DEFINING THE ROLE OF PIM IN REGULATING CYTOSKELETAL DYNAMICS AND TUMOR CELL INVASION IN HYPOXIA

by

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Dedication

I dedicate this work to all the underdogs, the neglected, the underserved, and the disabled students who are thinking about pursuing science. I was all of those things and am honored to have the opportunity to be where I am today. I hope that my work and my journey show you that no matter where you were born, how you grew up, and what obstacles you face, you can accomplish more than you ever thought possible. It just takes one person to give you an opportunity. Never stop fighting and working toward your dreams because great success occurs when great preparation meets great opportunity.

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0 Abstract

Invasion of tumor cells out of the primary site is an essential early step in metastasis, which is responsible for the majority of cancer deaths. Therefore, distinguishing key factors that drive the switch from indolent to invasive disease will make a significant impact on guiding the treatment of prostate cancer (PCa) patients. Here, we identify a novel signaling pathway that links hypoxia and Proviral Integration site for Moloney murine leukemia virus (PIM) 1 kinase to the actin cytoskeleton and cell motility. PIM1 is upregulated in hypoxia and known to promote tumor survival and growth, but its role in invasion and metastasis has not been established. An unbiased proteomic screen identified Abl-interactor 2 (ABI2), an integral member of the Wave Regulatory Complex (WRC) that controls cytoskeletal dynamics, as a novel PIM1 substrate. Biochemical studies demonstrated that phosphorylation of ABI2 at Ser183 by PIM1 increased ABI2 protein levels, which enhanced WRC-mediated activation of actin-related protein (ARP) 2/3, resulting in increased protrusive activity and cell motility. Knockout of ABI2 diminished protrusive activity and negated the sufficiency of hypoxia and/or PIM1 to induce cellular protrusion. In vivo (skeletal) muscle invasion assays showed that overexpression of PIM1 significantly increased the depth of tumor cell invasion in an ABI2-dependent manner, and treatment with a clinically available PIM inhibitor significantly reduced intramuscular PCa invasion. This research uncovers a HIF-1-independent signaling axis that is critical for hypoxia-induced invasion and establishes a novel role for PIM1 as a key regulator of the actin cytoskeleton.

1 Background

1.1 Abstract

Hypoxia, or low oxygen tension, is a universal feature of solid tumors. The tumor microenvironment consists of regions of chronic and acute hypoxia that develop due to the irregular distribution of tumor vessels and limited oxygen diffusion within tumor tissue. While exposure to prolonged or severe hypoxia can be toxic to both normal and cancer cells, cancer cells take particular advantage of their ability to initiate a transcriptional program that allows them to adapt and thrive under these harsh conditions. Importantly, hypoxia upregulates a wide array of signaling that drive angiogenesis, cell survival, metabolism, immune evasion, invasion, and metastasis. For these reasons, the presence of intratumoral hypoxia is an adverse prognostic factor for patients with solid tumors and is a key component contributing to therapeutic resistance. As a result, understanding of the signaling mechanisms and physiological changes that occur in response to hypoxia will lead to alternative and more efficient therapeutic approaches.

1.2 Introduction

The tumor microenvironment refers to the local biological environment in which solid tumors are located that includes cancer cells and neighboring stromal cells, including fibroblasts, various immune cells, and blood and lymphatic cells, all of which are embedded within extracellular matrices. Solid tumors experience harsh physical microenvironments, which includes changes in their exposure to oxygen. Molecular oxygen is a vital part of cellular homeostasis, as it serves as both a cell signaling molecule and as a metabolic substrate. Fluctuations in oxygen levels are highly regulated during normal development and have been implicated in the onset and progression of various pathological conditions, among the most

notable of which is cancer (Thomlinson and Gray, 1955). Tissue hypoxia results from an inadequate supply of oxygen (O_2), and it is a universal characteristic shared by all solid tumors, regardless of the primary organ site. Hypoxia in the tumor microenvironment can be attributed to two primary factors that limit oxygen delivery to tumor tissue. First, rapid proliferation of tumor cells and expansion of tumor tissue leads to an environment wherein oxygen demand is surpassed by oxygen supply. As a result, the distance between cells and the existing vasculature increases so that diffusion of oxygen is no longer sufficient to reach areas within the growing tumor. Second, solid tumors are constantly developing new blood vessels to obtain sufficient levels of oxygen and nutrients, a process termed angiogenesis (Nacov, 1990). However, these newly formed blood vessels are frequently leaky due to their discontinuous endothelium, resulting in vascular hyperpermeability and enhanced permeation. Hence, in contrast to the precise distribution of vasculature in normal tissues, tumor-induced angiogenesis produces unorganized vasculature that does not distribute oxygenated blood evenly throughout the tissue. It is generally accepted that the oxygen level in hypoxic tumor tissues is significantly lower compared to the oxygenation of the respective normal tissues. On average, the tissue within solid tumors generally displays oxygen concentrations between 1.0% – 2.0% O_2, or below (Muz et al., 2015). However, tumor oxygen levels are highly variable and depend on the initial oxygenation of the tissue and the size and stage of the tumor.

In addition to heterogeneity among the cellular constituents of solid tumors, there is also vast heterogeneity within the extent of hypoxia depending on tissue type. For instance, arterial blood has an oxygen concentration of around 13%, O_2 whereas the oxygen concentration of the liver is typically 5% O_2 (Koh and Powis, 2012). Thus, tissue context is important, as some tissues

are at a higher propensity for hypoxia naturally, such as the bone (2.0% O_2). The response of cancer cells to hypoxia, ultimately deciding whether to undergo cell death or survival, in large part depends on the extent and duration of oxygen deprivation. Two main classifications exist for describing hypoxia in clinical oncology: acute and chronic. However, there is no clear consensus as to the amount of time or O_2 concentration that are necessary to induce acute vs. chronic hypoxia, and there is great variation as to how these are modeled in clinical and experimental studies. The lack of consistency regarding the definition of acute versus chronic hypoxia in experimental oncology is problematic, as these often result in vastly different biological consequences. Acute hypoxia is described as 'perfusion-limited' hypoxia that it is caused by a temporary and local disturbance in oxygen diffusion that occurs due to the occlusion of blood vessels and transient loss of blood flow to a region of tissue (Vaupel et al., 1990). Thus, acute hypoxia is an abrupt and brief exposure to short-term hypoxia that lasts for at least several minutes and up to 72 h. In addition, tissues experiencing acute hypoxia often undergo frequent cycles of hypoxia and reoxygenation, termed cyclical hypoxia. Tumor cells are usually able to survive acute hypoxia by activating a diverse set of signaling pathways that allow them to adapt and maintain homeostasis. Chronic hypoxia is defined as 'diffusion-limited' and occurs in tissue that is too far removed from blood vessels to received diffused oxygen (Rey et al., 2017). Changes in blood flow and low oxygen availability that result in chronic hypoxia are more prevalent in larger tumors and often result in cell death via necrosis. However, chronic hypoxia can also contribute to long-term cellular adaptations and clonal selection. In experimental settings, chronic hypoxia is typically modeled by exposing cells to periods of low oxygen ranging from several hours up to several weeks. While both chronic and acute hypoxia

have been observed in human cancer and *in vivo* tumor models, cyclical hypoxia likely represents the most common physiological representation of oxygenation in tumor tissues. Due to the constantly evolving tumor microenvironment, oxygen fluctuation occurs at irregular intervals in cancer due to dysfunctional tumor vasculature and arbitrary alterations to the blood supply. Thus, it is clear that instances of both chronic and acute hypoxia in cancer directly impact tumor progression and responses to therapy by influencing tumor growth, survival, and metastasis.

1.3 Signaling in hypoxia

Hypoxia drives many transcriptional and enzymatic signaling pathways that have profound effects on tumor biology by suppressing apoptosis (Erler et al., 2004), enhancing angiogenesis and vasculogenesis (Kioi et al., 2010; Semenza, 2000), increased generation of reactive oxygen species (ROS) (Guzy et al., 2005), altering cellular metabolism (Cairns et al., 2011), suppression of immune activity (Yotnda et al., 2010), and increased invasion and metastasis (Chang et al., 2011; Hill et al., 2009; Pennacchietti et al., 2003). The combined effect of these changes alters how tumors grow, survive, and spread, as well as providing a multitude of routes to evade anti-cancer therapy. As a result, understanding the basics signaling mechanisms that govern the physiological response to hypoxia is essential to understanding how and why the tumor microenvironment promotes disease progression and therapeutic resistance. In this section, we will discuss the underlying mechanisms that activate key signaling pathways responsible for the pro-tumorigenic effects of tumor hypoxia.

Hypoxia inducible factor-1

Many of the changes in gene expression observed in hypoxia can be attributed to the Hypoxia Inducible Factor (HIF) transcription factor. HIF is a heterodimeric transcription factor composed of an alpha subunit, of which there are three known isoforms (HIF-1α, HIF-2α or endothelial PAS domain containing protein 1 (EPAS1), and HIF-3α) and a beta subunit (HIF-1β; encoded by the Ary Hydrocarbon Receptor Nuclear Translocator (ARNT) gene), all of which are basic helix-loop-helix proteins (Wang et al., 1995). The protein expression of HIF-α subunits are tightly regulated in an oxygen-dependent manner, whereas the HIF-1β subunit is a constitutively expressed nuclear protein (Majmundar et al., 2010). Both HIF-1α or HIF-2α are able to heterodimerize with HIF-1β to form functional HIF transcription factor complexes (often referred to as HIF-1 and HIF-2, respectively) responsible for shifting in gene expression profiles in response to hypoxia. In contrast, HIF-3α has not been described to form an active transcription factor, but instead behaves as a negative regulator of HIF-mediated transcription by dimerizing with HIF-1β and sequestering HIF-1β from HIF-1αα (Gu et al., 1998; Makino et al., 2001). When tissue oxygen levels are adequate, a family of prolyl 4-hydroxylases (PHD1, PHD2, PHD3, along with a recently discovered and less well studied PHD4) hydroxylate two conserved proline residues (P402 and P564) in the oxygen dependent degradation domain (ODDD) of HIF-1α (Jaakkola et al., 2001). This hydroxylation event serves as a signal to initiate proteasomal degradation of HIF-1α. Von Hippel-Lindau (VHL), the E3 ubiquitin ligase component of an SCF E3-ligase complex, binds directly to hydroxylated HIF-1α and adds ubiquitin molecules that cause rapid degradation of HIF-1α via the 26S proteasome (Ohh et al., 2000) (Fig. 1). The canonical degradation of HIF-2α is governed by the same ubiquitin-mediated degradation pathway, so I will discuss regulation in the context of HIF-1α going forward. While stabilization

of HIF-1α and subsequent formation of HIF-1 heterdimer is responsible for most of the gene expression changes in hypoxia, the true sensors of cytosolic oxygen levels are the PHD proteins. PHDs belong to a superfamily of iron- and 2-oxoglutarate-dependent dioxygenases, meaning that molecular oxygen, 2-oxoglutarate (2-OG), and iron (II) are required for their catalytic activity (Pollard et al., 2014). Thus, under hypoxia, PHD activity decreases due to their need for molecular oxygen as a co-substrate. All PHD isoforms have the ability to hydroxylate conserved proline residues within HIF-α proteins, but they differ in their substrate specificity and their distribution in tissues and cells. It has been reported that PHD2 is more active toward HIF-1α than HIF-2α, whereas PHD1 and PHD3 are thought to hydroxylate HIF-2α more efficiently (Appelhoff et al., 2004; Koivunen et al., 2004). Thus, HIF-1α is stabilized in conditions of low oxygen because PHDs no longer hydroxylate proline residues within the ODDD, leading to accumulation of HIF-1α in the cytosol. Then, HIF-1α is free to translocate to the nucleus, where it binds to HIF-1β, and forms an active transcription factor. HIF-1 promotes the transcription of genes that have Hypoxia Response Elements (HREs) containing a 5'-(A/G)CGTG-3' consensus sequence (Semenza and Wang, 1992). Most HIF-1 targets contain HRE sites in the promoter region; however, there are some HRE-independent modes through which HIFs can control signaling pathways as well. For example, in colon cancer lines, HIF-2α activates the caveolin 1 gene promoter independent of HRE binding (Xie et al., 2014). This interaction instead occurs through HIF-2α binding GC-rich regions in the promoter and activation through the Myc Associated Zinc Finger (MAZ) transcription factor (Xue et al., 2013). Further genome wide mapping of HIF binding sites using ChIP-seq revealed that HIF-1 was bound to promoters outside of canonical RCGTG sequences, but primarily at regions that were CG-rich (Schödel et

al., 2011). A common HRE-independent mode of modulating signaling pathways includes HIF-1 interactions with Myc family members; these interactions are discussed in more detail later.

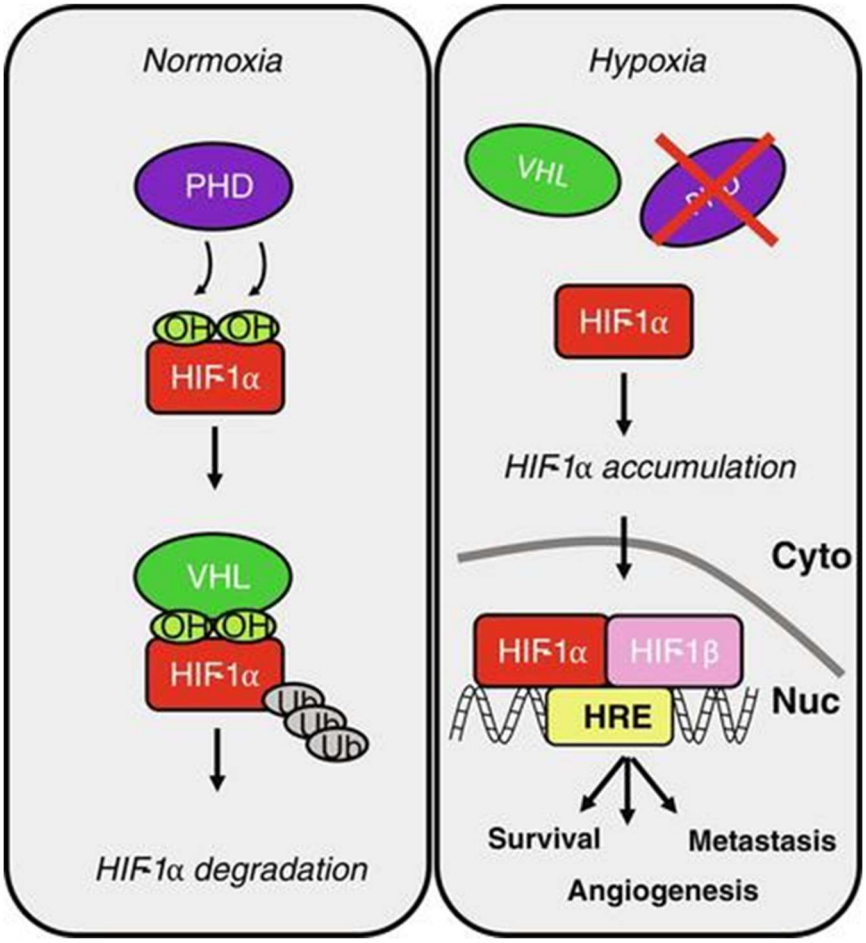

Figure 1 Oxygen regulates HIF-1α degradation. In normoxic conditions, HIF-1α is hydroxylated by PHDs and is recognized by VHL for ubiquitination and degradation through the 26S proteasome. PHDs are inactivated in hypoxia, allowing HIF-1α to accumulate and translocate to the nucleus where it dimerizes with HIF-1β to form an active transcription factor.

In addition to its regulation by oxygen and its canonical proteasomal degradation pathway, the transcriptional activity of HIF-1 is also controlled through interactions with transcriptional co-factors. Once HIF- 1 is assembled in the nucleus and binds to an HRE, it must recruit transcriptional co-activators to form an intact initiation complex. This process is controlled by two distinct transactivation domains present within HIF- 1/2α proteins, an oxygen-regulated C-

terminal transactivation domain (CAD) and a centrally located N-terminal transactivation domain (NAD) (Pugh et al., 1997) (Fig. 2). These transactivation domains are highly conserved amongst species, indicative of the critical role of these domains for robust transcriptional activation of HIF-1. Both the CAD and NAD recruit CBP/p300 and other transcriptional co-activators, linking HIF-1 with transcriptional machinery of the cell (Arany et al., 1996; Ema et al., 1999). While studies have shown that some HIF-1 target genes are exclusively dependent on the NAD, the CAD contributes to the regulation of most HIF-1 target genes and is considered the predominant transactivation domain (Jiang et al., 1997). Evidence suggests that the NAD contributes to the specificity of target genes between HIF-1 (composed of HIF-1α) and HIF-2 (composed of HIF-2α) (Hu et al., 2007). Swapping the NAD domains of HIF-1α and HIF-2α in chimeric proteins demonstrated that the CAD between had no effect on the target gene expression, whereas substitution of the NAD caused HIF-1α to behave as HIF-2α, and vice versa. Additionally, differences in sequence homology in the DNA binding and heterodimerization domains suggest that HIF-2 has unique target genes compared to HIF-1, and unique target sequences among the HIF-1 and HIF-2 transcription factors have been detailed (Keith et al., 2012). In general, HIF-1α appears to have a stronger influence in controlling acute hypoxic responses, whereas and HIF-2α is responsible for sustained signaling during chronic hypoxia (Saxena and Jolly, 2019). HIF-1 transcriptional activation is also influenced by post-translational modifications. For example, Factor Inhibiting HIF-1 (FIH-1) hydroxylates an asparagine residue in the CAD, which prevents the interactions with p300, a HIF transcriptional co-activator (Mahon et al., 2001). HIF-1 is more sensitive to FIH-1-mediated inhibition than HIF-2, so activation of FIH-1 promotes signaling through the HIF-2 axis over HIF-1 (Khan et al., 2011).

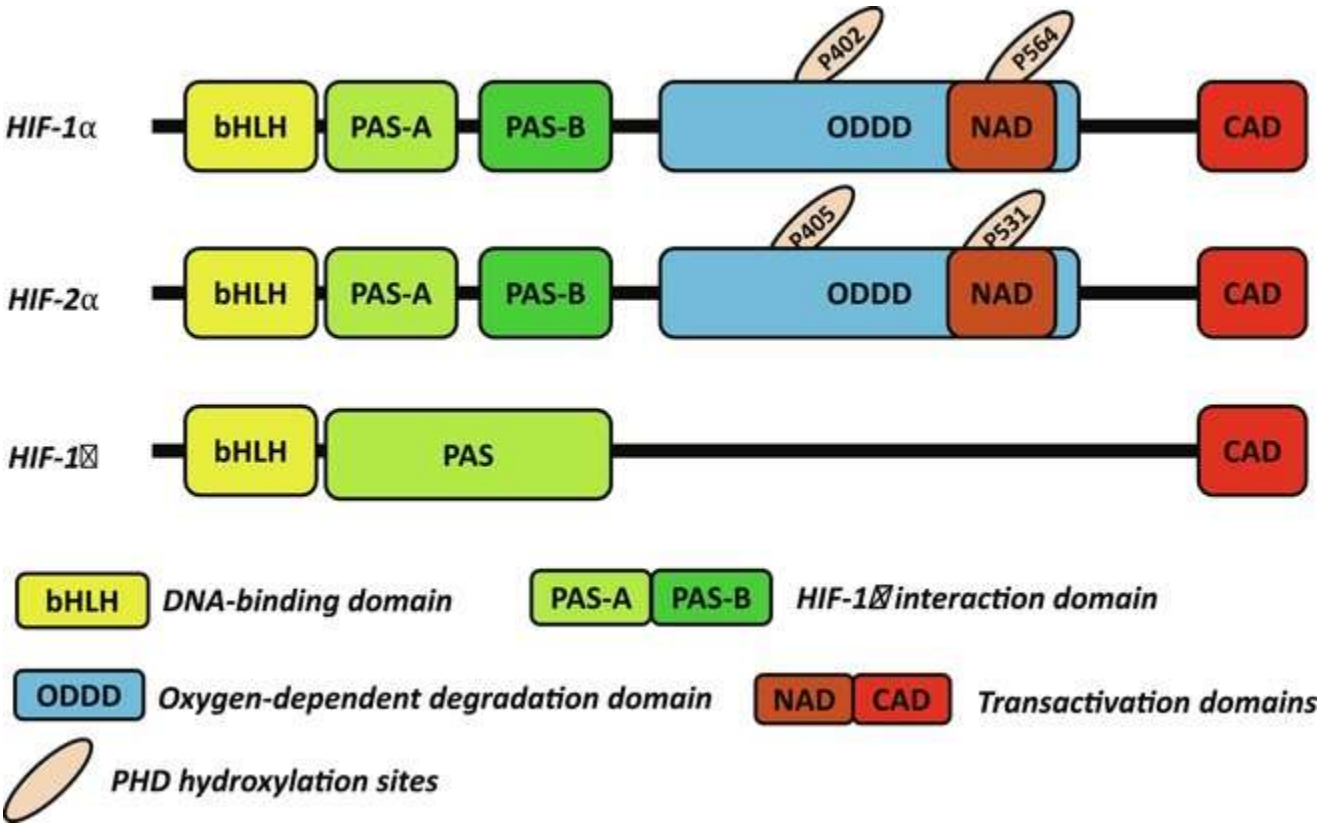

Figure 2 Schematic overview of the domain structures of HIF-1α and HIF-1β isoforms. All HIF isoforms contain the bHLH domain and PAS which are required for DNA binding and dimerization, respectively. HIF-1α and HIF-2α contain an ODDD, which contains conserved prolines that initiate the canonical degradation pathway.

Constitutive expression of HIF-1α is common in human cancers, regardless of oxygen tension. It is important to note that while the vast majority of HIF-1 activity is regulated by the hypoxia-driven hydroxylation mechanisms mentioned above; there are other non-hypoxic stimuli that can regulate HIF-1. Stabilization of HIF-1α in normoxia has been attributed to genetic alterations, most notably, loss of VHL (Krieg et al., 2000), as well as transcriptional upregulation due to the activation of oncogenic signaling pathways, such as NF-κB (van Uden et al., 2008), STAT3 (Niu et al., 2008), and Sp1 (Iyer et al., 1998). Various growth factors, hormones, cytokines, and other microenvironmental factors can activate pathways that increase the transcription of HIF-1α to the extent that protein generation outpaces its degradation, leading

to detectable levels of HIF-1α protein, even in normoxic conditions. In recent years, multiple new degradation pathways that control HIF-1α independent of oxygen have been discovered. This includes additional E3-ubiquitin ligases that target HIF-1α for proteasomal degradation, including RACK1, CHIP, HSP90/70, hypoxia-associated factor (HAF) (Koh et al., 2008), and SHARP1 (also known as BHLHE41) (Montagner et al., 2012). Moreover, a body of evidence indicates that HIF-1α can also be degraded by the lysosome through the chaperone-mediated autophagy pathway (Ferreira et al., 2013; Hubbi et al., 2013). Another critical event that controls HIF-1α activation is phosphorylation. Numerous phosphorylation sites and upstream kinases have been identified and shown to modulate HIF-1α protein stability and cellular localization. For example, Direct phosphorylation by ERK blocks the nuclear export of HIF-1α and promotes its accumulation in the nucleus, resulting in higher transcriptional activation (Mylonis and Simos, 2012). It has also been reported that phosphorylation of HIF-1α by various kinases can control its protein stability. Phosphorylation by glycogen synthase kinase 3 and Polo-like kinase 3 promote HIF-1α degradation (Flügel et al., 2007; D. Xu et al., 2017; Xu et al., 2010). Phosphorylation of HIF-1α by CDK1 and CDK2 help to promote proliferation by providing signals to stabilize and degrade HIF-1α, respectively, at different stages of the cell cycle (Warfel et al., 2013). Direct phosphorylation by PIM1, ATM, and PKA have also been reported to stabilize HIF-1α (Bullen et al., 2016; Cam et al., 2010; Casillas et al., 2018) (Reviewed extensively in (Kietzmann et al., 2016). Regardless of the mechanism, stabilization of HIF-1α in normoxia results in the constitutive upregulation of genes that initiate and sustain signal transduction pathways that drive many cellular processes associated with tumor growth and metastasis (Hartwich et al., 2013).

PI3K/Akt

Another oncogenic signaling pathway that is upregulated in response to hypoxia is phosphatidylinositol-3 kinase (PI3K)/ protein kinase B (Akt). PI3K is a family of lipid kinases that regulate a variety of cellular processes through the production of lipid second messengers that initiate signaling pathways involved in proliferation, cell survival, metabolism, and cell motility(Jean and Kiger, 2014). PI3K is frequently activated in oncogenesis due to mutations within PI3K itself or downstream of receptor tyrosine kinases (RTKs), which stimulate PI3K activity. Additionally PI3K sinalling can be upregulated by the loss of its negative regulator PTEN, as is commonly seen in prostate cancer. The PI3K family can be broken down into 3 classes (PI3K Classes I-III), the most well studies of which is Class IA PI3K. This kinase is composed of a heterodimer of p110 and a p85 subunits(Carpenter et al., 1990). The p100 subunit serves as the catalytic subunit, and the p85 subunit mediates receptor binding and localization. The p110 subunit contains many important binding domains, including a Ras binding domain, proline rich domains, membrane binding domain, and kinase domain. The p85 subunit contains SH2 and SH3 domains, which are vital to interactions with downstream substrates(Yoakim et al., 1994). Class I PI3Ks are responsible for the conversion of Phosphatidylinositol 4,5-bisphosphate (PIP2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which serves as a lipid second messenger for numerous proteins that contain a pleckstrin homology (PH) domain, most notably Akt (Woodgett, 2005). Akt is a serine/threonine kinase with an N-terminal regulatory region and kinase domain and C-terminal regulatory domain. Engagement of the PH domain of Akt with PIP3 leads to conformational changes that exposes key regulatory sites that can then be phosphorylated by several upstream kinases to fully activate the enzyme (Noel A Warfel et al., 2011; Xie et al., 2011). Growing evidence

suggests that hypoxia itself can activate the PI3K pathway, as demonstrated by increased and sustained of phosphorylation of Akt. Akt activation initiates a signaling cascade by interacting with a variety of downstream targets, one of which is the mammalian target of rapamycin (mTOR) kinase. mTOR is a central regulator of cellular growth, protein synthesis, and translation. As a result, it is highly sensitive to cellular stress, including hypoxia. The mTOR protein complexes are composed of mTOR complex 1 (mTORC1) and complex 2 (mTORC2), which react differently to hypoxia and will be discussed further. While the mechanisms have not been fully elucidated, biochemical studies using various inhibitors of both Akt and mTOR clearly demonstrate that both kinases positively regulate HIF-1α expression. PI3K inhibitors, LY294002 and Wortmannin, both reduce the expression of HIF-1α protein levels (Li et al., 2008; Yang et al., 2009). Constitutive activation of PI3K and/or Akt are sufficient to stabilize HIF-1α in both normoxia and hypoxia (Jiang et al., 2001). Moreover, the activation of HIF-1 downstream of growth factor signaling has been shown to be dependent on the PI3K/Akt pathway in some cases, such as stimulation with EGF(Zhong et al., 2000) and insulin (Dekanty et al., 2005). Regulation of HIF-1α via PI3K has been described in both normoxic and hypoxic conditions. However, in hypoxia the connection is more established. PI3K increases the stability of HIF-1α and in certain cases has been shown to upregulate HIF-1α via increased protein synthesis. Namely, in the MCF-7 breast cancer line, stimulation of HER2 leads to a dramatic increase in the rate of HIF-1α protein synthesis mediated through the PI3K/Akt/mTOR pathway (Laughner et al., 2001). PI3K and Akt have also been shown to enhance the heterodimerization of HIF-1α and HIF-1β subunits (Li et al., 2005). However, regulation of HIF-1α protein levels remains the primary mechanism for modulating HIF-1 activation. There are likely numerous complementary

mechanisms by which PI3K/Akt mediates the induction of HIF-1 in hypoxia or downstream of signaling agonists. One likely mechanism through which Akt increases HIF-1α is through the upregulation of mammalian target of rapamycin (mTOR) kinase (Pore et al., 2006).

PIM Kinases

While a majority of the hypoxic response can be attributed to HIF-1 as well as its ability to alter the previously described transcriptional networks, it is important to note that hypoxia also induces cell signaling pathways that are independent of HIF-1 transcription. One such example is the Proviral Integration site for the Moloney murine leukemia virus (PIM) family of Serine/Threonine kinases. PIM kinases promote tumorigenesis by impacting cell cycle progression, survival, proliferation, and angiogenesis and their expression is elevated in many solid tumors, including colon, breast, and lung cancer (Chen et al., 2009a). All PIM isoforms, particularly PIM1 and PIM2, are stabilized at the protein level in response to hypoxia. In response to hypoxia, PIM protein levels increase, while PIM mRNA levels stay relatively unchanged, suggesting that post-transcriptional regulation controls PIM expression in hypoxia and that this is independent of HIF-1-mediated transcription. Immunohistochemical staining has confirmed that PIM1 is highly elevated in hypoxic tumor tissue compared to normoxic tissue (Casillas et al., 2018). Importantly, PIM kinases are unique among kinases, as they lack regulatory domains. Thus, PIM isoforms are constitutively active upon translation and protein levels correlate with activity (Warfel and Kraft, 2015a). To date, little is known about the mechanisms controlling PIM kinase degradation, however recent work in our lab has identified one possible mechanism. Specifically, the deubiquitinase ubiquitin-specific protease 28 (USP28) regulates both PIM1 and PIM2. Upregulation of USP28 results in increased PIM protein stability

and total levels of PIM protein in normoxia and hypoxia, and knockdown of USP28 has the reverse effect (Toth et al., 2022). Another potential mechanism through which hypoxia could alter PIM stability is through upregulation of protein phosphatase 2A (PP2A) and downregulation of HSP70, both of which are known to promote PIM1 degradation (Petersen Shay et al., 2005). Thus, PIM represents an oncogenic signaling pathway in hypoxia that appears to be largely distinct from HIF-1.

The most well described role for PIM kinases in cancer is in promoting cell survival and providing an escape route for cancer cells to evade apoptosis. Importantly, hypoxic cancer cells are significantly more sensitive to small PIM inhibitors compared to normoxic cells (Warfel et al., 2016a). Thus, blocking PIM represents a HIF-independent approach to selectively target and kill hypoxic cancer cells. Several PIM targets, including Bcl2 agonist of cell death (Bad) and apoptosis signaling kinase 1 (ASK1), function directly in the intrinsic pathways of apoptosis. All three PIM isoforms phosphorylate Bad at Ser112, which blocks its binding with BCL-X_L and impedes its pro-apoptotic function (Macdonald et al., 2006). ASK1 plays an important role in the mitogen-activated protein kinase signaling that regulates stress-induced cell death, and phosphorylation by PIM1 inactivates this pathway (Gu et al., 2009). Recent studies indicate that PIM also prevents cell death by supporting tumor cell survival during oxidative stress. This is critically important for survival in hypoxic conditions, as hypoxia by nature causes an increase in the generation of reactive oxygen species (ROS). PIM kinases reduce ROS in cancer cells through multiple mechanisms. First, PIM1 supports the activation of nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), a transcription factor that is the master regulator of the cellular antioxidant response, by increasing its nuclear localization and protein levels (Warfel et al.,

2016a). Under conditions of oxidative stress, Nrf2 activates a transcriptional program that upregulates antioxidant molecules that reduce ROS. Small molecule PIM kinase inhibitors blocks Nrf2 nuclear accumulation, leading to excessive accumulation of ROS that is cytotoxic. PIM1 overexpression is sufficient to increase Nrf2 signaling in normal mouse prostate cells and prostate cancer cells (Song et al., 2018). Inhibition of PIM alone significantly increases intercellular ROS, and this effect is magnified in hypoxia, where ROS generation is already heightened. Second, PIM kinases maintain mitochondrial integrity and mitochondrial membrane potential, which is an integral aspect of the apoptotic response and can also contribute to elevated cellular ROS levels in hypoxia. Overexpression of PIM1 or PIM2 increases mitochondrial membrane potential, and a dominant-negative PIM1 worsens mitochondrial dysfunction (Fox et al., 2003; Lilly et al., 1999). PIM kinases also regulate dynamin-related protein 1 (DRP1), the key regulator of mitochondrial fission. PIM inhibition leads to increased levels of DRP1 as well as increased activation of DRP1, which promotes mitochondrial fragmentation (Chauhan et al., 2020). Thus, the upregulation of PIM in hypoxia appears to counteract the negative physiological changes associated with hypoxia, allowing tumor cells to adapt and maintain homeostasis in this harsh environment.

Data from outside of our group further demonstrates the role of PIM in cancer and specifically prostate cancer. PIM1 expression is higher in prostate tumor samples than in matched benign prostate hyperplasia samples (He et al., 2009). Overexpression of PIM1, both *in vitro* and *in vivo*, also enhances the tumorigenicity of DU145 and LNCaP prostate cancer cells (Kim et al., 2010); as does PIM2 (Ren et al., 2013). Furthermore, in vivo data shows PIM1 as a contributor to tumor invasion, migration, and metastasis (Santio et al., 2015a; Tursynbay et al., 2016) as

well as being associated with aggressiveness and drug-resistance (Jiménez-Garciá et al., 2016). PIM also works in coordination with other oncogenes such as c-MYC, p27, and BAD to promote more aggressive prostate cancer (Morishita et al., 2008; Wang et al., 2010). Interestingly, PIM seems to also paradoxically stabilize the prostate-specific tumor suppressor NKX3.1 (Padmanabhan et al., 2013) highlighting the complexity of targeting PIM in prostate cancer.

To date, there are several commercially available chemical inhibitors of PIM, some of which have undergone use in clinical trials. Most of these inhibitors generally use an ATP-competitive inhibition mechanism to prevent phosphorylation of downstream effectors of PIM. Perhaps the most well-known PIM-inhibitor is AZD1208, which inhibits all three isoforms of PIM. Treatment with AZD1208 was shown to inhibit cell growth, induce apoptosis and sensitize cells to radiation (N. Kirschner et al., 2015). However, phase 1 clinical trials using AZD1208 showed no functional response and resulted in adverse effects posttreatment for these patients, resulting in termination of the trial (Cortes et al., 2018). Other PIM inhibitors met a similar fate during phase 1 trials with SGI-1776 also being terminated and withdrawn due to toxicity (ClinicalTrials.gov Identifier NCT00848601). Second generation pan-PIM inhibitors have improved potency and decreased toxicity and are currently being evaluated in the clinic for use in prostate and other types of cancer. These include SGI-9481, which displays some selectively for the various isoforms of PIM depending on dosage, and PIM447 which has shown to be efficacious in large B-cell lymphoma patients (Foulks et al., 2014; Peters et al., 2016). Inhibition of PIM remains an active area of study as does the discovery of ways to reduce the toxicity that is accompanied by the use of these inhibitors. The PIM447 compound by Novartis is of

particular interest as it has bene shown to be effective and more tolerated and as such is the subject of several ongoing clinical trials either as a single agent therapy or in combination.

1.4 Biological effects of hypoxia

Hypoxia is a major signaling stimulus that effects a broad array of signal transduction pathways. As a result, the effect of hypoxia on cellular physiology and function is extensive. The biological capabilities acquired during the development of human tumors have been described by Hanahan and Weinberg as the hallmarks of cancer, which rationalize the complexity of neoplastic disease (Hanahan and Weinberg, 2011). Hypoxia is established as a fundamental feature of solid tumors that that influences or even cause these hallmarks of cancer, including survival, angiogenesis, metabolism, evading immunosurveillance, and tumor invasion and metastasis. As a result, the biological changes caused by hypoxia are correlated clinically with tumor progression, an aggressive phenotype, and poor outcome in patients with solid tumors.

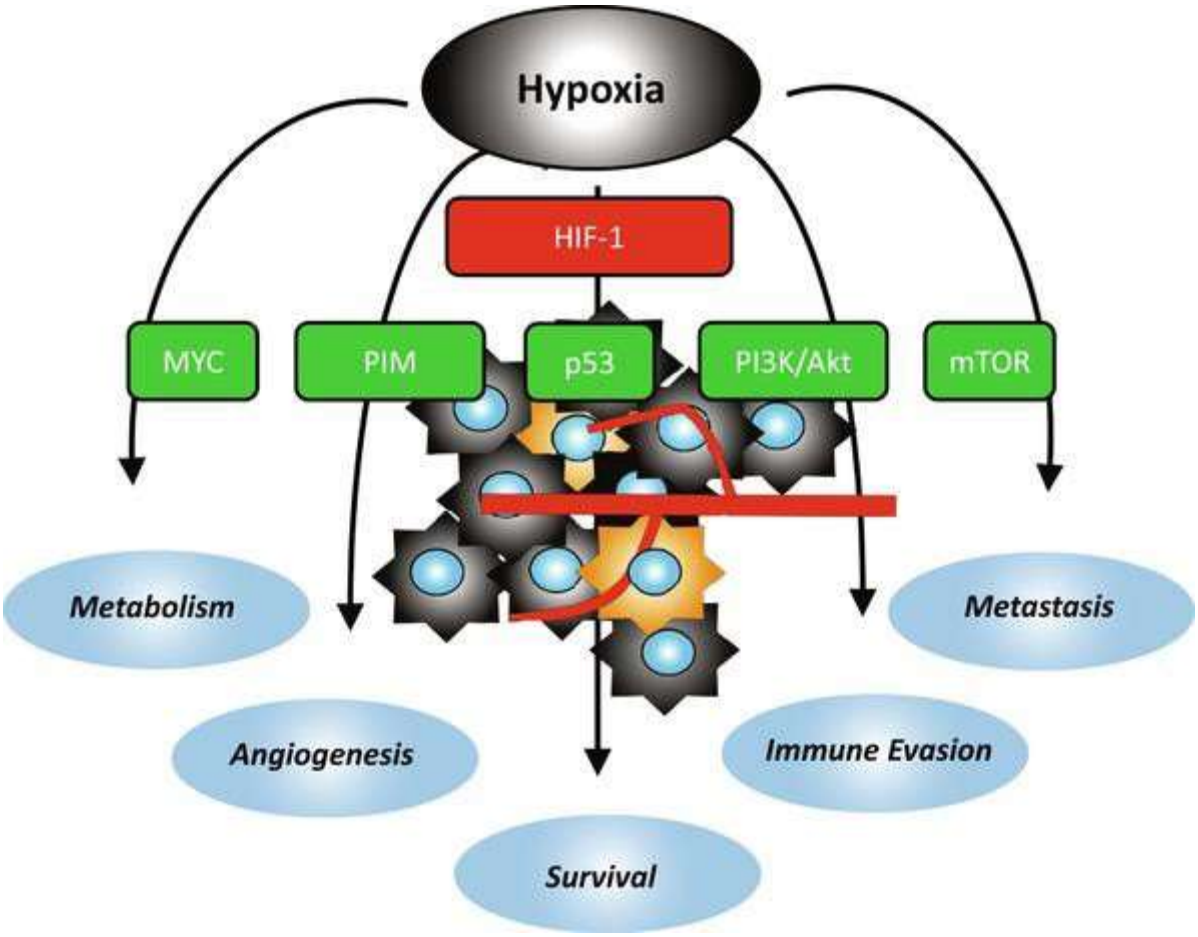

Figure 3 Hypoxia activates multiple signaling pathways to induce physiological changes that promote tumorigenesis. Activation of oncogenic kinases and transcription factors through HIF-1 dependent and HIF-1 independent mechanisms result in changes that drive cell metabolism, angiogenesis, survival, immune evasion, and metastasis that ultimately contribute to tumor progression, resistance to therapy, and worsen patient outcome.

Increased cell motility

Motility is an essential process during normal development and plays an important role in the onset and progression of many pathologies, including cancer. For tumor cells to metastasize, they must migrate out of the primary tumor and invade through surrounding stroma. Cell motility and migration is initiated in response to various cellular stimuli, such as chemical or physical cues in the surrounding microenvironment. These cues also serve to direct cell movement to an intended location (known as chemotaxis when directed by chemical signaling and durataxis when directed by mechanical signaling). Cell migration and invasion involve

interactions between cells and the extracellular matrix (ECM). The main difference between these modes of cell movement is that migration occurs along the ECM, whereas invasion involves moving through the ECM. The ECM is composed of various proteins, including collagen, laminin, and fibronectin. These ECM proteins form a network of direct and indirect interactions with microfilaments, phospholipids, proteoglycans, and integrins comprising the basement membrane which supports cell adhesion and structure. Cells in stasis display a high level of adhesion to the ECM, but during migration, the level of adhesion and the points of adhesion are highly dynamic. Differences in adhesion occur concert with alterations in cell shape, as motile cells are constantly sending out protrusions into the surrounding environment. Cells can migrate and invade either as single cells or as a group, a process known as collective migration. The basic mechanisms underlying single cell and collective migration are similar. In both cases, the formation of focal adhesions establishes a level of polarity that is crucial in sensing the environment and establishing directionality. In single cells, this establishes a leading edge, while in collective migration this distinguishes cells as “leaders” and “followers” (Friedl and Wolf, 2003). In single cell migration, much of this polarity is mediated by interaction with the actin cytoskeleton and Rho family GTPases. Namely, Rac and cdc42 induce actin polymerization and the formation of protrusions, such as filipodia and lamellipodia, at the leading edge. In the rear of the cell, Rho signaling controls actomyosin contractility that ultimately moves the body of the cells along the ECM during migration (Pollard and Cooper, 2009). These Rho family GTPases work in coordination with integrins, focal adhesion proteins, and cell-cell adhesion proteins to regulate migration. The basic process of single cell migration can be broken down into distinct steps: i) initial lamellipodia formation, ii) focal adhesion establishment, iii) cell contraction, and

iv) loss of adhesion points at the lagging edge. Collective migration follows many of these same principles; however, collective migration involves a concerted effort from a cluster of cells and relies on crosstalk amongst cells, strong cell-cell adhesion, and the establishment of leader cells. Collective migration increases the efficiency and ability of cells to migrate, as single cells typically experience less persistent migration (Mayor and Etienne-Manneville, 2016). As such, much of the physiological and pathological migration and invasion associated with cancer occurs via a collective manner rather than as single cells. Leader cells are named primarily because of their relative location rather than any inherent properties of the cells themselves, as cells inside of a cluster can migrate to the edge and become leader cells during collective migration (Poujade et al., 2007). The location of the leader cells on the periphery does however allow the cells to experience a higher level of external stimuli and more interaction with the ECM and microenvironment. Interaction with the ECM is primarily driven by integrin signaling, with integrin β1 being a major regulator in both endothelial and epithelial cells. Integrin binding to ECM recruits Rho GTPases and the downstream Rho effectors in the Scar-WAVE complex that ultimately lead to actin polymerization (Yamaguchi et al., 2015). As leader cells protrude and move through the ECM, they modify and change the ECM around them by altering matrix fibers. The organization and direction of various matrix fibers, such as collagen, has been shown to impact the speed and directionality as cell invasion (Alexander et al., 2008). Leader cells not only alter the direction of matrix fibers, but also actively cut and remodel the fiber through the secretion of Matrix Metalloproteases (MMPs). The secretion of MMPs, collagenases, and other proteolytic enzymes degrade and alter the ECM, paving the way for leader and follower cells behind them. As leader cells move along the ECM, they generate traction forces through

actomyosin contraction, much like in single cell migration. Unlike single cell migration, the high level of cell-cell adhesion, which is mediated by E-Cadherin, allows leader cells to pull follower cells behind them. The ability of follower cells to sense this pulling is known as mechano-sensing, and this is one of the key mechanisms of crosstalk between them and the leader cells during collective migration (Pruitt et al., 2014). Follower cells also exert forces on the cells around them by generating their own traction forces (Trepat et al., 2009). Notably, follower cells do more than just follow, as they have been shown to be important in generating the polarity of the leader cells, establishing the direction of leader cell protrusion, and amplifying chemotaxis through paracrine signaling.

Hypoxia has been shown to modulate and control several steps involved in both single cell and collective cell migration and invasion. Directly, hypoxia impacts both ECM remodeling and ECM degradation. As stated above, the alignment, composition and structure of the ECM are major factors that regulate cell migration. Hypoxia has been shown to control collagen 1 fiber remodeling and alignment, as well as increase ECM stiffness, which leads to increased adhesion and cell motility (Kakkad et al., 2010). These effects can be attributed to HIF-1-mediated increases in procollagen prolyl hydroxylases (P4H) A1 and A2 and lysyl hyoxylases (PLOD2). (Hofbauer et al., 2003; F. Xu et al., 2017), which modifies matrix stiffness and organization. Alternatively, hypoxia also promotes ECM degradation through multiple mechanisms. For example, hypoxia upregulates Cathepsins, urokinase-type plasminogen activator receptor (uPAR), and MMPs 1, 2, and 9 (Lewis et al., 1999; Sun et al., 2019). HIF activates the transcription of lysyl oxidase (LOX), which increases focal adhesion kinase activity and ECM remodeling through regulating collagen fiber crosslinking (Gilkes et al., 2014). In addition, HIF

upregulates chemokine and cytokine receptors, such as c-met and CXC chemokine receptor-4 (CXCR4), both of which play critical roles in chemotaxis and invasion (Ishikawa et al., 2009; Pennacchietti et al., 2003). Additionally, Hypoxia also affects the formation of focal adhesions on the ECM by increasing the expression of integrins. The expression of ITGα1, ITGα5, and ITGα6 which bind collagen, fibronectin, and laminin, respectively, have all been shown to increase under hypoxic conditions (Brooks et al., 2016; Ju et al., 2017). The upregulation of integrins downstream of HIF-1 enhances cell migration and invasion. Hypoxia has been shown to regulate integrin αvβ3, αvβ5, and the PI3K/Akt, mTOR, and HIF-1 pathways can regulate β1 integrins (Cowden Dahl et al., 2005; Lee et al., 2011; Ryu et al., 2010). Lastly, HIF-1 induces the expression of carbonic anhydrase 9 (CAIX), which degrades ECM, allowing for increased migration and invasion.

Beyond ECM modulation, hypoxia plays a role in determining the mode of tumor cell migration. HIF-1 induces a switch away from collective migration to an alternative form of single cell migration, known as amoeboid migration, through the upregulation of genes associated with epithelial to mesenchymal transition (EMT) (Lehmann et al., 2017). EMT is the process by which cells lose characteristics of epithelial cells, such as adhesion (through downregulation of E-Cadherin, claudins and cytokeratins), while gaining a more plastic and motile phenotype that resembles mesenchymal cells (upregulation of N-Cadherin, Vimentin, and Snail). Two of the major transcriptional proteins upregulated during EMT are Snail and Twist. Both of these proteins transcriptionally repress the expression of the CDH1, the gene that encodes the calcium-dependent adhesion molecule, E-Cadherin. Several of the major upstream inducers of EMT include Notch signaling, Transforming Growth Factor β1 (TGFβ1), NF-κB, Sonic Hedgehog

(SHH), Wnt, and Epidermal Growth Factor Receptor (EGFR) signaling pathways (Zhang et al., 2016). Hypoxia is a major factor in the TME that drives EMT in tumor cells (Higgins et al., 2007; Liu et al., 2014). Hypoxia promotes EMT by directly increasing many EMT transcription factors, including like Zinc Finger E-box binding homeobox (ZEB), Snail, Slug, and Twist (Luo et al., 2011; Yang et al., 2008; Zhang et al., 2015). In fact, a HIF-1 binding site was identified in the promoter region of Twist, allowing for direct induction of EMT by HIF-1. Crosstalk between HIFs, Wnt, and NF-κB have also been shown to enhance hypoxia induced EMT. Studies have also correlated loss of the HIF regulator, VHL, with the downregulation E-Cadherin in a HIF-1-dependent manner (Esteban et al., 2006). Hypoxia also promotes EMT through HIF-1-independent mechanisms. The activation of Notch 1 in hypoxia increases the expression of Snail and Slug through direct binding of the Notch intracellular domain to the Snail promoter or, indirectly, through LOX- mediated increases in Snail (Sahlgren et al., 2008). Hypoxia can also lead to the upregulation of NF-κB signaling through the Ras pathway, which ultimately increases Snail and Twist expression. TGFβ1 is also activated in hypoxia, where it can directly induce an EMT switch (Copple, 2010). Furthermore, TGFβ1 has been shown to downregulate PHD proteins, leading to accumulation of HIF-1α. This starts a feed-forward loop, as HIF-1α also promotes TGFβ1 signaling. Thus, hypoxia can increase both TGFβ1 and HIF-1α, which both directly and indirectly promote EMT. In addition, hypoxia induces EMT through changes in kinase signaling via PI3K/Akt and PIM signaling via their downstream regulation of GSK-3β. GSK-3β negatively regulates the stability and transcriptional activation of Snail. Thus, activation of Akt and PIM1/2 in hypoxia inactivates GSK-3β and relieves this inhibition. Overall, the multifaceted impact of hypoxia on ECM proteins, integrin expression, and cellular gene expression is sufficient to

switch tumor cells away from collective cell migration and toward single cell migration. This promotion of single cell migration and invasion is important for metastasis, which is considered the lethal step in the progression of solid tumors.

Increased invasion and metastasis

By nature, increased cell motility is associated with tumor metastasis. There is a prevailing theory that EMT drives invasion and metastasis by promoting detachment of cells away from the primary tumor. However, metastasis is a complex process that can occur through many different routes. The primary mode of metastasis occurs when cells invade into the blood stream or the lymphatic system, whereby they travel to colonize tumors at distant sites. Metastasis, however, requires more than just the physical movement of the cell to other sites; the metastatic cells also need to adhere to the ECM of the new tissue, survive in a new environment, and actively proliferate to progress from a micro metastasis to a macro metastasis. Many studies and analyses of clinical samples have revealed that intertumoral hypoxia is associated with metastasis and that HIF-1α overexpression is common in metastatic tumors. This is apparent in breast cancer, where 29% of primary tumors displayed HIF-1α overexpression compared to 69% of metastatic breast tumors with HIF-1α overexpression. Similar results have been reported for nearly all types of solid tumor, providing strong clinical evidence that HIF-1 is a critical factor stimulating tumor metastasis (Zhong et al., 1999).

As described above, the mechanisms involved in EMT and invasion promote the initial degradation of the ECM and basement membrane, allowing for intravasation into either the vasculature or lymphatic systems. Many of the hypoxia-induced proteins involved with ECM remodeling and degradation also play crucial roles during intravasation. For example,

knockdown of prolyl and lysyl hydroxylases (P4H and PLOD2) and LOX significantly reduced the ability of breast cancer cells to invade and intravasate into the blood stream and are essential for breast cancer metastasis (Gilkes et al., 2013a, 2013b). As described previously, hypoxia results in the upregulation of these enzymes. Many of the hypoxia-regulated proteins that are involved in angiogenesis also promote intravasation, including angiopoietins, VEGF, MMPs, uPAR, and VCAM. Physiologically, the "new" vasculature formed by tumor angiogenesis lends itself to intravasation for several reasons. First, growing tumors increase the number of vessels and their proximity to aggressive tumor cells, providing more access. Second, blood vessels that form in tumors are immature and tend to be leaky and weak (Nagy et al., 1989). Thus, it is easier for tumor cells to successfully penetrate the basement membrane in tumor-derived vessels as compared to the normal vasculature. Third, increased secretion of enzymes such as MMPs during the process of tumor angiogenesis further weakens the vessel walls. While gaining access to the bloodstream is an important step in the metastatic cascade, it is not enough for tumor cells to get into the blood stream or lymphatic system; they must also get out to form metastatic colonies. The first step in this process is margination, or the process by which circulating tumor cells (CTCs) halt circulation and adhere to the inner surface of the vasculature or lymphatic endothelium. One of the major molecules involved in margination of CTCs is L1 cell adhesion molecule (L1CAM), which is a direct target of HIF-1. Once margination occurs, CTCs must escape the vessel walls and undergo extravasation. A major factor in this process is angiopoietin-like 4 (ANGPTL4). Tumor cells secrete ANGPTL4 to interfere with endothelial cell-cell interactions allowing for the tumor cell to invade through the vessel wall. Much like L1CAM, ANGPTL4 is induced in hypoxia through a HIF-dependent mechanism (Zhang

et al., 2012). Thus, cells preconditioned in hypoxia have inherently higher levels of L1CAM and ANGPTL4, providing an advantage in their ability to establish at new sites of metastasis. Once cells extravasate, the disseminated tumor cell (DTCs) must then survive and grow in the new metastatic site. One prevailing thought is that the extravasation and subsequent growth of metastatic tumor cells is dependent on the formation of a premetastatic niche. There is much research demonstrating that before tumors metastasize to distant organs, such as the bone or the lungs, bone marrow derived hematopoietic progenitor cells (BMDPCs) first arrive to the metastatic site and makes the microenvironment more favorable for tumor cells. BMDPCs produce chemokines and modify the ECM to stimulate extravasation and facilitate the establishment of tumors cells. Preclinical and clinical studies demonstrate that both HIF-1α and VEGFR play critical roles in establishing the premetastatic niche. In breast cancer, HIF-1α expression was shown to be a key regulator of niche formation. One study showed that upregulation of LOX proteins in lung cells increased collagen crosslinking, as described previously (Wong et al., 2011). This increase in stiffness resulted in the recruitment of BMDPCs to the lung, priming the lung as a metastatic niche, which then recruited breast tumor cells to this metastatic site. Similar effects have been described in the bone as well as other tissues (Cox et al., 2015). BMDPCs expressing VEGFR were found to hone to metastatic sites and form cell clusters before the arrival of tumor cells, and that ablation of either HIF-1α or VEGFR in these cells prevented metastasis (Kaplan et al., 2005). There is also evidence that crosstalk among tumor and lymphatic cells exists that serve to promote the premetastatic niche in breast, prostate, melanoma, and colon cancer patients (Lee et al., 2014). Moreover, hypoxic tumor cells secrete endocrine factors, such as IL-6, which can activate a host of downstream targets,

including STAT3, within endothelial cells, such as lymphatic endothelial cell (LECs). Once these LECs are stimulated by STAT3, the LECs induce HIF-1 to produce VEGF and secreted chemo-attractants, such as SDF-1, which recruit tumor cells (Lee et al., 2014). These data suggest that tumor cell secretions, or possibly secretory exosomes, at the primary site can affect distant endothelial cells to recruit tumor cells that have broken away and intravasated in both the vasculature and the lymphatic system. Notably, each step in this process is enhanced by HIF-1 and other hypoxic signaling pathways.

The environment of the metastatic cell is drastically different from that of the primary tumor. Thus, many disseminated tumor cells (DTCs) rely on the hypoxia-induced survival mechanisms described previously, notably autophagy, evasion of apoptosis, alterations in metabolism, immune evasion, and angiogenic stimulation. Recent work indicates that autophagy facilitates CTC survival post-extravasation. There is also evidence that autophagy plays a role in the survival of dormant tumor cells; which describes the process in which tumors promote cellular senescence until a more favorable environment is established that allows for outgrowth and establishment of macro-metastases (Peng et al., 2013; Sosa et al., 2014). This is a major cause of recurrence in patients. The role of autophagy in metastasis has been reviewed in detail (Mowers et al., 2017). Survival of DTCs is also mediated by modulating metabolic requirements. Breast tumors that metastasize to the liver have been shown to be much more dependent on glycolysis than breast tumors that metastasize to the bone or lungs. To accomplish this metabolic switch to glycolysis, liver metastatic breast cells rely on HIF to induce PDK1 and repress PDH, halting progression into the TCA (Dupuy et al., 2015; Papandreou et al., 2006). Metabolism is not the only biological process required for the successful growth of DTCs.

Similar to the primary tumor sites, DTCs rely on the vasculature for the delivery of oxygen and other nutrients. Once DTCs grow in number and size, hypoxia sets in and HIF-induced VEGF is secreted to promote angiogenesis at the metastatic site. Lastly, for a macro-metastasis to form, DTCs must evade detection by the immune system. HIF signaling promotes resistance to T cells, and utilization of many of the immune evasion mechanisms described earlier. Additionally, HIF upregulation of programmed death-ligand 1 (PD-L1) expression on tumor cells which suppresses the adaptive immune response and further promotes immune evasion during metastasis (Palazon et al., 2014). Taken together, the biological contributions of HIF-1 are key to the survival and promotion of metastasis.

Metastasis is a major clinical problem, as the vast majority of cancer mortality can be attributed to metastasis. Outside of fast growing and highly lethal tumors such pancreatic cancer, or tumors in vital primary sites such as the brain, the primary tumor is typically not lethal. One of the best examples of this is prostate cancer. In patients where the tumor has not invaded and metastasized the five-year survival rate is an astonishing 99.8%. However, once the tumor has metastasized to other organs the survival rate drops to around 28% (Miller et al., 2016). This trend occurs in other solid tumors such as breast cancer and skin cancer, although not quite to the extent reported in prostate cancer. Hypoxia is a major driver of metastatic and aggressive disease. For this reason, targeting hypoxia and its associated pathways has been a goal of anti-cancer therapy for decades.

1.5 WAVE regulatory Complex

As briefly mentioned in the previous section on cell motility; the WAVE regulatory complex (WRC) is a major component of cellular protrusion and lamellipodia formation. These are both

key early events in the process of cell migration and necessary first steps in the invasion process. A better understanding of the WRC and its downstream effect on actin dynamics may prove useful in better understanding one of the mechanisms by which hypoxia promotes tumor invasion. The WRC is a pentameric complex made of ABI2, HSPC300, Nap1, Sra1, and the protein for which the complex is named after: WAVE. WAVE (WASP family Verprolin homolog), also known as SCAR (Suppressor of cAMP Receptor) belongs to a family of proteins known as the Wiskott-Aldrich Syndrome Protein family (WASP). The WASP family contains seven major members with WASP, WAVE, and WASH the most well studied (Gautreau et al., 2004). Each WASP family member contains a domain that binds to actin-related protein (Arp) facilitating the nucleation and branching of actin; although the mechanisms that regulate this are unique for each member. The WRC is of particular interest as it is essential for membrane protrusion and migration while also being deregulated in various human diseases.

How the WRC assembles the subunits and regulates the levels and stoichiometry of the individual WRC remains an active area of research. What is known however, is that the individual subunits and partially assembled complexes lacking all 5 members tend to be very unstable (Wu et al., 2012). Therefore, some level of regulation and stabilization of the individual members is needed prior to the formation of an intact WRC. Some work suggests that they may form homodimers or homotrimers as is the case with HSPC300 (Derivery et al., 2008) or be stabilized by outside proteins such as Nudel binding to the Sra1-Nap1-Abi1 sub complex to help stabilize its interaction with HSPC300 (Wu et al., 2012). In vitro data suggests that the large Sra1 and Nap1 dimer binds the smaller WAVE-Abi2-HSPC300 trimer. Specifically, the WAVE homology domain of WAVE1, the amino-terminal SNARE-like domain of Abi2 and the

entirety of HSPC300 bind the Sra1-Nap1 dimer. Once this binding occurs small portions of WAVE and Abi form tail like structures that loosely contact and interact with Sra1 and Nap1 respectively. The Abi2 tail contains an SH3 domain which can bind proteins such as Abl and a nap1-binding region. The WAVE tail contains a meander region that loosely interacts with Sra1 and a VCA (or more recently WCA) domain which is found in all WASP-family members. The V and C portions bind to Sra1 and WAVE to auto-inhibit the complex. When the complex is activated the VCA region of the WAVE tail dissociates and allows for Arp to bind the C and A regions with the V region serving as an actin-binding sequence (Chen et al., 2010) (Fig 4).

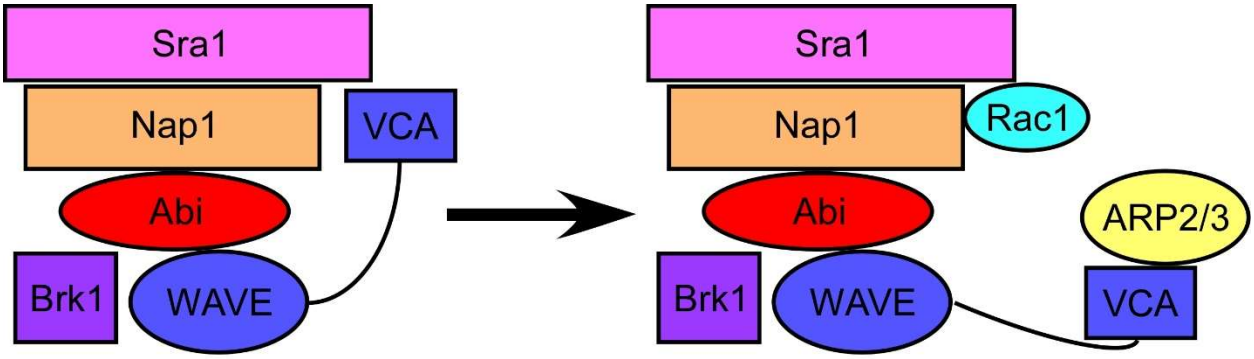

Figure 4 Schematic diagram of the WAVE regulatory complex in an auto-inhibited state (Left) or activated state (Right)

One of the major activators of the WRC, that allows for the freeing of the VCA domain, is the Rho-family GTPase Rac1. Rac1 binds to two sites of Sra1 which allosterically releases the VCA domain, allowing for the interaction with Arp. Other GTPases such as Arf1, RhoG and Cdc42 have also been shown to either activate the complex or cooperate with Rac1 to activate the WRC. Additionally, various kinases such as Abl, Src, and Cdk5 have also been shown to regulate the WRC's activity. Phosphorylation of the meander region of WAVE destabilizes VCA binding and leads to activation of the complex and the VCA domain itself is often phosphorylated leading to altered complex kinetics (Chen et al., 2017; Mendoza, 2013).

The final step of the process is translocation of the WRC to the plasma membrane. Recently Phosphatidylinositol (3,4,5)-trisphosphate and other transmembrane proteins have been thought to provide spatiotemporal control of WRC localization as well as activation of WRC. PIP_3 is known to enhance the activation of the WRC via interaction with the positively charged surface of the WRC (Chen et al., 2013). Transmembrane proteins also can directly recruit the WRC to the membrane via the WRC interacting receptor sequence (WIRS) motif. This motif is composed of amino acids from both Sra1 and Abi2, indicating that only the pentameric form of the WRC is able to fully bind and translocate to the membrane.

1.6 Conclusions and major gap in knowledge

In conclusion, the advances in our understanding of the biochemical and biological mechanisms by which cells sense and respond to hypoxia has launched new avenues of study in cancer biology and medicine. As our knowledge of tumor hypoxia has expanded, it has shed light on the complex crosstalk that exists between the regulatory dioxygenase enzymes sense oxygen, the transcription factor networks that control gene expression, and key signaling enzymes that are responsible for driving physiological changes. As a result, the far-reaching impact of hypoxia in cancer presents many opportunities and challenges for therapeutic intervention in solid tumors. Continuing research will undoubtedly produce effective ways to oppose the oncogenic effects of tumor hypoxia and overcome hypoxia-mediated therapeutic resistance.

One of the major gaps in our knowledge outlined above is how does PIM kinase signaling promoting tumor invasion and metastasis? There are many known PIM substrates that effect cell cycle, chemoresistance, and apoptosis; yet no identifiable substrate that could account for the invasive and metastatic behavior regulated by PIM. Through work that I will outline in the

next section, we identified ABI2 as a novel PIM substrate. The connection between PIM and ABI2 provide a direct link between PIM and regulation of the actin cytoskeleton that could bridge our gap in knowledge. Specifically, we hypothesize that PIM-1 activates the WRC via phosphorylation of ABI2 at Ser183 leading to increased protrusion and invasion.

2 PIM1 phosphorylates ABI2 to enhance actin dynamics and promote tumor invasion

By Corbin C Jensen, Amber N Clements, Hope Liou, Lauren E Ball, Jennifer R Bethard, Paul R Langlais, Rachel K Toth, Shailender S Chauhan, Andrea L Casillas, Sohail R Daulat, Andrew S Kraft, Anne E Cress, Cindy K Miranti, Ghassan Mouneimne, Greg C Rogers, and Noel A Warfel.

2.1 Introduction

The ability of cancer cells to migrate and invade surrounding tissue is an early and essential step for metastasis, a lethal progression of solid tumors that accounts for over 90% of cancer-related deaths. This is particularly true for prostate cancer (PCa), where patients have a good prognosis if the tumor remains confined to the primary organ. However, when tumor cells escape the gland, there is a dramatic decrease in the 5-yr survival (from nearly 100% to 30%)(Zhang et al., 2021). Although the metastatic process is complex, it is entirely dependent on the ability of cells to migrate. Therefore, identifying new, druggable targets to inhibit cancer cell migration represents a promising strategy to block disease progression and improve prognosis in PCa patients.

Hypoxia, or low oxygen concentration, is a hallmark of solid tumors that is strongly associated with invasion and metastasis (Jensen and Warfel, 2021). In PCa, increased expression of hypoxia-induced proteins correlates with treatment failure, independent of tumor stage, Gleason score, and prostate-specific antigen (PSA) levels (Deep and Panigrahi, n.d.). Higher expression of hypoxia-induced genes, such as hypoxia-inducible factor (HIF)-1, vascular endothelial growth factor, and osteopontin, is associated with early biochemical treatment failure and disease recurrence in patients with clinically localized PCa (Vergis et al., 2008). Moreover, hypoxia is an independent predictor of biochemical recurrence and is a better predictor of local recurrence of PCa than PSA levels or Gleason score (Milosevic et al., 2012). Recent work has established Proviral Integration site for Moloney murine leukemia virus (PIM) 1 kinase as a hypoxia-inducible pro-survival protein (Casillas et al., 2018). PIM1 is a member of a family of oncogenic Ser/Thr kinases whose levels are elevated in many types of solid tumors, particularly PCa. PIM1 levels are elevated in high-grade prostatic intraepithelial neoplasia relative to normal tissue and further elevated in castration-resistant prostate cancer (Dhanasekaran et al., 2001), and high PIM1 expression is associated with resistance to therapy (Logothetis et al., 2018). Notably, PIM1 levels are upregulated in hypoxia in a HIF-independent manner due to increased protein stability (Toth et al., 2022). Functionally, PIM1 has been shown to promote therapeutic resistance and enhance proliferation, survival, and angiogenesis (Toth et al., 2019; Toth and Warfel, 2021). Here we identify a novel role for PIM1 in tumor cell invasion and metastasis.

We previously reported that treatment with small-molecule PIM inhibitors significantly reduces metastasis in orthotopic models of prostate and colon cancer (Casillas et al., 2018), but the

mechanisms responsible have not been established. Here, we identify PIM1 as a potent driver of hypoxia-induced migration and invasion, key initial steps in the metastatic process. An unbiased proteomic screen performed in hypoxia identified Abl-interactor 2 (ABI2) as a novel substrate for PIM kinases. ABI2 plays an essential role in regulating actin dynamics and cell motility as an integral member of the WAVE regulatory Complex (WRC) (Dai and Pendergast, 1995). The WRC is a stable heteropentamer of isoforms of the following five proteins: WASp-family verprolin homologous protein (WAVE), Abl-interacting protein (ABI), NCK-associated proteins (NAP), specifically Rac-associated 1 (SRA1), and hematopoietic stem progenitor cell 300 (HSPC300) (Rottner et al., 2021). The WRC is held in an inactive conformation until the cell receives signals that initiate Rac binding or phosphorylation by protein kinases, which cause a conformation change in WAVE2 that releases the VCA (verprolin, cofilin, acidic) domain(Mendoza, 2013). This results in the recruitment of the actin-related protein (ARP) 2/3 complex, which stimulates actin branching and cellular protrusions (Echarri et al., 2004). Our findings reveal that phosphorylation by PIM1 increases ABI2 protein stability, which leads to stabilization of the WRC and enhances tumor cell protrusive activity and invasion. This work provides the first direct link between hypoxia, PIM kinases, and the regulation of the actin cytoskeleton. The identification of this signaling axis poises PIM1 as a critical driver of cancer cell invasion and metastasis and provides a novel target to oppose the pro-metastatic effect of hypoxia in PCa.

2.2 Results

PIM1 is critical for hypoxia-induced invasion and cellular protrusions

Hypoxia is known to promote an aggressive phenotype in solid tumors, as demonstrated by clinical association with heightened metastasis (Rankin and Giaccia, 2016). Although many hypoxia-induced factors can influence metastasis, it is the effect of hypoxia on early events, such as protrusive activity, that is particularly of interest to us. Therefore, we first established the effect of hypoxia on the invasive potential of PCa cell lines. Boyden chamber Transwell assays were performed using PC3-LN4 and DU145 PCa cells. Exposure to hypoxia significantly increased both the migration (uncoated) and invasion (laminin-coated) of both cell lines compared to cells maintained in normoxia (Fig. 1A). Next, we examined whether hypoxia increased cellular protrusion. To this end, we stably transfected DU145 cells with GFP-Lifeact to label actin. These cells were starved (DMEM with 0.5% FBS) for 4 hours and then imaged every 15 seconds over the course of the experiment. To establish a baseline level of protrusive activity, cells were imaged for 5 minutes prior to stimulation with either EGF (5 nM) or IGF (5 nM). Protrusion was measured as the change in cell area over time relative to the established baseline. We found that cells conditioned in hypoxia displayed significantly larger increases in cell area in response to EGF or IGF stimulation than cells in normoxia (Fig. 1B). Additionally, the changes in area occurred more rapidly and were sustained over the course of the experiment in hypoxic cells, whereas the area of normoxic cells quickly returned to baseline or decreased (Fig. 1B). Because cell area is influenced by both protrusion and retraction events, we modified publicly available code from the Danuser lab (Lee et al., 2015) to more accurately quantify cellular protrusions. In this approach, we created an algorithm to trace individual cells, assign unbiased sampling windows along the edge of the cell (10-µm wide), and determine protrusion vectors based on changes in the location of the cell edge between each frame. The resulting

data were quantified as the protrusive velocity over time for each cell and displayed as a 3D heatmap. To determine whether hypoxia altered the basal frequency and amplitude of protrusion events, DU145-GFP-Lifeact cells were cultured overnight in normoxia or hypoxia, then grown in starvation media for 4 hours, and imaged every 15 seconds. Strikingly, hypoxic cells displayed significantly more frequent and intense protrusive events under basal conditions than normoxic cells (Fig. 1C). Quantification of these protrusion vectors revealed a significant increase in the protrusive activity of DU145 cells in hypoxia. Similar results were observed in MCF7 breast cancer cells, indicating that the effect of hypoxia on protrusive activity is not specific to PCa cells (Fig. 1D). Taken together, our findings indicate that hypoxia increases the basal levels of membrane protrusion and primes cancer cells to respond to growth factors.

Previous work in our lab demonstrated that PIM1 is upregulated in hypoxia, independent of HIF-1 signaling (Chen et al., 2009; Warfel and Kraft, 2015), and treatment with a small-molecule PIM inhibitor reduces tumor metastasis in orthotopic models of prostate and colon cancer (Casillas et al., 2018). Based on these findings, we reasoned that PIM1 may play an important role in mediating the pro-invasive effects of hypoxia. To test this hypothesis, we implemented a 3D invasion assay where PC3-LN4 or PC3-LN4 cells lacking PIM1 (CRISPR knockout) were seeded into Matrigel as part of an inner core surrounded by a collagen matrix. Then, the chambers were cultured in normoxia (20% O_2) or hypoxia (1% O_2), and tumor cells were allowed to invade into the outer matrix. After 48 hours, the cells were fixed and stained for DNA. The number of invading cells and the distance from the edge of the core to each nucleus was measured (Fig. 1E). Strikingly, the number of invading cells was significantly reduced in cells lacking PIM1 (Fig. 1F). The fold-change decrease in invasion depth (11.2-fold decrease) observed in PIM1-KO cells

was identical in normoxia and hypoxia, suggesting that basal levels of PIM1 are important for invasion, regardless of oxygen tension. The average distance of invasion was significantly increased in hypoxia compared to normoxia, and this effect was significantly blunted in PC3-LN4 cells lacking PIM1 (Fig. 1G). To confirm that PIM activation is critical for hypoxia-induced invasion, we performed Boyden chamber invasion assays using PC3-LN4 cells. As expected, hypoxia significantly increased the number of invading cells compared to normoxia. Strikingly, treatment with a PIM inhibitor (AZD1208) blocked the increase in invasion seen in hypoxia, providing further evidence that PIM activity is required for the pro-invasive effect of hypoxia (Supplemental Fig. 1A). Next, we assessed the role of PIM1 in cell migration by live cell microscopy using PC3-LN4 cells with PIM1 overexpression or CRISPR knockout. Each cell line was plated sparsely and conditioned in normoxia or hypoxia for 4 h, and the distance traveled by single cells over a 24-hour time course was measured. Both exposure to hypoxia and overexpression of PIM1 significantly increased the average distance traveled by each cell (Supplemental Fig. 1B). Notably, hypoxia did not further increase motility in cells overexpressing PIM1. Importantly, knockout of PIM1 reduced migration compared to that in control cells in normoxia, and loss of PIM1 completely negated the hypoxia-induced increase in cell migration (Supplemental Fig. 1B). Wound healing assays using WT and TKO MEFs further demonstrated the necessity of PIM signaling for proper migration (Supplemental Fig. 1C). To further establish the importance of PIM in regulating protrusion dynamics, we measured spreading area over time as a dynamic measurement of leading-edge protrusion. During cell spreading, actin dynamics play a significant role in pushing the leading edge, resulting in protrusion and cell spreading. WT and TKO MEFs were plated on laminin-coated dishes in

serum-free media, and images were taken every 1 minute for 2 hours. The area of individual cells (n=20/cell line) was measured to determine the rate of cell spreading. Within 90 minutes, WT MEFs reached maximum area (approximately 3000 μm^2), whereas spreading was significantly slower in TKO MEFs, only reaching approximately 1700 μm^2 by 120 minutes (Supplemental Fig. 1D). Together these data demonstrate that PIM plays an important role in regulating cellular protrusion and motility, particularly in hypoxia.

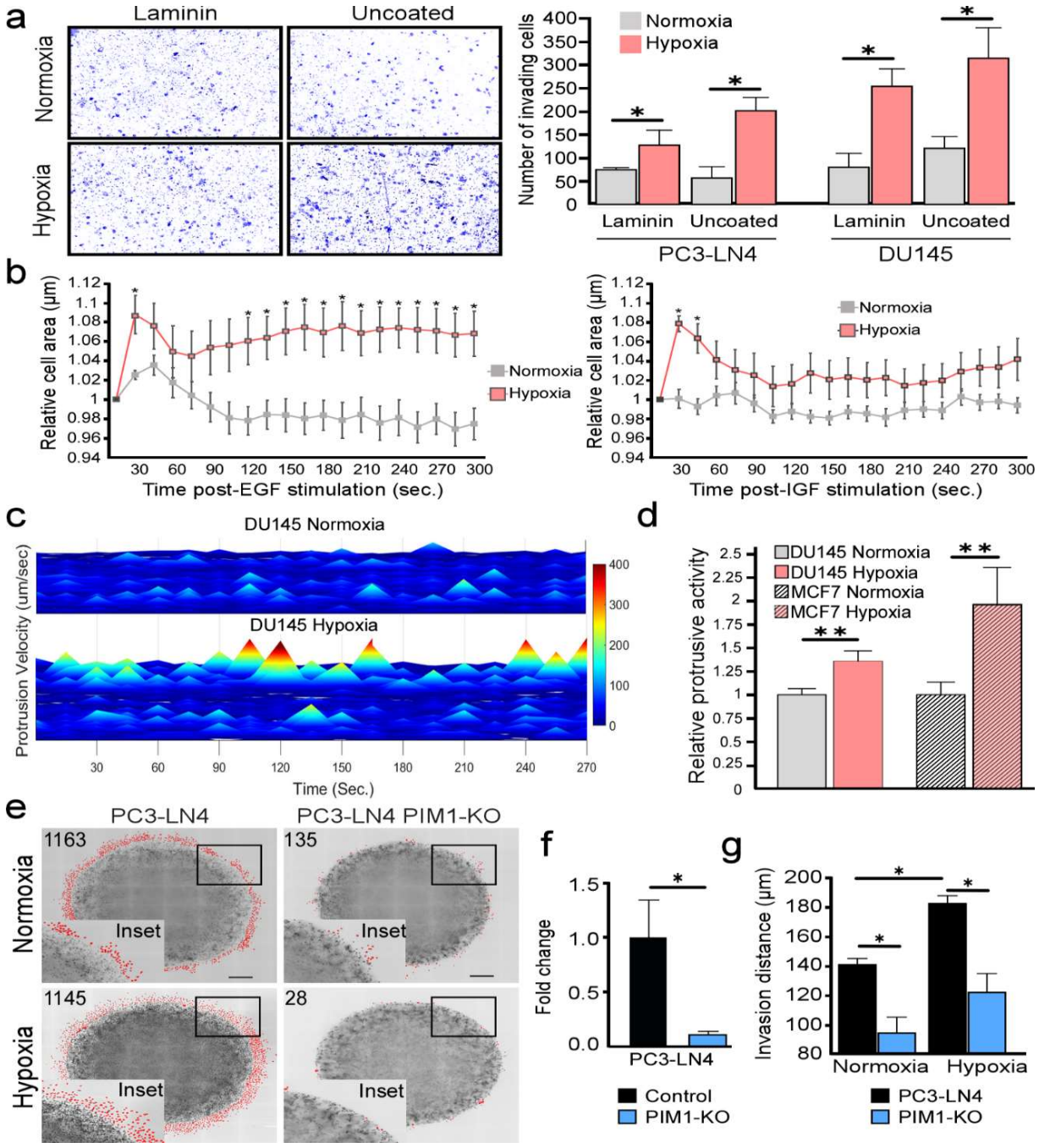

Figure 1. Hypoxia increases tumor cell invasion and protrusive activity. (A) Representative images of Boyden chambers invasion assay in normoxia (Nx) or hypoxia (Hx; 1% O2), with accompanying quantification of cell number (n=3). (B) Quantification of DU145 cell area following stimulation with EGF (5 nM) and IGF (5 nM) (n>10 cells/condition). Values are relative to cell area prior to stimulation. (C) Representative heatmap of basal (unstimulated) protrusive velocity of DU145 cells in normoxia (n=1637, N=5) and hypoxia (n=1286, N=12). (D) Quantification of relative protrusion activity in normoxia and hypoxia for DU145 (n=14) and MCF7 (n > 9) cells. Error shown as 95% CI by Student's t-test (*p<0.05, **p<0.01). (E) Representative images of 3D invasion assays using PC3-LN4 and PC3-LN4 PIM1-KO cells in normoxia and hypoxia. Invading cells are marked in red, and the number of invading cells is indicated in the upper left corner. (F) Quantification of the total number of invading cells and (G) Average invasion distance from the core of PC3-LN4 (n=2864 Nx and n=2079 Hx) and PC3-LN4 PIM1-KO (n=255 Nx and n=143 Hx) cells. N=3 biological replicates for all conditions.

ABI2 is a direct substrate of PIM kinase

Although an extensive list of PIM substrates has been described (Warfel and Kraft, 2015), none directly link PIM1 to regulation of the actin cytoskeleton. Therefore, we performed an unbiased proteomics screen incorporating SILAC to identify new PIM substrates, particularly in hypoxia. PC3-LN4 PCa cells were grown subconfluently with isotopically distinct forms of arginine. After validating SILAC incorporation, cells were conditioned in normoxia or hypoxia for 8 hours and then treated with AZD1208 (3 μM) or DMSO for 15 minutes. This time point was chosen to capture potential direct substrates of PIM kinases, as this was the earliest time point at which we observed loss of phosphorylation of eIF4B (Ser406), a known PIM1 substrate. Biological replicates were performed, and protein from each treatment group was combined and processed for mass spectrometric analysis. Phosphopeptides were isolated, and LC-MS/MS was used to identify potential PIM targets (Fig. 2A). We identified 75 phosphopeptides that were significantly reduced in hypoxia after the addition of AZD1208. Interestingly, the most significantly downregulated phosphopeptides upon inhibition of PIM in hypoxia belonged to ABI2 (Fig. 2B). ABI2 is an integral member of the WRC, which is responsible for recruiting ARP to the cell membrane, where it facilitates actin nucleation and the subsequent formation of lamellipodia. Specifically, we identified several peptides between Ile147 and Pro185 in ABI2 with differential phosphorylation upon PIM inhibition in hypoxia. To validate that ABI2 is a direct substrate of PIM1, we performed *in vitro* kinase assays. Recombinant ABI2 and PIM1 proteins were incubated along with GST (negative control) and MBP (positive control) in the presence or absence of a pan-PIM kinase inhibitor (PIM447). Autoradiography readily demonstrated that ABI2 was phosphorylated in the presence of PIM1, and inhibition of PIM

completely abrogated phosphorylation of ABI2 (Supplemental Fig. 2A). Subsequent quantitative mass spectrometry analysis revealed a dually phosphorylated peptide containing Ser183 and Ser187 in ABI2. Notably, phosphorylation of this peptide was almost completely lost upon the addition of PIM447 (Fig. 2C). To further confirm which site(s) are phosphorylated by PIM1, we performed in cell kinase assays using immunoprecipitated ABI2 from cells and mixed with GST-PIM1 or GST alone. Mass spectrometry analysis identified Ser183 as the most consistently phosphorylated PIM1 site both *in vitro* and *in vivo* (Fig. 3D; spectra from *in vivo* analysis). Sequence alignment of ABI2 indicated that S183 is conserved among species, suggesting functional relevance of this residue (Fig. 2D). Indicative of the importance of ABI2 in PCa metastasis, analysis of data from the NCBI Gene Expression Omnibus database (Bartha and Győrffy, 2021) showed that *ABI2* expression is significantly higher in prostate tumors than in normal tissue, and it is further upregulated in metastatic castrate-resistant PCa (Fig. 2E). Notably, the expression of an ABI2 homolog, ABI1, was not correlated with PCa progression, suggesting that they are not compensatory (Supplemental Fig. 2B). Further analysis of ABI2 in PCa using the Prostate Cancer Transcriptome Atlas (PCTA)(You et al., 2016) revealed an inverse correlation between ABI2 expression and biochemical recurrence-free survival (Fig. 2F). These data identify ABI2 as a novel substrate of PIM kinases and provide a potential link connecting PIM activity to regulation of the actin cytoskeleton.

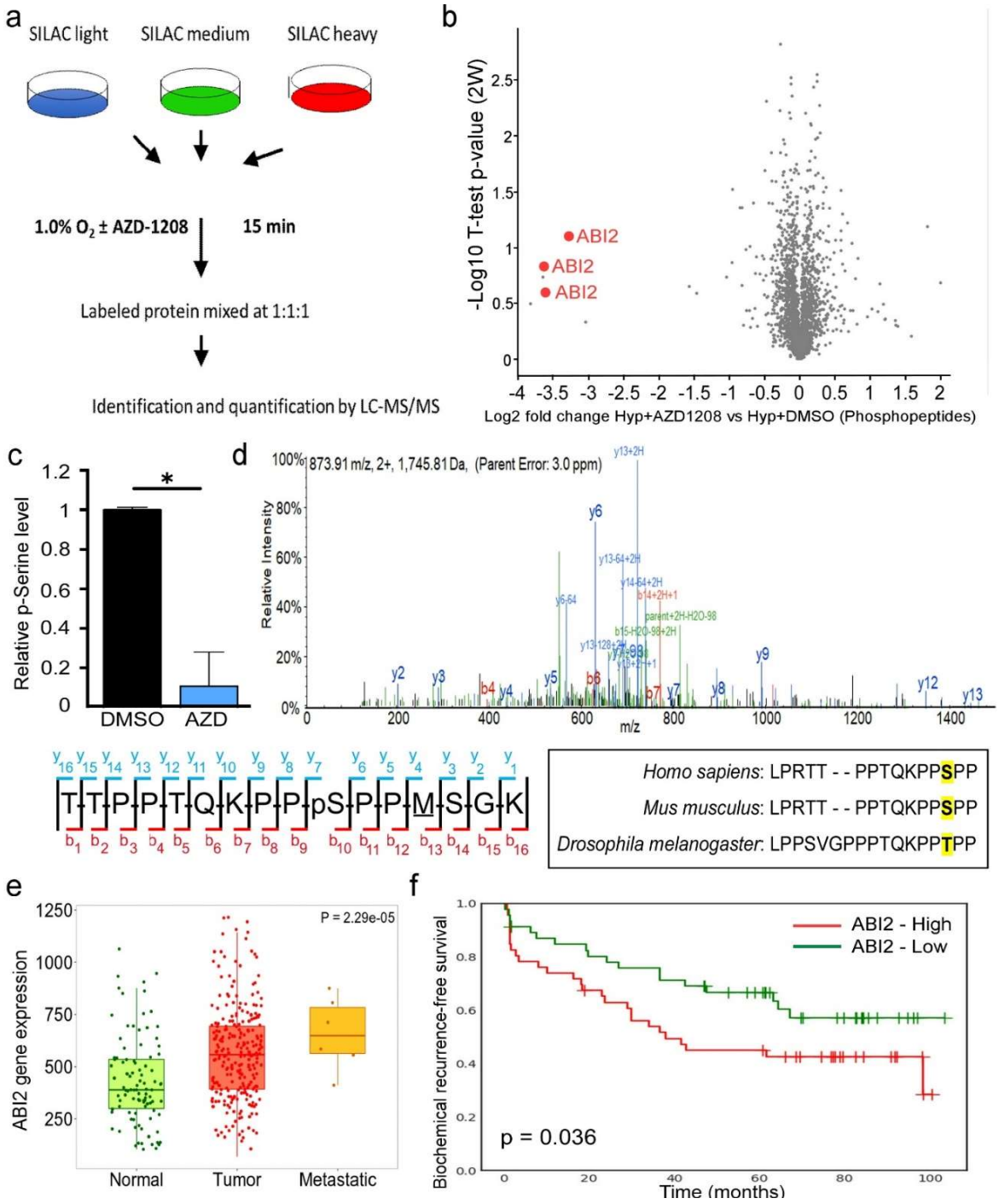

Figure 2. Identification of ABI2 as a novel PIM substrate. (A) Experimental strategy for the identification of novel PIM substrates using SILAC. (B) Volcano plot of the phosphopeptides that were significantly increased and decreased, as determined by a one-sample T-test in hypoxia after AZD1208 treatment. (C) Graph depicting relative serine phosphorylation of peptides containing Ser183 in the presence and absence of PIM inhibition. Error displayed as 95% CI (*p<0.05). (D) Spectra from mass spectrometry analysis of ABI2 from in vivo kinase assay showing phosphorylation of Ser183 by PIM1. Inset = sequence alignment across species of ABI2 Ser183 (highlighted). (E) ABI2 gene expression in human prostate tumor samples from the NCBI GEO database (TNMplot web analyzer). (F) Kaplan-Meier analysis of biochemical recurrence-free survival in PCa patient cohorts based on expression of ABI2.

Phosphorylation of ABI2 by PIM1 promotes its stability

Next, we tested the effect of PIM-mediated phosphorylation at Ser183 on ABI2. To this end, we generated phospho-mimetic (S183D) and phospho-null (S183A) mutants of ABI2. Then, WT and phospho-mutant ABI2 constructs were transiently transfected into 293T cells, and cycloheximide chases were performed to assess differences in ABI2 protein stability. Densitometry analysis of western blotting revealed that the S183D (half-life = 7.3 ± 0.2 h) and S183A (half-life = 1.8 ± 0.2 h) mutants were significantly more and less stable, respectively, than WT ABI2 (half-life = 3.2 ± 0.3 h) (Fig. 3A). To determine whether PIM1 controls ABI2 protein stability, cycloheximide chase experiments were performed in the previously described PC3-LN4 cell model with stable PIM1 overexpression or knockout. In normoxia, overexpression of PIM1 significantly increased the half-life of ABI2 compared to control (half-life = 7.6 ± 0.3 h vs. 3.1 ± 0.2 h, $p<0.05$) (Fig. 3B). Pre-conditioning in hypoxia significantly increased ABI2 stability (half-life = 4.6 ± 0.2 h, $p<0.05$) compared to vector control in normoxia (Fig. 3C), and PIM1 knockout significantly reduced ABI2 stability (half-life = 2.0 ± 0.1 h, $p<0.05$) compared to control in hypoxia (Fig. 3C). To test whether phosphorylation at Ser183 is required for PIM1 to stabilize ABI2, we overexpressed GFP-ABI2 WT or the S183A mutant in DU145 cells stably expressing vector or PIM1 and ABI2 levels were monitored by western blotting. We found that PIM1 overexpression increased WT ABI2, whereas the S183A mutant was refractory to PIM1 overexpression (Fig. 3D), suggesting that phosphorylation of S183 is required for PIM1 to increase ABI2 levels. Lastly, to determine whether the upregulation of ABI2 in hypoxia requires PIM1 activity, we performed cycloheximide chase experiments in DU145 cells in the presence or absence of PIM447. Hypoxia increased the stability of ABI2 compared to normoxia (half-life =

4.4 ± 0.2 h vs. 2.9 ± 0.1 h), whereas inhibition of PIM in hypoxia reduced ABI2 protein stability below that observed in normoxia (half-life = 1.7 ± 0.2 h) (Fig. 3E). Interestingly, another member of the WRC, WAVE2, displayed similar kinetics to ABI2, showing increased stability in hypoxia that was negated by PIM447 treatment (Fig. 3E). These data demonstrate that upregulation of PIM1 in hypoxia increases ABI2 levels via direct phosphorylation of Ser183.

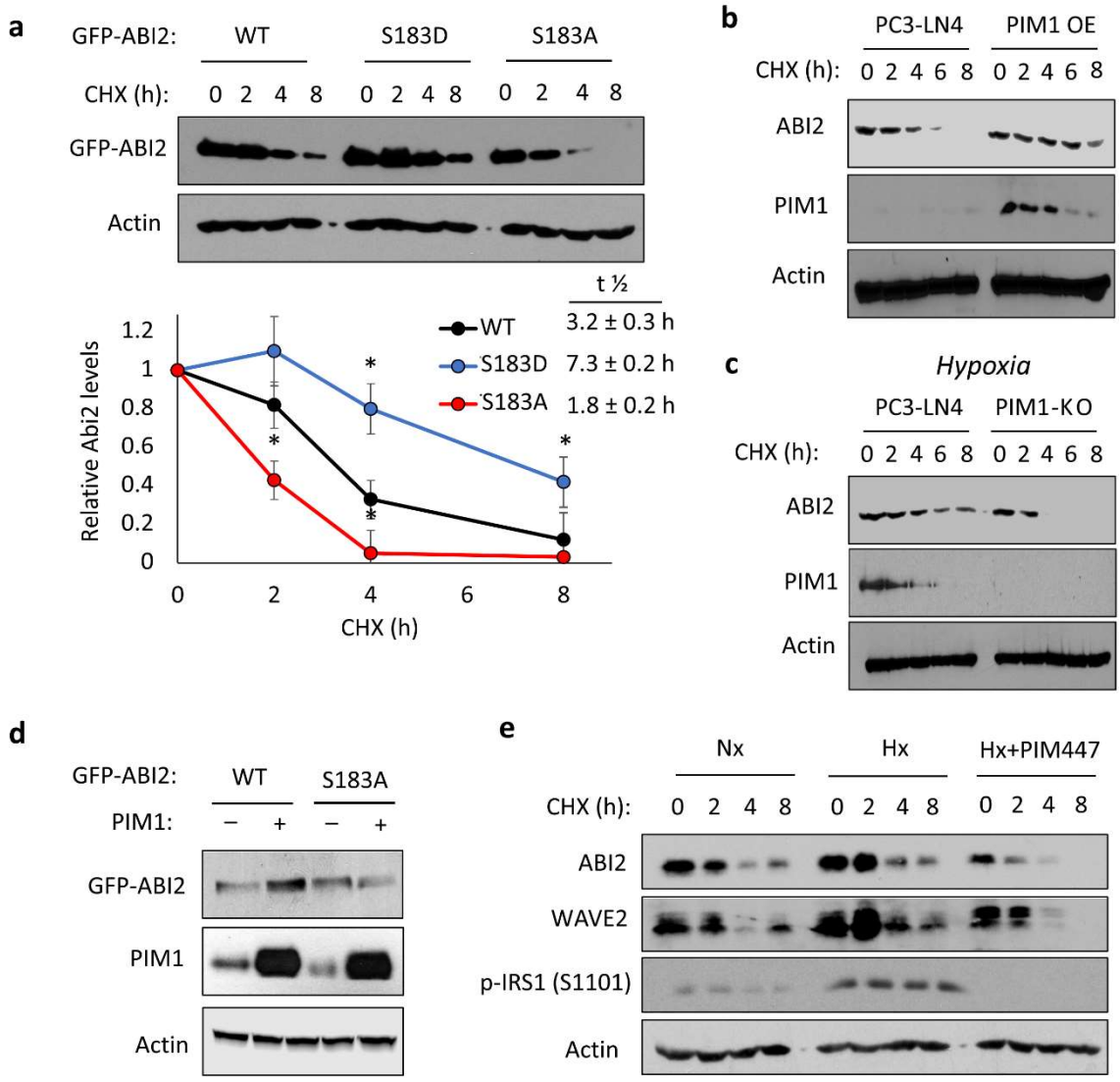

Figure 3. Phosphorylation at Ser183 by PIM1 increases ABI2 stability. (A) 293T cells were transfected with GFP-ABI2 WT, S183A, or S183D prior to treatment with cycloheximide (CHX, 10 µM), and GFP levels were assessed by western blotting. (B) PC3-LN4 cells transfected with vector or PIM1 or (C) PC3-LN4 and PIM1-KO cells were treated with cycloheximide for the indicated times, and ABI2 levels were assessed by western blotting. Densitometry was used to determine the rate of protein decay. (D) PC3-vec or PC3-dox PIM1 cells were transfected with GFP-ABI2 WT or GFP-ABI2 S183A and treated with dox (100 ng/mL) for 24 h, and protein levels were measured by western blotting. (E) DU145 cells were incubated in hypoxia (1.0% O2) for 4 h prior to treatment. DMSO or PIM447 was added 30 min prior to CHX, and cells were lysed at the indicated time points. *p<0.05, n=3 for all experiments, and error bars = SEM

PIM1 promotes WRC formation and activation in an ABI2-dependent manner

Previous reports suggest that the expression level of any single component of the WRC could impact the stability of other WRC members (Rottner et al., 2021). Therefore, we hypothesized

that PIM1-mediated stabilization of ABI2 in hypoxia could increase the amount of the WRC. Our previous results show that hypoxia increased WAVE2 levels in a PIM-dependent manner (Fig. 3E). To determine whether the upregulation of WAVE2 by PIM1 was dependent on ABI2, we generated two DU145 cell lines with CRISPR-knockout of ABI2 (A20 and B76). DU145 and A20 cells with or without stable overexpression of PIM1 were cultured in normoxia and hypoxia, and the expression of ABI2 and WAVE2 was assessed by western blotting. Hypoxia increased WAVE2 in DU145 but not A20 cells (Fig. 4A). Notably, overexpression of PIM1 increased WAVE2 levels in DU145 cells in both normoxia and hypoxia, whereas no change was observed in A20 cells in either condition (Fig. 4A). These results were confirmed in B76 cells (Supplemental Fig. 3). These data indicate that ABI2 is required for PIM1 to increase WAVE2 levels. Next, we asked whether hypoxia increased the relative levels of ABI2 bound to WAVE2, suggesting formation of the WRC. To this end, HA-ABI2 and GFP-WAVE2 were transfected into DU145 cells cultured in normoxia or hypoxia for 16 hours. WAVE2 was immunoprecipitated using the GFP-tag, and ABI2 binding was assessed by immunoblotting for HA. Both ABI2 and WAVE2 levels were increased by hypoxia, and the total amount of WAVE2 that co-immunoprecipitated with ABI2 under hypoxic conditions was greater than that from cells cultured in normoxia (Fig. 4B).

Activation of the WRC and the initiation of actin branching occurs upon WAVE2 binding to ARP2/3. Therefore, we used co-immunoprecipitation as a readout of relative WRC activation. GFP-WAVE2 and RFP-ARP3 were overexpressed in PC3-LN4 cells stably expressing dox-inducible PIM1. Following transfection, cells were treated with doxycycline and/or PIM447 for 24 hours to induce or inactivate PIM1, respectively. Hypoxia increased the relative amount of ARP3 pulled down with WAVE2 (Fig. 4C, lane 1 vs. 4). Overexpression of PIM1 further increased WRC

activation in both normoxia and hypoxia, and PIM inhibition blocked the increased association between WAVE2 and ARP3, particularly in hypoxia (Fig. 4C).

Another indicator of WRC activation is localization of ARP3 to the leading edge of cellular protrusions. Hence, we next investigated whether loss of PIM altered the localization of ARP2/3 in response to growth factor stimulation. WT and TKO MEFs were stably transduced with GFP-Lifeact (F-actin) and RFP-ARP3. Cells were then serum-starved and placed in hypoxia for 4 hours prior to the addition of PDGF for 30 minutes to stimulate ARP2/3 activation. Upon stimulation, ARP3 was highly localized to sites of actin nucleation (the leading edge of lamellipodia) in WT MEFs, whereas ARP3 remained evenly distributed throughout the cell in TKO MEFs (Fig. 4D). Representative line scans of lamellipodia indicate that ARP3 intensity spiked at the leading edge in WT MEFs compared to the remaining cell body. In contrast, there was no significant difference in ARP3 intensity between the leading edge and cell body in TKO MEFs (Fig. 4D). Quantification of ARP3 fluorescence intensity in 0.5-μm increments across multiple cells ($n > 15$) demonstrates that ARP3 localization to the leading edge was deficient in TKO MEFs compared to WT MEFs (Fig. 4E). Taken together, our results indicate that PIM1 increases WRC levels and promotes ARP activation at the leading edge of PCa cells.

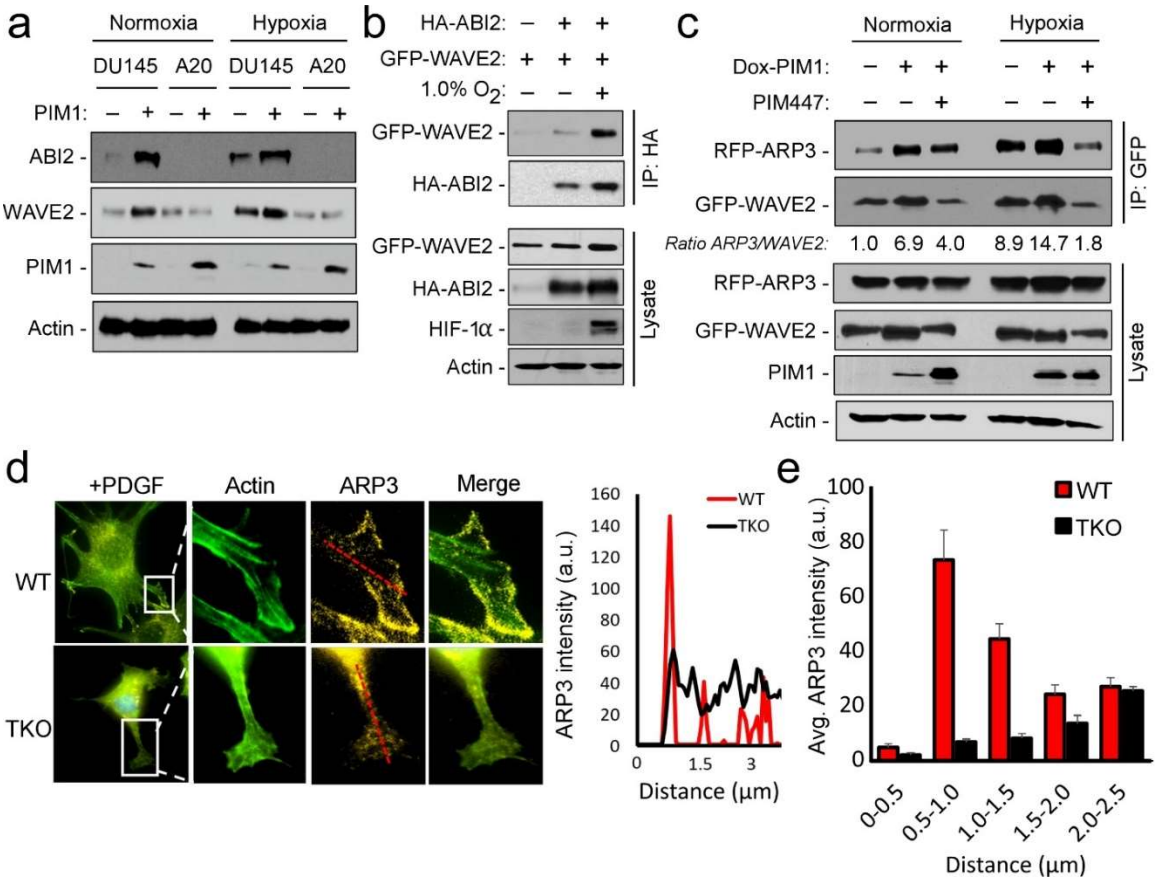

Figure 4. Hypoxia and PIM1 increase WRC levels and activation. (A) DU145 or A20 cells stably expressing vector or PIM1 were cultured in normoxia or hypoxia (1.0% O_2) for 16 h, and western blotting was used to assess protein levels. (**B**) 293T cells were transfected with HA-ABI2 and GFP-WAVE2 and cultured in normoxia or hypoxia for 4 h. ABI2 was immunoprecipitated using anti-HA beads, and WAVE2 binding was assessed by western blotting for GFP. (**C**) PC3-dox PIM1 cells were transfected with GFP-WAVE2 and RFP-ARP3. Then, cells were placed in normoxia or hypoxia and treated with dox (100 ng/mL) for 4 h in the presence of DMSO or PIM447 (3 μM). Five minutes prior to harvest, all samples were stimulated with EGF (50 nM). WAVE2 was immunoprecipitated using the GFP tag, and ARP3 binding was assessed by western blotting for RFP. (**D**) WT or TKO MEFs stably expressing GFP-Lifeact and RFP-ARP3 were starved for 4 h prior to stimulation with PDGF for 15 min. Cells were fixed for imaging, and ARP3 intensity was measured from the edge of cellular protrusions toward the center of the cell body (red dotted line). (**E**) Quantification of ARP3 intensity at given increments of distance from the leading edge ($n > 15$ cells/group). * $p < 0.05$, error = SEM.

ABI2 is essential for hypoxia and PIM1-driven protrusions

Based on our biochemical data showing more WRC formation in hypoxia, we reasoned that hypoxia may sensitize cells to chemotaxis. To test this, DU145 cells were stimulated with low (5 nM) and high (50 nM) concentrations of EGF. In normoxia, stimulation with 5 nM EGF did not

significantly increase cell area, whereas 50 nM EGF caused a rapid and pronounced response (Fig. 5A). Strikingly, treatment with a low dose of EGF (5 nM) significantly increased the speed and amplitude of protrusions in hypoxic cells to a greater extent than that observed in normoxic cells stimulated with a high dose of EGF (Fig. 5A). Next, we asked whether ABI2 is required for the hypoxia-induced sensitization to growth factor stimulation. To this end, we measured changes in cell area following stimulation with low or high doses of EGF in DU145 and A20 (lacking ABI2) cells conditioned in normoxia and hypoxia. In contrast to DU145 cells, A20 cells did not respond to either a low or high concentration of EGF, regardless of oxygen concentration (Supplemental Fig. 4A). To confirm the effect of ABI2 loss on cell migration, we performed wound healing assays using a non-targeting siRNA (si-NT), si-ABI2, or si-NT + PIM447. Knockdown of ABI2 and chemical inhibition of PIM significantly reduced wound closure to the same extent compared to controls (Supplemental Fig. 4B-D).

Next, we assessed the impact of PIM1 on protrusive activity. PIM1 was stably overexpressed in DU145-GFP-Lifeact cells, and basal protrusive activity, lacking growth factor stimulation, was measured as described previously. Overexpression of PIM1 significantly increased cellular basal levels of protrusion in normoxia compared to empty vector controls (Fig. 5B). Contrastingly, treatment with PIM447 significantly decreased protrusive activity (Fig. 5B). Exposure to hypoxia significantly increased the protrusive activity of both control and PIM1-overexpressing cells, and treatment with PIM447 significantly blunted the ability of hypoxia to enhance protrusive activity (Fig. 5B). We then asked whether the increased protrusive activity resulting from hypoxia and PIM1 requires ABI2. DU145 and A20 cells were stably transfected with vector or PIM1 and cultured in hypoxia for 16 hours prior to the addition of DMSO or PIM447. Loss of

ABI2 blunted the increase in protrusive activity observed with hypoxia and overexpression of PIM1 (Fig. 5C). To better understand how hypoxia alters membrane dynamics, we superimposed the cell outlines at each timeframe onto a single image as a function of time. In normoxia, we observed evidence of prolonged lamellipodia-like protrusions (Fig. 5D, region 1), retractions (region 2), and other protrusions (region 3) that were more transient in nature. In contrast, hypoxic cells displayed larger and more dynamic protrusion events, as evidenced by larger lamellipodia-like protrusions that extended further away from the cell body than those occurring in normoxia. Overexpression of PIM1 was sufficient to increase the frequency and extent of protrusions in normoxia and further increased protrusive activity under hypoxia. Treatment with PIM447 significantly decreased protrusive activity in both normoxia and hypoxia with a striking loss of almost all protrusions in normoxia. Cell edge traces of A20 cells show very little movement compared to that of DU145 cells in all conditions. Surprisingly, hypoxia was still able to increase the protrusive activity of A20 cells compared to normoxia (Fig. 5C). However, the protrusive phenotype was drastically different in cells lacking ABI2 (Fig. 5D). DU145 cells displayed broad lamellipodia that are typical of WRC-mediated protrusions, whereas A20 cells produced rapid, spike-like protrusions that are typical of filopodia (Fig. 5D). These data demonstrate that PIM1 and hypoxia drive actin dynamics and lamellipodia formation through stabilization of ABI2.

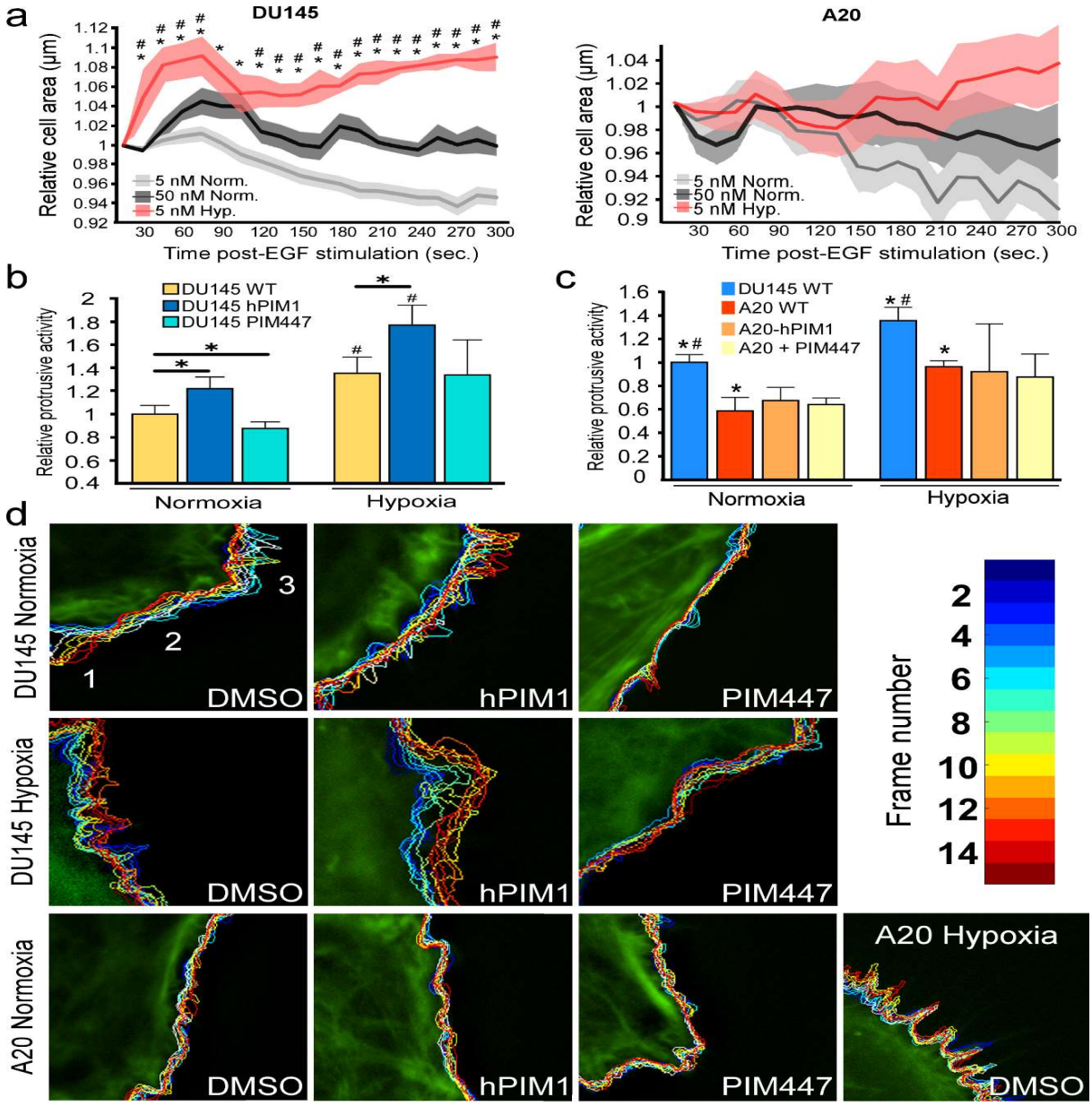

Figure 5. ABI2 is necessary for hypoxia- and PIM-induced protrusions. (A) DU145 and A20 cells were stimulated with the indicated doses of EGF in normoxia or hypoxia, and cell area was plotted relative to the pre-stimulated average area. The shaded area represents 95% CI (n=6). (B) DU145 cells were imaged in normoxia and hypoxia in the presence or absence of PIM447 (3 μM). The relative proportion of randomly sampled windows in DU145 or DU145-PIM1-overexpressing cells actively protruding during progressive frames (15-s intervals): DU145 WT (n=1637, N=12), DU145-hPIM1 (n=1395, N=12), and DU145-PIM447 (n=1682, N=11). In hypoxia, DU145 WT (n=1268, N=5), DU145-hPIM1 (n=1333, N=10), and DU145-PIM447 (n=2056, N=10). Error shown as 95% CI, (*p<0.05, #p<0.05 between normoxic and hypoxic conditions within each cell line). (C) Quantification of unstimulated cell protrusive activity in A20 (n=2116 Nx, N=9 and n=1279 Hx, N=6), A20-hPIM1 (n=1213 Nx, N=7 and n=2183 Hx, N=10), and A20-PIM447 (n=1899 Nx, N=10 and n=2133 Hx, N=10) cells. Error = 95% CI, (*p<0.05 between normoxic and hypoxic samples, #p<0.05 between DU145 and A20 WT samples). (D) Representative cell traces depicting changes in actin structure over time in DU145 and A20 cells overexpressing PIM1 or treated with PIM447 under normoxic and hypoxic conditions. Regions representative of changes in actin structure are marked: 1 = lamellipodia, 2 = retraction, 3 = transient protrusion.

PIM inhibition blocks PCa invasion *in vivo*

In order to escape the gland, PCa cells must traverse through a layer of smooth muscle. To accurately represent the human course of PCa metastasis, we utilized an *in vivo* smooth muscle invasion assay (Mccandless et al., 1997; Rubenstein et al., 2019) to validate the role of the PIM1-ABI2 signaling axis in tumor invasion. First, DU145 cells (with or without stable overexpression of PIM1) were injected into the peritoneum of male SCID mice and allowed to seed onto the diaphragm for 2 weeks. Then, the mice were randomly segregated into groups for treatment with vehicle or PIM447 (30 mg/kg, p.o., daily). The mice were sacrificed 4 weeks post-injection, and diaphragms were harvested for immunohistochemistry. Across all samples, PIM1 was strongly expressed in tumor cells seeded onto the peritoneal side of the diaphragm, and expression was retained in invading cells (Fig. 6A). We then measured tumor burden (i.e., the amount of diaphragm with tumor present on it). Mice injected with DU145-hPIM1 cells had a significantly increased tumor burden compared to mice injected with DU145-Vec or A20-Vec cells (Fig. 6C). Conversely, inhibition of PIM in mice injected with DU145 cells significantly reduced the tumor burden, confirming the established antitumor effect of PIM inhibitors (Fig. 6C). Next, we quantified the average distance tumor cells invaded into the diaphragm by measuring the distance from the nuclei of individual tumor cells to the thoracic tumor/diaphragm interface (Fig. 6B; white dotted lines). DU145 cells successfully invaded into the diaphragm, with an average depth of 116 µm, but few were able to invade entirely through the diaphragm muscle and into the thoracic cavity (Fig. 6B). Overexpression of PIM1 significantly increased the average depth of invasion (135 µm, $p<0.05$) and markedly increased the number of cells that fully invaded through the diaphragm (Fig. 6B). Strikingly, treatment

with PIM447 in parental cells significantly reduced the average depth of invasion (105 μM, $p<0.05$) and prevented invasion through the diagram (Fig. 6B). These data demonstrate that PIM1 enhances tumor cell invasion, and blocking PIM is sufficient to reduce the invasive potential of PCa cells.

Next, we tested the importance of ABI2 for PIM-induced invasion using A20 cells with or without stable overexpression of PIM1. Compared to parental DU145 cells, A20 cells were significantly less invasive into the muscle, and we observed no invasion into the thoracic cavity (Fig. 6B). Surprisingly, the overall volume of A20 tumors was significantly smaller than that of DU145 tumors (Fig. 6C). We observed a modest but significant reduction in proliferation of A20 cells *in vitro* compared to DU145 cells (Supplemental Fig. 5A), but immunohistochemical assessment of Ki67 revealed no difference in staining between A20 and DU145 cells (Supplemental Fig. 5B). Therefore, reduced cell growth is unlikely to account for the smaller tumor volume observed with A20 cells. Tumor cell interaction may explain why loss of ABI2 impacts tumor growth, as ABI2 knockout has been implicated in reduced matrix adhesion and cell-cell adhesions (Li et al., n.d.; Ryu et al., 2009). To ensure that the deficit in invasion was not due to a decreased ability of A20 cells to seed on the diaphragm, we measured tumor penetrance, which indicates the proportion of seeded cells that went on to successfully invade the muscle layer. No significant difference in tumor penetrance was observed between DU145 and A20 cells, indicating that tumor burden did not affect depth of invasion (Fig. 6D). Importantly, A20 cells also had a significantly lower average depth of invasion than DU145 cells (95 μm vs. 115 μm, $p<0.01$), and overexpression of PIM1 in A20 cells did not increase the depth of invasion (PIM1-overexpression vs. vector: 92 μm vs. 97 μm) (Fig. 6E). To determine whether

increased protrusive activity resulted in more invasive tumors *in vivo*, we plotted the relative protrusive activity of each cell line versus depth of invasion. This analysis showed a significant correlation between the basal rate of cellular protrusion and invasive potential *in vivo* (Fig. 6F). Further data mining of the PCTA database revealed a higher correlation between PIM and ABI2 expression in metastatic castration-resistant PCa than in benign prostate tumors (Fig. 6G). Taken together, these data demonstrate that PIM1 promotes a muscle-invasive phenotype in an ABI2-dependent manner, and inhibiting PIM significantly reduces the invasive potential of PCa cells.

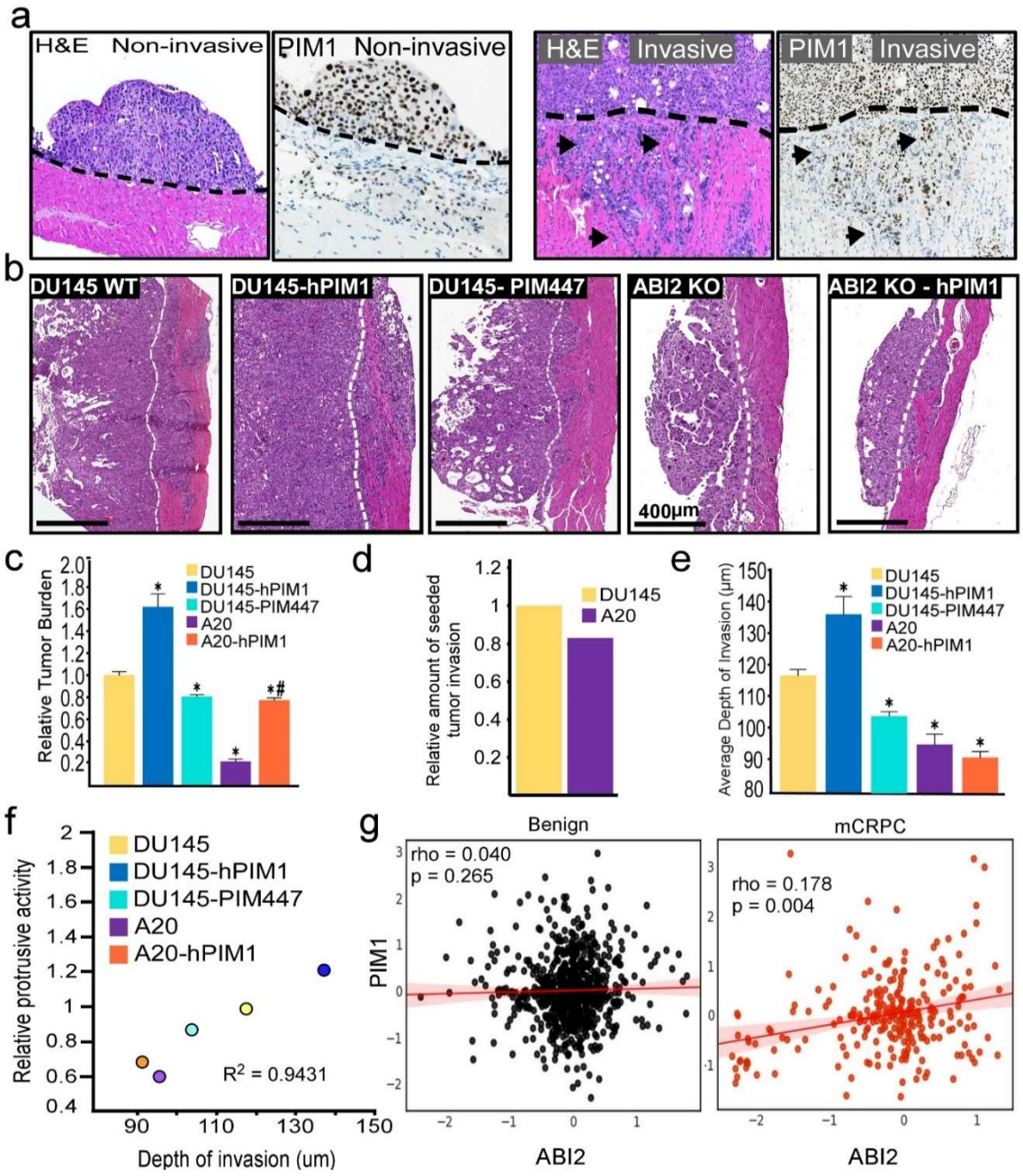

Figure 6. PIM1-ABI2 signaling controls tumor cell invasion in vivo. (A) Representative images of PIM1 expression in non-invasive and invasive tumor seeded on the diaphragm (black dotted line indicates the tumor/diaphragm interface). (B) Representative images of DU145 and A20 tumors with PIM1 overexpression or PIM447 treatment (white dotted line indicates the tumor/diaphragm interface). (C) Relative tumor burden was quantified based on H&E staining of the total tumor area compared to parental DU145 tumors (n=12, N=3). Error = 95% CI (*p<0.05 compared to DU145 vector, #p<0.05 compared to A20 vector) (D) Tumor penetrance was calculated as the relative amount of diaphragm surface area seeded with tumor (DU145 and A20: n=12 and 7, respectively, N=3) (E) Depth of invasion from the tumor/diaphragm interface of individual tumor cells was measured in DU145 (n=11,951, N=12), DU145-hPIM1 (n=12,314, N=12), DU145-PIM447 (n=12,547, N=12), A20 (n=1942, N=7), and A20-hPIM1 (n=5275, N=12) tumors. (F) Plot depicting the depth of tumor cell invasion in vivo vs. relative protrusive activity. (G) Gene expression data of PIM1 and ABI2 from benign and metastatic PCa patient samples.

2.3 Discussion

Due to their heightened expression in a variety of solid tumors and established role in promoting angiogenesis (Casillas et al., 2018), survival (Yan et al., 2003), and cell proliferation (Wang et al., 2002), PIM kinases represent a promising anti-cancer target. Clinical studies demonstrate a positive correlation between PIM1 expression and tumor metastasis in breast (Gapter et al., 2006), lung (Cao et al., 2019), pancreatic (Xu et al., 2016), and prostate tumors (Santio et al., 2015b). Here, we uncover a new signaling axis that poises PIM1 as a key driver of cell motility and invasion, the early steps of metastasis. This work is particularly relevant to solid tumor biology, as PIM1 is known to be increased in response to hypoxia, and our results indicate that upregulation of PIM1 is critical for hypoxia-induced invasion (Fig. 1). However, knockout of PIM1 was sufficient to significantly reduce tumor cell protrusive activity and invasion regardless of oxygen tension, suggesting that basal levels of PIM1 are important for proper cell migration. The striking decrease in the number of invaded cells and depth of 3D invasion in PIM1 KO cells suggests that PIM2 and PIM3 are not sufficient to compensate in our system (Fig 1). However, PIM2, and to a lesser extent PIM3, are also upregulated in PCa and induced by hypoxia (Warfel et al., 2016), so further experiments are warranted to determine if blocking all three PIM genes would provide additional anti-migratory effect.

Through an unbiased screening effort, we identified ABI2 as a novel substrate of PIM kinase that facilitates the regulation of cellular protrusions in both normoxia and hypoxia. *In vitro* and *in vivo* kinase assays demonstrated that PIM1 phosphorylates ABI2 at Ser183 (Fig. 2). Notably, phosphorylation of Ser183 has been observed in numerous large proteomic screens in breast, lung, and ovarian cells (Klammer et al., 2012; Mertins et al., 2014; Schweppe et al., 2013), but

its functional relevance has not been reported. Phosphorylation of ABI2 has been shown to regulate its stability via ubiquitin-mediated degradation (Dai et al., 1998), but no specific sites have been identified. Ser183 is located immediately downstream of the Homeobox Homology domain of ABI2 (Dai and Pendergast, 1995; Sato et al., 2012), a region that is disordered and lacks known functional significance. ABI2 and ABI1 (a homologue) were initially identified as proteins that interact with the Abl tyrosine kinase (Dai and Pendergast, 1995). Previous studies have shown that ABI1/2 serve as a scaffold for Abl tyrosine kinase. Abl can directly phosphorylate ABI1/2 at multiple sites that differentially affect ABI1 and ABI2 protein stability, and Abl phosphorylation of WAVE2 at Y150 is sufficient to activate the WRC (Leng et al., 2005). However, Abl is unlikely to increase ABI2 protein stability or its incorporation into the WRC downstream of PIM1, because modulation of endogenous Abl activity through serum starvation, growth factor stimulation, and treatment with Abl kinase inhibitors does not alter ABI2 levels or WRC formation (Innocenti et al., 2004; Stuart et al., 2006).

Using genetic and chemical approaches, we demonstrated that phosphorylation of ABI2 at Ser183 suppresses protein turnover. Interestingly, even though Ser183 is conserved in ABI1 according to sequence alignment, ABI1 peptides were not identified in our SILAC screen as a PIM substrate in hypoxia. Moreover, ABI1 expression was decreased in PCa compared to normal prostate tissues, whereas ABI2 levels significantly increased with disease progression and predicted worse survival (Fig. 2E). These clinical data indicate a distinct and important role for ABI2 in PCa. Our data indicate that the ability of PIM1 to stabilize ABI2 is important for the formation of the mature WRC. ABI2 is known to be critical for the stability and integrity of the WRC, and loss of ABI2 is associated with decreases in the WAVE, NAP, and SRA proteins

(Dubielecka et al., 2011). However, the reverse is not well established, as overexpression of ABI2 has not been previously shown to increase the levels of other WRC members. Here, we show that stabilization of ABI2 downstream of PIM1 increased WAVE2 protein levels (Fig. 4A). Furthermore, PIM1 overexpression or upregulation in hypoxia increased the relative amount of ABI2 bound to WAVE2 (Fig. 4C), suggesting that more WRC is present under these conditions. PIM1 also increased activation of the WRC, as indicated by increased WAVE2 binding to ARP3 and localization of ARP3 to the leading edge of protrusions (Fig. 4D and E). Importantly, the ability of PIM1 to increase WRC activity and cellular protrusion are entirely dependent on ABI2, as ABI2 knockout negated the effect of PIM1 on WRC formation and protrusion. These findings suggest a model where PIM1 upregulation in hypoxia stabilizes the WRC, which poises hypoxic cancer cells to respond to chemoattractants and increases their motility. While hypoxia is established as a driver of cancer metastasis, these data provide the first direct mechanism coupling hypoxia to the regulation of actin protrusions.

The biological and functional impact of PIM regulation of the WRC and plasticity of the actin cytoskeleton were profound. The negative impact of PIM inhibition on protrusive activity could be due to the relationship between ABI2 protein stability and WRC kinetics post-assembly. The WRC is assembled in the cytoplasm but remains inactive until bound by the Rho-family GTPase, Rac, at which point the VCA domain of WAVE is free to bind ARP2/3 and promote actin branching (Echarri et al., 2004; Mendoza, 2013). Once activated, the WRC translocates to the membrane, where it binds membrane receptors via a WRC interacting receptor sequence (WIRS) motif. The WIRS motif is composed of amino acids from both SRA and ABI2 and thus requires an intact WRC rather than individual subunits (Chen et al., 2013). Once bound to the

membrane, ARP can then facilitate acting nucleation, branching, and ultimately the promotion of lamellipodia and protrusions (Leng et al., 2005; Rottner et al., 2021). Thus, it is possible that having more ABI2 present in the cell increases binding of the WRC to the membrane via the WIRS motif. This is supported by the observation that localization of ARP3 at the leading edge was significantly impaired in cells lacking PIM, resulting in reduced protrusions, particularly in response to chemoattractants (Fig. 4D and E). Future work is needed to determine how hypoxia and PIM1 impact the kinetics of WRC formation and disassembly, as the details of this process remain largely unknown.

Hypoxia increases metastasis through various mechanisms, including increased tumor angiogenesis, modification of extracellular matrix, and altered metabolism(Jensen and Warfel, 2021). However, little is known about the effect of hypoxia on cytoskeletal dynamics. Our work provides the first mechanistic evidence that hypoxia increases tumor cell invasion by enhancing WRC activation and cellular protrusion. Hypoxia has been shown to impact the expression and activity of nearly every major oncogene, both directly and indirectly, through HIF-1-driven changes in gene expression(Jensen and Warfel, 2021). As a result, efforts to target hypoxia therapeutically have focused on blocking HIF-1 activation, which has been largely unsuccessful. Therefore, targeting HIF-1-independent hypoxia-inducible signaling proteins, such as PIM1, presents a promising opportunity for therapy. Our findings indicate that the ability of hypoxia to enhance the invasive potential of PCa cells is largely dependent on PIM1. While PIM inhibitors are currently being tested in anti-cancer clinical trials, the benchmarks of these trials are focused on blocking tumor growth and inducing tumor cell death. This work provides compelling evidence for testing PIM inhibitors as anti-metastatic agents in PCa. Further, the

ability to distinguish between invasive and indolent tumors remains a long-standing clinical problem in PCa, as monitoring PSA levels is not a reliable indicator of tumor aggression (Mccaffery et al., n.d.), and the field lacks well-defined subtypes. Our data suggest that PIM1 expression, in addition to ABI2, could serve as a potential biomarker to assess the invasive and metastatic potential of primary prostate tumors. Overall, this study provides exciting preclinical data that expands the potential utility of PIM inhibitors in PCa as anti-metastatic agents that could reduce the invasive potential of prostate tumors, resulting in lower patient mortality.

2.4 Materials and Methods

Cell lines and cell culture

Parental and genetically modified DU145 cells, HEK293T, and wild-type (WT) and triple knockout (TKO) (lacking all three PIM isoforms) mouse embryonic fibroblasts (MEFs)(Mikkers et al., 2004) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Corning; Cat# 10-017-CV) containing 10% fetal bovine serum (FBS) (Omega Scientific). DU145 ABI2-knockout cell lines (hereafter referred to as A20 and B76) were generated by CRISPR-Cas9-mediated knockout using two CRISPR sgRNAs 5'-GCTACACTACATAACAAAGG-3' and 5'-TGTGATTGAGATTTACGATT-3'. Knockouts were confirmed by PCR, and positive clones were expanded for further use. PC3-LN4, PC3-LN4-pCIP, PC3-LN4-hPIM1, and PC3-LN4 PIM1-KO cells were cultured in RPMI 1640 medium (Corning; Cat# Cat# 10-040-CV) containing 10% FBS. MCF7 cells were cultured in high glucose DMEM base media with sodium pyruvate and L-glutamine (Corning; Cat# 10-013-CV) supplemented with additional 100mM L-glutamine (Corning; Cat# 25-005-CI), 10% FBS (Gibco), and antibiotics (100 U/mL penicillin + streptomycin) (Life Technologies; Cat# 30-002-CI). All cells were cultured at 37°C in 5% CO_2 with ambient O_2 levels.

Cells were routinely screened for mycoplasma and authenticated by the University of Arizona Genetics Core Facility and were used for fewer than 30 passages. Cells were cultured at 37°C in a hypoxic environment (1% O_2, 5% CO_2, 94% N_2) using an InVivo2 400 hypoxia workstation (Baker Ruskinn) when stated. Lipofectamine 3000 (Thermo Fisher; Cat# L3000015) was used to carry out transient transfection according to the manufacturer's protocol. Immunoblotting and ubiquitination assays were performed as described previously(Noel A. Warfel et al., 2011). ImageJ analysis software (National Institutes of Health) was used to perform densitometric analysis.

Plasmids and siRNA

hPIM1 (short) was cloned into pCIP (lentiviral backbone) as previously described(Casillas et al., 2018). For generation of ABI2 phospho-mutants, sequences containing the desired wild-type or mutant amino acids were created by TWIST biosciences and cloned into pGCS-N6 (Addgene; Plasmid #85723) using Gateway cloning. siRNA for ABI2 was purchased from Thermo Fisher (Cat# 4392420, siRNA ID s19769). GFP-WAVE was purchased from Addgene (Plasmid #54314). GFP-Lifeact and RFP-ARP3 plasmids were gifts from Dr. Ghassan Mouneimne (University of Arizona).

Reagents and antibodies

Trypsin-EDTA (Cat# 25-053-CI), phosphate-buffered saline (PBS) (Cat# 21-031-CV) and laminin Cat# 354232) were purchased from Corning. Recombinant epithelial growth factor (EGF) was purchased from Life Technologies (Cat# PHG0311). Insulin growth factor (IGF) was purchased from Sigma (Cat# I3769). PIM447 was purchased from Selleck Chemicals (Cat# S7985). AZD1208

was acquired from AdooQ Bioscience (Cat# A13203-750). DMSO was purchased from Fisher Scientific (Cat# 97064-724). Cycloheximide was purchased from VWR (Cat# 97064-724), and doxycycline was purchased from Sigma (Cat# D9891-5G). Recombinant myelin basic protein (MBP) was a gift from Dr. Greg Rogers, and recombinant ABI2 protein was purchased from Origene (Cat# TP300637). Radio-labeled ATP was purchased from Perkin Elmer (Cat# BLU502A). The antibody to ABI2 was purchased from Bethyl Laboratories (Cat# A302-499A-M). GFP (Cat# 2956S), HA (Cat# 3724S), HIF-1a (Cat# 14179S), p-IRS1 [S1101] (Cat# 2385S), PIM1 (Cat# 3247S), and WAVE2 (Cat# 3659S) antibodies were purchased from Cell Signaling Technology. The antibody for actin was purchased from BD Biosciences (Cat# 61656). PIM1 (Cat# ab75776) and Ki67 (Cat#ab833) antibodies used for immunohistochemistry were purchased from Abcam.

Cell protrusion assays

Cells expressing GFP-Lifeact were starved (complete media with 0.5% FBS) for 4 hours prior to imaging and allowed to equilibrate in the environmental chamber on the Nikon Ti2 microscope either at 20% or 1% O_2 (37°C and 5% CO_2 constant). Images were then taken every 15 s for 5 min at 60× magnification to establish a baseline. Imaging was paused to add 500 µL of media containing EGF or IGF to obtain the desired final concentration and then immediately resumed for an additional 10 min at 15-s intervals. The area of the cells was measured using Nikon Elements software and averaged for each cell during the baseline readings. The area of the cells post-stimulation was also measured and plotted relative to that at baseline to determine protrusive activity of the cell membrane.

Image analysis

Unstimulated cells expressing GFP-Lifeact were starved (complete media with 0.5% FBS) and then imaged every 15 s for 15 min as described above. The resulting Nikon files were then uploaded into Matlab via the Bioformats package. Using code developed by Gaudenz Danuser(Lee et al., 2015), cell outlines were obtained, the edge of the cell was randomly divided into windows (10 microns wide), and protrusion vectors were generated based on the change in the cell outline between each frame. Each window of the cell was then sampled for the protrusion vectors that fell within it, and an average velocity per window was obtained. This was then displayed as a heat map. Further, the portion of the cell protruding at any given time could be determined based on the protrusion vector analysis, and a relative protrusive activity could be determined. We generated code to superimpose the outline of the cell at each given frame onto each other to form one image where each line represented the outline of the cell as a function of time.

SILAC-based Phosphoproteomics

SILAC was performed as described (Williams et al., 2016). Briefly, PC3-LN4 cells were grown subconfluently with isotopically distinct forms of arginine or lysine [(Arg0/Lys0), (Arg6/Lys4), or (Arg10/Lys8)] for approximately seven cell doublings. Once sufficient stable isotope labeling of amino acids in cell culture (SILAC) incorporation was confirmed by mass spectrometry, the cells were placed in normoxia or hypoxia for 8 h and then treated with AZD1208 (3 μM) or DMSO for 15 minutes. Biological replicates were performed with a label-swap control, and protein (5 mg) from each treatment group was combined at a 1:1:1 ratio and processed for Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS). Two biological replicates were performed per condition. A one sample t-test was performed to compare the relative

abundance of phosphopeptides between treatment conditions. To identify and quantify phosphopeptides, raw data were searched against a human Uniprot protein database downloaded May 31, 2013 using MaxQuant v.1.5.1.0 (Max Planck Institute).

Kinase assays and mass spectrometry

In vitro: Recombinant full-length GST-PIM1 was incubated with recombinant ABI2, GST (negative control), or MBP (positive control) in the presence or absence of AZD1208 (100 nM) and ^{32}P-labeled ATP in kinase assay buffer (20 mM MOPS [pH 7.0] containing 100 mM NaCl, 10 mmol/L $MgCl_2$, and 2 mmol/L dithiothreitol). Reactions were incubated for 1 hour at 37°C and subsequently separated by SDS-PAGE. Gels were stained with Coomassie and further analyzed via mass spectrometry or autoradiography.

In vivo: HA-ABI2 was immunoprecipitated with anti-HA antibodies. Immune complexes were washed with lysis buffer followed by kinase buffer and then incubated for 1 hour with 100 ng of recombinant PIM1 in the presence or absence of AZD1208. Mass spectrometry was done following separation by SDS-PAGE and Coomassie staining. Sample preparation, LC-MS/MS parameters, and follow-up database searching were all performed as previously described (Casillas et al., 2021).

Boyden chamber Transwell assays

Transwell inserts (Corning; Cat# 353292) with a high-density PET membrane and 3-micron pore size were treated with PBS alone or PBS containing laminin (10 µg/mL) overnight to coat the wells. Wells were washed with PBS, and PC3-LN4 or DU145 cells in medium with 0.5% FBS were seeded directly into the Transwell inserts. Medium with 10% FBS was placed inside the lower

chamber. The cells were allowed to invade overnight. The wells were then harvested, cells on the top side of membrane were removed, and the bottoms of the inserts were stained using crystal violet (Fisher Scientific; Cat# C581-25). The number of invaded cells was measured to determine cellular migration (uncoated) and invasion (laminin-coated).

3D invasion assays

The 3D invasion assay was performed as previously described (Padilla-Rodriguez et al., n.d.). Briefly, 1.0×10^5 PC3-LN4 or PC3-LN4 with CRISPR knockout of PIM1 (PIM1-KO) cells were embedded in a central Matrigel matrix. The internal matrix was encapsulated by an external collagen matrix, and the cells were allowed to migrate into the outer collagen matrix for 48 hours. The cells were then fixed with 4% paraformaldehyde and stained with Hoechst 33342 for 24 hours at 4°C. Confocal z-series of Hoechst and differential interference contrast images were acquired at 1.5-µm z-steps on a Nikon Ti-E inverted microscope. A large-stich composite image of the entire area, including the internal dot and invaded cells, was generated. The number of invaded cells was quantitated using Nikon Elements as was the invasion distance for each nucleus.

Smooth muscle invasion assay

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Arizona. Approximately one million prostate tumor cells (DU145 or A20) in sterile saline were injected into the peritoneal cavity of 6-8-week male severe combined immunodeficient (SCID) mice. After 2 weeks, mice were randomly segregated into groups for treatment with vehicle or the PIM inhibitor PIM447 (30 mg/kg, p.o. daily). Four weeks after

injection, mice were euthanized by cervical dislocation to avoid changes in tissue oxygenation due to artifacts of euthanasia by CO_2. After euthanasia, the diaphragm from each mouse was collected, fixed in formalin, and processed for histology. Stained slides were digitally scanned using an Aperio Image Scanner. Scanned slides were subsequently analyzed to obtain depth of invasion (distance nuclei traveled into the diaphragm), tumor burden (portion of diaphragm with tumor present), and tumor penetrance (portion of seeded tumor that exhibits invasion into the diaphragm).

ARP kymography assay

WT and TKO MEF cells with stably expressed GFP-Lifeact and RFP-ARP3 were serum-starved and placed in hypoxia for 4 hours prior to the addition of platelet-derived growth factor (PDGF) for 30 min. Kymographs were generated by starting at the cell edge and going proximal to center of the cell parallel to the actin filaments. The intensity of ARP3 staining was plotted relative to distance from the cell edge.

Immunoprecipitation assays

For co-immunoprecipitation assays, DU145 or PC3-LN4 cells were transfected with the indicated constructs. Then, cells were treated as stated and placed in hypoxia for the stated times. Cells were harvested in an IP lysis buffer (20 mM Tris HCl, pH 8; 137 mM NaCl; 10% glycerol; 1% Nonidet P-40; and 2 mM EDTA) with protease inhibitors and centrifuged at 15,000 RPM for 10 min. Lysates were incubated overnight at 4°C with HA magnetic beads (Pierce; Cat# 88836) or GFP magnetic beads (Chromotek) and analyzed by western blotting as described above.

Statistical analysis

A minimum number of three replicates were performed to ensure adequate statistical power for all *in vitro* experiments. One-way ANOVA and unpaired t-test was performed and $p<0.05$ was considered statistically significant. Confidence intervals are 0.95 ($\alpha = 0.05$). Figure legends contain all information regarding 'n' values and p values.

2.5 Acknowledgments

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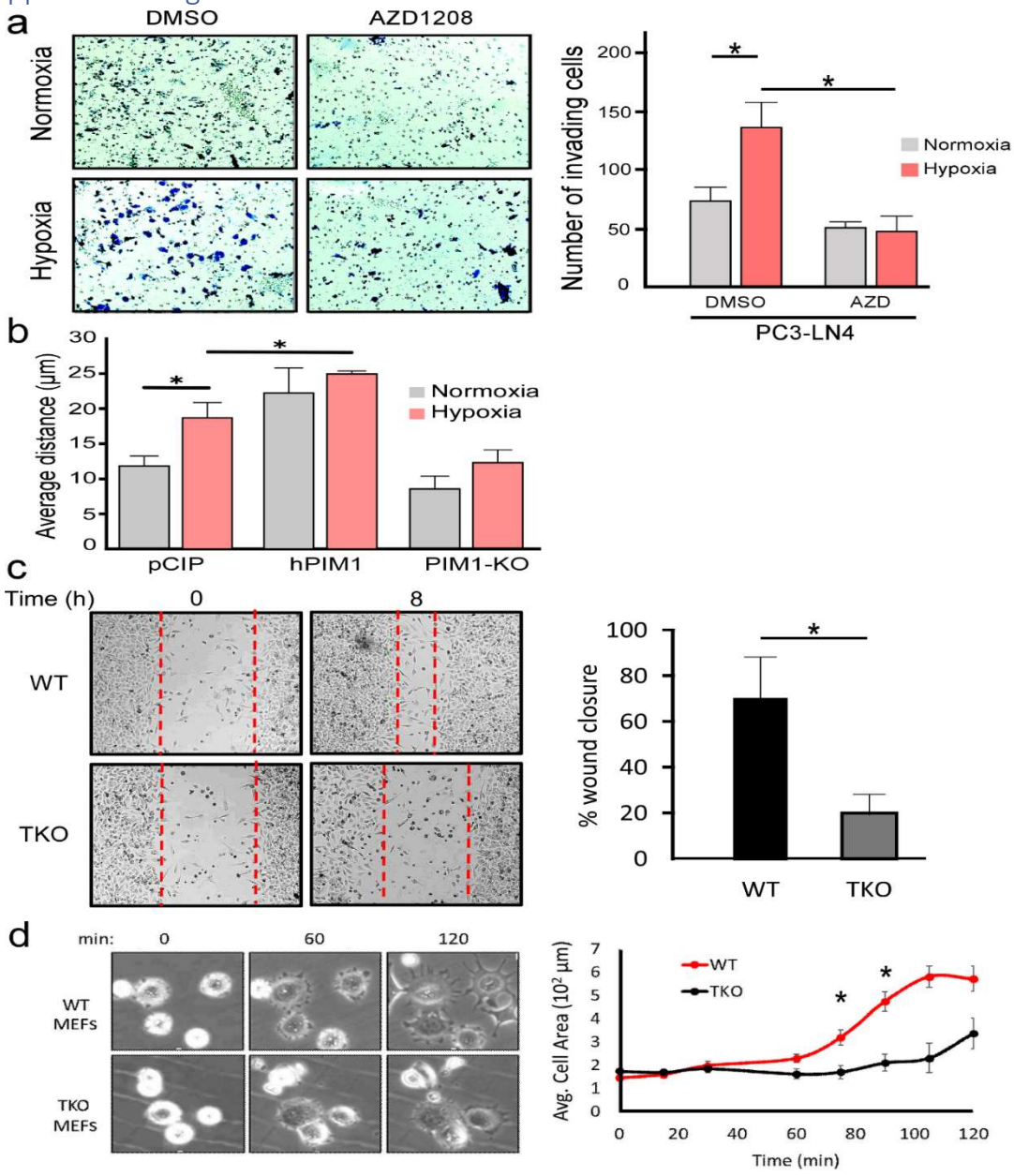
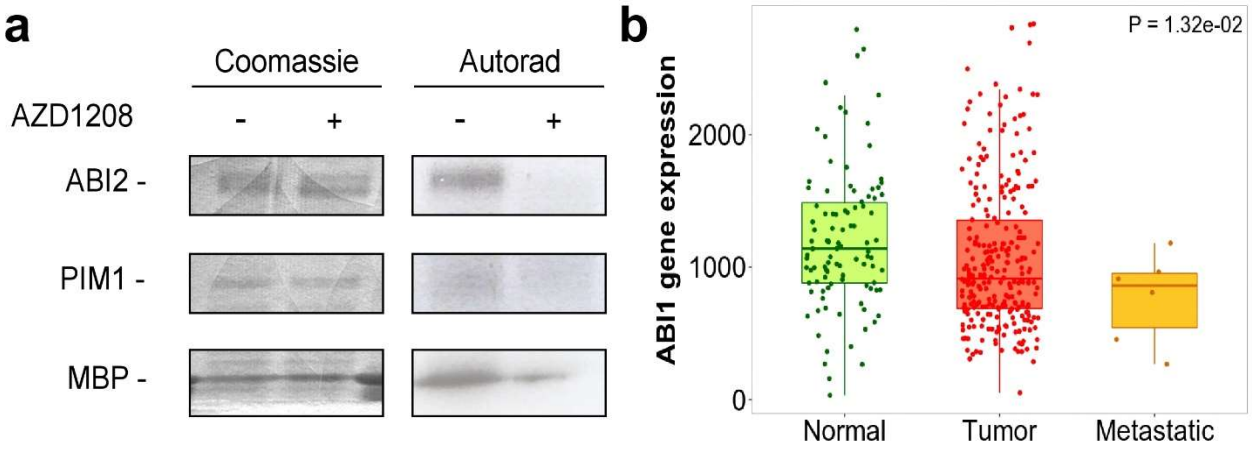
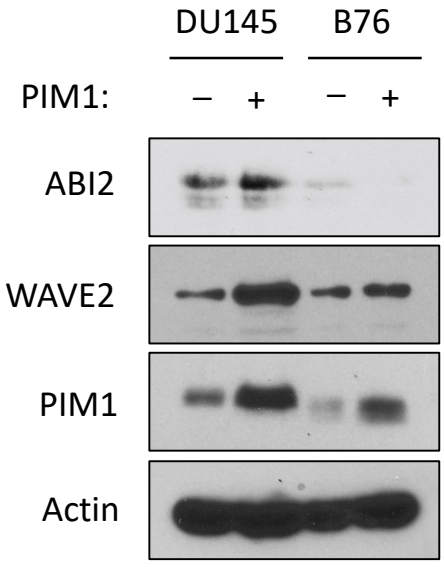
2.6 Supplemental Figures



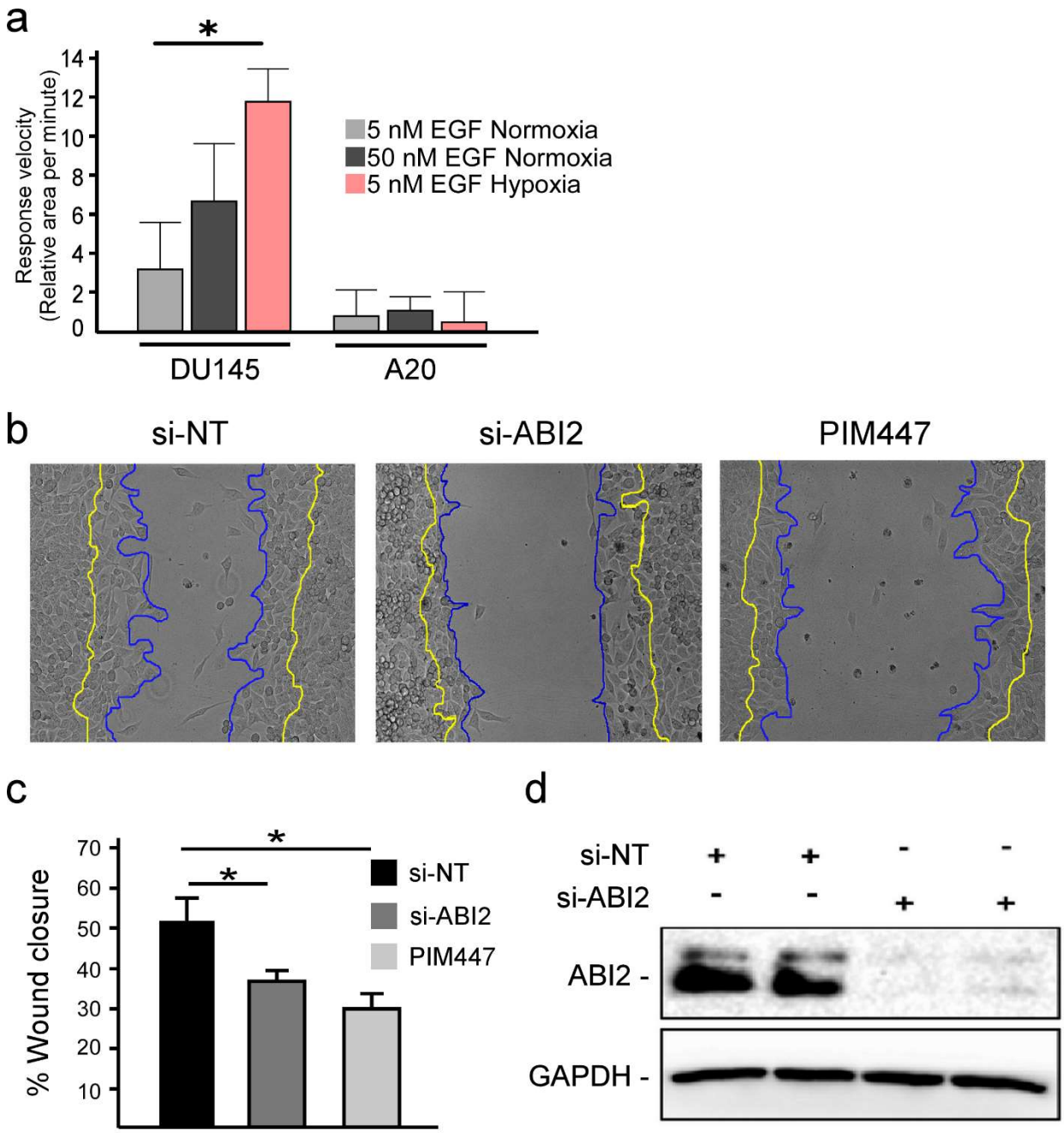
Supplemental Figure 1. Loss of PIM inhibits cell motility and spreading. (A) Boyden Chamber invasion assay of PC3-LN4 cells with or without PIM inhibition under normoxic and hypoxic conditions. Number of invading cells was quantified. Error displayed as SEM (n=3) (*p<0.05). (B) Quantification of single cell migration assays. PC3-LN4 (pCIP; n=27 Nx and n=45 Hx), PC3-LN4 + PIM1 (hPIM1, n=17 Nx and n=41 Hx), or PIM1-KO (n=15 Nx and n=36 Hx) cells were placed in normoxia or hypoxia, and the distance each cell traveled in a 16-h period was measured. (C) Wound healing assay and subsequent quantification of percent wound closure in MEFs with intact or complete loss of PIM kinase. Error is SEM (n=3) (*p<0.05). (D) WT and TKO cells were placed in dishes and allowed to seed. Cells were imaged every 20 min and average cell area was plotted over time. Error is SEM (n=3) (*p<0.05)



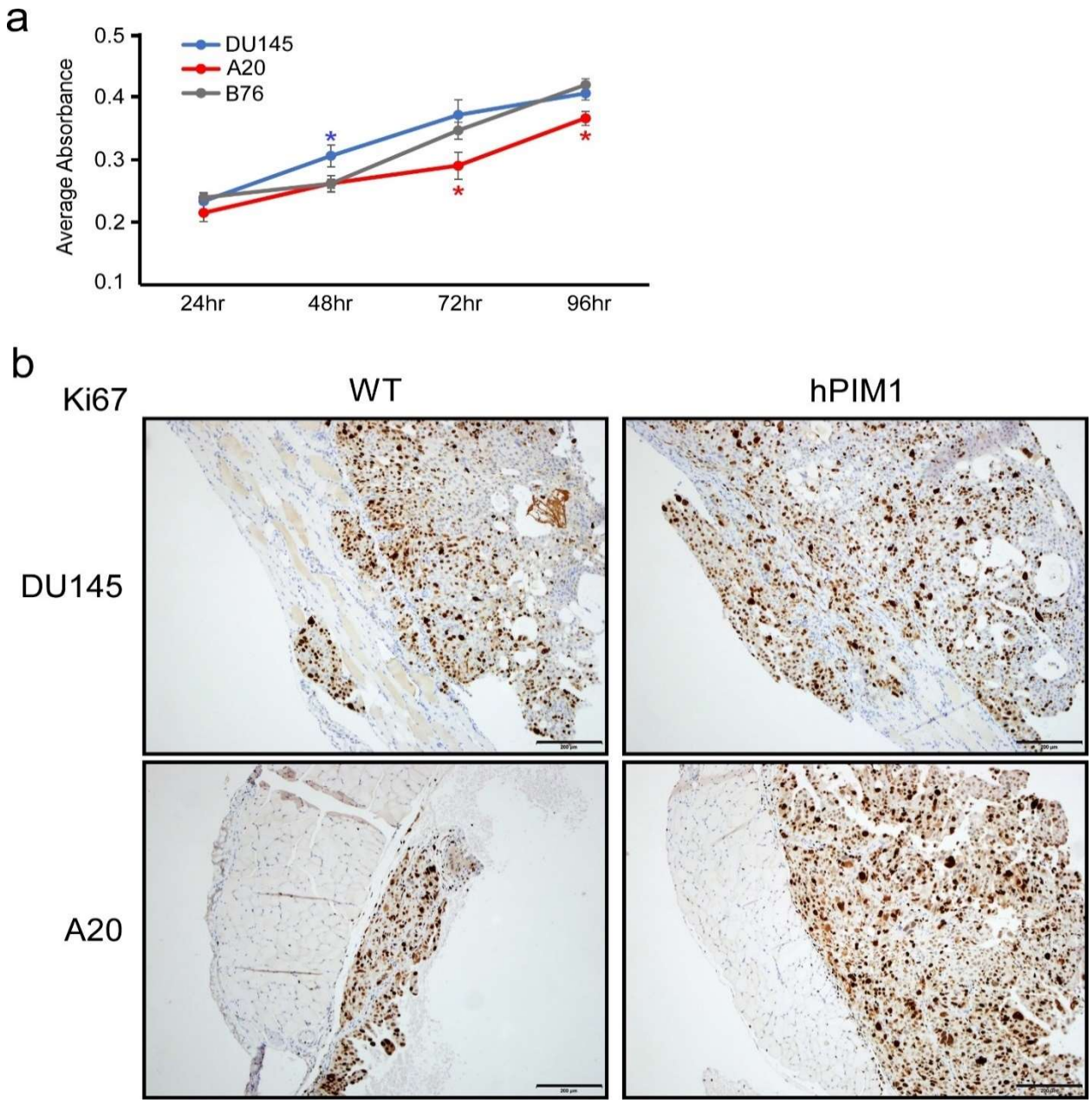
Supplemental Figure 2. PIM1 phosphorylates ABI2 *in vitro*. (A) Coomassie and autoradiography of *in vitro* kinase assay using recombinant ABI2, PIM1 and MBP (positive control) in the presence or absence of PIM inhibitor AZD1208. (B) *ABI1* gene expression in human prostate tumor samples from the NCBI Geo database (TNMplot web analyzer).



Supplemental Figure 3. PIM1 increases WAVE2 levels in a ABI2-dependent manner. DU145 or B76 cells stably expressing vector or PIM1 were cultured in normoxia for 16 h, and western blotting was used to assess protein levels.



Supplemental Figure 4. ABI2 and PIM are necessary for proper wound healing. (A) Quantification of the change in cell area per minute upon stimulation with EGF in different oxygen environments. Error = 95% CI (n=45 5 nM EGF Nx; n = 15, 50 nM EGF Nx; and n = 15, 5 nM EGF Hx for DU145; n=6 for A20 in all conditions) (*p<0.05). (B) Wound healing assay with PC3-LN4 cells with a non-targeting siRNA, ABI2-targeting siRNA, or chemical inhibition of PIM. Cell edge at time of scratch depicted in yellow and edge 8 hours post-scratch in blue. (C) Quantification of scratch assay. Error is SEM (n=3) (*p<0.05). (D) Western blot depicting efficacy of siRNAs targeting ABI2 or non-specific sequence (negative control).



Supplemental Figure 5. Loss of ABI2 does not affect cell proliferation. (A) Cells were plated in 96-well dishes and allowed to grow for 96hrs. Wells were stained with Crystal Violet and eluted using SDS. Average absorbance was measured as an indirect measure of number of cells present and plotted over time. Error is SEM (n=3) (*$p<0.05$; color indicates point is significant compared to other cell lines at indicated time). (B) IHC analysis of Ki67 expression in mouse diaphragms injected with DU145 and A20 cells with or without overexpression of PIM1.

3 Future Directions

There is still a lot to learn about the exact mechanisms that regulate how the WRC complex forms from the individual subunits and the regulation and translocation of the activated complex. Hypoxia may prove to be an important regulator of the WRC outside of just the PIM and ABI2 pathway. As previously discussed, hypoxia is known to enhance Akt signaling. Akt is known to upregulate Rac1 and has been shown to enhance actin dynamics (Enomoto et al., 2005; Toker and Yoeli-Lerner, 2006). Further, hypoxia converges with PI3K signaling which has the potential to play a role in the translocation and activity of the WRC via interactions with the WIRS motif of PIP_3. This could be particularly of interest in the context of prostate cancer when one of the most common oncogenic alterations is altered PI3K and PTEN signaling. Monoallelic loss of PTEN is present in up to 60% of localized prostate tumors with complete loss of PTEN implicated with more metastatic tumors (Phin et al., 2013). Thus, prostate cancers may have more WRC present at the membrane due to increased PI3K signaling, and in a feedforward mechanism, would also result in upregulation of Akt and thus increase Rac1. This may be further increased in hypoxic regions of PTEN null prostate tumors.

In coordination with the localization of WRC via PIP_3 there is much to learn about the actual mechanisms by which phosphorylation of ABI2 at Ser183 is altering the WRC. This phosphorylation, along with those observed at Thr174, Thr178 and Ser187, are located just outside of the HHR domain of ABI2 in a region with no known function. The introduction of 4 negatively charged phospho-groups within such close proximity may result in a conformational change in ABI2 that could affect its interactions with the other WRC members. Further, while not identified in our SILAC screen, *in vitro* and *in vivo* mass spec. analysis revealed that two

Threonines that were within and immediately adjacent to the WIRS motif portion of ABI2 were differentially phosphorylated upon PIM inhibition. We have not evaluated these other sites even in a preliminary manner but there is evidence to support further probing the exact effect of phosphorylation on ABI2.

The major translational conclusion of my work, in summation, is that PIM kinases should be targeted in the clinic for the reduction and possible prevention of tumor invasion and metastasis. While two major compounds chemically inhibiting the family of PIM kinase have been tested in the clinic; neither one of them had an end goal of metastatic reduction. One of these compounds, PIM447 from Novartis, remains an actively tested clinical compound. Based on my findings, further research testing the efficacy of PIM447 as an anti-metastatic agent is warranted. Additionally, ABI2 gene expression may serve as a novel biomarker in prostate tumors as higher ABI2 levels could be prognostic for increased risk of invasion and metastasis. This is supported based on clinical data in prostate cancer patients, as is the connection between PIM and ABI2 in tumor samples.

Additionally, these findings have the potential to extent outside of the confines of prostate cancer treatment. Hypoxia is a hallmark of all solid tumors. Solid tumors account for the majority of cancer-related deaths and a majority of those deaths can be attributed to metastasis and resistance to current therapies (Siegel et al., 2019). A better understanding of how hypoxia, PIM and cellular protrusions have the potential to significantly reduce the mortality rates of all solid tumors. This works establishes a groundwork for further evaluation of PIM for the use of clinical care of cancer patients.

4 References

Alexander, S., Koehl, G.E., Hirschberg, M., Geissler, E.K., Friedl, P., 2008. Dynamic imaging of cancer growth and invasion: a modified skin-fold chamber model. Histochem Cell Biol 130, 1147–1154.

Appelhoff, R.J., Tian, Y.-M., Raval, R.R., Turley, H., Harris, A.L., Pugh, C.W., Ratcliffe, P.J., Gleadle, J.M., 2004. Differential Function of the Prolyl Hydroxylases PHD1, PHD2, and PHD3 in the Regulation of Hypoxia-inducible Factor. Journal of Biological Chemistry 279, 38458–38465.

Arany, Z., Huang, L.E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M.A., Bunn, H.F., Livingston, D.M., 1996. An essential role for p300/CBP in the cellular response to hypoxia. Proceedings of the National Academy of Sciences 93, 12969–12973.

Bartha, Á., Győrffy, B., 2021. TNMplot.com: A Web Tool for the Comparison of Gene Expression in Normal, Tumor and Metastatic Tissues. Int J Mol Sci 22, 1–12.

Brooks, D.L.P., Schwab, L.P., Krutilina, R., Parke, D.N., Sethuraman, A., Hoogewijs, D., Schörg, A., Gotwald, L., Fan, M., Wenger, R.H., Seagroves, T.N., 2016. ITGA6 is directly regulated by hypoxia-inducible factors and enriches for cancer stem cell activity and invasion in metastatic breast cancer models. Mol Cancer 15, 26.

Bullen, J.W., Tchernyshyov, I., Holewinski, R.J., DeVine, L., Wu, F., Venkatraman, V., Kass, D.L., Cole, R.N., van Eyk, J., Semenza, G.L., 2016. Protein kinase A–dependent phosphorylation stimulates the transcriptional activity of hypoxia-inducible factor 1. Sci Signal 9, ra56–ra56.

Cairns, R.A., Harris, I.S., Mak, T.W., 2011. Regulation of cancer cell metabolism. Nat Rev Cancer 11, 85–95.

Cam, H., Easton, J.B., High, A., Houghton, P.J., 2010. mTORC1 Signaling under Hypoxic Conditions Is Controlled by ATM-Dependent Phosphorylation of HIF-1α. Mol Cell 40, 509–520.

Cao, L., Wang, F., Li, S., Wang, X., Huang, D., Jiang, R., 2019. PIM1 kinase promotes cell proliferation, metastasis and tumor growth of lung adenocarcinoma by potentiating the c-MET signaling pathway. Cancer Lett 444, 116–126.

Carpenter, C.L., Duckworth, B.C., Auger, K.R., Cohen, B., Schaffhausen, B.S., Cantley, L.C., 1990. Purification and Characterization of Phosphoinositide 3-Kinase from Rat Liver. Journal of Biological Chemistry 265, 19704–19711.

Casillas, A.L., Chauhan, S.S., Toth, R.K., Sainz, A.G., Clements, A.N., Jensen, C.C., Langlais, P.R., Miranti, C.K., Cress, A.E., Warfel, N.A., 2021. Direct phosphorylation and stabilization of HIF-1α by PIM1 kinase drives angiogenesis in solid tumors. Oncogene 2021 40:32 40, 5142–5152.

Casillas, A.L., Toth, R.K., Sainz, A.G., Singh, N., Desai, A.A., Kraft, A.S., Warfel, N.A., 2018a. Hypoxia-Inducible PIM Kinase Expression Promotes Resistance to Antiangiogenic Agents. Clinical Cancer Research 24, 169–180.

Chang, Q., Jurisica, I., Do, T., Hedley, D.W., 2011. Hypoxia Predicts Aggressive Growth and Spontaneous Metastasis Formation from Orthotopically Grown Primary Xenografts of Human Pancreatic Cancer. Cancer Res 71, 3110–3120.

Chauhan, S.S., Toth, R.K., Jensen, C.C., Casillas, A.L., Kashatus, D.F., Warfel, N.A., 2020. PIM kinases alter mitochondrial dynamics and chemosensitivity in lung cancer. Oncogene 39, 2597–2611.

Chen, B., Chen, Z., Brinkmann, K., Pak, C.W., Liao, Y., Shi, S., Henry, L., Grishin, N. v, Bogdan, S., Rosen, M.K., 2013. The WAVE Regulatory Complex Links Diverse Receptors to the Actin Cytoskeleton.

Chen, B., Chou, H.T., Brautigam, C.A., Xing, W., Yang, S., Henry, L., Doolittle, L.K., Walz, T., Rosen, M.K., 2017. Rac1 GTPase activates the WAVE regulatory complex through two distinct binding sites. Elife 6.

Chen, J., Kobayashi, M., Darmanin, S., Qiao, Y., Gully, C., Zhao, R., Kondo, S., Wang, Hua, Wang, Huamin, Yeung, S.-C.J., Lee, M.-H., 2009a. Hypoxia-Mediated Up-Regulation of Pim-1 Contributes to Solid Tumor Formation. Am J Pathol 175, 400–411.

Chen, J., Kobayashi, M., Darmanin, S., Qiao, Y., Gully, C., Zhao, R., Kondo, S., Wang, Hua, Wang, Huamin, Yeung, S.C.J., Lee, M.H., 2009b. Hypoxia-mediated up-regulation of Pim-1 contributes to solid tumor formation. Am J Pathol 175, 400–411.

Chen, Z., Borek, D., Padrick, S.B., Gomez, T.S., Metlagel, Z., Ismail, A.M., Umetani, J., Billadeau, D.D., Otwinowski, Z., Rosen, M.K., 2010. Structure and control of the actin regulatory WAVE complex. Nature 468, 533–538.

Copple, B.L., 2010. Hypoxia stimulates hepatocyte epithelial to mesenchymal transition by hypoxia-inducible factor and transforming growth factor-β-dependent mechanisms. Liver International 30, 669–682.

Cortes, J., Tamura, K., Deangelo, D.J., de Bono, J., Lorente, D., Minden, M., Uy, G.L., Kantarjian, H., Chen, L.S., Gandhi, V., Godin, R., Keating, K., McEachern, K., Vishwanathan, K., Pease, J.E., Dean, E., 2018. Phase I studies of AZD1208, a proviral integration Moloney virus kinase inhibitor in solid and haematological cancers. Br J Cancer 118, 1425.

Cowden Dahl, K.D., Robertson, S.E., Weaver, V.M., Simon, M.C., 2005. Hypoxia-inducible Factor Regulates αvβ3 Integrin Cell Surface Expression. Mol Biol Cell 16, 1901–1912.

Cox, T.R., Rumney, R.M.H., Schoof, E.M., Perryman, L., Høye, A.M., Agrawal, A., Bird, D., Latif, N.A., Forrest, H., Evans, H.R., Huggins, I.D., Lang, G., Linding, R., Gartland, A., Erler, J.T., 2015. The hypoxic cancer secretome induces pre-metastatic bone lesions through lysyl oxidase. Nature 522, 106–110.

Dai, Z., Pendergast, A.M., 1995. Abi-2, a novel SH3-containing protein interacts witli the c-Abl tyrosine kinase and modulates c-Abl transforming activity.

Dai, Z., Quackenbush, R.C., Courtney, K.D., Grove, M., Cortez, D., Reuther, G.W., Pendergast, A.M., 1998. Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway.

Deep, G., Panigrahi, G.K., n.d. Hypoxia-Induced Signaling Promotes Prostate Cancer Progression: Exosomes Role as Messenger of Hypoxic Response in Tumor Microenvironment.

Dekanty, A., Lavista-Llanos, S., Irisarri, M., Oldham, S., Wappner, P., 2005. The insulin-PI3K/TOR pathway induces a HIF-dependent transcriptional response in Drosophila by promoting nuclear localization of HIF- /Sima. J Cell Sci 118, 5431–5441.

Derivery, E., Fink, J., Martin, D., Houdusse, A., Piel, M., Stradal, T.E., Louvard, D., Gautreau, A., 2008. Free Brick1 Is a Trimeric Precursor in the Assembly of a Functional Wave Complex. PLoS One 3.

Dhanasekaran, S.M., Barrette, T.R., Ghosh, D., Shah, R., Varambally, S., Kurachi, K., Pienta, K.J., Rubin, M.A., Chinnaiyan, A.M., 2001. Delineation of prognostic biomarkers in prostate cancer. Nature 412, 822–826.

Dubielecka, P.M., Ladwein, K.I., Xiong, X., Migeotte, I., Chorzalska, A., Anderson, K. v., Sawicki, J.A., Rottner, K., Stradal, T.E., Kotula, L., 2011. Essential role for Abi1 in embryonic survival and WAVE2 complex integrity. Proc Natl Acad Sci U S A 108, 7022–7027.

Dupuy, F., Tabariès, S., Andrzejewski, S., Dong, Z., Blagih, J., Annis, M.G., Omeroglu, A., Gao, D., Leung, S., Amir, E., Clemons, M., Aguilar-Mahecha, A., Basik, M., Vincent, E.E., St.-Pierre, J., Jones, R.G., Siegel, P.M., 2015. PDK1-Dependent Metabolic Reprogramming Dictates Metastatic Potential in Breast Cancer. Cell Metab 22, 577–589.

Echarri, A., Lai, M.J., Robinson, M.R., Pendergast, A.M., 2004. Abl Interactor 1 (Abi-1) Wave-Binding and SNARE Domains Regulate Its Nucleocytoplasmic Shuttling, Lamellipodium Localization, and Wave-1 Levels. Mol Cell Biol 24, 4979–4993.

Ema, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J., Sogawa, K., Poellinger, L., Fujii-Kuriyama, Y., 1999. Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J 18, 1905–1914.

Enomoto, A., Murakami, H., Asai, N., Morone, N., Watanabe, T., Kawai, K., Murakumo, Y., Usukura, J., Kaibuchi, K., Takahashi, M., 2005. Akt/PKB regulates actin organization and cell motility via Girdin/APE. Dev Cell 9, 389–402.

Erler, J.T., Cawthorne, C.J., Williams, K.J., Koritzinsky, M., Wouters, B.G., Wilson, C., Miller, C., Demonacos, C., Stratford, I.J., Dive, C., 2004. Hypoxia-Mediated Down-Regulation of Bid and Bax in Tumors Occurs via Hypoxia-Inducible Factor 1-Dependent and -Independent Mechanisms and Contributes to Drug Resistance. Mol Cell Biol 24, 2875–2889.

Esteban, M.A., Tran, M.G.B., Harten, S.K., Hill, P., Castellanos, M.C., Chandra, A., Raval, R., O'Brien, T.S., Maxwell, P.H., 2006. Regulation of E-cadherin Expression by VHL and Hypoxia-Inducible Factor. Cancer Res 66, 3567–3575.

Ferreira, J.V., Fôfo, H., Bejarano, E., Bento, C.F., Ramalho, J.S., Girão, H., Pereira, P., 2013. STUB1/CHIP is required for HIF1A degradation by chaperone-mediated autophagy. Autophagy 9, 1349–1366.

Flügel, D., Görlach, A., Michiels, C., Kietzmann, T., 2007. Glycogen Synthase Kinase 3 Phosphorylates Hypoxia-Inducible Factor 1α and Mediates Its Destabilization in a VHL-Independent Manner. Mol Cell Biol 27, 3253–3265.

Foulks, J.M., Carpenter, K.J., Luo, B., Xu, Y., Senina, A., Nix, R., Chan, A., Clifford, A., Wilkes, M., Vollmer, D., Brenning, B., Merx, S., Lai, S., McCullar, M. v., Ho, K.K., Albertson, D.J., Call, L.T., Bearss, J.J.,

Tripp, S., Liu, T., Stephens, B.J., Mollard, A., Warner, S.L., Bearss, D.J., Kanner, S.B., 2014. A Small-Molecule Inhibitor of PIM Kinases as a Potential Treatment for Urothelial Carcinomas. Neoplasia 16, 403.

Fox, C.J., Hammerman, P.S., Cinalli, R.M., Master, S.R., Chodosh, L.A., Thompson, C.B., 2003. The serine/threonine kinase Pim-2 is a transcriptionally regulated apoptotic inhibitor. Genes Dev 17, 1841–1854.

Friedl, P., Wolf, K., 2003. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer 3, 362–374.

Gapter, L.A., Magnuson, N.S., Ng, K. yun, Hosick, H.L., 2006. Pim-1 kinase expression during murine mammary development. Biochem Biophys Res Commun 345, 989–997.

Gautreau, A., Ho, H.Y.H., Li, J., Steen, H., Gygi, S.P., Kirschner, M.W., 2004. Purification and architecture of the ubiquitous Wave complex. Proc Natl Acad Sci U S A 101, 4379.

Gilkes, D.M., Bajpai, S., Wong, C.C., Chaturvedi, P., Hubbi, M.E., Wirtz, D., Semenza, G.L., 2013a. Procollagen Lysyl Hydroxylase 2 Is Essential for Hypoxia-Induced Breast Cancer Metastasis. Molecular Cancer Research 11, 456–466.

Gilkes, D.M., Chaturvedi, P., Bajpai, S., Wong, C.C., Wei, H., Pitcairn, S., Hubbi, M.E., Wirtz, D., Semenza, G.L., 2013b. Collagen Prolyl Hydroxylases Are Essential for Breast Cancer Metastasis. Cancer Res 73, 3285–3296.

Gilkes, D.M., Semenza, G.L., Wirtz, D., 2014. Hypoxia and the extracellular matrix: drivers of tumour metastasis. Nat Rev Cancer 14, 430–439.

Gu, J.J., Wang, Z., Reeves, R., Magnuson, N.S., 2009. PIM1 phosphorylates and negatively regulates ASK1-mediated apoptosis. Oncogene 28, 4261–4271.

Gu, Y.Z., Moran, S.M., Hogenesch, J.B., Wartman, L., Bradfield, C.A., 1998. Molecular characterization and chromosomal localization of a third α- class hypoxia inducible factor subunit, HIF3α. Gene Expr 7, 205–213.

Guzy, R.D., Hoyos, B., Robin, E., Chen, H., Liu, L., Mansfield, K.D., Simon, M.C., Hammerling, U., Schumacker, P.T., 2005. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. Cell Metab 1, 401–408.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of Cancer: The Next Generation. Cell 144, 646–674.

Hartwich, J., Orr, W.S., Ng, C.Y., Spence, Y., Morton, C., Davidoff, A.M., 2013. HIF-1α activation mediates resistance to anti-angiogenic therapy in neuroblastoma xenografts. J Pediatr Surg 48, 39–46.

He, H.C., Bi, X.C., Zheng, Z.W., Dai, Q.S., Han, Z.D., Liang, Y.X., Ye, Y.K., Zeng, G.H., Zhu, G., Zhong, W. de, 2009. Real-time quantitative RT-PCR assessment of PIM-1 and hK2 mRNA expression in benign prostate hyperplasia and prostate cancer. Med Oncol 26, 303–308.

Higgins, D.F., Kimura, K., Bernhardt, W.M., Shrimanker, N., Akai, Y., Hohenstein, B., Saito, Y., Johnson, R.S., Kretzler, M., Cohen, C.D., Eckardt, K.-U., Iwano, M., Haase, V.H., 2007. Hypoxia promotes

fibrogenesis in vivo via HIF-1 stimulation of epithelial-to-mesenchymal transition. Journal of Clinical Investigation.

Hill, R.P., Marie-Egyptienne, D.T., Hedley, D.W., 2009. Cancer Stem Cells, Hypoxia and Metastasis. Semin Radiat Oncol 19, 106–111.

Hofbauer, K.-H., Gess, B., Lohaus, C., Meyer, H.E., Katschinski, D., Kurtz, A., 2003. Oxygen tension regulates the expression of a group of procollagen hydroxylases. Eur J Biochem 270, 4515–4522.

Hu, C.-J., Sataur, A., Wang, L., Chen, H., Simon, M.C., 2007. The N-Terminal Transactivation Domain Confers Target Gene Specificity of Hypoxia-inducible Factors HIF-1α and HIF-2α. Mol Biol Cell 18, 4528–4542.

Hubbi, M.E., Hu, H., Kshitiz, Ahmed, I., Levchenko, A., Semenza, G.L., 2013. Chaperone-mediated Autophagy Targets Hypoxia-inducible Factor-1α (HIF-1α) for Lysosomal Degradation. Journal of Biological Chemistry 288, 10703–10714.

Innocenti, M., Zucconi, A., Disanza, A., Frittoli, E., Areces, L.B., Steffen, A., Stradal, T.E.B., di Fiore, P.P., Carlier, M.F., Scita, G., 2004. Abi1 is essential for the formation and activation of a WAVE2 signalling complex. Nat Cell Biol 6, 319–327.

Ishikawa, T., Nakashiro, K.I., Klosek, S.K., Goda, H., Hara, S., Uchida, D., Hamakawa, H., 2009. Hypoxia enhances CXCR4 expression by activating HIF-1 in oral squamous cell carcinoma. Oncol Rep.

Iyer, N. v., Leung, S.W., Semenza, G.L., 1998. The Human Hypoxia-Inducible Factor 1α Gene:HIF1AStructure and Evolutionary Conservation. Genomics 52, 159–165.

Jaakkola, P., Mole, D.R., Tian, Y.-M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A. v., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J., 2001. Targeting of HIF-alpha to the von Hippel-Lindau Ubiquitylation Complex by O2-Regulated Prolyl Hydroxylation. Science (1979) 292, 468–472.

Jean, S., Kiger, A.A., 2014. Classes of phosphoinositide 3-kinases at a glance. J Cell Sci 127, 923–928.

Jensen, C.C., Warfel, N.A., 2021. Hypoxia. Reference Module in Biomedical Sciences.

Jiang, B.H., Jiang, G., Zheng, J.Z., Lu, Z., Hunter, T., Vogt, P.K., 2001. Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. Cell Growth and Differentiation 12, 363–369.

Jiang, B.-H., Zheng, J.Z., Leung, S.W., Roe, R., Semenza, G.L., 1997. Transactivation and Inhibitory Domains of Hypoxia-inducible Factor 1α. Journal of Biological Chemistry 272, 19253–19260.

Jiménez-Garciá, M.P., Lucena-Cacace, A., Robles-Friás, M.J., Narlik-Grassow, M., Blanco-Aparicio, C., Carnero, A., 2016. The role of PIM1/PIM2 kinases in tumors of the male reproductive system. Sci Rep 6.

Ju, J.A., Godet, I., Ye, I.C., Byun, J., Jayatilaka, H., Lee, S.J., Xiang, L., Samanta, D., Lee, M.H., Wu, P.-H., Wirtz, D., Semenza, G.L., Gilkes, D.M., 2017. Hypoxia Selectively Enhances Integrin α5β1 Receptor Expression in Breast Cancer to Promote Metastasis. Molecular Cancer Research 15, 723–734.

Kakkad, S.M., Solaiyappan, M., O'Rourke, B., Stasinopoulos, I., Ackerstaff, E., Raman, V., Bhujwalla, Z.M., Glunde, K., 2010. Hypoxic Tumor Microenvironments Reduce Collagen I Fiber Density. Neoplasia 12, 608–617.

Kaplan, R.N., Riba, R.D., Zacharoulis, S., Bramley, A.H., Vincent, L., Costa, C., MacDonald, D.D., Jin, D.K., Shido, K., Kerns, S.A., Zhu, Z., Hicklin, D., Wu, Y., Port, J.L., Altorki, N., Port, E.R., Ruggero, D., Shmelkov, S. v., Jensen, K.K., Rafii, S., Lyden, D., 2005. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 438, 820–827.

Keith, B., Johnson, R.S., Simon, M.C., 2012. HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression. Nat Rev Cancer 12, 9–22.

Khan, M.N., Bhattacharyya, T., Andrikopoulos, P., Esteban, M.A., Barod, R., Connor, T., Ashcroft, M., Maxwell, P.H., Kiriakidis, S., 2011. Factor inhibiting HIF (FIH-1) promotes renal cancer cell survival by protecting cells from HIF-1α-mediated apoptosis. Br J Cancer 104, 1151–1159.

Kietzmann, T., Mennerich, D., Dimova, E.Y., 2016. Hypoxia-Inducible Factors (HIFs) and Phosphorylation: Impact on Stability, Localization, and Transactivity. Front Cell Dev Biol 4.

Kim, J., Roh, M., Abdulkadir, S.A., 2010. Pim1 promotes human prostate cancer cell tumorigenicity and c-MYC transcriptional activity. BMC Cancer 10, 248.

Kioi, M., Vogel, H., Schultz, G., Hoffman, R.M., Harsh, G.R., Brown, J.M., 2010. Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. Journal of Clinical Investigation 120, 694–705.

Klammer, M., Kaminski, M., Zedler, A., Oppermann, F., Blencke, S., Marx, S., Müller, S., Tebbe, A., Godl, K., Schaab, C., 2012. Phosphosignature predicts dasatinib response in non-small cell lung cancer. Mol Cell Proteomics 11, 651–668.

Koh, M.Y., Darnay, B.G., Powis, G., 2008. Hypoxia-Associated Factor, a Novel E3-Ubiquitin Ligase, Binds and Ubiquitinates Hypoxia-Inducible Factor 1α, Leading to Its Oxygen-Independent Degradation. Mol Cell Biol 28, 7081–7095.

Koh, M.Y., Powis, G., 2012. Passing the baton: the HIF switch. Trends Biochem Sci 37, 364–372.

Koivunen, P., Hirsilä, M., Günzler, V., Kivirikko, K.I., Myllyharju, J., 2004. Catalytic Properties of the Asparaginyl Hydroxylase (FIH) in the Oxygen Sensing Pathway Are Distinct from Those of Its Prolyl 4-Hydroxylases. Journal of Biological Chemistry 279, 9899–9904.

Krieg, M., Haas, R., Brauch, H., Acker, T., Flamme, I., Plate, K.H., 2000. Up-regulation of hypoxia-inducible factors HIF-1α and HIF-2α under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene 19, 5435–5443.

Laughner, E., Taghavi, P., Chiles, K., Mahon, P.C., Semenza, G.L., 2001. HER2 (neu) Signaling Increases the Rate of Hypoxia-Inducible Factor 1α (HIF-1α) Synthesis: Novel Mechanism for HIF-1-Mediated Vascular Endothelial Growth Factor Expression. Mol Cell Biol 21, 3995–4004.

Lee, E., Fertig, E.J., Jin, K., Sukumar, S., Pandey, N.B., Popel, A.S., 2014. Breast cancer cells condition lymphatic endothelial cells within pre-metastatic niches to promote metastasis. Nat Commun 5, 4715.

Lee, K., Elliott, H.L., Oak, Y., Zee, C. te, Groisman, A., Tytell, J.D., Danuser, G., 2015. Functional Hierarchy of Redundant Actin Assembly Factors Revealed by Fine-Grained Registration of Intrinsic Image Fluctuations. Cell Syst 1, 37–50.

Lee, S.H., Lee, Y.J., Han, H.J., 2011. Role of hypoxia-induced fibronectin-integrin β1 expression in embryonic stem cell proliferation and migration: Involvement of PI3K/Akt and FAK. J Cell Physiol 226, 484–493.

Lehmann, S., te Boekhorst, V., Odenthal, J., Bianchi, R., van Helvert, S., Ikenberg, K., Ilina, O., Stoma, S., Xandry, J., Jiang, L., Grenman, R., Rudin, M., Friedl, P., 2017. Hypoxia Induces a HIF-1-Dependent Transition from Collective-to-Amoeboid Dissemination in Epithelial Cancer Cells. Current Biology 27, 392–400.

Leng, Y., Zhang, J., Badour, K., Arpaia, E., Freeman, S., Cheung, P., Siu, M., Siminovitch, K., 2005. Abelson-interactor-1 promotes WAVE2 membrane translocation and Abelson-mediated tyrosine phosphory lation required for WAVE2 activation. Proc Natl Acad Sci U S A 102, 1098–1103.

Lewis, J.S., Lee, J.A., Underwood, J.C.E., Harris, A.L., Lewis, C.E., 1999. Macrophage responses to hypoxia: relevance to disease mechanisms. J Leukoc Biol 66, 889–900.

Li, L., Qu, Y., Mao, M., Xiong, Y., Mu, D., 2008. The involvement of phosphoinositid 3-kinase/Akt pathway in the activation of hypoxia-inducible factor-1α in the developing rat brain after hypoxia–ischemia. Brain Res 1197, 152–158.

Li, Y., Clough, N., Sun, X., Yu, W., Abbott, B.L., Hogan, C.J., Dai, Z., n.d. BCR-ABL Induces Abnormal Cytoskeleton Remodeling, β1 Integrin Clustering, and Increased Cell Adhesion to Fibronectin Through ABL Interactor 1 Pathway.

Li, Y.M., Zhou, B.P., Deng, J., Pan, Y., Hay, N., Hung, M.-C., 2005. A Hypoxia-Independent Hypoxia-Inducible Factor-1 Activation Pathway Induced by Phosphatidylinositol-3 Kinase/Akt in HER2 Overexpressing Cells. Cancer Res 65, 3257–3263.

Lilly, M., Sandholm, J., Cooper, J.J., Koskinen, P.J., Kraft, A., 1999. The PIM-1 serine kinase prolongs survival and inhibits apoptosis-related mitochondrial dysfunction in part through a bcl-2-dependent pathway. Oncogene 18, 4022–4031.

Liu, Yang, Liu, Yawei, Yan, X., Xu, Y., Luo, F., Ye, J., Yan, H., Yang, X., Huang, X., Zhang, J., Ji, G., 2014. HIFs enhance the migratory and neoplastic capacities of hepatocellular carcinoma cells by promoting EMT. Tumor Biology 35, 8103–8114.

Logothetis, C., Morris, M.J., Den, R., Coleman, R.E., 2018. Current perspectives on bone metastases in castrate-resistant prostate cancer. Cancer Metastasis Rev 37, 189–196.

Luo, D., Wang, J., Li, J., Post, M., 2011. Mouse Snail Is a Target Gene for HIF. Molecular Cancer Research 9, 234–245.

Macdonald, A., Campbell, D., Toth, R., McLauchlan, H., Hastie, C.J., Arthur, J.S., 2006. Pim kinases phosphorylate multiple sites on Bad and promote 14-3-3 binding and dissociation from Bcl-XL. BMC Cell Biol 7, 1.

Mahon, P.C., Hirota, K., Semenza, G.L., 2001. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 15, 2675–2686.

Majmundar, A.J., Wong, W.J., Simon, M.C., 2010. Hypoxia-Inducible Factors and the Response to Hypoxic Stress. Mol Cell 40, 294–309.

Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., Cao, Y., Berkenstam, A., Poellinger, L., 2001. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature 414, 550–554.

Mayor, R., Etienne-Manneville, S., 2016. The front and rear of collective cell migration. Nat Rev Mol Cell Biol 17, 97–109.

Mccaffery, K., Nickel, B., Pickles, K., Moynihan, R., Kramer, B., Barratt, A., Hersch, J., n.d. Resisting recommended treatment for prostate cancer: a qualitative analysis of the lived experience of possible overdiagnosis.

Mccandless, J.R., Cress, A.E., Rabinovitz, I., Payne, C.M., Bowden, G.T., Knox, J.D., Nagle, R.B., 1997. A human xenograft model for testing early events of epithelial neoplastic invasion. Int J Oncol 10, 279–285.

Mendoza, M.C., 2013. Phosphoregulation of the WAVE regulatory complex and signal integration. Semin Cell Dev Biol 24, 272–279.

Mertins, P., Yang, F., Liu, T., Mani, D.R., Petyuk, V.A., Gillette, M.A., Clauser, K.R., Qiao, J.W., Gritsenko, M.A., Moore, R.J., Levine, D.A., Townsend, R., Erdmann-Gilmore, P., Snider, J.E., Davies, S.R., Ruggles, K. v., Fenyo, D., Kitchens, R.T., Li, S., Olvera, N., Dao, F., Rodriguez, H., Chan, D.W., Liebler, D., White, F., Rodland, K.D., Mills, G.B., Smith, R.D., Paulovich, A.G., Ellis, M., Carr, S.A., 2014. Ischemia in tumors induces early and sustained phosphorylation changes in stress kinase pathways but does not affect global protein levels. Mol Cell Proteomics 13, 1690–1704.

Mikkers, H., Nawijn, M., Allen, J., Brouwers, § Conny, Verhoeven, E., Jonkers, J., Berns, A., 2004. Mice deficient for all PIM kinases display reduced body size and impaired responses to hematopoietic growth factors. Mol Cell Biol 24, 6104–6115.

Miller, K.D., Siegel, R.L., Lin, C.C., Mariotto, A.B., Kramer, J.L., Rowland, J.H., Stein, K.D., Alteri, R., Jemal, A., 2016. Cancer treatment and survivorship statistics, 2016. CA Cancer J Clin 66, 271–289.

Milosevic, M., Warde, P., Meńard, C., Chung, P., Toi, A., Ishkanian, A., McLean, M., Pintilie, M., Sykes, J., Gospodarowicz, M., Catton, C., Hill, R.P., Bristow, R., 2012. Tumor hypoxia predicts biochemical failure following radiotherapy for clinically localized prostate cancer. Clinical Cancer Research 18, 2108–2114.

Montagner, M., Enzo, E., Forcato, M., Zanconato, F., Parenti, A., Rampazzo, E., Basso, G., Leo, G., Rosato, A., Bicciato, S., Cordenonsi, M., Piccolo, S., 2012. SHARP1 suppresses breast cancer metastasis by promoting degradation of hypoxia-inducible factors. Nature 487, 380–384.

Morishita, D., Katayama, R., Sekimizu, K., Tsuruo, T., Fujita, N., 2008. Pim kinases promote cell cycle progression by phosphorylating and down-regulating p27Kip1 at the transcriptional and posttranscriptional levels. Cancer Res 68, 5076–5085.

Mowers, E.E., Sharifi, M.N., Macleod, K.F., 2017. Autophagy in cancer metastasis. Oncogene 36, 1619–1630.

Muz, B., de la Puente, P., Azab, F., Azab, A.K., 2015. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia 83.

Mylonis, I., Simos, G., 2012. The Involvement of the ERK-Hypoxia-Angiogenesis Signaling Axis and HIF-1 in Hepatocellular Carcinoma. In: Hepatocellular Carcinoma - Basic Research.

N. Kirschner, A., Wang, J., van der Meer, R., Anderson, P.D., Franco-Coronel, O.E., Kushner, M.H., Everett, J.H., Hameed, O., Keeton, E.K., Ahdesmaki, M., Grosskurth, S.E., Huszar, D., Abdulkadir, S.A., 2015. PIM Kinase Inhibitor AZD1208 for Treatment of MYC-Driven Prostate Cancer. JNCI Journal of the National Cancer Institute 107, 1–11.

Nacov, E., 1990. Tumor angiogenesis formation of vessels de novo at germ cell tumors. Cancer 66, 916–922.

Nagy, J.A., Brown, L.F., Senger, D.R., Lanir, N., van de Water, L., Dvorak, A.M., Dvorak, H.F., 1989. Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer 948, 305–326.

Niu, G., Briggs, J., Deng, J., Ma, Y., Lee, H., Kortylewski, M., Kujawski, M., Kay, H., Cress, W.D., Jove, R., Yu, H., 2008. Signal Transducer and Activator of Transcription 3 Is Required for Hypoxia-Inducible Factor-1 RNA Expression in Both Tumor Cells and Tumor-Associated Myeloid Cells. Molecular Cancer Research 6, 1099–1105.

Ohh, M., Park, C.W., Ivan, M., Hoffman, M.A., Kim, T.-Y., Huang, L.E., Pavletich, N., Chau, V., Kaelin, W.G., 2000. Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel–Lindau protein. Nat Cell Biol 2, 423–427.

Padilla-Rodriguez, M., Parker, S.S., Adams, D.G., Westerling, T., Puleo, J.I., Watson, A.W., Hill, S.M., Noon, M., Gaudin, R., Aaron, J., Tong, D., Roe, D.J., Knudsen, B., Mouneimne, G., n.d. The actin cytoskeletal architecture of estrogen receptor positive breast cancer cells suppresses invasion.

Padmanabhan, A., Gosc, E.B., Bieberich, C.J., 2013. Stabilization of the prostate-specific tumor suppressor NKX3.1 by the oncogenic protein kinase Pim-1 in prostate cancer cells. J Cell Biochem 114, 1050–1057.

Palazon, A., Goldrath, A.W., Nizet, V., Johnson, R.S., 2014. HIF Transcription Factors, Inflammation, and Immunity. Immunity 41, 518–528.

Papandreou, I., Cairns, R.A., Fontana, L., Lim, A.L., Denko, N.C., 2006. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab 3, 187–197.

Peng, Y.-F., Shi, Y.-H., Shen, Y.-H., Ding, Z.-B., Ke, A.-W., Zhou, J., Qiu, S.-J., Fan, J., 2013. Promoting Colonization in Metastatic HCC Cells by Modulation of Autophagy. PLoS One 8, e74407.

Pennacchietti, S., Michieli, P., Galluzzo, M., Mazzone, M., Giordano, S., Comoglio, P.M., 2003. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 3, 347–361.

Peters, T.L., Li, L., Tula-Sanchez, A.A., Pongtornpipat, P., Schatz, J.H., 2016. Control of translational activation by PIM kinase in activated B-cell diffuse large B-cell lymphoma confers sensitivity to inhibition by PIM447. Oncotarget 7, 63362–63373.

Petersen Shay, K., Wang, Z., Xing, P.X., McKenzie, I.F.C., Magnuson, N.S., 2005. Pim-1 Kinase Stability Is Regulated by Heat Shock Proteins and the Ubiquitin-Proteasome Pathway. Molecular Cancer Research 3, 170–181.

Phin, S., Moore, M.W., Cotter, P.D., 2013. Genomic Rearrangements of PTEN in Prostate Cancer. Front Oncol 3.

Pollard, P., Yang, M., Su, H., Soga, T., Kranc, K., 2014. Prolyl hydroxylase domain enzymes: important regulators of cancer metabolism. Hypoxia 127.

Pollard, T.D., Cooper, J.A., 2009. Actin, a Central Player in Cell Shape and Movement. Science (1979) 326, 1208–1212.

Pore, N., Jiang, Z., Shu, H.K., Bernhard, E., Kao, G.D., Maity, A., 2006. Akt1 Activation Can Augment Hypoxia-Inducible Factor-1 Expression by Increasing Protein Translation through a Mammalian Target of Rapamycin-Independent Pathway. Molecular Cancer Research 4, 471–479.

Poujade, M., Grasland-Mongrain, E., Hertzog, A., Jouanneau, J., Chavrier, P., Ladoux, B., Buguin, A., Silberzan, P., 2007. Collective migration of an epithelial monolayer in response to a model wound. Proceedings of the National Academy of Sciences 104, 15988–15993.

Pruitt, B.L., Dunn, A.R., Weis, W.I., Nelson, W.J., 2014. Mechano-Transduction: From Molecules to Tissues. PLoS Biol 12, e1001996.

Pugh, C.W., O'Rourke, J.F., Nagao, M., Gleadle, J.M., Ratcliffe, P.J., 1997. Activation of Hypoxia-inducible Factor-1; Definition of Regulatory Domains within the α Subunit. Journal of Biological Chemistry 272, 11205–11214.

Rankin, E.B., Giaccia, A.J., 2016. Hypoxic control of metastasis. Science (1979) 352, 175–180.

Ren, K., Gou, X., Xiao, M., Wang, M., Liu, C., Tang, Z., He, W., 2013. The over-expression of Pim-2 promote the tumorigenesis of prostatic carcinoma through phosphorylating eIF4B. Prostate 73, 1462–1469.

Rey, S., Schito, L., Koritzinsky, M., Wouters, B.G., 2017. Molecular targeting of hypoxia in radiotherapy. Adv Drug Deliv Rev 109, 45–62.

Rottner, K., Stradal, T.E.B., Chen, B., 2021a. WAVE regulatory complex. Current Biology 31, R512–R517.

Rubenstein, C.S., Gard, J.M.C., Wang, M., McGrath, J.E., Ingabire, N., Hinton, J.P., Marr, K.D., Simpson, S.J., Nagle, R.B., Miranti, C.K., Warfel, N.A., Garcia, J.G.N., Arif-Tiwari, H., Cress, A.E., 2019. Gene editing of α6 integrin inhibits muscle invasive networks and increases cell-cell biophysical properties in prostate cancer. Cancer Res 79, 4703–4714.

Ryu, J.R., Echarri, A., Li, R., Pendergast, A.M., 2009. Regulation of Cell-Cell Adhesion by Abi/Diaphanous Complexes. Mol Cell Biol 29, 1735–1748.

Ryu, M.H., Park, H.M., Chung, J., Lee, C.H., Park, H.R., 2010. Hypoxia-inducible factor-1α mediates oral squamous cell carcinoma invasion via upregulation of α5 integrin and fibronectin. Biochem Biophys Res Commun 393, 11–15.

Sahlgren, C., Gustafsson, M. v., Jin, S., Poellinger, L., Lendahl, U., 2008. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. Proceedings of the National Academy of Sciences 105, 6392–6397.

Santio, N.M., Eerola, S.K., Paatero, I., Yli-Kauhaluoma, J., Anizon, F., Moreau, P., Tuomela, J., Härkönen, P., Koskinen, P.J., 2015a. Pim Kinases Promote Migration and Metastatic Growth of Prostate Cancer Xenografts. PLoS One 10.

Santio, N.M., Eerola, S.K., Paatero, I., Yli-Kauhaluoma, J., Anizon, F., Moreau, P., Tuomela, J., Härkönen, P., Koskinen, P.J., 2015b. Pim Kinases Promote Migration and Metastatic Growth of Prostate Cancer Xenografts. PLoS One 10, e0130340.

Sato, M., Maruoka, M., Takeya, T., 2012. Review Article Functional Mechanisms and Roles of Adaptor Proteins in Abl-Regulated Cytoskeletal Actin Dynamics. J Signal Transduct 2012, 13.

Saxena, K., Jolly, M.K., 2019. Acute vs. Chronic vs. Cyclic Hypoxia: Their Differential Dynamics, Molecular Mechanisms, and Effects on Tumor Progression. Biomolecules 9, 339.

Schödel, J., Oikonomopoulos, S., Ragoussis, J., Pugh, C.W., Ratcliffe, P.J., Mole, D.R., 2011. High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. Blood 117, e207–e217.

Schweppe, D.K., Rigas, J.R., Gerber, S.A., 2013. Quantitative phosphoproteomic profiling of human non-small cell lung cancer tumors. J Proteomics 91, 286–296.

Semenza, G.L., 2000. Hypoxia, Clonal Selection, and the Role of HIF-1 in Tumor Progression. Crit Rev Biochem Mol Biol 35, 71–103.

Semenza, G.L., Wang, G.L., 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 12, 5447–5454.

Siegel, R.L., Miller, K.D., Jemal, A., 2019. Cancer statistics, 2019. CA Cancer J Clin 69, 7–34.

Song, J.H., Singh, N., Luevano, L.A., Padi, S.K.R., Okumura, K., Olive, V., Black, S.M., Warfel, N.A., Goodrich, D.W., Kraft, A.S., 2018. Mechanisms Behind Resistance to PI3K Inhibitor Treatment Induced by the PIM Kinase. Mol Cancer Ther 17, 2710–2721.

Sosa, M.S., Bragado, P., Aguirre-Ghiso, J.A., 2014. Mechanisms of disseminated cancer cell dormancy: an awakening field. Nat Rev Cancer 14, 611–622.

Stuart, J.R., Gonzalez, F.H., Kawai, H., Yuan, Z.M., 2006. c-Abl interacts with the WAVE2 signaling complex to induce membrane ruffling and cell spreading. J Biol Chem 281, 31290–31297.

Sun, Y., Chen, G., Lv, Y., 2019. Effects of hypoxia on the biological behavior of MSCs seeded in demineralized bone scaffolds with different stiffness. Acta Mechanica Sinica 35, 309–320.

Thomlinson, R.H., Gray, L.H., 1955. The Histological Structure of Some Human Lung Cancers and the Possible Implications for Radiotherapy. Br J Cancer 9, 539–549.

Toker, A., Yoeli-Lerner, M., 2006. Akt Signaling and Cancer: Surviving but not Moving On. Cancer Res 66, 3963–3966.

Toth, R.K., Solomon, R., Warfel, N.A., 2022. Stabilization of PIM Kinases in Hypoxia Is Mediated by the Deubiquitinase USP28. Cells 2022, Vol. 11, Page 1006 11, 1006.

Toth, R.K., Tran, J.D., Muldong, M.T., Nollet, E.A., Schulz, V. v, Jensen, C.C., Hazlehurst, L.A., Corey, E., Durden, D., Jamieson, C., Miranti, C.K., Warfel, N.A., 2019. Hypoxia-induced PIM kinase and laminin-activated integrin α6 mediate resistance to PI3K inhibitors in bone-metastatic CRPC. Am J Clin Exp Urol 7, 297.

Toth, R.K., Warfel, N.A., 2021. Targeting PIM kinases to overcome therapeutic resistance in cancer. Mol Cancer Ther 20, 3–10.

Trepat, X., Wasserman, M.R., Angelini, T.E., Millet, E., Weitz, D.A., Butler, J.P., Fredberg, J.J., 2009. Physical forces during collective cell migration. Nat Phys 5, 426–430.

Tursynbay, Y., Zhang, J., Li, Z., Tokay, T., Zhumadilov, Z., Wu, D., Xie, Y., 2016. Pim-1 kinase as cancer drug target: An update. Biomed Rep 4, 140.

van Uden, P., Kenneth, N.S., Rocha, S., 2008. Regulation of hypoxia-inducible factor-1α by NF-κB. Biochemical Journal 412, 477–484.

Vaupel, P., Kallinowski, F., Okunieff, P., 1990. Blood Flow, Oxygen Consumption and Tissue Oxygenation of Human Tumors. pp. 895–905.

Vergis, R., Corbishley, C.M., Norman, A.R., Bartlett, J., Jhavar, S., Borre, M., Heeboll, S., Horwich, A., Huddart, R., Khoo, V., Eeles, R., Cooper, C., Sydes, M., Dearnaley, D., Parker, C., 2008. Intrinsic markers of tumour hypoxia and angiogenesis in localised prostate cancer and outcome of radical treatment: a retrospective analysis of two randomised radiotherapy trials and one surgical cohort study.

Wang, G.L., Jiang, B.H., Rue, E.A., Semenza, G.L., 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proceedings of the National Academy of Sciences 92, 5510–5514.

Wang, J., Kim, J., Roh, M., Franco, O.E., Hayward, S.W., Wills, M.L., Abdulkadir, S.A., 2010. Pim1 kinase synergizes with c-MYC to induce advanced prostate carcinoma. Oncogene 29, 2477.

Wang, Z., Bhattacharya, N., Mixter, P.F., Wei, W., Sedivy, J., Magnuson, N.S., 2002. Phosphorylation of the cell cycle inhibitor p21Cip1/WAF1 by Pim-1 kinase. Biochim Biophys Acta 1593, 45–55.

Warfel, N.A., Dolloff, N.G., Dicker, D.T., Malysz, J., El-Deiry, W.S., 2013. CDK1 stabilizes HIF-1α via direct phosphorylation of Ser668 to promote tumor growth. Cell Cycle 12, 3689–3701.

Warfel, N.A., Kraft, A.S., 2015a. PIM kinase (and Akt) biology and signaling in tumors. Pharmacol Ther 151, 41–49.

Warfel, N.A., Kraft, A.S., 2015b. PIM kinase (and Akt) biology and signaling in tumors. Pharmacol Ther 151, 41.

Warfel, Noel A, Niederst, M., Newton, A.C., 2011. Disruption of the Interface between the Pleckstrin Homology (PH) and Kinase Domains of Akt Protein Is Sufficient for Hydrophobic Motif Site Phosphorylation in the Absence of mTORC2. Journal of Biological Chemistry 286, 39122–39129.

Warfel, Noel A., Niederst, M., Stevens, M.W., Brennan, P.M., Frame, M.C., Newton, A.C., 2011. Mislocalization of the E3 ligase, β-transducin repeat-containing protein 1 (β-TrCP1), in glioblastoma uncouples negative feedback between the pleckstrin homology domain leucine-rich repeat protein phosphatase 1 (PHLPP1) and Akt. J Biol Chem 286, 19777–19788.

Warfel, N.A., Sainz, A.G., Song, J.H., Kraft, A.S., 2016a. PIM Kinase Inhibitors Kill Hypoxic Tumor Cells by Reducing Nrf2 Signaling and Increasing Reactive Oxygen Species. Mol Cancer Ther 15, 1637–1647.

Warfel, N.A., Sainz, A.G., Song, J.H., Kraft, A.S., 2016b. PIM Kinase Inhibitors Kill Hypoxic Tumor Cells by Reducing Nrf2 Signaling and Increasing Reactive Oxygen Species. Mol Cancer Ther 15, 1637–1647.

Williams, G.R., Bethard, J.R., Berkaw, M.N., Nagel, A.K., Luttrell, L.M., Ball, L.E., 2016. Exploring G protein-coupled receptor signaling networks using SILAC-based phosphoproteomics. Methods 92, 36–50.

Wong, C.C.-L., Gilkes, D.M., Zhang, H., Chen, J., Wei, H., Chaturvedi, P., Fraley, S.I., Wong, C.-M., Khoo, U.-S., Ng, I.O.-L., Wirtz, D., Semenza, G.L., 2011. Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation. Proceedings of the National Academy of Sciences 108, 16369–16374.

Woodgett, J.R., 2005. Recent advances in the protein kinase B signaling pathway. Curr Opin Cell Biol 17, 150–157.

Wu, S., Ma, L., Wu, Y., Zeng, R., Zhu, X., 2012. Nudel is crucial for the WAVE complex assembly in vivo by selectively promoting subcomplex stability and formation through direct interactions. Cell Res 22, 1270.

Xie, L., Xue, X., Taylor, M., Ramakrishnan, S.K., Nagaoka, K., Hao, C., Gonzalez, F.J., Shah, Y.M., 2014. Hypoxia-Inducible Factor/MAZ-Dependent Induction of Caveolin-1 Regulates Colon Permeability through Suppression of Occludin, Leading to Hypoxia-Induced Inflammation. Mol Cell Biol 34, 3013–3023.

Xie, X., Zhang, D., Zhao, B., Lu, M.-K., You, M., Condorelli, G., Wang, C.-Y., Guan, K.-L., 2011. I B kinase and TANK-binding kinase 1 activate AKT by direct phosphorylation. Proceedings of the National Academy of Sciences 108, 6474–6479.

Xu, D., Dai, W., Li, C., 2017. Polo-like kinase 3, hypoxic responses, and tumorigenesis. Cell Cycle 16, 2032–2036.

Xu, D., Yao, Y., Lu, L., Costa, M., Dai, W., 2010. Plk3 Functions as an Essential Component of the Hypoxia Regulatory Pathway by Direct Phosphorylation of HIF-1α. Journal of Biological Chemistry 285, 38944–38950.

Xu, F., Zhang, J., Hu, G., Liu, L., Liang, W., 2017. Hypoxia and TGF-β1 induced PLOD2 expression improve the migration and invasion of cervical cancer cells by promoting epithelial-to-mesenchymal transition (EMT) and focal adhesion formation. Cancer Cell Int 17, 54.

Xu, J., Xiong, G., Cao, Z., Huang, H., Wang, T., You, L., Zhou, L., Zheng, L., Hu, Y., Zhang, T., Zhao, Y., 2016. PIM-1 contributes to the malignancy of pancreatic cancer and displays diagnostic and prognostic value. Journal of Experimental & Clinical Cancer Research 35, 133.

Xue, X., Ramakrishnan, S., Anderson, E., Taylor, M., Zimmermann, E.M., Spence, J.R., Huang, S., Greenson, J.K., Shah, Y.M., 2013. Endothelial PAS Domain Protein 1 Activates the Inflammatory Response in the Intestinal Epithelium to Promote Colitis in Mice. Gastroenterology 145, 831–841.

Yamaguchi, N., Mizutani, T., Kawabata, K., Haga, H., 2015. Leader cells regulate collective cell migration via Rac activation in the downstream signaling of integrin β1 and PI3K. Sci Rep 5, 7656.

Yan, B., Zemskova, M., Holder, S., Chin, V., Kraft, A., Koskinen, P.J., Lilly, M., 2003. The PIM-2 kinase phosphorylates BAD on serine 112 and reverses BAD-induced cell death. J Biol Chem 278, 45358–45367.

Yang, M.-H., Wu, M.-Z., Chiou, S.-H., Chen, P.-M., Chang, S.-Y., Liu, C.-J., Teng, S.-C., Wu, K.-J., 2008. Direct regulation of TWIST by HIF-1α promotes metastasis. Nat Cell Biol 10, 295–305.

Yang, X.-M., Wang, Y.-S., Zhang, J., Li, Y., Xu, J.-F., Zhu, J., Zhao, W., Chu, D.-K., Wiedemann, P., 2009. Role of PI3K/Akt and MEK/ERK in Mediating Hypoxia-Induced Expression of HIF-1α and VEGF in Laser-Induced Rat Choroidal Neovascularization. Investigative Opthalmology & Visual Science 50, 1873.

Yoakim, M., Hou, W., Songyang, Z., Liu, Y., Cantley, L., Schaffhausen, B., 1994. Genetic analysis of a phosphatidylinositol 3-kinase SH2 domain reveals determinants of specificity. Mol Cell Biol 14, 5929–5938.

Yotnda, P., Wu, D., Swanson, A.M., 2010. Hypoxic Tumors and Their Effect on Immune Cells and Cancer Therapy. pp. 1–29.

You, S., Knudsen, B.S., Erho, N., Alshalalfa, M., Takhar, M., Ashab, H.A.D., Davicioni, E., Karnes, R.J., Klein, E.A., Den, R.B., Ross, A.E., Schaeffer, E.M., Garraway, I.P., Kim, J., Freeman, M.R., 2016. Integrated classification of prostate cancer reveals a novel luminal subtype with poor outcome. Cancer Res 76, 4948–4958.

Zhang, A.C., Rasul, R., Golden, A., Feuerstein, M.A., 2021. Incidence and mortality trends of metastatic prostate cancer: Surveillance, Epidemiology, and End Results database analysis. Canadian Urological Association Journal 15, E637.

Zhang, H., Wong, C.C.L., Wei, H., Gilkes, D.M., Korangath, P., Chaturvedi, P., Schito, L., Chen, J., Krishnamachary, B., Winnard, P.T., Raman, V., Zhen, L., Mitzner, W.A., Sukumar, S., Semenza, G.L.,

2012. HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs. Oncogene 31, 1757–1770.

Zhang, J., Tian, X.-J., Xing, J., 2016. Signal Transduction Pathways of EMT Induced by TGF-β, SHH, and WNT and Their Crosstalks. J Clin Med 5, 41.

Zhang, W., Shi, X., Peng, Y., Wu, M., Zhang, P., Xie, R., Wu, Y., Yan, Q., Liu, S., Wang, J., 2015. HIF-1α Promotes Epithelial-Mesenchymal Transition and Metastasis through Direct Regulation of ZEB1 in Colorectal Cancer. PLoS One 10, e0129603.

Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M.-M., Simons, J.W., Semenza, G.L., 2000. Modulation of Hypoxia-inducible Factor 1α Expression by the Epidermal Growth Factor/Phosphatidylinositol 3-Kinase/PTEN/AKT/FRAP Pathway in Human Prostate Cancer Cells: Implications for Tumor Angiogenesis and Therapeutics. Cancer Res 60.

Zhong, H., de Marzo, A.M., Laughner, E., Lim, M., Hilton, D.A., Zagzag, D., Buechler, P., Isaacs, W.B., Semenza, G.L., Simons, J.W., 1999. Overexpression of hypoxia-inducible factor 1α in common human cancers and their metastases. Cancer Res 59, 5830–5835.