPHOS genes that were shown to be highly expressed in HCC, had no significant difference in expression between pre-cancerous and normal livers. Collectively these results characterise a glycolytic shift early on in cirrhotic livers relative to PPP and OXPHOS metabolism.

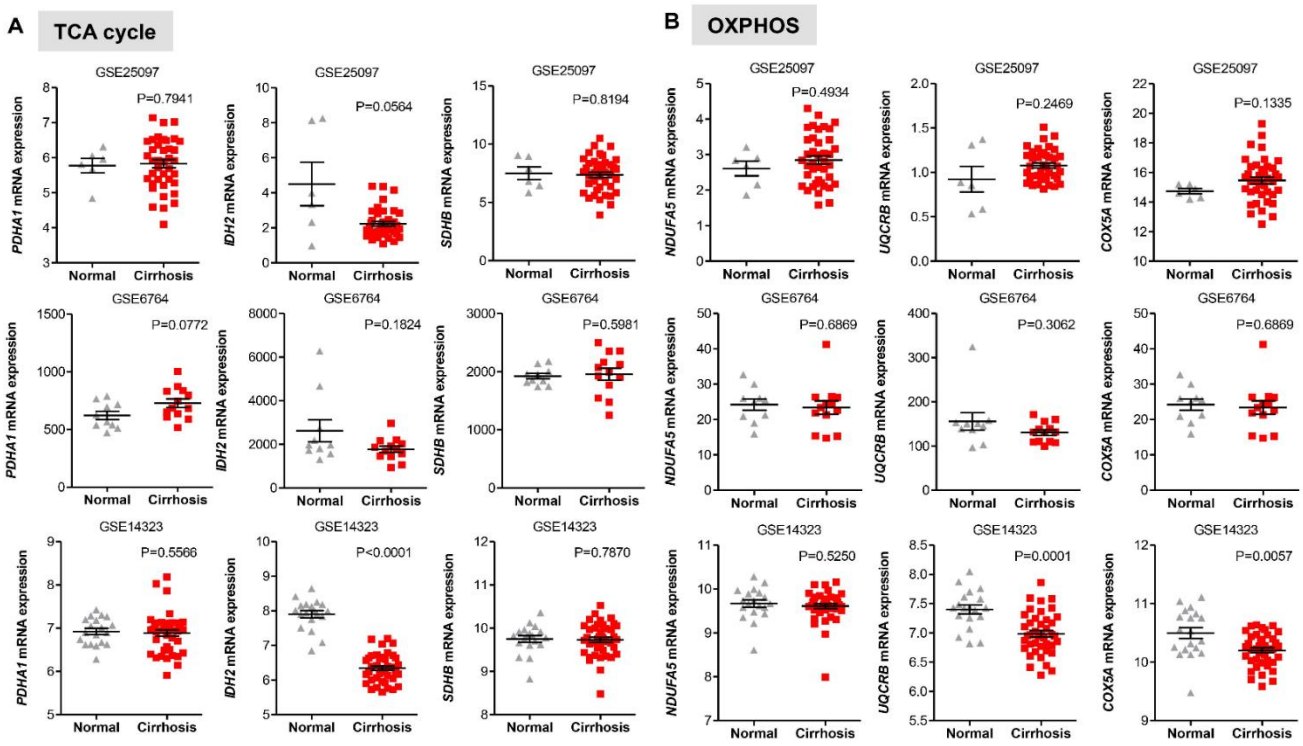


FIGURE 6.12. Gene expression of TCA and OXPHOS enzymes in cirrhotic livers. (A,B) Scatterplots showing homogenous expression of representative TCA and OXPHOS biomarkers in cirrhotic livers compared to normal healthy liver tissues in three independent clinical data sets GSE25097, GSE6764, and GSE14323 (Wurmbach et al., 2007; Sung et al., 2012; Levy et al., 2016). The horizontal lines indicate mean ± SEM P-values were calculated by non-parametric Mann–Whitney tests.

6.12 Increased risk of developing HCC in cirrhotic patients significantly correlates with high expression of glycolytic genes

Finally, we examined if genes involved in glycolysis, PPP, TCA cycle and OXPHOS were associated with the progression of cirrhosis to HCC and patient survival. This was done using the clinical data set GSE15654 that contains biopsies obtained from patients with hepatitis C-related child pugh A cirrhosis who were prospectively followed in an HCC surveillance program for a median of 10 years. These patients were then characterised as low (n=55) and high (n=60) HCC risk based on the rates of patient survival and incidence of developing HCC. When examining the expression of HK2, PFKL, ALDOA, PKM2, we found their mRNA expression to correlate positively with the progression of cirrhosis to HCC with a worse survival rate. The exception was LDHB, which showed no difference in expression between the two survival groups.

As for PPP enzymes in the oxidative phase, elevated G6PD was found associated with progression of cirrhosis to HCC with a reduced survival rate, whereas PGLS and PGD showed no correlation. Lastly, we demonstrated no increases in metabolic gene expression in both the TCA cycle and OXPHOS were found between high and low HCC risk groups but expression of IDH2, SDHB and COXA5 were actually inversely correlated with poor prognosis. Collectively this data highlights an increased risk of developing HCC by cirrhotic livers characterised with high glycolytic gene expression.

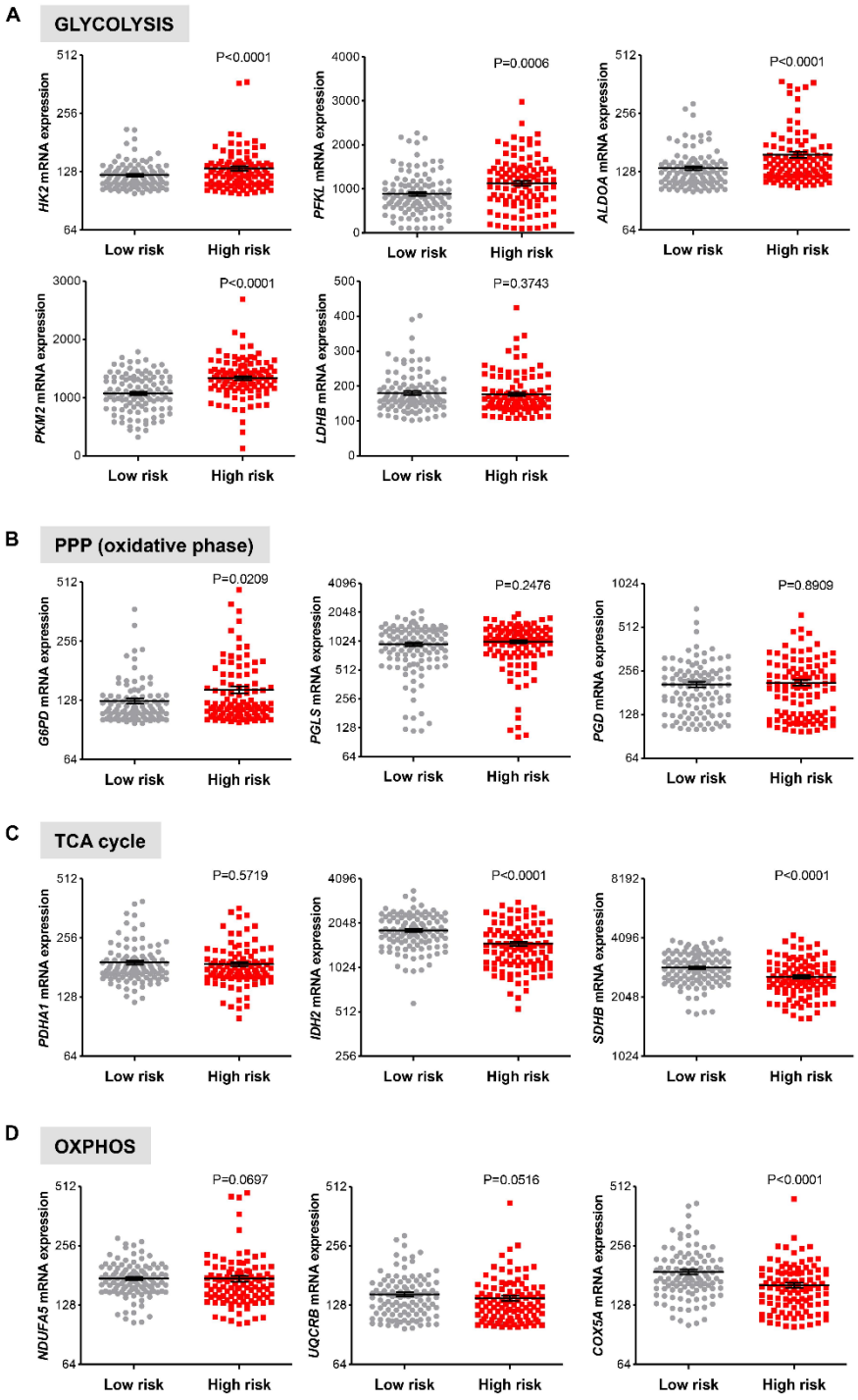


FIGURE 6.13. Increased risk of developing HCC in cirrhotic patients significantly correlates with high expression of glycolytic genes. (A–D) Levels of transcripts in the clinical data set GSE15654 consisting of 115 patients with newly diagnosed cirrhosis who were prospectively followed up in an HCC surveillance program and classified as having low (n = 55) and high (n = 60) HCC risk based on the rates of patient survival and risk of developing HCC (Hoshida et al., 2013). The horizontal lines indicate mean ± SEM P-values were calculated by non-parametric Mann–Whitney tests.

Chapter 7 – Discussion

7.1 Uncovering a post-translational interaction between JNK and PIN1 in cancer

To begin with, we identified an elevated expression of JNK proteins apparent across diverse liver cancer cell lines (Figure 4.1). Over-expression of JNK proteins occurs in a variety of cancer tissues suggestive that this same phenomenon maty fuel malignant signalling in liver cancer (*162, 180, 208*). However, unlike JNK expression, PIN1 expression fluctuated in its intensity detailing a lower PIN1 expression in SG231 and HuCCT-1 subsets but higher PIN1 expression in HEP3B and CCLP1 cell lines compared with normal tissues (Figure 4.1). Such variation may be an accurate reflection of the molecular heterogeneity found between liver cancers, reaffirming the need to sub-classify liver cancers based on their molecular signatures(*40, 54*). In fact, reports of striking variation in PIN1 expression are common in human cancer and found to be highly dependent for both the tissue environment and the manifestation of clinical development (*309*).

Upon interrogation with targeted shRNA, we reported that silencing of JNK expression within our selected cell lines resulted in subsequent PIN1 protein reduction (Figure 4.2). As prior investigation into the effects of JNK expression on the regulation of PIN1 transcription were shown to have no effect (results not shown), we hypothesised that the knock down of JNK was inducing a post-translational response on the protein expression of PIN1. Much evidence surrounds the post-translational regulation of PIN1 by upstream effector proteins in cancer (*20, 210*), however there are limited studies describing its post-translational interaction with JNK. One author describes the need for PIN1 as a rate-limiting activation step following the dual phosphorylation of both threonine and tyrosine residues in JNK's activation loop (*211*). However, this line of enquiry details PIN1 as an upstream activator for JNK and is not supportive of the novel post-translational effect observed on PIN1 when silencing JNK expression shown here (Figure 4.2).

In support of this post-translational effect observed on PIN1, we quantified the interaction of JNK with PIN1 in liver cancer subsets (Figure 4.2). Through over-expression of tagged-protein constructs in mammalian cell lines, we confirmed through co-immunoprecipitation that the interaction of constitutively active JNK with PIN1 occurs within mammalian cells. (Figure 4.3). JNK's are routinely activated by upstream MAP2Ks, *MKK7* and *MKK4*, resulting in their subsequent phosphorylation of a conserved tri-peptide activation loop (*159*). As master protein kinases, once activated, JNK proteins undergo conformational changes enabling further interactions with downstream substrates. The interaction of JNK with downstream substrate PIN1 outlined here, provides evidence of another mechanism that may potentially contribute to important physiological processes within the cell yet to be defined. Of note, immunoprecipitation of inactive JNK with PIN1 did not result in an interaction taking place (Figure 4.7). Given most downstream JNK interactions are dependent on the active state of the protein kinase, this comes as no surprise and further supports the need for JNK's activity to post-translational interact with PIN1 (*155, 159, 160, 174, 307*).

To substantiate the specificity of JNK isoform binding, we conducted over-expression studies with both active tagged JNK isoforms in the presence of PIN1. Co-immunoprecipitation analysis identified that PIN1 interacts with both JNK1 and JNK2 isoforms in mammalian cells (Figure 4.4). This specificity of JNK isoforms and their related functions has previously caused much confusion regarding the overall JNK signalling response in both normal physiological and diseased states. Of the many studies focusing on JNK functions in cancer, conflicting findings are common, with some studies reporting pro-oncogenic functions of JNK whilst others provide sufficient evidence of JNK's roles as a tumour suppressor. Over time, it has become clear that JNK isoforms often play diverse and opposing effects in cancer, providing insight into their capabilities to be central to multifarious signalling pathways critical for the cell's response (*174, 181*). The functionality of JNK isoforms is also dependent on the tissue context. Amongst malignant tissues that give rise to a variety of cancers, both JNK1/2 isoforms are shown to have multiple functions that depend on the signalling environment. For example, JNK1 in liver cancers is regularly overexpressed and understood to be predominately oncogenic, providing opportunities for cancers

to grow and survive. However, the same cannot be said for JNK1 mechanisms in MM, with studies reporting its involvement in apoptotic signalling which is routinely suppressed by its sister isoform JNK2 (*154*). It is therefore of high importance to establish which JNK isoforms are interacting with PIN1. The results in this study outlined PIN1 to interact with both JNK isoforms, consequentially this may provide cause for PIN1's wide-spread oncogenic effects throughout a range of cancer tissues.

We further demonstrated that the interaction between active-JNK and PIN1 occurs through a direct manner. Through a combination of recombinant protein production and pull-down assays we confirm that *in vitro* analysis of JNK's interaction with PIN1 requires no additional scaffold proteins for this interaction to occur (Figure 4.6). Activated by a range of external stimuli, the JNK signalling pathways are intricate to protein-protein interactions (PPIs) involving a variety of cytoplasmic and nuclear based substrates through both direct and indirect mechanisms (*307*). The characteristics of PPIs can provide subsequent information surrounding the protein functions in the signalling pathway. Reports of indirect interactions with JNK include functionality for lysosome transportation, protein stability, nuclear and cytoplasmic translocation and the indirect activation of downstream substrate proteins. In comparison, direct interactions of JNK more commonly surround the actions of JNK's kinase domain to directly phosphorylate downstream substrates, in turn effecting substrate protein function (*157, 159, 160, 187, 251, 307*). Based on the direct interaction between JNK and PIN1, we proposed that JNK's kinase domain may be conferring phosphorylation mechanisms to PIN1 through this mutual interaction.

To date, proteomic studies have revealed phosphorylation, N-acetylation and oxidation mechanisms to occur at a collection of residues on PIN1's protein structure influencing its isomerase activity, stability, and subsequent downstream interactions (*200*). Adding to these post-translational modifications, we describe here that active JNK phosphorylates PIN1 directly *in vitro* (Figure 4.8). This was achieved by the use of recombinant active JNK1 and His-PIN1 analysis through a kinase assay involving the incorporation of radio-actively labelled phosphate groups onto the His-PIN1 protein structure. On reflection, it would have been helpful to have included inactive JNK1 into this experiment as a control to confirm

that no phosphorylation occurs with JNK1. In Figure 4.9, we confirmed that this was the case, however for consistency between experiments, it would have been correct to provide this control again in Figure 4.10. This was apparent for both active forms of JNK, indicative that under *in vitro* conditions PIN1 can be directly phosphorylated by both isoforms (Figure 4.10). Of the established post-translational modifications on PIN1 already identified in literature, we find that there is evidence for PTMs that both inactivate and activate PIN1 functions in the cell. For instance, phosphorylation at Ser16 of PIN1's WW domain abolishes its ability to mediate substrate interactions and cellular localization(*21*). This residue is reportedly phosphorylated by master protein kinases including protein kinase A, ribosomal S6 kinase 2 and Aurora kinase A. Other studies detail PIN1 phosphorylation and subsequent inactivation by death-associated protein kinase 1 (DAPK1) at the Ser71 position also acting as a tumour suppressor through suppression of MYC and E2F induced oncogenic transformation in cancer. This occurs as a result of Ser71 phosphorylation closing PIN1's binding pocket in the active site, subsequentially abolishing PIN1's PPIase activity and inhibiting its nuclear translocation (*21*). Furthermore, PIN1's catalytic activity and substrate binding abilities are inhibited by sumoylation of Lys63 in the PPIase domain and Lys6 in the WW domain respectively as well as oxidative modifications on Cys113 known to inhibit PIN1s enzymatic activity (*21*). In addition to these inactivating modifications, polo-like kinase 1 (PLK1) phosphorylates Ser65 in PIN1's catalytic domain increasing its stability by inhibiting its ubiquitination. Whereas both catalytic activity and nuclear translocation is increased by phosphorylation of PIN1's Ser138 residue by Mixed-lineage kinase 3 (MLK3). Finally, PIN1's substrate-binding, catalytic activity and oncogenic functions are further increased by desumolylation of Lys6 and Lys63 by SUMO1 / sentrin specific peptidase 1 (SENP1). Collectively, these known PTM's of PIN1 and the effect on its function in normal tissue functions as well as cancer environments, implore us to consider how our discover of active-JNK's direct phosphorylation of PIN1 will have on its biological function in the cell.

With the aid of a novel detection method for phosphorylated proteins (Phos-Tag™) (Figure 4.10), we elucidated that active JNK phosphorylates PIN1 at one major protein site (Figure 4.11). Upstream kinases regularly inflict

phosphorylation modifications to a range of downstream substrates, to which the specificity of phosphorylation will ultimately dictate the overall substrate functions such as subcellular translocation, activation, and alterations to the substrate's stability. The specificity of this outcome is in turn, largely dictated by the combination of the number of phosphorylation modifications carried out by upstream effector protein, as well as the site specificity of the post-translational modifications themselves. For instance, activation of JNK is dictated by upstream kinases MKK7 and MKK4, that cause dual phosphorylation of both threonine and tyrosine residues in JNK's activation loop. This consequentially results in a structure change to expose the active kinase site of JNK allowing for its downstream effects to be carried out. In this case, we show that active JNK's phosphorylation of PIN1 points to one major post-translational site. This site was confirmed to occur through phosphorylation as treatment with calf intestine phosphates (CIPs) reversed the presence of the phosphorylated band. Furthermore, we showed that active JNK is necessary for the phosphorylation of PIN1, as inactive JNK under the same conditions appeared to have no post-translational effect on PIN1 (Figure 4.11). To support these studies, we confirmed this post-translational outcome to occur in the context of liver cancers (Figure 3.12). Both the inhibition of JNK activity (SP600125) and the knock down of JNK expression (*shJNK1/2)* was shown to reduce the presence of the PIN1 phosphorylation form in CCLP1 cell lines. SP600125 treatment of JNK further supports the role of the JNK kinase in the action of PIN1 phosphorylation (*310*).

To identify the specific phosphorylation site of PIN1 by active JNK, we collaborated to carry out mass spectrometry analysis of our recombinant PIN1 proteins in the presence and absence of active JNK proteins (Figure 4.13). Collectively, this analysis pointed to one PIN1 site deemed to be phosphorylated by JNK activity, namely the serine at position 115 (s115). Many known post translational modifications on PIN1 have already been detailed in the literature and are regularly implicated to play specific roles in PIN1's intricate functions within the cell's machinery. Of these, serine at position 138 (s138) is worthy to note. Phosphorylated by mixed-lineage kinase 3 (MLK3), a MAP3K family member, phosphorylated s138 results in the increased catalytic activity and nuclear translocation of PIN1 shown to drive cell cycle activity and promote

centrosome amplification (*311*). Given the structure of PIN1 is relatively small (18 kDa) with numerous serine residues present on the protein structure, including s114 adjacent to the identified s115, we sought to confirm the mass spectrometry analysis using ulterior methods to ensure the validity of our claim. Using a combination of mutagenesis and over-expression studies, we confirmed that s115 was the site of JNK-directed phosphorylation on PIN1 (Figure 3.14). Furthermore, using recombinant protein production we confirmed this phosphorylation to occur directly through *in vitro* analysis of the protein-protein interaction (Figure 4.15). Together, these results added a novel PTM of PIN1 to the literature yet to be deciphered and led us to question the importance of this PTM on the biological function of PIN1 in cancer. Of note, Figure 3.14 has a number of additional bands that may highlight off target effects of MKK7 on the serine 155 residue. On further repetition of this data, it would be useful to include controls for MKK7 alone to better understand whether our MKK7-JNK1 linked molecule is causing phosphorylation due to the presence of MKK7.

7.2 Altered PIN1 mechanisms have oncogenic functions

With the identity of the phosphorylation mechanism by JNK on PIN1 now known, we sought to further understand the effect of JNK's phosphorylation on PIN1's functions within the cell. Of note, PIN1 has distinctive roles in sustaining proliferative signalling in cancer. Despite both intracellular and extracellular signalling mechanisms being under tight control in normal cells (*12, 13*), both PIN1's enhanced activity and over-expression in cancer notably contribute to the overall proliferative function of the tumour. These examples include PIN1's ability to promotes proliferation through enhancing ERα expression in breast cancer by increasing its transcriptional activity (*205, 214, 215*). Furthermore, cytokine stimulation is known to induce PIN1's binding to NF-kb subunits in glioblastoma, endometrial carcinoma, acute myeloid leukaemia and hepatocellular carcinoma, all of which result in the enhanced proliferation that is beneficial to the tumour's progression (*202, 204, 217*).

To this end, we initially carried out rescue experiments in liver cancer cell lines to understand the effect on both the presence and absence of active JNK's

expression on PIN1's proliferative role within cancer cells (Figure 5.1). We found that silencing of JNK expression in liver cancer cell resulted in a dramatic decrease in the proliferative function of the cancer cells compared to controlled conditions. What's more, reconstitution of PIN1 in the absence of JNK expression did not recover the full proliferative function of the tumours, despite the strong evidence surrounding PIN1's oncogenic roles in cancer cell proliferation. Combined with our results surrounding the phosphorylation of PIN1 by active JNK signals in cancer, we hypothesised that active JNK may be conferring stability to PIN1 through its phosphorylation at the s115 residue. By blocking protein synthesis and over-expressing a combination of PIN1 with or without active JNK, we confirmed this assumption by showing that over a 9-hour time course, PIN1 stability was enhanced in the presence of active JNK (Figure 5.2). We later confirmed this mechanism of stability to also be true within liver cancer cells, by observing PIN1 stability in *shJNK* cell lines vs-controlled conditions (Figure 5.3). This is not the first time active JNK signals are reported to regulate protein stability in cancer. In fact, JNK's kinase activity is common mechanism used to stabilise substrates in cancer, contributing to the likes of p53 and c-Myc stability causing alterations in their functions in cancer. This seems to be also apparent in the case of this study, with further experiments in liver cancer cells depicting the kinase activity of JNK to be responsible for PIN1 stability (Figure 5.14). With these findings in mind, we turned our attention to uncover the effect of JNK's stability on PIN1 in cancer (*177, 178, 187, 306, 308*). On reflection of the results shown in Figure 5.2, it would be interesting to determine the half-life of PIN1 expression without our selected cell lines. Although we have proved PIN1's expression is dependent on active JNK, by synchronising the cells to ensure expression of PIN1 from all cells were in the same stages of the cell cycle, we would have been able to more accurately determine the half-life of PIN1 in the presence and absence of active-JNK expression. Through synchronising the cells, we would have ensured all cells were at the same process of cell division allowing for consistent PIN1 upregulation, opposed to each cell upregulating PIN1 within its own cell cycle time. As a result, PIN1 half-life from the population of cells would have more consistent, allowing us to determine how the presence of JNK affects PIN1 protein expression across the cell population.

Following on from our findings that active JNK stabilises PIN1 protein expression in cancer, we returned to build on our former rescue experiment results (Figure 5.1). Given high active JNK expression is present within liver cancer cell lines, we showed that silencing of PIN1 dramatically reduced the overall proliferation capacity of the tumours. What's more, reconstitution with a shRNA- resistant exogenous PIN1 completely restored the proliferative phenotype, providing evidence that PIN1 in the presence of active-JNK signals allows for full PIN1 proliferative functions to be maintained (Figure 5.5). Collectively with our previous findings, these data show that active JNK's stability on PIN1 allows for its proliferative functions to be maintained in liver cancer.

Many studies within the literature discuss how the aberrant expression of PIN1 in cancer tissues acts as a critical step for proliferative abnormalities of tumour cells to supersede. However, almost all of these studies focus on the effect of PIN1's expression to cause oncogenic effects via downstream substrate alterations. For example, silencing of PIN1 in HepG2 liver cancer cells is well known to result in enhanced tumour cell proliferation through TP53 function (*312*). In addition, cancer cells knocked down of PIN1 expression have been shown to reduce Grb7 stability causing proliferative dysfunctions(*313*). PIN1-deficient mice are more widely shown to have a range of cell-proliferative abnormalities (*314*), most of which these functions can be explained through PIN1's known roles in regulating cell cycle functions. Acting as a molecular timer of cell-cycle progression, the absence of PIN1 is strongly associated with the progression of hyperproliferative diseases (*21, 199*). Indeed, we confirmed this within our own subsequent studies showing that silencing of PIN1 resulted in a reduced expression of key cell cycle component cyclin D1, a downstream target for PIN1. The expression of cyclin D1 was then re-established through exogenous shRNA- resistant PIN1 expression in the liver cancer cell lines (Figure 5.6). However, it is worth noting that few studies have linked the post-translational regulation of PIN1's expression to be critical to the ability of PIN1 to carry out oncogenic effects within the cell. These findings surrounding the master regulator of PIN1 stability in these cells can be advantageous for therapeutical targeting in clinics (*199, 200, 252, 312*) .

Through over-expression studies with PIN1 s115 mutations, we further showed that active JNK's phosphorylation of PIN1 at residue s115 was responsible for

the stability of PIN1's protein expression (Figure 5.7). It is not uncommon to see JNK's kinase activity result in phosphorylation and stabilisation of downstream substrates, in fact, this mechanism is regularly used to alter the cellular outcome of tumour cells (*153, 155, 159, 163, 166, 173, 306*). However, here we identify a novel post-translational phosphorylation mechanism of JNK on PIN1, adding to our understandings of how PIN1 is regulated in both normal tissues and oncogenic conditions (*20*). In an attempt to mimic the phosphorylation of JNK on PIN1's s115 residue, we conducted experiments using a S115E phosphorylation mimic methodology. These results did not depict the maintained stability of PIN1 (Figure 4.8) despite their successes elsewhere (*315, 316*). This outcome may be suggestive of the need for active JNK proteins to not only carried out phosphorylation at S115 but also to add structural configurations that ensure the stability of PIN1's protein expression. These findings are worth exploring in future studies using protein X-ray crystallography methods.

Applying our findings that active JNK phosphorylates PIN1 at S115 resulting in its stability, we conducted further rescue experiments in liver cancer cell lines to understand the role of this PTM on PIN1's proliferative function. Using an exogenous shRNA-resistant WT PIN1 vs the equivalent shRNA-resistant S115A PIN1, we showed that JNK's phosphorylation of PIN1 at S115 is critical for the full proliferative function of PIN1 in cancer cells (Figure 5.9). Of the known post-translational regulation of PIN1, there are four established mechanisms of phosphorylation already detailed in the literature. Those that inhibit cancer, include DAPK1 and protein kinase A (PKA), ribosomal S6 kinase 2 (RSK2) and Aurora kinase A (AURKA). PKA, RSK2, and AURKA are all capable of phosphorylating PIN1 at Ser16 in the WW domain, consequentially abolishing PIN1's ability to mediate substrate interaction and cellular localization (*252, 317-319*). Furthermore, DAPK3 can phosphorylate and inactivate PIN1 at Ser71 by resulting in a conformational change that closes the phosphate binding pocket of PIN1's active binding site (*320, 321*). As a result, DAPK3 phosphorylation of PIN1 inhibits its nuclear localization and abolishes its PPIases activity preventing cell transformation and centrosome amplification. Of the phosphorylation mechanics on PIN1 that promote cancer, Polo-like kinase 1 (PLK1) and Mixed-lineage kinase 3 (MLK3) are regularly discussed. PLK1 phosphorylates PIN1 at Ser65, found in

the catalytic domain of the protein that subsequentially inhibits PIN1 ubiquitylation and increases its stability(*322, 323*). On the other hand, MLK3 increases PIN1's catalytic activity and nuclear translocation by phosphorylation at S138 (*311, 324*). To these known phosphorylation sites regulating PIN1, we can now add Ser115, a PTM carried out by active JNK to increase PIN1's proliferative function. This mechanism of action is shown to happen in part by a subsequent increase in expression of PIN1's downstream cell cycle substrates of which we outlined in further experiments (Figure 5.11).

In addition to phosphorylation at S115 ensuring the proliferative function in cancer, we also uncovered an enhancement of PIN1's isomerase activity through the same PTM. Using phosphorylated recombinant proteins in an isomerase assay, we concluded that sustained phosphorylation of S115 also increased PIN1's isomerase potential (Figure 5.11). These findings were significant given the range of protein groups that PIN1 is known to target through its isomerase activity, that can affect the overall outcome of the substrate's functions within the cell. Indeed, in cancer alone, PIN1 is known to activate over 40 oncogenic substrates and at least 20 tumour suppressor substrates through its isomerase activity, many of which are shown to have well-established roles in cancer(*200*). Collectively these findings point to a master regulation by active JNK on PIN1 at s115, that contribute to an enhanced isomerase activity and proliferative function in cancer.

7.3 JNK protects PIN1 from ubiquitination in cancer

With our understanding of active JNK's stability of PIN1 in cancer, we next showed that this stability prevents PIN1 from degrading via the 26s proteasome. The findings that active JNK enhances PIN1 stability within the cell suggests a mechanism by which active-JNK prevents either the ubiquitination of PIN1 or re-localization of PIN1 to subcellular spaces such as lysosomal degradation. Given approximately 80% of all cellular proteins are at some point in their life cycle, targeted for degradation by the 26s proteasome machinery we first explored the possibility that PIN1 was being degraded via the proteasome. Using lentiviral transfections in liver cancer cell lines, we outlined that silencing JNK expression

results in an enhanced degradation of PIN1 via the 26s proteasome machinery (Figure 5.12). In addition, we showed that this enhanced proteasomal degradation of PIN1 occurs when the activity of JNK is subsequentially blocked, further proving that active JNK is required for the stability of PIN1 within the cell.

Next, using immunoprecipitation and over-expression studies, we showed that active JNK protects PIN1 from mono-ubiquitination via the 26s proteasome (Figure 5.15). This protection was further shown to occur through active JNK's phosphorylation at s115 of the PIN1 structure (Figure 5.16), indicative that the mechanism of stability by JNK prolongs the half-life of PIN1 within the cell prior to destruction by the proteasome. Under normal conditions, the process of ubiquitination ensures that all transcribed proteins are ultimately recycled by the cell. These mechanisms function in part to prevent aberrant protein expression from disrupting key cellular functions, in turn maintaining the overall homeostasis of the cell. JNK proteins have been shown to use ubiquitination mechanisms to both positively and negatively regulate downstream effector proteins (*306, 325*). In non-stressed cells, JNK is known to target p53 ubiquitination, reducing p53 expression within the cell. Here, we find that JNK targets p53 and carries out phosphorylation to the proteins structure. As a result, p53 phosphorylation, inhibits the association of Mdm2, resulting in the ubiquitination of p53 reducing its half-life within the cell (*306*). In other cases, JNK phosphorylation of a substrate ultimately sequesters the substrate protein from ubiquitination mechanisms. JNK signalling in neuronal cells results in the phosphorylation of dual leucine zipper-bearing kinase (DLK). These phosphorylation events increase DLK abundance by reducing the overall DLK ubiquitination within the cell. This mechanism is shown to be critical in the promotion of neuronal apoptosis(*325*). In the case of the current study, we observe that under oncogenic conditions, active JNK's phosphorylation of PIN1 at s115 prolongs the half-live of the protein by protecting PIN1 from ubiquitin mechanisms at large within the cell. Despite initial technical difficulties surrounding the ubiquitination of PIN1 (Figure 4.14), we eventually established that the activity of JNK was protecting PIN1 from mono-ubiquitination.

7.4 Phosphorylation of PIN1 affects cancer metabolic functions

Upon exploring the biological effect of PIN1 in cancer, we found that active JNK's phosphorylation of PIN1 at s115 resulted in a reduced glycolytic function in liver cancer cells. The JNK proteins play pivotal roles in a cell's metabolic functions and have recently been shown to regulate the redirecting of energy harvest to glycolysis in both malignant and highly proliferative cells (*155*). Highly dependent on cell type, the JNK proteins contribute to the metabolic outcome of the cell in a variety of manners. In liver cancers, JNK1 activity is suppressed by the antiapoptotic protein PARP14. This suppression is a key determinant for a Warburg-like phenotype required for the enhanced survival that is beneficial for liver cancer cells (*154, 158, 159*). The inhibition of JNK1 by PARP14 was also shown to support antioxidant capacity of liver cancer cells by increasing NADPH and glutathione levels. At a mechanistic level, JNK1 is shown to stimulate PKM2 activity by enhancing its affinity of PKM2 to its substrates. Here, we detail an additional mechanism by which JNK alters the glycolytic landscape within liver cancers. Leading on from our previous stability experiments surrounding JNK's activity on PIN1, we find that a reduced expression of PIN1 in liver cancer consequentially reduces the glycolytic enzyme expression of HK2 (Figure 6.1). HK2 acts as a rate limiting step in glycolysis metabolism, trapping glucose within the cell to be used in a variety of metabolic pathways. Thus, the subsequent reduction of HK2 expression by reduced expression of PIN1, resulted in an overall reduced glucose metabolism phenotype by liver cancer cell lines (Figure 6.2). Alterations in HK2 expression in liver cancer cells, was further linked to the JNK-directed phosphorylation of PIN1 at s115 (Figure 5.3). Collectively, these results outline active JNK's phosphorylation of PIN1 at s115 to be critical in the glucose consumption and glycolytic landscape of liver cancer (Figure 6.4). However, it is worth noting that the studies outlined in Figure 6.1 were unable to conclude whether silencing of PIN1 affects the total glucose consumption per cancer cell. As the overall glucose consumption was measured from a population of cells, there is the possibility that reduced PIN1 expression resulted in a reduced number of proliferating cells. As a result these experiments would need to be

repeated by analysing the total glucose consumption after reseeding cells as a specific number.

Following our findings on PIN1's role regarding the glycolytic metabolism of liver cancer cells, we opted to explore more broadly the link between metabolic enzyme expressions on the overall altered metabolic signature of hepatocellular carcinoma (HCC). The dramatic reduction in oncogenic glycolytic function through decreased HK2 expression highlighted the potential of metabolic enzymes to contribute substantially to the hepatocarcinogenetic process. Notably, metabolic enzymes from glycolysis, PPP, TCA and OXPHOS pathways have all shown to have altered expression within HCC cells in order for malignant hepatocytes to carry out oncogenic functions like high levels of proliferation that require an increased bioenergetic demand. We therefore were curious to understand the extent of a range of metabolic enzymes from glycolysis, PPP, TCA and OXPHOS pathways and whether there was a distinct pattern of expression within HCC phenotype. As more than 80% of HCC cases arise from cirrhosis of the liver we extended this analysis to compare metabolic landscapes between biopsies derived from patients with cirrhosis as well as HCC.

Using publicly available genomic patient data sets to carry out wide-spread analytics of a liver cancer cohort, we first compared the expression of key metabolic genes (Figure 6.5) in both non-tumour and HCC patients. Compared to normal livers, elevated mRNA expression of key metabolic genes in glycolysis, PPP, TCA, and OXPHOS were found in liver samples from patients with HCC, implying a pathogenic role of glucose metabolism for this disease (Figure 6.6). In normal hepatocytes, conversion of carbon intermediates including lactate and pyruvate into glucose are modulated by the gluconeogenesis pathway. Under normal circumstances gluconeogenesis enzymes including the glucose-6-phosphatase (G6Pase), fructose-1,6-bisphosphatase (FBP1), and PEP carboxykinase (PEPCK) can bypass the first, second and third irreversible reactions of the glycolytic pathway, respectively, allowing for a reduced rate of glycolysis to maintain cellular homeostasis (*130*). However malignant hepatocytes in HCC are found to suppress these aforementioned enzymes as well as the low affinity hexokinase 4 (HK4) whilst simultaneously overexpressing the high-affinity hexokinase HK2 resulting in an enhanced rate of glucose

consumption beneficial for the extended bioenergy requirement of the tumour (*130, 152*) .

Interestingly, through analysis of publicly available microarrays, we found that HK2 is not only highly expressed in HCC but also in cirrhosis as compared to normal liver samples, indicative of a molecular need for high HK2 expression early in the hepatocarcinogenetic process (Figure 6.10). The same could be said for glycolytic enzymes ALDOA and PKM2 within cirrhosis as well as HCC. Of the three known isoforms of the glycolytic aldolase (ALDOA, ALDOB, and ALDOC), ALDOA is known to most efficiently catalyse the conversion of Fructose 1,6-bisphosphate (F1,6BP) to Dihydroxyacetone phosphate (DHAP) and Glyceraldehyde 3-phosphate (G3P). As a result, the rate of glycolysis is dramatically increased causing ALDOA expression to be highly favourable for the proliferation of malignant hepatocytes (*130, 149*). Our finding that the levels of ALDOA are up regulated in cirrhotic and HCC samples suggests that a high glycolytic phenotype is a remarkable feature of both precancerous and cancerous conditions. To add, much research highlights the role of pyruvate kinase M2 isoform (PKM2) as a critical driver of the Warburg effect. PKM2 is responsible for catalysing the synthesis of pyruvate and ATP using phosphoenolpyruvate (PEP) and ADP as substrates (*139, 327, 328*). Indeed, we find that PKM2 is highly expressed in HCC like many other cancers used to maintain a low pyruvate kinase activity to aid the build-up of carbon intermediates beneficial for the tumour's needs (reviewed in (*159*)). The low activity of PKM2 allows the accumulation of glycolytic intermediates that can be diverted into the biosynthetic pathways to form amino acids, nucleic acids, and lipids (*138, 326, 227*). While promoting these biosynthetic pathways, low PKM2 activity also contributes to boost the levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and antioxidant reduced glutathione (GSH), which serves to detoxify reactive oxygen species (ROS) whose accumulation would result in apoptosis in chronically injured and tumour tissues (*159*). As for the expression of LDHA and/or LDHB, the glycolytic enzymes responsible for converting pyruvate into lactate, it was apparent that there was no pattern of HCC associated risk from the cirrhosis cohort (Figure .12). As a result, it is accurate to assume that not all

pyruvate is converted into lactate in cirrhosis damaged livers instead allowing for carbon intermediates to be pushed into other metabolic pathways.

Our analysis also revealed a high expression of genes relevant to mitochondrial metabolism in HCC compared with cirrhosis biopsies, a requisite for ATP production and macromolecule synthesis in both malignant and healthy hepatocytes. Despite previous studies indicating the Warburg effect was predominantly due to impaired mitochondrial oxidative metabolism, more recent research demonstrates the need for both glycolytic and mitochondrial metabolism in HCC samples (*20, 329*). This phenomenon was not apparent in cirrhotic livers which showed heightened expression of glycolytic enzymes including HK2, ALDOA, PKM2, and LDHB but no changes to the expression levels of TCA or OXPHOS genes. As a result, it is prudent to assume that the metabolic readjustment surrounding glycolytic phenotypes of HCC, can be traced back to the cirrhotic routes. On a molecular level, the metabolic readjustment of glycolysis pathways within cirrhosis may explain why cirrhotic livers regularly trigger mechanisms against ROS-inflicted cell death over mitochondrial metabolism that also contributes to the generation of ROS (*162, 190, 330*). Cancer cell survival benefits from the production of ROS by the electron transport chain as it ultimately contributes to the level of DNA damage within the tumour resulting in Kras-induced cellular transformation (*329, 331*). With this in mind, glycolytic pathways that generate protective mechanisms against ROS production can counterbalance the chronic injury, giving rise to a need for early glycolytic functions in cirrhosis.

Early detection of HCC still provides the best clinical outlook for patient's overall survival by allowing therapeutical intervention to have effect prior to the rise of large levels of mutations within malignant hepatocytes. (*333-335*). As such, it is clinically relevant to assess where possible, the metabolic phenotype of patients with cirrhotic livers given 80% of HCC develops from early cirrhosis of the liver. Interestingly, HK2, ALDOA and PKM2 expression levels appear to have important clinical implication for patients with cirrhosis, as analysis of cirrhotic livers from patients followed up during a span of 10 years showed a positive correlation between high expression of glycolysis genes and progression of cirrhosis to HCC. These findings suggest that these glycolytic enzymes over TCA

or OXPHOS enzyme expression could behave as relevant biomarkers for the early detection of HCC. This metabolic shift from OXPHOS to glycolysis is shown to be true in early studies of rat cirrhotic livers vs normal rat livers that continue to use OXPHOS (*263, 336, 337*). Further studies in humans are required support our observations to prevent HCC development and recurrence. Furthermore, this form of analysis opens up the opportunity to better match individual HCC patients with viable drug options. By using this analysis to identify individual gene expression patterns, it would be feasible to plan the best course of therapies that would reduce the likelihood of drug resistance. This analysis provides an opportunity to reduce the level of relapsed/refractory disease

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Chapter 8 – Future Impact

The intricate signalling mechanisms of JNK proteins and how they contribute to cancer has long been of great interest. Seen to be regularly over-expressed in tumours (*159, 162, 170, 178, 185, 187, 307*), the JNK proteins play diverse roles in mediating cellular transformation, proliferation, death and survival in cancers. Unforeseen downstream mechanisms often confer clinical resistance to JNK inhibitors, and as such, the use of successful JNK inhibitors in clinics will be determined by correctly identifying suitable patients based on their disease biology . In this study, we characterize a novel JNK signalling pathway involving the peptidyl-prolyl cis-trans isomerase (PIN1). Through a combination of cell culture, over-expression studies, recombinant protein analysis, immunoprecipitation, mass spectrometry, metabolic-function studies and datamining of genomic patient data, we rigorously detail how this intricate JNK-PIN1 interaction contributes to the development of cancer. Given both JNK and PIN1 are independently topics of extensive research (*21, 159, 160, 170, 185, 187, 197-199, 206, 259, 309*), these findings will help towards the development of targeted JNK therapies. By screening patients with gene expression analysis, such as those used in this study, we can better identify sub-populations of patients where JNK therapies would confer beneficial results. This work supports the need for further translation of JNK inhibitors to clinical trials, with an increasing focus on the patient stratification to account for downstream mechanism such as the JNK-PIN1 role outlined in this study

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Chapter 9 – References

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